Two Heads Are Better Than One:
The Combination of Dendritic Cells & B Cells Yields An Improved T Cell Response Over Dendritic Cells Alone When Presenting Tumour Lysate Antigens

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Abstract

BACKGROUND

The best APC for tumour immunotherapy is yet to be defined. The dendritic cell (DC) is well established as the key professional antigen-presenting cell (APC) for priming naïve T cells against tumours and for this reason it is the APC of choice in adoptive cell therapy (ACT) for cancer. However, robust clinical results with DC ACT remain disappointing and researchers are beginning to investigate the utility of alternative APCs, particularly B cells. DCs have been used as vaccines clinically to present tumour antigens in the form of tumour cell lysates, also with limited success. Tumour cell lysates represent an attractive source of tumour-associated antigens (TAAs) for cancer immunotherapy as their defined and undefined TAAs have the potential for simultaneous activation of CD4+ and CD8+ cytotoxic T lymphocytes (CTLs). Although their self-antigenic nature requires overcoming of inherent tolerance mechanisms as well as caution with regards to induction of autoimmunity.

METHODS

In this study the ability of three professional APCs, DCs, macrophages (MΦs) and B cells, to stimulate an anti-tumour T cell response against tumour lysate (TL) antigens was compared. The activation profile and IL-12 response of the APCs to soluble freeze-thaw lysate (s-L) and oxidized whole freeze-thaw lysate (ox-L) was examined. Also investigated was the lysate-loaded APCs' ability to induce T cell proliferation and effector functions, whether individually or in combination.

RESULTS

The three APCs displayed differing responses to the two TLs indicative of differing T cell activation capacities.
MHC-II and CD40 were upregulated on DCs in response to s-L and ox-L while MHC-II and CD86 were upregulated on B cells in response to s-L and ox-L. A synergistic increase in IL-12 production was observed in the lysate-loaded DC+B cell groups. No proliferation response was observed in lysate-primed CD4+ T cells. However, significant increases in CD8+ T cell proliferation were observed when T cells were primed with combinations of s-L-loaded APCs compared to a DC alone. The greatest proliferation was observed when CD8+ T cells were primed by the ox-L-loaded DC+B cell combination. The greatest IFN-γ levels were also observed in CD8+ T cells primed with lysate-loaded DC+B cells. The greatest *in vivo* cytotoxicity was achieved by ox-L-primed CD8+ T cells activated by the DC+B cell combination.

SIGNIFICANCE

Both DCs and B cells are currently being investigated as APCs in adoptive cell therapy for cancer. This study demonstrates that combining DCs and B cells yields an improved CD8+ T cell response in a pre-clinical model of melanoma. Given that, *in vivo,* DCs do not present TAA in isolation, these results suggest that a single APC approach may be unnecessarily limiting the potential of ex-vivo APC adoptive cell therapy.

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Abbreviations

ACK	Ammonium Chloride
APC	Allophycocyanin (context-dependent)
APC	Antigen presenting cell (context-dependent)
Bc	B cell
BM	Bone marrow
BMMΦ	Bone marrow MΦ
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CTL	Cytotoxic T lymphocyte
CLIP	Class II-associated invariant peptide
cm	centimetre
CpG	Cytosine phosphodiester Guanine
dH_20	Distilled water
DC	Dendritic cell
dLN	Draining lymph node
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's Phosphate Buffered Saline
FACS	Fluorescence Activated Cell Sorter
FBS	Foetal Bovine Serum
FcR	Fragment crystallisable receptor
FCS	Foetal Calf Serum
FITC	Fluorescein isothiocyanate
Flt3	FMS-like tyrosine kinase 3
Flt3L	FMS-like tyrosine kinase 3 ligand
FSC	Forward scatter
FVS450	Fixable Viability Stain 450© (BD Biosciences)
g	Gram
GM-CSF	Granulocyte macrophage-colony stimulating factor
GMDC	GM-CSF-differentiated dendritic cell
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
Ii	Invariant chain
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL4DC	DC differentiated in GM-CSF+IL-4
IMDM	Iscove's Modified Dulbecco's Medium
IU	International units
kDa	Kilodalton
LN	Lymph node
LPS	Lipopolysaccharide
MΦ	Macrophage
MPS	mononuclear phagocyte system
µg	Micro-gram
µl	Micro-litre
mg	Milli-gram
MHC	Major Histocompatibility Complex
MHC-I	Major Histocompatibility Complex Class I
MHC-II	Major Histocompatibility Complex Class II
mL	Milli-litre
MPS	Mononuclear phagocyte system
MQ	Milli-Q water
nm	nanometer
ODN	oligodioxinucleotide
OT	Ovalbumin transgenic
Ox-L	Oxidised whole freeze-thaw lysate
PBS	Phosphate Buffered Saline
pDC	Plasmacytoid DC
p-MHC	peptide-MHC
PE	Phycoerythrin
pg	Pico-gram
RBC	Red blood cell
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute (medium)
SSC	Single Stained Control
SSC	Side scatter
s-L	Soluble fraction of freeze-thaw lysate
TAA	Tumour Associated Antigen
TCF	Tissue Culture Flask
TCR	T Cell Receptor
Th1	T helper type 1 cell
Th2	T helper type 2 cell
TL	Tumor lysate
Tu dLN	Tumour draining lymph node
U	Units
VPD450	Violet Proliferation Dye 450® (BD Biosciences

1 Introduction

"Adoptive cell therapy (ACT) using autologous tumour-infiltrating lymphocytes has emerged as the most effective treatment for patients with metastatic melanoma and can mediate objective cancer regression in approximately 50% of patients"

Rosenberg *et al*, 2008[1]

1.1 Melanoma in the New Zealand Context

New Zealand's incidence of melanoma is the highest in the world[2]. With early diagnosis and surgical removal, melanoma has a 50% cure rate and an 80-100% 5-year survival expectation[3]. However, in cases of advanced melanoma, the 5-year life expectancy drops to 10% with most patients surviving 6-8.5 months[4]. Advanced melanoma is refractory to chemotherapy treatment[5,6], but owing to a lack of suitable alternatives, treatment with Dacarbazine (DTIC) remains the 'gold standard'[7], in spite of DTIC receiving 'placebo' results in recent clinical trials and having a very low overall response (OR) and overall survival (OS) rates. Combinations of different chemotherapeutic agents are no more effective than DTIC[8], but currently alternative options are limited.

1.2 Current Cancer Therapies

From the 1950s until very recently the standard treatments for cancer fell broadly into the three categories of surgery, chemotherapy and radiation therapy. In the context of melanoma, surgical excision of the primary tumour is the first treatment. Lymph node mapping, sentinel node biopsies, and sometimes lymph node removal depending on the level of lymph node involvement, follow excision of the primary.

Metastatic melanoma is treated with regional lymph node therapy, which, if unsuccessful is followed up with chemotherapy with drugs such as Dacarbazine. The outlook for Stage IV melanoma patients is poor with a 6 – 9 month median survival and a 5-year survival of 5 – 10% depending on various prognostic factors, serum LDH levels, sites and number of metastases[9]. DTIC treatment results in short duration response rates of 5 – 20%, with only 5% complete responses[9]. Combination chemotherapies in metastatic melanoma do not increase survival. Radiotherapy only

improves local symptoms and is not curative[9]. There is a real need for therapeutics that can complement current approaches and target metastases not removed by surgery, drugs or radiation.

Recent advances in our understanding have identified that, rather than simple elimination of rapidly dividing cells, one of the reasons for the success of chemotherapy and radiotherapy is their capacity to generate non-targeted innate and adaptive immune activation via immunologic tumour cell death and disruption of immune suppression and tolerance (reviewed in[10,11]). Immunoadjuvant pathways, such as the binding of high mobility group box protein 1 (HMGB1) to TLR4, or translocation of calreticulin to the cell surface, can be triggered by chemotherapeutic tumour cell death[12–14]. Cyclophosphamide has been observed to lift immune suppression by selectively depleting regulatory T cells (T_{REG}s)[15], while a comparative study identified the immune-enhancing role of DNA-damaging alkylating chemotherapeutic agents and the reduced immunostimulatory capacity of cytarabine, an inhibitor of DNA polymerase[16]. Radiotherapy's success in cancer eradication can also be attributed to its induction of immunogenic forms of cell death and destruction of immune barriers, which results in increased immune cell infiltration, increased tumour associated antigen (TAA) presentation and T cell activation[17–21]. Thus, in addition to their roles as debulking and cytotoxic agents, certain chemotherapies as well as radiotherapy, are increasingly viewed as potentially useful primers or adjuncts for immunotherapy for cancer[10,22].

The immune-stimulating features of chemotherapy and radiotherapy[23,24] have been documented (reviewed in[10,11,17,25–27]) Chemotherapy and radiotherapy can essentially create a form of tumour lysate *in situ*, exposing the entire range of tumour-associated elements to the attentions of the immune system. However, chemotherapy and radiotherapy are non-specific treatments, resulting in side effects that bring about significant patient suffering. A more targeted approach that minimises collateral damage to healthy tissue is required.

The advent of new immunotherapies for cancer has introduced treatments that are able to fulfill these criteria to varying degrees. Checkpoint blockade inhibitors such as

monoclonal antibodies (mABs) against the T cell inhibiting molecules CTLA-4 and PD-1 are enhancing survival rates, and in many cases the suffering experienced by patients is greatly reduced compared to standard treatments[28]. Several of these immune modulators have been approved for the treatment of different cancers, usually in combination, and often in conjunction with standard therapies[17,29,30]. The use of chimeric antigen receptor (CAR) T cells with hybrid monoclonal antibody (mAB) T cell receptors (TCRs) has also entered the clinic as important tools for the treatment of certain lymphocyte malignancies and will be discussed further under Immunotherapy for Cancer.

The introduction of these new treatments has only been possible due to advances in understanding of how the immune system responds to malignant cells and how tumour cells modulate the immune response. It is now well understood that cancer is a highly heterogeneous disease ([31], reviewed in[32]), of varying and complex etiology. The cancer of each patient is likely to be unique to the point that the possibility of a generic 'one size fits all' approach to treatment is highly improbable[11]. Given that cancer is a highly individual disease an individualised approach to treatment is required. Thus any cancer therapy must address the cancer threat in a manner that is responsive to the uniqueness of the cancer in each patient.

1.3 Antigen Presenting Cells: Initiating the Adaptive Immune Response to Tumours

Antigen presenting cells (APC) stand at the interface between innate and adaptive immunity, directing key decision-making processes in the immune response repertoire, from tolerance of self-antigens, to rejection of anything foreign or dangerous. They guide the response to a wide range of threats including infectious organisms, potential allergens, autoimmunity, graft rejection and cancer.

Major Histocompatibility Complex (MHC) is also called the human leukocyte antigen (HLA) in humans. HLA complexes A, B and C correspond to MHC Class I and are the molecules used for endogenous peptide presentation. HLA DR, DP and DQ are MHC Class II. Cells with antigen-presentation capacity process exogenous antigen into peptides and load them into MHC Class II molecules for presentation to CD4+ T

cells. APCs possessing cross-priming ability can also present exogenous antigen on Class I molecules to CD8+ T cells. All nucleated cells express MHC Class I (MHC-I), therefore MHC-I-expressing tumours can also be targeted directly by antigen-specific class I-restricted CD8+ T cells[33–35].

A paper by the Restifo group reporting on adoptive cell therapy (ACT) for melanoma demonstrates the crucial role of APCs[36] in the anti-tumour response. Beta2 microglobulin (β2m) is a component of the MHC class I molecule on the surface of cells. In order to assess whether the MHC-I+ tumours or APCs were providing the antigen source to T cells, β2m-/- and β2m WT chimeric mice were generated and the effect of adoptively transferred early effector T cells observed. In the mice with ineffective MHC-I on the surface of the tumour cells the effect of ACT was ablated, showing that APC antigen presentation was necessary for the anti-tumour response.

Three cell types, dendritic cells (DCs), macrophages (MΦs) and B cells, are known as professional APCs. DCs are the foremost APC and they have been utilised in most APC-mediated immune-based cancer therapies. However the success of these treatments has been limited. Therefore this thesis has examined whether or not combining the three professional APCs can augment the presentation of lysate tumour antigens to T cells.

1.3.1 Antigen Acquisition

Endocytosis is the general term for intake of material from the extracellular environment. Endocytotic mechanisms fall into the broad categories of phagocytosis ('cell eating', or the acquisition of larger particles) and pinocytosis ('cell drinking', which refers to uptake of liquid and solutes). Phagocytosis occurs in phagocytes, while virtually all cells conduct some form of pinocytosis[37]. Pinocytosis is further subdivided into at least four mechanisms, including macropinocytosis, clathrin-dependent endocytosis, caveolae-dependent endocytosis and clathrin- and caveolae-independent endocytosis. These mechanisms have been extensively reviewed[38].

Immature DCs efficiently endocytose material via receptor-mediated endocytosis and non-specific phagocytosis and macropinocytosis[39]. Apoptotic cells, soluble proteins, bacterial or viral particles can all be taken up by non-specific sampling of the

environment, or triggered by engagement of specific receptors. Many receptors have been described that specifically mediate antigen endocytosis, including Fc receptors, heat shock protein receptors, scavenger receptors and C-type lectin receptors. These receptors internalize antigen into endosomes where their outcome varies according to the receptor involved[40]. DCs excel at receptor-mediated endocytosis, whereas MΦ appear to be superior at phagocytosis[149].

DCs, MΦs[38] and B cells all take in particles and macromolecules via phagocytosis, pinocytosis and receptor-dependent endocytosis. MΦs, like DC, express a variety of phagocytic receptors rendering them highly efficient at clearance of pathogens, and infected or stressed cells. These include the mannose receptor, C-type lectin receptors, NOD-like receptors, scavenger receptors, retinoic-acid-inducible gene 1 (RIG1)-like helicase receptors (RLRs) and toll-like receptors (TLRs)[41,42].

TLRs on MΦs recognise the extracellular presence of molecules normally encountered intracellularly, such as ATP[43], high-mobility box group box 1 protein (HMGB1)[44], which increases their phagocytic clearance of necrotic cell debris. Complement receptors[42,45] and Fc receptors, which bind complement- and Ig-opsonised antigens respectively, are also avidly phagocytosed by MΦs[42], while phosphatidyl serine on the cell surface triggers MΦs to engulf apoptotic cells[46].

The common misconception that mature DCs downregulate all forms of antigen uptake has been clarified recently. While macropinocytosis is briefly upregulated[47].and then downregulated following inflammatory signaling[48], mature DCs retain the capacity to form clathrin-coated vesicles[48], and clathrin-dependent acquisition of viral antigen[49] and immune complexes[50] demonstrating that inward transport of surface molecules continues[51]. Platt and colleagues also demonstrated that Fcγ-receptor and DEC-205-mediated endocytic internalization continued, as did phagocytosis, in the face of abrogated macropinocytosis[51].

1.3.2 Antigen Processing

Loading of exogenous peptides onto MHC-II molecules is the result of degradation of acquired exogenous proteins by proteolytic and other enzymatic processes.

Extracellular antigen is taken up into endocytic vesicles that fuse with lysosomes to form endolysosomes or phagolysosomes. Protein degradation into peptides occurs inside the phagolysosome.

MHC-II molecules are formed in the endoplasmic reticulum (ER), and complexes with the invariant chain (CD74) in the Golgi. CD74 assists trafficking of the MHC-II molecule to the endolysosomal path, resulting in cathepsin-mediated degradation of CD74 that leaves only the peptide-binding portion of CD74 (the CLIP peptide) attached to MHC-II. Fusion of the phagolysosome with the MHC-II-vesicle results in CLIP being replaced with an antigenic peptide with the assistance of the chaperone HLA-DM[40,52]. Finally the vesicle containing peptide-loaded MHC-II traffics to the plasma membrane for presentation to CD4+ T cells.

MHC Class I is expressed on all nucleated cells, allowing the presentation of intracellular antigens to CD8+ T cells. Six steps leading to presentation on Class I have been well-characterised. One: antigen acquisition; 2: ubiquitin-tagging of peptides for destruction; 3: proteolytic degradation; 4: peptide delivery to the ER; 5: peptide-loading into MHC-I molecules; and 6: traffic of the MHC-I-peptide complex to the cell surface[40,53]. During normal homeostatic processes intracellular proteins destined for destruction are tagged with ubiquitin molecules, which targets them for break down into short peptide sequences inside the barrel-shaped proteasome[54].

Like the MHC-II chains, MHC-I heavy chains are also synthesized in the ER. Peptides translocated from the cytosol into the ER lumen via Transport Associated with Antigen Processing (TAP)-mediated transport bind to MHC-I in the ER during assemblage of the heavy chain β2m dimer (reviewed in[53]). TAP connects with tapasin and calreticulin to form the Peptide Loading Complex (PLC), which, in conjunction with other molecules, chaperones and edits the loading of peptides onto MHC-I, stabilizing the MHC-I-β2m complex[53]. Competition for high affinity binding occurs, and only stable MHC-I-peptide complexes traffic to the surface for presentation to CD8+ T cells.

1.3.3 Cross-presentation

DC have the capacity to present exogenous antigen on MHC class I molecules, a process termed cross-presentation, enabling them to activate CD8+ T cells with exogenous antigen that has accessed the MHC Class I pathway. One of the key challenges in cancer therapy design is orchestrating the delivery of exogenous tumour antigen into the DC cross-presentation machinery.

Cross-presentation allows exogenous antigens to cross from the MHC Class II presentation pathway in to the MHC-I pathway for presentation to CD8+ T cells (Figure 1). Our current understanding of cross-presentation mechanisms comes primarily from *in vitro* experiments that have demonstrated diverse means of antigen transfer from tumours to DCs to for Class I presentation. These include antigens from dead (apoptotic or necrotic) tumour cells; soluble antigens; scavenger-receptor-mediated uptake of chaperone-bound soluble antigens; uptake of tumour-derived exosomes; gap junction transfer of peptides; and "nibbling" of plasma membranes of live tumour cells by DCs (reviewed in[26]).

CD8+ DCs are recognised as superior to CD8- DCs at cross-presentation[55], and are also the superior DCs in the anti-tumour response[56]. Cross-presentation is constitutively active in CD8+ DCs due to their low degradation endosomes which have been shown to be responsible for the increased cross-presentation efficiency compared to CD8- DCs[57]. In addition CD8+ DCs possess phagosome to cytosol transport that permits antigen entry to the cytosol, which enables loading onto MHC-I[58,59], and other mechanisms that foster antigen entry to the cytosol have also been described[59,60].

While the majority of researchers argue that cross-presentation of tumour antigens is the "only important natural mode of presentation"[26,61–64] the question has been asked whether or not the importance of physiological cross-presentation may have been overstated[65]. Zinkernagel argues that the importance of cross-presentation has been overemphasized. He views cross-presentation as an experimental artefact of *in vitro* experiments, which use higher than physiologically-achievable amounts of antigen, and of *in vivo* experiments using artificially high numbers of antigen-specific T cells in TCR transgenic mice[65,66]. An alternative view to cross-priming suggests that any

"antigen-expressing cell", including tumour cells, that arrive at peripheral lymphatic organs can induce effector T cells[65,67,68]. These views are supported by studies demonstrating both TAP-dependent and TAP-independent mechanisms of cross-presentation[69–73].

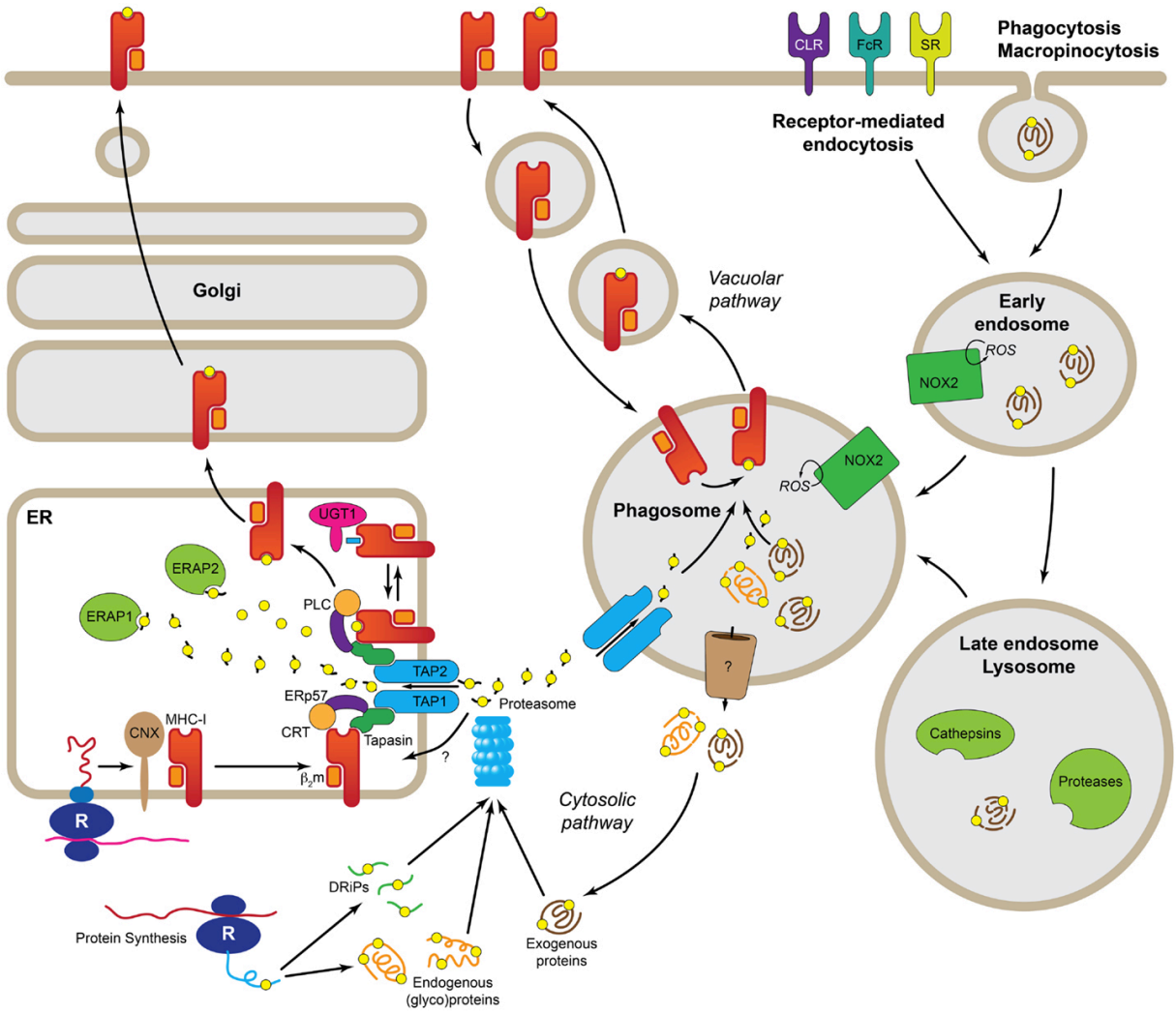

Figure 1 **Molecular Pathways Leading to Cross-Presentation in DCs.** DCs take up Ag by three general mechanisms, receptor-mediated endocytosis, phagocytosis, or macropinocytosis. Once the Ag reaches the endolysosomal pathway, depending of the specific routing, it may be degraded by the concourse of the mild pH and different types of cathepsins and other proteases. At this point, properly degraded Ag can be directly loaded into recycling MHC-I in the phagosome (Vacuolar pathway). Ag that still needs further processing must be transported to the cytosol (Cytosolic pathway) where it is degraded, together with endogenous proteins and DRiPs, by the proteasome. The peptides generated by the proteasome are transported by TAP or a yet uncharacterized transporter into the ER where they are loaded into MHC-I with the help of the peptide-loading complex. Further trimming in the ER prior to loading, it is possible by the presence of ER-localized endopeptidases (ERAP1 and 2). R, ribosome; CNX, calnexin; CRT, calreticulin; b2m, b2microglobulin; UGT1, UDP-glucose:glycoprotein glucosyltransferase 1; ERAP1/2, ER-aminopeptidases 1/2; PLC, peptide-loading complex; ERp57, protein disulfide isomerase 3; TAP1/2, transporter associated with antigen-presenting 1/2; DRiPs, defective ribosomal products; ROS, reactive oxygen species; NOX2, NADPH oxidase 2; CLR, C-type lectins; FcR, Fc receptors; SR, scavenger receptors. Understanding the biology of antigen cross-presentation for the design of vaccines against cancer, Fehres et al, Frontiers in Immunology, 2014. Reprint permitted under the terms of the Creative Commons Attribution License (CC BY).

In vivo cross-presentation of tumour antigens is much less understood and relies on work with model antigens outside the cancer field. Studies in autoimmune diabetes, for example, demonstrated that while high OVA expression resulted in cross-presentation, low OVA expression resulted in a lack of cross-presentation[74], pointing to the amount of antigen as being important[45]. Furthermore the maturation state of the cross-presenting DC is also critical. DC maturation during stimulation of a primary immune response includes upregulation of MHC and costimulatory molecules, and migration of the DCs to secondary lymphoid organs. In the lymph nodes interactions with naïve T cell clones results in T cell activation and clonal expansion. The maturation status of the DC determines the outcome of cross-presentation with activated DCs activating strong CTL responses[26,64,75] and immature DCs stimulating tolerance[76]. In the absence of DC activation the cross-presentation of OVA as a model self-antigen in autoimmune diabetes resulted in deletion of OVA-specific (self-reactive) CD8+ T cells[77].

While pathogens induce strong DC activation, cancer cells are likely to lead to weaker DC activation, in spite of the presence of tumour associated inflammation and danger signals[78]. The PRR pentraxin 3 has been shown to suppress CD8+ T cell activation initiated by DCs cross-presenting TAAs, but not pathogenic antigens[79] and the amount of apoptosis occurring in growing tumours has been described as too low to generate sufficient uric acid or hsp-bound tumour antigens to effectively prime DCs[26]. This may explain the immature, tolerising DCs associated with many cancers.

1.3.4 Cross dressing

Cross-dressing differs from cross-presentation in that peptide-MHC-I (p-MHC-I) complexes acquired from dead cells are attached to the outside of the DC and have not been generated via uptake and processing mechanisms[80]. Cross-dressed DCs can activate CD8+ T cells in the absence of antigen processing[80] and can drive anti-viral effector and memory T cell activation *in vivo*[81,82]. In the context of tumour lysate that consisting of dead cell components this may be an important mode of CD8+ T cell priming.

1.3.5 Antigen Presentation

The final step in the antigen uptake and presentation process is the cell-cell contact between APCs and T cells, in which the T cell receptor (TCR) complex recognises peptide bound to MHC on the DC surface. This forms "Signal 1", which initiates the process that determines whether or not the T cell will react to or ignore the antigen being presented. "Signal 2" comprises co-stimulation by molecules on the surface of the antigen-presenting cell, initially CD28 on the T cell binding to CD80 and CD86 on the APC. CD4+ T cells "license" DCs to present antigen to CD8+ T cells, and this licensing is dependent in part on further costimulation in the form of CD40-CD40L and 4-1BB-4-1BBL interactions, which stimulate upregulation of "Signal 3" cytokines, such as IL-12, by APCs[83–85]. Successful interactions between APCs and T cells form an immunological synapse, or supramolecule activation cluster (SMAC)[86] the initiation event in T cell proliferation. The T cell response to presentation is discussed under "T Cells".

1.4 Dendritic Cells

In humans, dendritic cells (DCs) are the best known and most potent of the antigen presenting cells[87,88]. Immature DC (iDC), widely, but sparsely, dispersed throughout the tissues and in circulation, are highly phagocytic, constantly sampling the environment by taking up and processing antigen. The ability to generate large numbers of DCs from bone marrow and blood[89–91] greatly facilitated the study of DCs and they have been well established as the most proficient presenters of antigen[69,89–92].

Immature DC (iDC) can be differentiated *in vitro* into mature DC (mDC) by exposure to stimuli such as bacterial lipopolysaccharide (LPS), interleukin 1 (IL-1), polyinosinic polycytidylic acid, CD40L or tumour necrosing factor alpha (TNF-alpha)[93,94] . DCs are well equipped with receptors that recognise both pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs)[95,96]. Their receptor repertoire includes TLRs, RLRs, nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and receptor for advanced glycation end products (RAGE) receptors for recognition of a wide range of pathogenic and danger-associated molecules. When iDC encounter an appropriate stimulus, such as pathogenic antigens, they undergo a maturation process during

which cell surface markers such as CD40, CD80, CD86 and migration markers such as CCR7 are up-regulated, along with IL-12 production. The immunostimulatory or immunoregulatory outcome of antigen presentation by DC depends on the maturation state of the DC presenting the antigen, and that state is mediated by inflammatory signals that accompany antigen acquisition and processing. Thus the maturation status of the TAA-presenting DC is critical due to its role in directing the immune response stimulated, and ensuring that DCs presenting undefined tumour antigens are properly activated is one of the goals of tumour immunotherapy.

Maturation enables the DC to migrate efficiently to the site of antigen presentation (most commonly the lymph node or spleen) and proficiently activate cognate naïve CD4+ and CD8+ T cells via presentation of antigenic peptide on MHC class II and class I molecules respectively[97–99]. In this regard DCs are key initiators of the innate immune response. They also regulate the T cell response, polarizing the T cell response into a Type 1 or Type 2 helper T cell response. This polarization may be mediated by distinct DC subsets[100]. The differentiation of CD4+ T cells into T helper 1 and T helper 2 cells is discussed under CD4+ T cell differentiation.

1.4.1 Peripheral Myeloid DC

There are several populations of DCs. In mice, myeloid or conventional DC (cDC) are found in both peripheral and lymphoid tissue and are broadly grouped into $CD11b^{HI}$ and $CD11b^{LO}$ cells. CD11b+CD11c+ antigen-presenting Langerhans cells (LC) are found in the external, epidermal layer of the skin and their primary role may be one of immunoregulation in the skin[101]. In the next layer down, the dermis, CD11c+ dermal DC (dDC) are distinguished from LC by their expression of CD103[102,103]. Only the $CD11b^{LO}$ dDC subset demonstrate cross-presentation[104]. In the pulmonary setting CD11b-CD103+ DCs migrate with lung-derived antigen to the LNs to initiate antigen responses and are able to cross-present antigen[105], but their presence is also required in the lung for the maintenance of CD8+ T cell function[106]. CD11b+ lung DC on the other hand primarily present to CD4+ T cells on MHC-II and foster antibody production[112].

1.4.2 Lymph Tissue DC

Two subpopulations of DC are encountered in murine lymph tissue: those DCs such as mature LC that migrate into lymph tissue from the periphery, and the permanent residents. Whereas peripheral DC are discriminated by their CD11b expression, in lymph tissue they are grouped by CD4 and CD8α expression (reviewed in [107]). Four subpopulations are found in the spleen on their basis of CD4, CD8, CD11b and DEC205 expression[107]. These populations are also found in the LNs along with two more groups that are $CD8^{LO}DEC205^{INT}$, and $CD8^{LO}DEC205^{HI}$[108]. CD8+ and CD8- DC can both present antigens but the CD8+ DCs are the primary subset that cross-present antigen to CD8+ T cells[75,109–112]. Two exceptions appear to be the migratory CD8+langerin-CX3CR1 plasmacytoid DC-related subgroup that are unable to cross-present[113] and migratory CD103+ lung and dermal DC, which cross-present antigens in the LNs[104,114].

Each of these DC subsets possess varying antigen presentation capabilities. CD8+ DC efficiently cross-present antigen to CD8+ T cells[55] while marginal zone CD8- DC preferentially present on Class II to CD4+ T cells[115115], for example the presentation of *Sacchromyces cerivisiae* antigen[116]. However it is the CD8+ DCs with cross-presenting capacity that are the target in DC-mediated immunotherapies. In the clinic the focus is on harnessing the human equivalents of murine peripheral CD103+ and lymph-resident CD8+ DCs for their CTL-stimulating capacity.

1.4.3 Plasmacytoid (pDC)

$CD11c^{LO}B220^{HI}$ pDC are best-known for their Type I IFN-α response in viral infections (reviewed in[117]). TLR ligation by viruses upregulates pDCs powerful, TRAIL-mediated cytotoxic functions and cross-presentation machinery[118]. The role of pDC in cancer has generally shown an association with poorer outcomes[119,120] however work with the adjuvant CpG has shown that this TLR9 agonist can be harnessed to activate anti-tumour immunity in these cells[121].

1.4.4 Human DCs

The divisions of pDC and cDC exist in murine and human systems, though human DC do not express CD8α. In humans the Type I IFN anti-viral function of pDC is conserved[122], as are the tolerogenic functions of epidermal LC[123]. However the easy

categorisations end there with correspondences between mouse and human peripheral and lymph-resident DC less well defined. Blood-derived human cDC are normally identified as $CD11c^{HI}MHC\text{-}II^{HI}$ lineage marker negative cells. This group is further partitioned into 3 sub-populations: CD1c+, CD16+ or CD141+[124].

CD11c+CD141+ were recently defined as the equivalent of murine CD8α+ cross-presenting cells[125,126], with further characterization adding XCR1[127] and Clec9A (DNGR-1)[128,129] to their surface markers. However some differences still exist between CD141+ human DC and murine CD8α+ DC. The latter express TLR4 and TLR9 whereas CD141+ huDC do not. $CD1c^{+}$ DCs express all TLR except TLR9, whereas $CD141^{+}$ DCs diplay a restricted TLR configuration with high expression of TLR3 and TLR10, some expression of TLRs 1,2, 6 and 8, and absence of TLRs 4, 5, 7 and 9[130]. All activated huDC subsets can produce IL-12 and cross-present antigen[124], whereas in mice only the CD8+ DC have demonstrated superior IL-12 and cross-presentation capacity[131]. However the current consensus is that the CD141+ huDC compartment possess potent CTL activating capacities and are the appropriate target for immunotherapies.

1.5 Macrophages

Metchnikoff identified and named these cells 'Big Eaters' due to their high phagocytic capacity[132], but they are much more than mere scavengers or garbage disposal cells. MΦ activities are numerous due to the various roles they play in different tissues. They possess a full complement of pattern recognition receptors[133] to defend against threats including infection, toxins, ischaemia, tissue trauma and sterile wounds. They carry out tissue remodeling and remove dead or dying cells and cell components. They can be cytotoxic in their own right and secrete an extensive range of cytokines, growth factors, coagulation factors, lysozymes, proteases, complement components and prostaglandins[134]. They are found in high numbers at sites of infection, trauma or inflammation and, like DC, they are able to present antigen and express co-stimulatory molecules. However it has been suggested that the panoply of MΦ functions can be summarised under four headings denoted with the acronym SHIP: Sample, Heal, Inhibit, Present[135] (Figure 2).

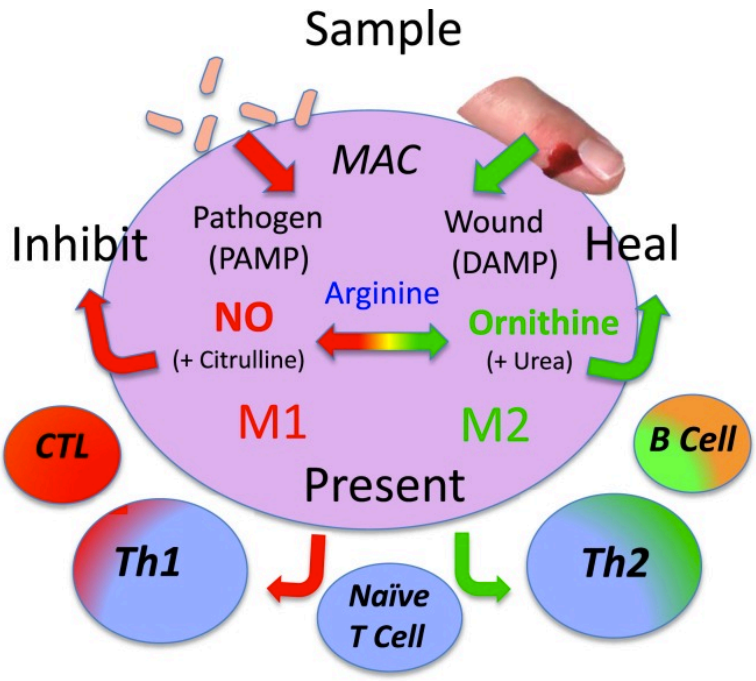

Figure 2 **Macrophages have four basic SHIP functions [sample, heal, inhibit and present (antigen] that allow them to recognize pathogens or injury, and respond directly (or indirectly by presenting antigens) to engender responses that provide optimal host protection**. From Ref. (1) with permission from S. Karger AG, Basel. Anatomy of a Discovery: M1 and M2 Macrophages, Charles Dudley Mills, Front Immunol. 2015; 6: 212. Published online 2015 May. doi: 10.3389/fimmu.2015.00212 PMCID: PMC4419847. Reproduction permitted with acknowledgement.

Tissue-resident MΦs are yolk-sac derived, tissue-based, and primarily self-regenerate *in situ*, except in cases of inflammation[136]. Myeloid-derived MΦs are generated in the bone marrow as immature monocytes and migrate to the periphery where they differentiate into lymph and non-lymph, tissue-resident MΦs. *In vivo* MΦs, and certain DC subsets, can be replenished from circulating monocytes under both steady state and inflammatory conditions[137].

Tissue-specific MΦs include liver-resident Kupfer cells[138], longer-lived alveolar MΦs[139], and shorter-lived interstitial MΦs in the lung, bone-resorptive osteoclasts[140], migroglia in the brain[141], histiocytes of connective tissue, gastrointestinal, splenic, lymph node and peritoneal MΦs[142,143] . At least four types of MΦ have been identified in the mouse spleen based on their location and combinations of surface markers: red pulp MΦs (F4/80++CD11b+), marginal zone MΦs (CD209b+F4/80-), marginal zone metalophilic MΦs (CD169+F4/80-) and monocyte-derived MΦs (F4/80+CD11b+)[144]. CD169+CD11c+ subcapsular MΦs in the lymph nodes are positioned to sample afferent lymph flow and capture dead tumour cell antigens that accumulate in the subcapsular and paracortical sinuses. Meanwhile lymph node medullary MΦs cluster around lymphatic endothelium[145]. In the peritoneal cavity large

F4/80++CD11b+MHC-II- MΦs and small F4/80+CD11b+MHC-II+ MΦs have been described[146]. Thus MΦs with a variety of surface markers have been described among peripheral and lymph tissues.

In cancer, and in other fields[147] there has been a tendency to broadly vilify MΦs as pro-tumourigenic and their role in tumour development was seen as inherently negative. Over the years this view merged to a more ambiguous position however the consensus is now one of distinct MΦ subsets (just like DCs), which may be pro- or anti-tumourigenic in nature depending on polarization. The three broad subsets are classically activated M1 inflammatory MΦ; the alternatively activated immunosuppressive or tolerising M2 MΦ, and the more recently described regulatory MΦs[148]. In addition another subpopulation of immature myeloid cells known as myeloid derived suppressor cells (MDSCs) comprise innately suppressive monocyte- or granulocyte-derived cells whose role in tumour suppression has been extensively reviewed[149,150]. MDSCs directly impact on the progression of tumours by promoting angiogenesis, inhibiting T cell activation and proliferation, disrupting T cell homing to LNs and intratumoural CTL penetration. Their cytokine profile also promotes expansion of naturally occurring T_{REG}s and the differentiation of inducible T_{REG}s (reviewed in[149]). The tumour-promoting nature of MDSCs has brought them to the attention of investigators as therapeutic targets in cancer treatment[150].

1.5.1 M1 Versus M2 Macrophages

MΦs are broadly polarized as M1 or M2[151–153] with the former having a pro-inflammatory phenotype and the latter having an anti-inflammatory, healing phenotype (Figure 3). The M1/M2 concept stems from the ability of MΦs to use a single amino acid, arginine, to conduct polar-opposite functions (Figure 4). Conversion of arginine to growth-stimulating ornithine and growth-regulating nitric oxid (NO) is achieved by the same cell, via nitric oxide synthase or arginase conversion of this substrate[153–158]. The combination of IFN-y with a TLR agonist provides canonical induction of MΦ activation[159,160] and induces enhanced antigen presentation via exchange of three proteasome subunits[228,229161,162]. IFN-γ produced by T_H1 activation downregulates M2 activity, while T_H2- or tumour-induced IL-4 ameliorates M1 actions.

Culture of bone-marrow precursor cells with granulocyte-macrophage-colony stimulating factor (GM-CSF) and interleukin-3 (IL-3) induces differentiation of M1-type MΦs with a $CD11c^{LOW\text{-}INT}IL\text{-}12^{HI}IL\text{-}23^{HI}IL\text{-}10^{LO}$ phenotype[151]. Culture of the same precursors with macrophage-colony stimulating factor (M-CSF) generates anti-inflammatory M2-type MΦs ($IL\text{-}12^{LO}IL\text{-}23^{LO}IL\text{-}10^{HI}$)[152].

The M1/M2 paradigm is a debated area with many saying this concept oversimplifies the immunological context of stimuli source, and the fact that polarising stimuli exist together in tissues[163]. Opponents of the M1/M2 concept contend that simplified representations of macrophage active states can't account for the degree of heterogeneity observed *in vivo*. Although distinct macrophage subsets with unique functional abilities have been described, it is generally believed that macrophages represent a spectrum of activated phenotypes rather than discrete stable subpopulations[143]. They adopt context-dependent phenotypes that either promote or inhibit host antimicrobial defence, antitumour immune responses and inflammatory responses.

However it has also been argued that the classification of MΦs into numerous subpopulations[41] on the basis of surface markers, many of which are observed *in vitro* and not *in vivo*, is an over complication, which clouds the fact that MΦs possess two prime activities. They 'inhibit' the progress of pathogens, and cancer, and cause tissue damage, (M1-type). And they 'heal', repair and maintain tissue integrity and promote cell proliferation (M2-type)[153]. While acknowledging the on-going debate, for the purposes of this thesis the use of the terms M1 and M2 will suffice.

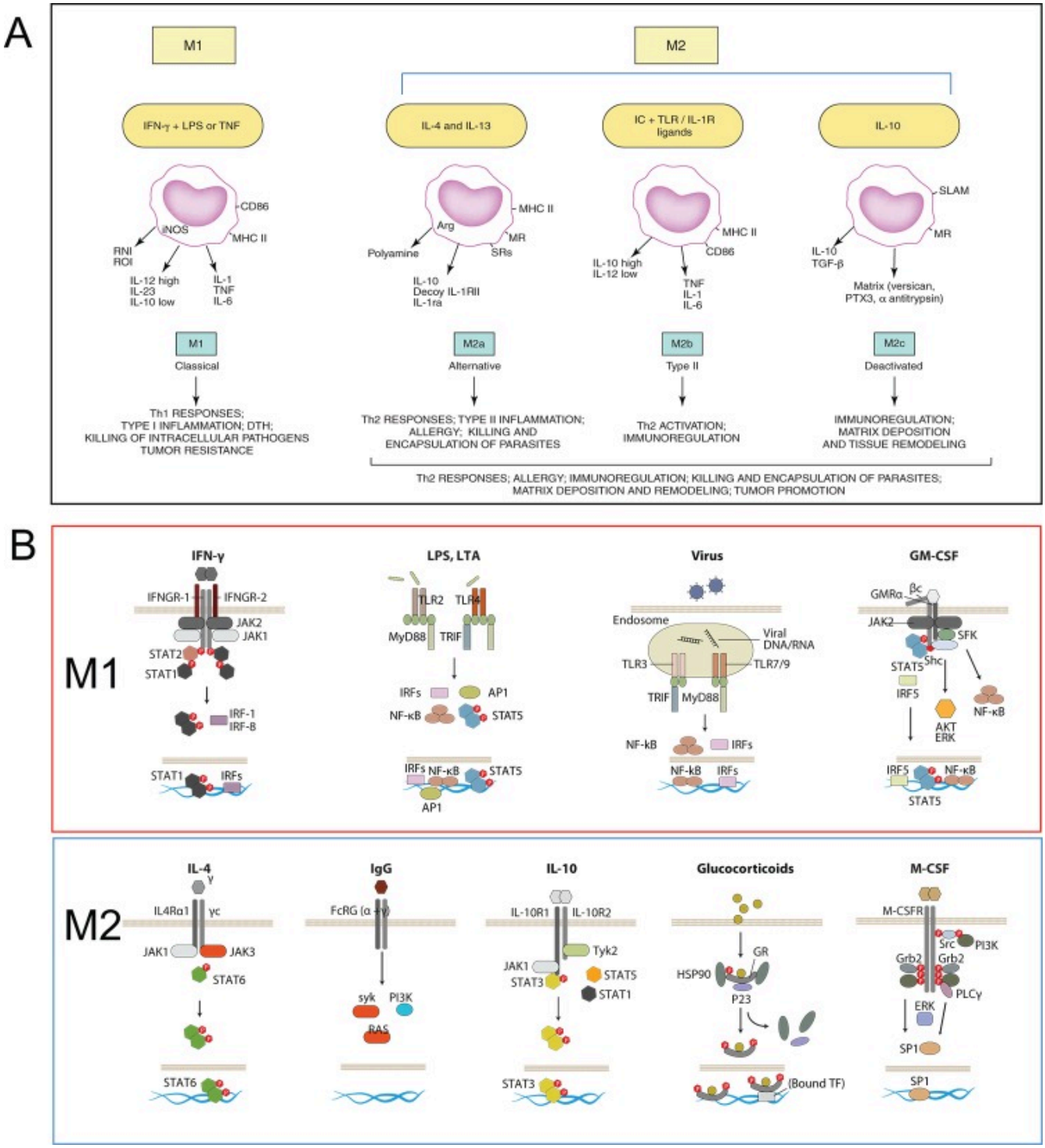

Figure 3 **The M1/M2 paradigm, origin, and molecular basis (A)** 4 years after Mills proposed the M1/M2 macrophage model Mantovani and colleagues published this M1-M2 macrophage model, in which M1 included interferon-gamma (IFN-γ) + lipopolysaccharide (LPS) or tumour necrosis factor (TNF) and M2 was subdivided to accommodate similarities and differences between interleukin-4 (IL-4) (M2a), immune complex + Toll-like receptor (TLR) ligands (M2b), and IL-10 and glucocorticoids (M2c). Diagram reproduced with the permission of Elsevier. **(B)** The signaling behind the effects of M1 and M2 stimuli in macrophages has gained clarity in recent years. Here, we highlight receptors and key signaling mediators in common and distinct pathways, explained in the text. The diagram includes granulocyte macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) as M1 and M2 stimuli. From: The M1 and M2 paradigm of macrophage activation: time for reassessment, Fernando O. Martinez and Siamon Gordon. Permission to reprint in thesis granted by Martinez, email correspondance 5.11.15 All F1000Prime Reports articles are distributed under the terms of the Creative Commons Attribution-Non Commercial License, which permits non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

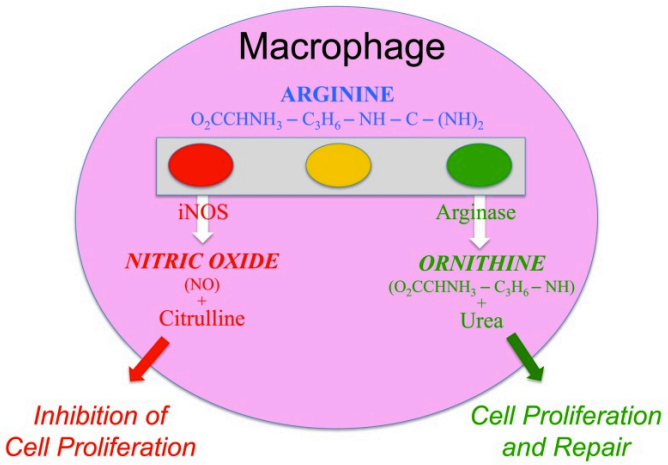

Figure 4 **Macrophages have both iNOS and arginase enzymes that can convert arginine to NO or ornithine, respectively**. Products of each reaction inhibit the opposing reaction, promoting preferential NO or ornithine production. Anatomy of a Discovery: M1 and M2 Macrophages, Charles Dudley Mills, Front Immunol. 2015; 6: 212. Published online 2015 May. doi: 10.3389/fimmu.2015.00212 PMCID: PMC4419847. Reproduction permitted with acknowledgement.

Although murine M1- and M2-polarized macrophage subsets are relatively easy to distinguish on the basis of combinatorial gene expression profiles, the identification of equivalent subsets in humans has been less clear. Much progress has been made in unraveling MΦ and DC subsets with the use of lineage-specific genes[41,59,164–166] which may lead to more refined use of surface markers in future. However currently the issue remains complex, particularly for the neophyte to MΦ DC research.

1.5.2 Antigen processing by Macrophages

MΦ antigen processing follows the same trafficking patterns described under Antigen Processing (1.3.2), with transfer of ingested items transitioning from early through late endosomes and lysosomes, or early phagosomes through to phagolysosomes[167]. One key difference between DCs and MΦs lies in the increased degradative potential of the MΦ processing pathway. While DCs have both degradative and non-degradative machinery[168,169]MΦs have higher lysosomal protease activity and degrade internalized antigen more rapidly compared to DC and B cells[169,170].

1.5.3 Antigen Presentation By Macrophages

The phagocytic ability of MΦs, combined with their antigen presentation capacity, allows them to link innate with adaptive immunity. MΦs bearing internalized antigen have been shown to stimulate T cell microtubule organizing centres and repositioning of F-actin cytoskeletal fragments to the point of contact with the MΦ[42]. When loaded with the same dose of antigen (1 μM) MΦs bearing phagocytized antigen formed productive immune synapses with T cells with greater efficiency than peptide-loaded

MΦs[42]. These data suggest that lysate antigen-loaded MΦs may have the potential to stimulate effective T cell responses from lysate antigens.

However another study found that uptake of apoptotic cells by MΦs did not result in presentation to T cells[171]. Inhibition of MΦ antigen presentation was the result of apoptotic DNA binding to MHC-II, which led to TGF-β production, and which also inhibited antigen presentation by DCs in the immediate vicinity. Freeze-thaw lysate is usually described as generating necrotic-type cell death[172–174], however some have argued that freeze-thaw lysis does not mimic *in vivo* necrotic death[175], therefore we were interested to see whether or not MΦs loaded with TAAs generated from freeze-thaw-generated lysate could activate T cells.

The consensus appears to be that MΦs are excellent scavenger cells in many situations but that while they possess antigen presenting ability[167], and can even generate T cell epitopes[248], their T cell stimulation capacity is limited[176–179]. Nevertheless some researchers have shown in both humans and mice, *in vivo,* that MΦs and DCs can have equal effects on T cell activation[180,181]. These data demonstrate the ability for MΦ function to be modulated, such as has been shown in other models in which IFN-γ and TNF-α have been shown to overcome the effects of suppressive molecules such as prostaglandin E2[182].

1.5.4 Cross-presentation By Macrophages

Their steady-state role as regulators of inflammatory-mediated tissue damage means that unactivated MΦs do not produce meaningful quantities of inflammatory factors. This changes upon exposure to activation stimuli such as LPS binding to TLR4, which activates the MyD88 signal path, triggering Type 1 interferons and leads to the production of various pro-inflammatory cytokines[152]. In addition to cytokines, the uptake of necrotic or apoptotic cell components exerts differential activation programming on MΦs, with phagocytosis of apoptotic cells leading to a non-inflammatory, non-immunogenic response and phagocytosis of necrotic cells resulting in tumour cell cytotoxicity[183], the release of inflammatory cytokines and upregulation of costimulatory molecules[179,184,185].

Just as the value of MΦs in the tumour environment is debated so too is the value of using MΦs to generate anti-cancer T cell responses. Nonetheless the ability of MΦs to present tumour antigen and prime naïve CD8+ T cells has been demonstrated[42,180,181]. Efficient MΦ cross-presentation of OVA has been demonstrated in murine models[177], and the ability of human MΦs to cross-present naturally occurring TAAs taken from apoptotic or necrotic cells has also been investigated [180].

Dead cells from the periphery are one source of tumour antigen[26] but prior to 2011 little was known about the role of APCs in the clearance of dead cells and the presentation of dead cell-associated antigens in peripheral tissues or LNs. A study by Asano *et al* suggests that apoptotic cells in the periphery are cleared and processed differently from blood-borne apoptotic cells in the spleen. Dead cells accumulate in the sinuses of draining LNs where resident CD169+ MΦs phagocytose and cross-present dead cell-associated antigens to CD8+ T cells[177]. Mice that were depleted of CD169+ MΦs failed to cross-prime CD8+ T cells, demonstrating that these MΦs played a key role in the induction of antitumour immunity via cross-presentation of dead tumour cell antigens. Asano's work showed that migratory DCs were not primarily involved in delivery and cross-presentation of dead cell antigens. CD169+ MΦs residing in the LN sinus took up dead tumour cells and cross-presented this source of TAAs to CTLs. By contrast, CD8α+CD103+ DCs phagocytosing dead cell antigen entering the spleen via blood flow after i.v. injection induced a tolerogenic response after cross-presenting these antigens.

Continuing with the CD169+ MΦ theme, Bernhard *et al* showed in 2015 that these APCs were able to prime CTLs against epitopes that cross-priming DCs had failed to present[178]. Using adenoviral vectors that do not infect DCs but accumulate specifically in CD169+ MΦs, they demonstrated that, in the absence of DCs, CD169+ MΦs were able to prime CTLs against all epitopes tested. When DCs alone were used to prime CTLs the only epitopes selected were strong-binding MHC-I peptides, demonstrating that DCs prime CTLs against a more restricted repertoire. These two studies were important since they question the absolute necessity of DC-mediated cross-presentation and highlighted the role of particular MΦ subsets in T cell priming, which can generate CTLs with broader repertoires. These may be precisely the kinds

of APCs that are ideal for use in ACT for cancer to minimise the possibility of escape variants due to the failure of DCs to prime T cells against the full range of the patient's TAAs.

1.6 A Comment On Dendritic Cell and Macrophage Generation and Classification

Arising as they do from common myeloid precursors, MΦs and DCs inevitably share surface marker expression. It has been a common practice to distinguish solely DCs on the basis of CD11c expression, or the combination of CD11c and MHC-II expression. However, as described in the following section, most if not all MΦs also express CD11c, to differing degrees, just like DCs, and this has led to obfuscated experimental conclusions. In a similar manner F4/80 has been over-utilised as a single MΦ marker when certain DC populations express F4/80[186] and some MΦ subsets lack F4/80 expression (for example marginal zone and white pulp MΦs in the spleen, and subcapsular sinus and cortical MΦs in the LNs[145]). Therefore it has been noted that identifying APCs on the basis of their cell surface markers merely identifies mononuclear phagocytes with the functional characteristics of DCs or MΦs, which may stimulate naïve T cells to the same degree[41,186].

Adding to the confusion are more recent acknowledgements in the literature that the ubiquitous differentiation of murine bone-marrow precursors in GM-CSF results in a heterogeneous population of MΦs, DCs and other granulocytes[187,188]. This has led to some passionate debate in the literature about what constitutes a 'real DC'[189,190]. Due to their complexity and heterogeneity classifying MΦ on the basis of gene expression profiles and surface markers has been described as "genuinely futile and misleading" (D Hume, personal correspondence). Jenkins and Hume argue that MΦs and DCs arise from separate lineages and are distinct functional entities. Moreover, they take the position that the name DC should be applied to cells arising from Flt3+ pre-DCs, and that a monocyte-derived cell is a MΦ irrespective of its ability to conduct antigen presentation[191]. Hume, Summers and Rehli divide bone marrow-derived cells in to monocyte-derived MΦs (if cultured in CSF1); monocytes (if cultured in GM-CSF, +/- IL-4); and DCs (if cultured in Flt-3 ligand)[192]. Thus according to that argument this

thesis has not compared MΦs and DCs but rather two different populations of antigen-presenting MΦs.

1.7 B cells

The B cell is the third, and least-recognised, member of the professional APC triumvirate. Anyone with a rudimentary knowledge of B cell biology may be surprised by the selection of B cells as an APC for comparison with DCs given the DCs' reputation of superior antigen presentation. Naïve B lymphocytes are, as a rule, thought to be inadequate APCs and there is incomplete understanding of their role in activation of CD8+ T cells. However B cells, which recognise native protein antigen as well as peptide fragments, take up protein antigen comparably to[176], or better than DCs[193] and since this study is looking at protein-packed tumour lysate it was important to compare B cell ability to present tumour lysate antigen.

B cells are best known for their antibody-production role but early studies failed to identify their antigen-presenting capabilities and this aspect of their biology has been underappreciated. This led to the misunderstanding, which persists today, that antibody production is their primary immune function. However, under the right conditions B cells efficiently present, and even cross-present, antigen to CD4+ and CD8+ T cells respectively. In this way cytokine-secreting, antigen-presenting B cells actively direct and regulate immune responses.

B cells arise from lymphoid progenitors known as haematopoietic stem cells in the bone-marrow and undergo a transitional maturation and functional rearrangement of immunoglobulin (Ig) loci[194] before exiting to the periphery as mature, naïve, IgM-expressing B cells. Activation occurs in secondary lymphoid tissue when antigen-induced somatic hypermutation, or "class switching", genetic rearrangement that leads to production of antibodies with the same specificity but of a different class[195], results in IgM and IgD-expressing B cells[196]. The classes IgA, IgE and IgG can also arise from class switching, depending on the immune response required. B cell VJD gene rearrangements and somatic hypermutation that result in the generation of single specificity B cell receptors (BCRs) have been extensively reviewed and will not be discussed in this thesis (reviewed in[197,198]).

In secondary lymphoid organs hubs of proliferating B cells, known as germinal centres, develop along the interface of the T cell zone in B cell-populated regions called follicles. It is here that B cells differentiate into high affinity, antibody-secreting plasma cells and memory B cells of the primary humoral response. B cells are broadly categorized as being either B-1 or B-2[199], or as B-1, B-2 and marginal zone B cells (MZB)[200]. B-1 and MZB cells are described as innate-like B cells with regulatory functions (B_{REG}s) including secretion of IgM and IL10[200]. They recognise self-antigen and produce immunoglobulins (Ig) in response to repetitive epitope patterns associated with specific pathogens[199]. The germline-encoded, semi-invariant BCRs of innate-like B cells recognise a limited range of epitopes. They provide a link between rapid immune responses and the more protracted T cell-dependent adaptive response with their prompt T cell-independent production of low affinity antibodies[200–202]. MZB can undergo rapid, short-lived T-independent responses to antigenic stimuli, for example in response to the TLR4 ligand, LPS[203,204].

Resident B cells of the secondary lymphoid organs that mediate adaptive immunity include the B-2 follicular B cells (fB cell), which make up the majority of the LN- and spleen-resident B cells, and the less populous marginal zone (MZ) B cells. Blood flow into the spleen brings blood borne antigens and T cells into contact with MZB, as well as MZMΦs and metalophilic MZMΦs, facilitating the initiation of T cell-dependent and independent immune responses[205]. Lymph flow into the LNs is an alternative source of circulating lymphocytes, as well as tissue-derived antigens and cellular debris. Cancer cells also flow in via afferent lymphatics, allowing for potential recognition by immune cells, but also mediating metastasis[206].

Both fB cells and MZ B cells can undertake Ig class switching and transition into memory cells[199]. B2 cells generate immune responses against T cell-dependent antigens and require T cell help to undergo germinal centre formation that results in the production of high-affinity antibodies[200].

The observation that B cell-deficient mice display aberrant secondary lymphoid structure led to understanding of the importance of B cells in both development as well as maintenance of these tissue structures and their associated cell

populations[205,207]. Thus, in addition to antibody production B cells are key players in immune development, maintenance, antigen presentation and regulation of T-dependent and T-independent immune responses. Their presence has been shown to be a positive prognostic factor in colorectal cancer[208], while in a melanoma model their absence results in loss of tumour control[209]. In addition to antibody-dependent cell-mediated cytotoxicity (ADCC), the fact that B cells can kill tumour cells directly via antibody-independent mechanisms has also been under-recognised. Activated B cells express a variety of the death receptor ligands including Fas-L, tumour necrosis factor-related apoptosis-inducing ligand (TRAIL), as well as the granzyme system(reviewed in[210,211]). Li has shown that tumour-primed CD40-activated B cells can mediate primary and metastatic tumour regression in a model of ACT[212,213].

1.7.1 Antigen Acquisition by B cells

Like DCs and MΦs, B cells also utilise phagocytosis, pinocytosis and possess numerous receptors for conducting receptor-mediated endocytosis. They include the B cell receptor (BCR), as well as Fc receptors, heat shock protein receptors, scavenger receptors and C-type lectin receptors.

The BCR comprises surface-bound immunoglobulin heavy (IgH) Ig light (IgL) chains complexed with the Igα and Igβ signaling chains[214]. Antigen cross-linking is the first step in breaking quiescence in a B cell[215]. It activates signaling through the immunoreceptor tyrosine-based activation motifs (ITAMs) on the BCR, which leads to the B cell to progress from G0 to G1 phase, making the B cell 'competent'. Antigen binding to the BCR triggers endocytosis and an activation-inducing signal cascade[216]. Interaction with T cell ligands and cytokines is the second signal that pushes the B cell from G1 to S phase[217]. The BCR has specificity, and high affinity, for a single antigen, which allows concentration and efficient presentation of small amounts of antigen[218,219]. The high affinity nature of BCR-peptide binding also means that processing of antigen sequences that have interacted with the BCR is limited, leading to enhanced presentation of particular epitopes[219–221].

Different subpopulations of B cells express distinct TLR profiles[222], allowing B cells to recognise various PAMPs and DAMPs. The combination of BCR signaling and TLR ligation facilitates their cytokine production, induces upregulation of surface

molecules and enhances their presentation of antigen (reviewed in[223]). The binding of CpG to TLR9 is particularly relevant to this thesis due to our use of CpG as an immune stimulant. IL-12p70 production by human B cells has been reported to only occur in the presence of both TLR9 ligation and CD40 signaling, and interestingly this occurred in the absence of BCR cross-linking[224]. The carbohydrate-binding C-type lectin DEC205 is a surface receptor specific for CpG Class B, though not for the CpG motif that binds to intracellular TLR9[225]. DEC205 facilitates CpG uptake by B cells and DCs, and transport to the internally located TLR9[225]. The CpG TLR9 interaction is strongly mitogenic and induces costimulatory molecule upregulation in B cells, along with increased levels of the Fcγ receptor[226–228]. Human B cells upregulate expression of not only CD80 and CD86 but also CD40 and CD54 in response to CpG[229]. Certain CpG motifs (CpG-A) are especially potent at activating NK cells and inducing IFN-alpha production by pDCs, while other motifs (CpG-B) are especially potent B cell activators[227,230], inducing production of T_H1-like proinflammatory cytokines, interferons, and chemokines[231]. In mature peripheral B cells, low concentrations of CpG DNA binding to TLR9 strongly synergize with signals through the BCR, leading to an approximate tenfold increase in B cell proliferation and antigen-specific Ig secretion and IL-6 secretion[228]. While CpG is a known immune stimulant tumour lysate also contains other unspecified immune-stimulating DAMPs, such as heat shock proteins and intracellular components that alert immune cells when encountered in the extracellular setting. These will contribute to APC activation in ways that were not specifically measured in this study.

Cross-linking of antigen on the BCR triggers internalisation[219,232] and rapid reorganisation of antigen presentation vesicles[233]. The BCR-antigen complex enters the endocytic trafficking pathway to commence peptide-MHC-II loading in MHC-II-rich compartments[234–237]. B cell processing machinery is coupled to BCR-mediated antigen recognition in a manner that mediates its preferential presentation over that of non-specifically pinocytosed material[219,234]. This feature results in T cell presentation of BCR-bound antigen with 10^3 to 10^4-fold efficiency over antigen entering in a non-BCR-mediated manner[238,239].

Finally, expression of the non-classical MHC-II molecule HLA-DO (H-20 in mice) distinguishes B cells from other APCs[240]. Another non-classical MHC-II molecule HLA-DM mediates peptide-MHC-II loading in all APC. HLA-DM operates in acidic lysosomes whereas HLA-DO is active in the more basic pH of the endosomes. It is postulated that HLA-DO inhibition of HLA-DM at higher pH also serves to favour presentation of BCR-antigenic material, and to limit B cell-mediated autoimmunity induced by presentation of pinocytosed self antigen[241].

T cell-independent (TI) antigens do not require contact with T_H cells in order to activate the B-lymphocytes. TI-1 antigens, such as bacterial lipopolysaccharides are polyclonal activators of B cells. They are capable of non-specific activation of B cells at high concentrations and stimulating both transitional and naïve B cells. TI-2 antigens require cytokines secreted by T helper cells for their activation, but not direct contact with them. Bacterial polysaccharides can also be TI-2 antigens as can polymeric bacterial flagellin, which have repeating antigenic determinants. The response to a TI antigen is weak, usually produces only IgM and a poor recall response (reviewed in[217]).

With T-dependent antigens interactions between B cells and T_H cells cause maturation of primary follicles into secondary follicles. Germinal centres arise from secondary follicles and it is in these germinal centres that B cells go through massive proliferation[217]. During proliferation genetic mutations in the variable region of the Ig gene sees the formation of B cells with low, medium and high affinity for the antigen that was encountered by the originating cell. These cells migrate to areas of high follicular DC (fDC) density where those with high-affinity BCRs will bind antigen-antibody complexes on fDCs, receiving survival signals from fDC and T_H cells. Thus T cell-dependent antigen recognition by the BCR also initiates differentiation into antibody-secreting plasmocytes and initiation of germinal centre (GC) formation for high affinity antibody production. However the presentation of antigen to CD4+ T cells is required for GC formation to proceed (reviewed in[242]). For the purposes of this thesis we will focus on the B cells' antigen presentation role, rather than antibody production.

1.7.2 Antigen Presentation by B Cells

Despite B cell antigen presentation being less widely known, the ability of B cells to activate antigen specific T cells has been recognised by immunologists since the early 1980s[243–248]. While resting B cells are poor at antigen presentation[238,249–251] , or induce T cell tolerance[252], properly activated B cells upregulate expression of MHC-II and costimulatory molecules[253] after BCR cross-linking with antigen[254], and consequently act as effective APCs[249]. In a similar manner to DCs and MΦs antigen-stimulated B cells activated under certain circumstances also play regulatory roles inducing tolerance and T_{REG}s[255].

The ability of B cells, MΦs and DC to stimulate Class-II-restricted T cell responses *in vivo* was compared in an early study employing a mouse chimera model[256]. That work appeared to demonstrate that antigen presenting B cells were not involved in T cell priming but could reactivate previously primed T cells. Those data suggested that in an immune response DCs first present antigen to naive T cells, and only afterwards can primed T cells be activated by antigen presented by B cells. However it was shown shortly thereafter that naïve CD4+ T cells can be primed *in vivo* by activated B cells[193,219,257–259], though this concept was initially not fully understood and remained controversial for some time.

B cells encounter both their specific antigen and T cells specific for that same antigen in the spleen and LNs[219]. The epitopes on the same antigen recognized by the T and B cells are very different[217]. After antigen encounter B cells participate early in T cell priming. Following chemokine gradients, they traffic toward T cell zones where antigen-specific B cell-CD4+ T cell interactions occur soon after the entrance of antigen into the lymph tissue[204,219,260–262].

Expression of CD86, one of the two CD28 ligands, is induced following cross-linking of the BCR[257,263] thus the Signal 1 and Signal 2 criteria for inducing CD40L expression on T cells are met by B cells encountering antigen. Rodríguez-Pinto demonstrated that during activation B cells can induce CD40L expression on T cells enabling full CD4+ T cell activation[219]. CD40-CD40L interactions play a vital role in T cell priming[264]. CD40 signals also effect multiple functions in B cells including

sustaining activation, enhancing antigen processing[264], increasing MHC-II upregulation, inducing CD80 expression and sustaining CD86 on the cell surface[219,265–268]. Inhibition of CD40L-CD40 signaling with mABs or genetic deletion abolishes B cell antigen presentation or renders the B cells tolerogenic[261,269–272].

In addition to T-independent functions, MZB have been shown to have increased T cell presentation and activation capacity by virtue of greater MHC-II, CD80 and CD86 expression, relative to follicular B cells[273]. MZB have also been observed to traffic blood-borne antigen from the marginal zone sinus to follicular B cells and DC in a manner suggestive of enhancing antigen presentation[274].

The final requirement for complete T cell activation is Signal 3, cytokine production, which is also ably fulfilled by B cells. Immune stimulatory B cells and B_{REG}s secrete distinct cytokine profiles in response to antigen encounter. B cells participating in T_H1 T cell responses produce IL-12, which assists in escalating the Type 1 response. B cells can also drive T_H2 responses by their secretion of IL-4 and IL-13, while B_{REG}s secrete elevated IL-10 to regulate immunity[275,276]. During T-dependent B cell activation cytokines secreted by T helper cells direct the B cell response. IL-4 known previously as B cell differentiation factor 1, induces B cell activation and differentiation, as well as T_H2 differentiation. IL-2 and IFN-γ secreted by T_H1 cells drives a feedback loop that enhances T_H1 activities[217].

Many studies delineated the current understanding, which is that the type and form of antigen dictates the degree of B cell participation in T cell priming (reviewed in[219]). Two studies confirmed that B cells can prime naïve CD4+ T cells in the absence of other APC[277,278]. Rodríguez-Pinto suggests that each circumstance of antigen presentation involves the different APCs to varying degrees but we can now be assured that B cells are indeed major players in particular instances[285].

1.7.3 Cross-Presentation by B cells

The B cell role as cross-presenters to CD8+ CTLs is an underappreciated fact[279]. While B cell ability to prime CD4+ cells and drive a T_H2 response was established in the 1980s and 1990s[193,245,280] little was known about B cell priming of T_H1 responses until a 2002 study established that when exposed to CpG-conjugated antigen B cells

can also carry out this function, which was previously uniquely assigned to DCs[231]. Since then further studies have confirmed the ability of conjugated and unconjugated CpG to induce cross-presentation by both murine and human B cells[281–283].

B cell cross-presentation to CD8+ T cells is required for the clearance of *Salmonella* infection[279]. Clearance is mediated in the absence of DC and in the absence of antibody but not in the absence of B cells. B cell cross-presentation has also been observed in the pathogenesis of diabetes[284], after uptake of OVA and immune-stimulating complexes incorporating OVA[285], and after gene gun vaccination with influenza and CMV DNA[286]. Jiang *et al* has shown that CpG-activated B cells can cross-present soluble tetanus toxoid and MV antigen[282]. Heit *et al* also demonstrated upregulation of costimulatory molecules and IL-12 production in B cells, accompanied by cross-presentation of SIINFEKL to CD8+ T cells, after uptake of CpG-conjugated OVA, albeit at a reduced efficiency compared to DC[281]. The cross-presentation of viral particles has been variously shown to occur[287,288] and not to occur[289]. However these different examples probably reinforce Rodriguez-Pinto's view that the role of B cells in antigen presentation, cross or otherwise, varies according to the circumstances[219].

Using a recombinant E-3-defective human adenovirus type 7 (HAdV7) vector transduced to express the colorectal cancer carcinoembryonic antigen (CEA) Peng and colleagues demonstrated transduction of naïve human B cells and presentation to CEA-specific CD8+ autologous T cells with accompanying B cell signal transduction, cytokine production and T cell cytotoxic capabilities[290]. These effects were eliminated in the presence of TLR9 inhibition. Castiglioni *et al* had demonstrated similar findings earlier with CD8+ CTL-mediated immunity to influenza generated by B cells transduced to express influenza epitopes[291]. Finally, CD40-activated B cells have been used to activate human CD8+ T cells in the cancer immunotherapy setting[292,293]. Interestingly, in LaPointe's study using CD40-activated B cells to present undefined TL antigens CD4+ T cell expansion was limited whereas successful induction of CD8+ T cells occurred in more than 80% of patients in two other studies[294,295]. These studies demonstrate significant possibilities for increased use of B cells in the cancer vaccine and immunotherapy fields to activate both T helper and CTL responses.

1.8 Evidence For APC Cooperation

While it is important to isolate individual cell types, especially in the initial period of research, to tease out their various qualities and capabilities, it hardly needs to be stated that *in vivo* individual cell types involved in immune responses do not operate in isolation. As such, work remains to be done on groups or combinations of cells in order to gain a better understanding of how these cells work together – the systems biology approach. The cooperative nature of the anti-tumour response is not surprising given the complexity of systems biology, however that very complexity makes defining the relationships and interactions painstakingly difficult.

Another factor to be considered is that antigen presentation does not always occur in the secondary lymphoid organs, and this brings other cells into the antigen presentation scenario. A key observation of Ma's 2013 study was that effective anti-tumour presentation may take place in the tumour bed itself, rather than in tertiary lymph structures of spleen or nodes[296]. They observed that ablation or removal of draining lymph nodes (dLNs) or abolition of tertiary lymphoid structures did not impact on the activation of tumour-infiltrating CD8+ T cells or adoptively transferred tumour-antigen-specific T cells. In addition, chemotherapy did not increase the numbers of CD11c+MHC-II+ cells in the dLNs. They observed accumulation of both CD169+F4/80+ MΦs and CD11b+Ly6C+ granulocytes in tumours after treatment with the anthracycline-based chemotherapies doxorubicin or mitoxantrone. This recruitment was driven by myeloid cells attracted by ATP released from dying cancer cells, which then stimulated local differentiation of CD11b+CD11c+Ly6C^{HI}cells. These cells then presented tumour antigens to T cells[296]. Thus significant levels of T cell activation was occurring externally to the secondary lymph tissue.

Campisano *et al* sorted CD11c+ and CD11c- cells from their heterogeneous GM-CSF-differentiated bone marrow cultures and showed that the CD11c+ cells alone were insufficient to generate an anti-tumour response in mice. They found that all the cell types present in the heterogeneous mix were necessary for *in vivo* anti-tumour protection[187]**.** In recent years an increasing number of studies have reported that cells such as neutrophils, basophils and eosinophils, previously thought to be exclusively involved in innate immunity, in fact play an accessory role in adaptive immunity[297–301].

The Parish group also found that eosinophil degranulation inside the tumour was required for OVA-specific CD4+ T_H2 cells to clear tumours, but interestingly not T_H1[302].

Some clinical investigators are also widening the net and looking beyond DCs and T cells. A Phase I trial in metastatic melanoma utilising autologous dying tumour cells transduced to express GM-CSF analysed the DC, MΦ, B cell, eosinophil and T cell infiltrates in patients[303]. DCs MΦs and eosinophils quickly infiltrated vaccination sites and high concentrations of CTLs, B cells and Ig-secreting plasma cells entered tumour tissues.

While acknowledging that other cell types all play a part in the anti-cancer response, this study focused on the ability of the three professional APCs to activate T cells against the TAAs found in tumour lysate. An orchestrated set of responses among DCs, MΦs and B cells results in the proliferation and differentiation of a variety of mature effector T cells. These responses are mediated by intercellular communications including cell-to-cell contact, cytokines and antigen presentation. Each APC conducts particular functions via their distinct APC uptake and processing machinery. The varying temporal and spatial dynamics of each APC trigger diverse signaling information that is integrated to coordinate the final immune outcome.

In one case of multi-APC cooperation Näslund *et al*, reported that tumour protein-loaded DC-derived exosomes (dexosomes) were superior to peptide-loaded dexosomes[304]. They showed that the CTL response was totally dependent on CD4+ T cells and, interestingly, also on B cells, suggesting that proper activation of CD4+ T cells and B cells needs to be considered, in addition to the activation of DCs and CTLs, when designing cancer vaccines to ensure full potential of the treatment.

In an effort to delineate the relative contributions of two APCs Kleindienst and Brocker used a transgenic mouse model in which B cells or DCs or both expressed MHC Class II. They showed that DCs alone are sufficient to prime naïve T cells *in vivo* after immunization with a peptide antigen. In their hands, neither naïve nor activated B cells alone could activate naïve CD4+ T cells with peptide antigen,

however if DCs were present B cells supported and enhanced CD4+ T cell priming. While antigen presentation by DC was sufficient for *in vivo* CD4+ T cell activation, DC and B cell together produced enhanced T cell proliferation, expansion and cytokine production[305]. Interestingly, despite documented examples demonstrating divergent T cell activation by B cells and DCs delivering peptide versus protein[193,306], Kleindeinst and Brocker did not observe T cell activation after B cell-mediated protein presentation in their system. Once again, the type of antigen dictated the immunological outcome.

DC uptake machinery comprises both degradative and non-degradative pathways, enabling them to present antigen to both B and T cells[168]. Native antigen is presented to the BCR by DCs that have endocytosed material via the FcγRIIB receptor, which enters a non-degradative vesicle that subsequently returns to the plasma membrane. DCs can thus present antigen to B cells in the splenic marginal zone. Additional work has shown that migratory DCs store antigen acquired by macropinocytosis and after entry into LNs transfer antigen from late endocytic vesicles to B cells[169]. Certain B cell subsets also respond to T-independent antigens and this requires interactions with particular DC populations[201,307,308].

On the other hand, B cells have been shown to inhibit DC-induced CD8+ T cell effector function via a mechanism that depends on B cell MHC-II expression[309]. The results of this and the studies described above was the basis of our interest in examining whether the addition of B cells could enhance DC-mediated presentation of lysate antigens to T cells, particularly since those studies imply MHC-II-dependent and independent outcomes.

Cooperative interactions between B cells and MΦs have also been described in the literature. B cells are essential for protection against VSV infection due to their provision of lymphotoxin α1β2 signals to MΦs[310]. When B cells were excluded the MΦ protection was lost. Conversely, when MΦs were eliminated, there was likewise no protection from B cells. Antibodies, an adaptive immune response, were not required; it was the lymphotoxin activating the MΦs that led to the protection. Thus B cells play multiple roles and understanding of this is required when analysing B cell-

mediated disease responses. Indeed, studies of cancer have also demonstrated B cell-mediated responses in the absence of ABs in the patient sera[311].

In the presence of antigen-specific B cells MΦs have been shown to acquire more antigen than in their absence[312]. MΦ activation of OVA-specific T cells occurred after MHC-independent transfer of B cell-captured antigen. The transfer of BCR-acquired antigen to other APCs escalates the frequency of antigen presentation and in this manner B cells amplify or regulate the immune response to specific antigen[377]. The transfer of antigen from B cells to DC, and subsequent DC-mediated presentation of B cell-captured antigen has also been described[313].

As with the DC-B cell interaction negative outcomes in the anti-cancer response have also been described for MΦ-B cell collaborations. B cell-derived antibodies and immune complexes can also drive MΦ polarization via ligation of FCγ receptors on the surface of MΦs[379] leading to malignant progression and carcinoma development in a murine model of inflammation-driven squamous carcinoma[314].

As can be seen some combinatorial studies have been conducted, which highlight inter-cell cooperation and provide a basis for examination of the ability of combined APCs to present undefined TAAs to T cells. We are not aware of any studies that have combined three APCs, or of any combinatorial studies using tumour lysate as the source of TAA.

1.9 T cells

T cells are implicated in all aspects of immune responses to pathogens or transformed cells, and as such are indispensable in the adaptive immune response to cancer. The three main subsets, CD4+ CD8+ and NK T cells are discussed in the context of cancer.

1.9.1 Self Antigen and Immunological Tolerance

Our immune systems are finely tuned to distinguish 'self' from 'non-self'. During development in the thymus T cells undergo selection processes that lead to central tolerance against self antigens. Developing T cells first undergo "positive" selection to eliminate T cells that fail to bind MHC Class I or II molecules[315]. This step is

followed by "negative" selection to eliminate those T cells that bind with high affinity to self-antigen peptide in the MHC molecule[316]. T cells that react to self antigen become anergic[317], or are eliminated by apoptosis,[318] and the mature non-self-reactive T cells complete development into single positive CD4 T helper cells (T_H) or CD8 positive cytotoxic T lymphocytes (CTLs).

These selection processes are not perfect and low frequencies of self-reactive T cells may be found in the periphery[319]. However, peripheral tolerance mechanisms also exist as a second layer of protection against inappropriate immune responses by mature T and B lymphocytes to benign antigens such as foods and gut microflora. Peripheral tolerance mechanisms include immunologically privileged sites such as the eye, the testes, the female reproductive tract and the central nervous system (Shirai, 1921, Murphy and Sturm 1923, Medawar 1948[320]), that restrict entry of lymphocytes by mechanisms such as FasL-induced apoptosis[321]. Other peripheral tolerance mechanisms include anergic T cell responses when MHC-peptide is encountered without accompanying costimulation[322]. Immune regulation also occurs via naturally arising or induced regulatory T cells (T_{REG}s)[323], whose regulatory systems target both T cells and APCs (reviewed in[324]). T_{REG}s secrete TGF-β and IL-10, suppressive cytokines that lead to T cell cycle arrest[324,325]. T_{REG}s also bind via CTLA4 to CD80 and CD86 on T_{EFF} cells, switching off the effector T cell response. Activated T_{REG}s are also capable of direct cell-cell granzyme-mediated cytolysis of activated CD4+ and CD8+ T cells[326].

Finally there is also presentation of antigen by tolerogenic DCs[327]. Cross-presentation of tumour peptides by DCs that are insufficiently activated, either due to immune-suppressive factors in the tumour environment, or due to the fact that tumour cells are self cells, will not lead to a robust anti-tumour T cell response, but rather to T cell anergy or activation induced cell death (AICD)[328], resulting in immune tolerance to these antigens.

1.9.2 CD4+ T Cells Priming: Activation Signals 1 & 2

Priming refers to the first time a naïve T cell encounters its cognate antigen in the context of the MHC molecule and becomes activated against that antigen. Antigens in the body, self or otherwise, flow through blood and lymphatic channels. Antigen

entering secondary lymphoid tissue and presented by APCs are ceaselessly surveyed by TCRs. CD4+ T cells interact with APCs via the peptide-MHC-II complex on the surface of mature APC such as DCs, macrophages (MΦ) and B cells. Recognition of the peptide-MHC complex (p-MHC) constitutes Signal 1 of T cell activation and triggers intracellular phosphorylation-mediated signaling cascades[329].

The duration of the TCR connection with the antigen-MHC complex is important[330,331], with long engagements of the TCR, approximately 20 hours in the case of naïve CD4+ T cells, required for the T cell to commit to proliferation[332]. This commitment time, and the amount of antigen required, are both reduced in the presence of CD28 costimulation[333]. By contrast, primed effector T_H cells have reduced activation requirements (reviewed in [334]) and are able to begin proliferating and secreting cytokines after TCR engagement of approximately 30 minutes, even in the absence of CD28.

A CD4+ T cell that recognises antigen in the context of the MHC-II molecule, and also receives Signal 2 - co-stimulation - will become activated against that specific antigen, including TAAs. Initial co-stimulation involves ligation of the T cell's CD28 molecule[253,335] with CD80 (B7.1)[336] and, or CD86 (B7.2)[335,337], which are upregulated on APCs after PAMP or DAMP receptor or inflammatory cytokine engagement. CD28 binding to CD80 and CD86 delivers a costimulation signal necessary for naïve T cell activation and survival[338–341].

The importance of costimulation was demonstrated by experiments in which antigen presentation in the absence of costimulation resulted in T cell anergy[342,343]. Diminished CD4+ T cell proliferation in CD28-deficient mice demonstrated the critical role of this co-stimulatory molecule, but also identified redundancy in the pathway that allows for other co-stimulatory paths. Ligation of CD28 and the T cell receptor (TCR) delivers a potent signal for proliferation and production of cytokines, especially IL-2[340] and IL-6[344].

One of the additional costimulatory mechanisms involves CD40L on T cells ligating with CD40 on activated APC[345,346]. This sends a potent activating signal to DC that, in

combination with IL-1, mediates pro-inflammatory cytokine production, which in turn drives T_H1 differentiation of naïve T cells.

CD27 is another of the costimulatory molecules involved in T cell activation[347–350] and is required for the generation and long-term maintenance of T cell immunity[351]. CD27 stimulates both growth signals for naïve T cell antigen-specific proliferation as well as pro-apoptotic signals, such as binding to Siva protein, the proapoptotic intracellular ligand of CD4[347–353]. CD70 on DCs binding to CD27 on CD4+ T cells indirectly promotes T_H1 differentiation[354]. Furthermore, CD27 binding to CD70 on B cells plays a key role in regulating B cell activation and immunoglobulin synthesis[352]. CD27 ligation leads to the activation of NF-κB and MAPK8/JNK signaling. Finally, the CD28 homologue inducible T cell costimulator (ICOS)[355], along with 4-1BB and OX-40 (all members of the TNF receptor family), also provide further, less powerful costimulation.

Inhibitory molecules are also upregulated in response to T cell activation. CTLA-4 is another homologue of CD28 and functions to switch off the T cell response once the pathogenic condition has been resolved. CTLA4 binds with higher affinity than CD28 to CD80 and CD86, inhibiting IL-2 production and proliferation[356]. It is for this reason that mABs against CTLA4 have seen success in the treatment of some cancers (reviewed in [357]).

1.9.3 CD4+ T cell Proliferation and Cytokine Secretion

Clonal expansion of the antigen-experienced T cell is the result of a successful priming interaction. In addition to proliferating, activated T cells integrate the signals received via TCR ligation, co-stimulation and the cytokine milieu to differentiate into different subpopulations capable of addressing the threat that has been identified. Differentiation provides the T cells with effector functions[358,359], mediated primarily via the secretion of immune-modulatory chemokines and cytokines[360].

Cytokines, secreted by APCs and the T cells themselves, comprise Signal 3 in T cell activation[361] and their requirement has been demonstrated for T cell proliferation, differentiation into effector and memory cells[362], as well as T cell survival[183–85]. IL-2 is

a pleiotropic growth factor that drives T cell proliferation[363]. Successful resolution of tumours have been observed in mice[364–368] and humans[369,370] after treatment with IL-2, highlighting the important role of this cytokine in the immune system's anti-tumour arsenal. The clinical use of IL-2 is constrained by dose-limiting toxicities in patients, though more than twenty years of clinical experience has resulted in refined administration and patient care which can ameliorate the morbidity observed in early years of IL-2 treatment[371,372].

IL-12, secreted by APCs, drives T_H1 differentiation and the IL-12 IFN-α/β combination has been identified as the key third signal for CD8+ T cells[373]. Cytokines from activated APCs and innate immune cells provide preliminary information to the T cells. Cytokines from T cells provide feedback to the APCs, inducing positive and regulatory feedback loops that amplify, direct and ultimately switch off the immune response. The nature of the presented antigen along with the cytokine setting in which presentation occurs dictates the phenotype that the CD4+ T cell will develop as well as the type of immune response[337,374,375]. The cytokine messages received by the T cells control the expression of transcription factors that dictate differentiation and lymphokine production.

IL-12's T cell-independent mechanism of MΦ activation was first delineated in *L. monocytogenes*-infected SCID mice that lack functional T and B cells, but whose APCs produce IL-12. This cytokine is responsible for inducing IFN-γ production from activated T cells, which in turn drives phagocytic cell activation, increasing their cytokine production and bacteriocidal properties[376]. Shortly after the SCID mice experiments IL-12's production by APCs and its polarizing effects on CD4+ T cells were demonstrated[377,378]. The importance of IL-12 in driving T cell proliferation and directing CD4+ T cells toward the T_H1 phenotype was confirmed in an artificial i*n vitro* system using MHC-peptide complexes immobilized on microspheres in the absence of confounding APC cytokines[83] (reviewed in). IL-12 from activated APCs and innate cells promotes activation of Tbet, driving the production of IFN-γ, but not IL-4 or IL-17, resulting in a T_H1 phenotype[379]. By contrast high levels of IL-4 in the activation environment switches on GATA-3, sending the cells into T_H2 mode resulting in the production of more IL-4 but not IFN-γ or IL-17[380]. Paracrine IFN-γ

feedback[381], along with signaling from IL-15[382] and CD40-CD40L exchanges[383], amplify IL-12-mediated responses (reviewed in [378]).

As with the costimulatory molecules of Signal 2, the balance of cytokines helps determine the differentiation fate of naïve T cells, and the ultimate effector functions, of the differentiated cell[84,384]. Understanding of IL-12's role in the anti-tumour response was enhanced with the observation that mice deficient in IL-12 or IL-12 receptors showed induced susceptibility to chemically –induced carcinomas and lymphoma[385–387], while in humans IL-12 deficiency endows a genetic predisposition to glioma[388]. While IL-12's anti-tumour capacity has been clearly demonstrated in pre-clinical models (reviewed in[378]), and vector-mediated co-delivery of antigen and IL-12 has been shown to enhance anti-tumour effects[389], as with IL-2, dose-limiting toxicities and low response rates have plagued clinical trials of IL-12 as an adjuvant therapy for cancer immunotherapy.

Our interest in IL-12 lies in its polarization of naïve CD4+ T cells toward the T_H1 differentiation cycle[390], primarily via its role in the induction of IFN-γ[391–394]. Unmodified freeze-thaw lysates have been reported to suppress DC IL-12 production[175,395]. By contrast studies with appropriately activated, lysate-loaded DCs have demonstrated effective IL-12 productio[395–398], therefore we hypothesized that with the appropriate activation our lysate-loaded APCs would produce IL-12 and drive a T_H1 response in the lysate-primed T cells.

Early studies of the Type II interferon IFN-γ indicated that it was secreted only by T cells[399] but it is now known that this cytokine is produced by innate NK cells[400], as well as adaptive T_H1 CD4+, CD8+ T cells and APCs, including activated B cells[401,402]. IFN-γ further directs the immune response by aiding IL-12-mediated promotion of CD4+ T cell differentiation into T_H1 cells[403] and by blocking IL-4-mediated B cell isotype switching[404].

The critical functions of IFN-γ in the anti-tumour immune response were revealed in mouse studies that demonstrated elevated risk of spontaneous and chemically-stimulated tumours in IFN-γ-/- or IFN receptor -/- mice[405–407]. Radiation-induced

intratumoural IFN-γ production has been shown to cause upregulation of MHC-I on tumour cells that aided T cell tumour infiltration and tumour cell targeting[408]. In addition to direct killing of tumour cells by CD8+ and CD4+ T cells[409], IFN-y mediates other anti-tumoural effects such as inhibition of tumour-promoted angiogenesis[409–413], and migration of T cells to tumour sites[414].

IFN-γ-treated melanoma cells have been characterised as a way of enhancing whole tumour vaccines for use in DC-based immunotherapy[415]. In a Phase II clinical trial patients received a vaccine of DCs that were loaded with surgically excised melanoma cells that had been briefly grown as cells lines and treated with IFN-γ prior to loading onto DCs. Patients also received adjuvant GM-CSF. The vaccine was safe and effective and resulted in a "remarkable 5-year survival rate of more than 50%". Reduced MHC-II expression is one method tumours use to evade the immune response. In this study pre-treatment of the patient melanoma cells with IFN-γ resulted in increased MHC-II, MHC-I and ICAM-1 expression, but reduced expression of the TAAs mel-4, Mart-1 and tyrosinase.

The upregulation of MHC-II was in contrast to a study by Dranoff in 1993, which found that genetically engineering B16 to express IFN-γ did not increase MHC-II expression[416]. Loading patient DCs with the IFN-γ-treated melanoma cells resulted in a small but significant increase in CD83. Treating the melanoma cells with IFN-γ resulted in slightly reduced tumour cell viability (85% compared to 89% in untreated cells) and the production of apoptotic melanoma cells correlated with increased survival in patients. When the melanoma cells were pre-treated with IFN-γ, immunostimulatory heat shock proteins (hsps) remained present in the cell lysates and the immunostimulatory proteins calreticulin (CRT) and HMGB-1 were upregulated. Also significantly upregulated was ICAM-1, a lymphocyte adhesion molecule that is associated with sensitivity to lymphocyte-mediated cytotoxicity.

Cornforth posed the interesting theory that DCs exposed to IFN-γ-treated melanoma cells, which have begun the DC maturation process, may be more effective *in vivo* after transfer as they may continue their maturation in a more 'natural', effective, manner, than those DCs 'pre-matured' with cytokine cocktails prior to transfer. It has

been postulated that immature DCs stimulate more T_{REG}s but Cornforth says that may not necessarily be true. He suggests that the presence of hsps, CRT, HMGB-1 in lysate phagocytosed by the DCs may provide a more bo*na fide* maturation signal as opposed to the commonly used cytokine cocktails.

The well-characterised immunoregulatory cytokine interleukin 10 (IL-10) is best-known as an anti-inflammatory mediator, inhibiting T cell responses by blocking MHC-II upregulation[417], costimulatory molecule expression[418] and inflammatory cytokine production in APCs[419,420] (reviewed in[421]). IL-10 is important in maintenance of peripheral tolerance via its induction of T_{REG}s[422] and is also involved in directing CD4+ T cells toward T_H2 differentiation[421], though this may be situation dependent. Other direct IL-10 effects on T cells include suppression of activation and cytokine secretion[423]. The role of IL-10 in the anti-tumour response is not clear-cut with some studies demonstrating clear pro-tumoural effects, and others demonstrating anti-tumoural benefits, such as inhibition of tumour stroma formation[410]. On the "IL-10 Is A Bad Guy" side of the argument is a study showing that IL-10 produced by MΦs suppresses IL-12 expression in intratumoural DCs, thereby blocking CD8+ T cell-dependent responses to chemotherapy[424]. In other studies, the removal of IL-10 with mABs resulted in increased tumour eradication after intratumoural CpG injection[425], while in an IL-10 knockout model enhanced Type 1 T cells responses to anti-tumour vaccines were observed[426].

Arguing for IL-10's more nuanced role in the regulation of anti-cancer responses are many studies in which IL-10 mediated cancer regression. Methodologies employed included release of IL-10 from tumour cells[427–430] and adjuvant administration of IL-10 in mouse models[431–434]. Intriguingly IL-10's anti-tumour effect was CD8+ T cell-mediated, inducing CD8+ CTL proliferation and cytolysis and behaving as a chemoattractant for CD8+ T cells *in* vitro[427–429,432,433]. Recent *in vivo* work in several mouse models has demonstrated activation and intratumoural expansion of CD8+ CTLs that resulted in regression of large, established tumours mediated. Tumour elimination was dependent on high expression of the IL-10 receptor on intratumoural CD8+ T cells[432], without involvement of IL-10R-expressing CD4+ T cells, NK cells or DCs . Thus the fascinating functions of IL-10 in cancer immunotherapy remain to be fully clarified.

1.9.4 CD4+ T Cell Differentiation

During immune responses CD4+ T cells differentiate into various phenotypes, which are characterised by the transcription factors they express and the cytokines they produce[390]. These are primarily T-bet, IFN-γ, IL-2 and TNF-α in the case of T_H1; GATA-3, IL-4, IL-5, IL-10 and IL-13 in the case of T_H2; and RORγt and IL-17 in the case of T_H17. Further subsets of CD4+ T lymphocytes include naturally-occurring and inducible T_{REGS}, and follicular helper T cells (T_{FH}) that provide crucial help to B cells in germinal centres[435,436]. The T_H1, T_H2 and T_H17 subsets are the most well-characterised, however the γδ T cells' role in cancer is also recognised[437] and we acknowledge that T_H9[438] and T_H22[439] cells have also been described. Figure 5 outlines the primary cytokines, signal paths and transcription factors that influence the differentiation of naïve CD4+ T cells into T_{REG}s, T_H1, T_H2 and T_H17 cells.

CD4+ T_H2 cells are generally responsible for protective responses against extracellular prasites such as helminths, as well as allergic responses. T_H2 cells are also involved in the humoral immune response, which may be useful in certain cancer types, however these cells will not be focused on in this thesis.

CD4+ T_H1 cells, on the other hand, provide "help" for activation of CD8+ T cells, mediate protection against intracellular pathogens such as viruses, and are also key cells in the removal of transformed cells[440–442]. Recent advances in understanding of CD4+ T cell plasticity of function may be harnessed in future to provide a more robust T cell anti-tumour attack[443].

Factors such as the ratio of costimulatory molecules on APCs, or the balance between costimulation and cytokines influence T cell differentiation fate. IL-2 and the B-7 molecules both drive T_H1 and tolerising T_{REG} responses. The balance between the two determines which one prevails[444]. The ratio of CD80 to CD86 is another factor in determining whether CD4+ T cells will undergo massive clonal expansion or whether they will differentiate into proliferation suppressing T_{REG}s. High CD80 to CD86 ratios support T_{REG} induction whereas high CD86 to CD80 levels suppress induction of T_{REG}s[337]. In addition the cell cycle and division number determines the timing of release of particular cytokines by activated T cells. IFN-γ is released early and

increases with division number, while IL-4 is released only after four rounds of division[358].

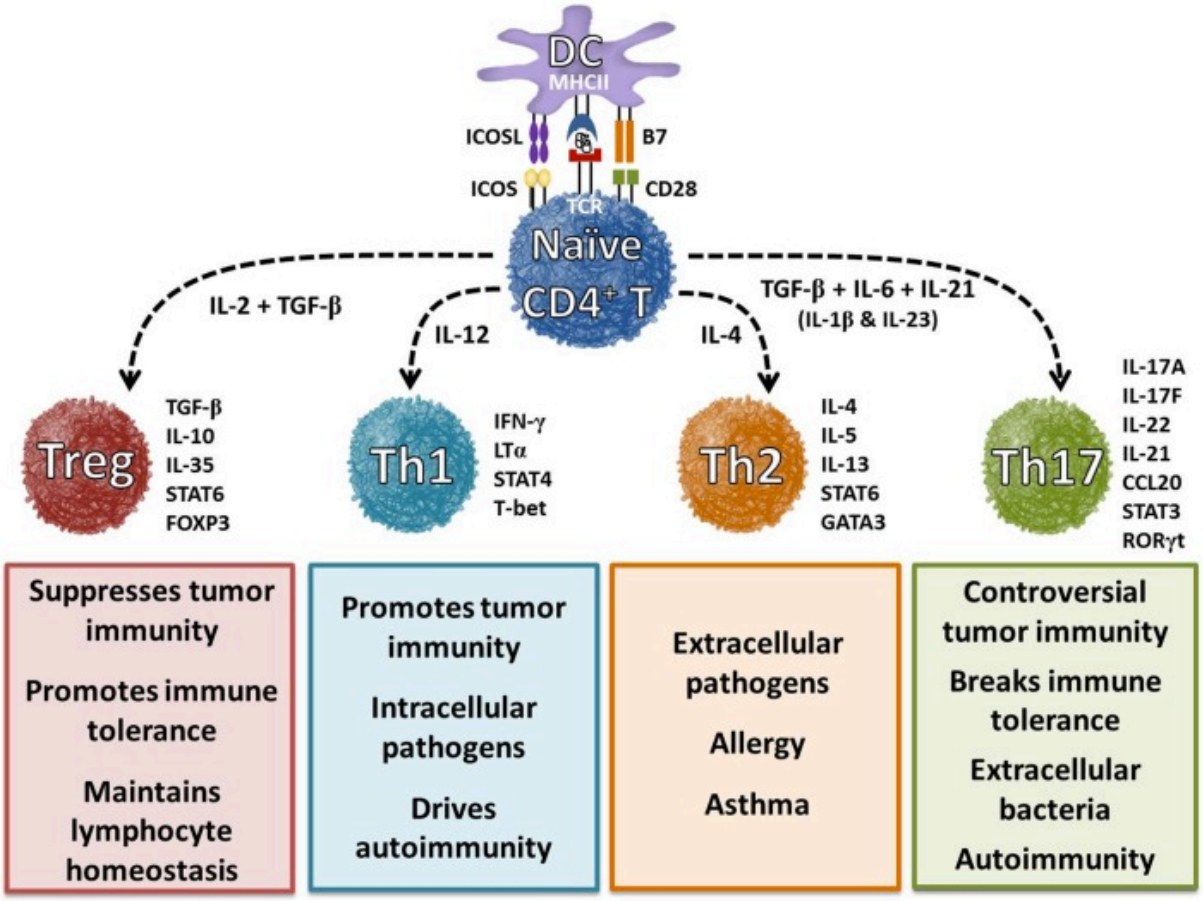

Figure 5 **Differentiation of helper T cell subsets is determined by cytokines**. In the presence of interleukin-6 (IL-6), IL-21, and transforming growth factor-beta (TGF-β), naïve CD4+ T cells differentiate into a Th17 cell phenotype, which is characterized by the expression of transcription factors retinoic acid receptor-related orphan receptor-γt (RORγt) and signal transducer and activator of transcription 3 (STAT3). IL-1β and IL-23 cytokines can promote and stabilize this phenotype during cell expansion. Once programed, these cells secrete IL-17A, IL-17F, IL-21, and IL-22, which play a key role in enhancing autoimmunity and host defense. Cytokines IL-12, IL-4, and TGF-β and transcription factors T-bet, GATA3, and FoxP3 have been shown to regulate Th1, Th2, and Treg cell development, respectively. These distinct subsets regulate immune response to foreign, self, and tumour antigens. From: Th17 Cells in Cancer: The Ultimate Identity Crisis510, Bailey et al, Frontiers In Immunology, Copyright 2014. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice.

1.9.5 CD4+ T Cell Effector Functions in the Anti-Tumour Immune Response

Activated CD8+ cytotoxic T lymphocytes (CTLs) were once thought to be the key cancer eliminating cells, primarily due to their direct cytotoxic functions, however it

has been known for some time that CD4+ T cells are more involved in tumour clearance than originally realized[441–443,445–447]. In addition to providing "help" for CD8+ CTLs, specific effector functions mediated by CD4+ T cells include direct recognition of endogenous antigen on the surface of melanoma cells, accompanied by IFN-γ production[448–450], and direct tumour killing[451]. Both CD4+ and CD8+ T cells exhibit IFN-γ-mediated inhibition of angiogenesis[413], and Class II-restricted CD4+ T cells specific for NY-ESO-1 have been shown to mediate durable remission and reactivity against other melanoma antigens in one patient[452]. Expanded CD4+ TILs have recently been successfully utilised in ACT for melanoma[449,453] and epithelial cancer[454]. IFN-γ production by CD4+ T cells also mediates the upregulation of MHC-I on some tumours leading to their increased recognition by CD8+ CTLs[455], or their elimination by IFN-γ-induced M1 MΦ[456]. As such it was important to investigate the differences in CD4+ versus CD8+ T cell response to TAA presentation by APC.

1.9.6 CD4+ T Cell "Help" for CTLs

CD4+ T_H cells fulfill vital functions during CD8+ CTL development and maintenance. Cytokines secreted by activated CD4+ T cells play a crucial role in activating naïve CD8+ T lymphocytes[457,458] and B cells. "Help" provided by CD4+ T cells enhances CTL clonal expansion, assists in effector entry into peripheral tissues, and promotes maintenance of memory T cells[454–456]. These functions are only undertaken after CD4+ "licensing" of APCs, which facilitates the functional activation of the APC, enabling the APC to independently prime memory CTL. Ligation of CD40 with CD40L on APCs forms part of the CD4+ T cell-mediated APC activation that provides initial "help" for CTLs[346]. CD40 binding to CD40L on CD4+ T cells stimulates expression of CD70, the only known ligand of CD27, on DC. Activated DC are then able to bind CD27 on CTL, catalyzing a full-fledged CTL response, including secondary expansion and circumvention of TRAIL-mediated AICD on antigen re-exposure[459].

1.9.7 CD8+ T cell Activation

Activated CD8+ T cells are crucial in the host defense against viral infections and cancer. CD8+ CTLs recognize short, 8–10-mer amino acid peptides localized in MHC class I molecular complexes on APCs and on cells ubiquitously distributed in the

body. Hydrophobicity of the TCR contact residues on both self and foreign antigens has recently been identified as a key element in determination of immunogenicity of peptides[460]. These cells eliminate infected or transformed cells via secretion of perforin and granzyme, or Fas/FasL-mediated activation of caspases, and secretion of IFN-γ. For this reason they are known as cytotoxic T lymphocytes (CTLs). Like naïve CD4+ T cells, naïve CD8+ T cells primarily reside secondary lymphoid organs and after activation also divide into functionally distinct subpopulations.

Like CD4+ T cells CD8+ T cells recognise peptide in the context of MHC, receive appropriate costimulation, and integrate cytokine signals from activated APCs and activated CD4+ T cells to become activated. However CD8+ CTLs require significantly less time than CD4+ T cells to trigger their cytotoxic functions, less than 30 minutes[334,461,462].

Some evidence exists demonstrating that given a strong enough signal via the TCR, or high enough numbers of responding T cells, costimulation is dispensable in CD8+ T cell activation[462–464], though whether this is a physiologically plausible scenario may remain to be resolved. Experiments with CD28-/- mice have demonstrated that CD28 is not absolutely required for T cell activation *in vivo*, suggesting that alternative costimulation pathways may exist[465].

1.9.8 CD8+ T cell Proliferation and Cytokine Secretion

The combination of IL-12 and IFN-α/β are the key Signal 3 cytokines for CD8+ T cell activation and differentiation into cytotoxic T lymphocytes (CTLs)[373]. DC IL-12 production is focused around the immunological synapse[466], as is T cell IFN-γ and IL-2 production[467], promoting CTL synapse formation[468] and additional STAT-4-mediated IFN-γ production[469]. The adhesion molecule ICAM-1 is an important stabilizing factor in APC-T cell synapses and its activity is required for the formation of CD8+ T cell memory[470].

Upon activation CTLs follow chemokine gradients and migrate to peripheral sites of malignancy where they, ideally, eliminate cancerous cells by various mechanisms. These mechanisms include the secretion of cytokines (IFN-γ, TNF-α, IL-2, IL-12), which may be cytotoxic in and of themselves, but which also attract other cells, such

as MΦs, polymorphonuclear cells and NK cells, to assist in the elimination process. One of the common γ chain (γc) cytokines, IL-2's anti-tumour role has been reported to lie in its ability to activate and expand CTLs in host tissues[370], and studies are in progress to elucidate the regulation of IL-2 with its additional contradictory regulatory roles in the induction of T_{REG}s and AICD. IL-15 induces DC production of IFN-y, which is part of DC-induced stimulation of CD8+ T cell proliferation[471].

1.9.9 CD8+ T Cell Differentiation

Like CD4+ T cells, CD8+ T cell differentiation is T-bet-mediated but is supported by an additional transcription factor Eomesodermin (Eomes)[472]. Figure 6 shows a simplified model of the environmental cues and cell-intrinsic factors that drive T cell fates. However there are other markers not identified in Figure 6 that are also used to identify differentiated T cells.

Whereas naïve T cells circulate continually through the peripheral blood and lymph, entering and exiting secondary lymph organs, activated effector and memory T cells must migrate from peripheral tissues and secondary lymph organs respectively, to the site of infection or malignancy to execute their effector functions[473,474]. Differentiation programmes induce the expression of homing molecules that foster the migration of effector cells to inflamed or malignant regions, where the cytokines released by effectors assist in antigen removal. The chemokine receptor CCR7 on the surface of naïve CD4+ T cells allows entry to lymph nodes via high endothelial venules (HEV)[475] where they enter T cell areas before returning to circulation. Thus CCR7 downregulation is a key differentiation feature identifying T_H1 and T_H2 cells whose chemokine receptors and cytokine profiles promote migration to sites of delayed-type hypersensitivity (DTH) or allergic inflammation respectively[606–608].

CD28 is constitutively expressed on naïve T cells and loss of CD28 expression, as part of a multi-marker profile, is one indicator of an antigen-experienced T cell. Whereas CD28 stimulation is generally thought to be required for CD4+ T cell activation CD28-B7 ligation is not absolutely required for activation of memory CD8+T cells[478]. Some have argued that given a strong enough signal via the TCR, or high enough numbers of responding T cells, costimulation is dispensable in T cell activation[462–464].

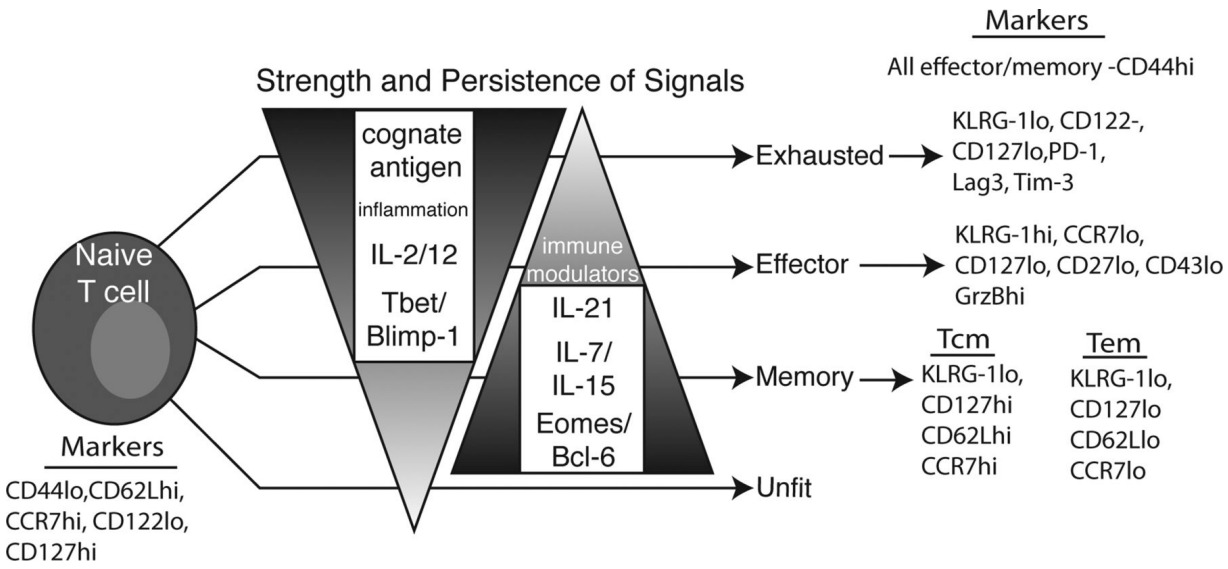

Figure 6 **T cell memory. T cells may assume many phenotypes in response to stimulation**. The eventual fate of a T cell depends on many environmental queues, including, but not limited to, cytokines, inflammatory and immune-modulatory products, and tissue-specific factors. These signals, in turn, influence the transcriptional profile of the T cell, leading to developmental choices. Generally, IL-7/15/21 and Eomes/Bcl-6 are considered to tip the scale toward memory. In contrast, IL-2/12, inflammatory products, and T-bet/Blimp-1 weigh toward terminal differentiation and effectors. Exhaustion can result when T cells experience these factors too intensely or for too long. A proper balancing of all these factors will lead to long-lived, protective memory. Markers commonly used to identify cells within a particular functional grouping are given on the right. KLRG-1, killer cell lectin-like receptor subfamily G member 1; PD-1, programmed cell death-1; Lag3, lymphocyte activation gene 3; Tim-3, T cell Ig mucin-3. From: T cell responses: naïve to memory and everything in between, Pennock et al, Advances In Physiological Education. Published 1 December 2013 Vol. 37 no. 4, 273-283 DOI: 10.1152/advan.00066.2013. Permission not required for reproduction in thesis.

CD27-CD28+ T cells are terminally differentiated T cells that fail to persist in vivo in adoptive cell therapy whereas CD27+CD28+ are early antigen-experienced T cells with strong proliferation potential that demonstrate good long-term persistence in adoptive cell therapy[476] Persistence at one month of CD8+CD27+ infused cells correlates highly with anti-tumour response in clinical trials of adoptive cell therapy for melanoma[477].

CD27, expressed on resting B[352] and T cells[348,350], is a member of the tumour necrosis factor (TNF) receptor family that does not express a death domain in its cytoplasmic tail region. CD27 has been demonstrated to promote activated T cell survival, to be necessary for maintenance of memory populations and to rescue CD28-/- cell death[351,479]. CD70 on DCs binding to CD27 on CD4+ T cells provides help to CD8+ T cells[459] and aids CD8+ memory formation[459,480]. In mice loss of CD27 is observed on CD8+ T cells after antigenic stimulation, therefore its presence and absence and can be used to identify memory T cells and effector T cells respectively[481,482]. Persistence

of CD27+CD8+ T cells has been highly correlated with anti-tumour responses in ACT for metastatic melanoma[483,484].

CD122 is the β chain of the trimeric IL-2 receptor on T cells. Different combinations of the α, β and γ protein chains form the IL-2 receptor. The three receptor chains are expressed separately and differently on various cell types and can assemble in different combinations to generate low, intermediate and high affinity IL-2 receptors[485]. The combination of β and γ binds IL-2 with intermediate affinity primarily on memory T cells and NK cells. The combination of α, β and γ binds IL-2 with high affinity on activated T cells[486] and T_{REG}s[487]. IL-2 binding to the IL-2 receptor also promotes the differentiation of T cells into effector T cells and memory T cells, as well as being required for memory cell maintenance[488,489]. Both IL-2 and IL-15 mediate their effects by docking on to the common gamma chain CD122[490].

CD127, the IL-7 receptor α (IL-7Rα), is expressed by most resting T cells and downregulated following T cell activation. Long-living T cells are characterized by constitutive CD127 expression[491,492]. The combination of CD122 and CD127 are used to assess survival potential and responsiveness of cells to homeostatic cytokine signaling *in vivo*. Cells that are negative for CD122 display unresponsiveness to IL-2, which is indicative of reduced long-term persistence *in vivo*. In the same manner cells that are negative for CD127 display unresponsiveness to IL-7, which is also indicative of reduced long-term persistence *in vivo*.

CD44, (homing cell adhesion molecule; HCAM) is a cell-surface glycoprotein involved in cell-cell interactions, cell adhesion and migration[493]. CD44 binds to its ligands hyaluronic acid and matrix metalloproteinases and participates in a wide variety of cellular functions including lymphocyte activation, recirculation and homing, hematopoiesis and tumour metastasis.

CD62 ligand (CD62L), also known as L-selectin, is a homing receptor that, along with CCR7[494], allows lymphocytes to enter secondary lymphoid tissues via high endothelial venules (HEV)[495]. Ligands on endothelial cells bind CD62L, slowing rolling cells for entry into lymphoid organs. CD62L is highly expressed on naïve T

cells, allowing these cells access to secondary lymph tissue where they can encounter antigen. CD62L is shed from the cell membrane after antigen encounter[496,497] and this is accompanied by CD107 upregulation, which allows for the activated T cells lytic activity[497].

1.9.10 CD8+ T cell Effector Function in the Anti-Cancer Immune Response

It is the CD8+ CTLs that ACT usually seeks to activate against the patient's tumours. It has been proposed that CTLs become activated against tumours via TAA-presentation in either the tumour environment or in the dLNs. In the periphery CTLs can engage MHC-I-peptide complexes on tumour cells and, if sufficient costimulation and cytokine signaling is also received, these peripherally activated CTLs can lyse tumour cells *in situ*. Alternatively, tumour cells that may have reached the dLNs via lymphatic flow may also directly present antigen to CTLs. Tumours do not express costimulatory molecules, therefore unless costimulation from APCs in the vicinity can also be engaged, this form of direct CTL priming will be ineffective.

Whereas other cytokines such as TNF-α are secreted by T cells, monocytes, MΦs, and NK cells IL-2 is principally secreted by activated T cells[498] and its presence is used clinically as indicative of T cell activation and killer capability. The combined actions of IFN-γ and TNF force cancer cells into senescence via growth arrest at G1/G0, and phosphorylation of molecules in the STAT1 pathway[498] which ignite inflammatory pathways.

The same group that demonstrated IL-12's role in driving CD4+ T cells into the T_H1 phenotype showed more recently that IL-12 signaling, induced by CD40L-CD40 interactions between DCs and CD4+ T cells, is required to induce effector cytolytic function in CD8+ T cells[499]. IL-12 also enhances CTL IFN-γ production[389], which has direct cytotoxic action on cancer cells. Due to successful Fas/FasL and perforin/granzyme-mediated killing of T cells by CTL in *in vitro* cytotoxic assays it was assumed that CTLs killed tumour cells directly via these mechanisms *in vivo*. However IFN-γ deficient T cells seldom mediate tumour regression, even if their lytic function is intact (reviewed in [410]). The efficacy of adoptively transferred TILs in

immunotherapy has been shown to correlate better with their cytokine production, particularly that of IFN-γ, and to a lesser degree TNF-α[410,447,500–502].

Other cell death mechanisms utilised by CTLs include activation of apoptotic, death-inducing pathways such as the Fas/FasL and perforin/granzyme methods. Secretion of perforin and granzyme-containing vesicles bring about the lysis of neighbouring cells – including the MHC-I-expressing cells presenting the antigen to the T cells, whether they be APCs or tumour cells. In the perforin-granzyme-mediated method of cell killing, lymphocyte-secreted perforin forms a pore in target cell membranes through which the serine protease granzyme can enter and activate intracellular caspases and mitochondrial death signaling[503,504]. CTLs also upregulate the death ligand CD95L (FasL), which forms a synapse with CD95 (Fas) on the surface of target cells, triggering a signal cascade that ultimately activates apoptotic, caspase-mediated death[505,506].

1.9.11 Development of T Cell Memory: The Key to Preventing Cancer Recurrence

Immunological memory empowers the immune response with an expedited, stonger response to antigen re-encounter. The majority of effector cells die during the post-activation contraction phase[507,508], but a proportion differentiate further to become long-lived, non-dividing memory T cells[507], which are able to self-renew and maintain themselves in the absence of antigen. Survival in the absence of the stimulating antigen is a key feature of memory cells. Naïve T cells require ongoing interactions with self-MHC complexes[509], but antigen-experienced memory T cells rely on cytokines[510] for their continued survival – IL-7 in the case of CD4+ memory cells[511,512] and IL-15 in the case of CD8+ memory cells[513].

Both CD4+ and CD8+ T cells differentiate into two main classes of memory cells: central memory (T_{CM}) and effector memory (T_{EM}). Irrespective of initial phenotype the memory pool differs from naïve cells in three key aspects. First, the frequency of memory T cells specific for a given antigen is dramatically higher than that of their very rare naïve precursors[514,515]. Second, their differentiation program renders them competent to access both secondary lymphoid organs and peripheral tissues. This is in

contrast to naïve T cells and T_{EM} whose surface molecules restrict their location to secondary lymphoid organs and peripheral tissues respectively (reviewed in[514]) where they await antigen encounter. Effector memory cells in peripheral tissues is one factor contributing to the markedly reduced response times in secondary responses. Finally, the activation requirements and thresholds for memory cells are less stringent than for naïve cells, enabling them to respond faster, to less antigen and with less costimulation, during antigen re-encounter, after which they secrete the full complement of polarised cytokine profiles.

T_H1 effector memory cells, commonly called T effector memory (T_{EM}) cells derive from T_H1 effector cells that survived the expansion contraction phase of immune activation[516,517]. Various factors have been implicated in the T_{EM} development program and include strong TCR signaling; a switch toward glycolysis; IL-2-receptor signaling; STAT-5-mediated upregulation of the β2 chain of the IL-12 receptor; and expression of the Tbet and Blimp-1 transcription factors (reviewed in[518]). Primarily found in peripheral tissues[519] T_{EM} provide immediate protection upon antigen challenge through, for example, the rapid, high production of inflammatory effector cytokines. Romero *et al* have identified four discrete CD8+ T_{EM} cell populations on the basis of their expression of CCR7 as well as CD27 and CD28[520], though whether this describes cells throughout a differentiation spectrum is unclear.
T_{CM}, by contrast, preferentially reside in secondary lymphoid organs, mounting recall responses to antigens[494]. Even though T_{CM} lack immediate effector functions, they rapidly proliferate and differentiate into effector T cells following antigen stimulation[514,521]. Figure 7 outlines the current understanding of human memory T cell subset formation.

Exactly what drives the T_{CM} development program remains unclear. The role of weaker TCR signaling is being elucidated[522,523] with more recent work demonstrating a role for IFN-γ signaling in blocking memory T cell precursors that respond to weak TCR agonists, thus supporting the creation of high affinity T_{CM} cells[524]. Other recent studies have highlighted the roles of mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) in differential regulation of T_{EM} and T_{CM} generation. Mice with constitutive mTORC1 expression generated highly effective T_{EM} cells that were

unable to transition into memory cells. By contrast, mTORC1-deficient mice retained memory characteristics but did not differentiate into effector cells. Finally, inhibition of mTORC2 also enhanced T_{CM} cell generation[525]. Kim *et al* reported that while CD4+ help enhances CD8+ cell effectiveness and memory expansion, the CD8+ memory programme is a cell-intrinsic feature that can occur in the absence of CD4+ cells[526].

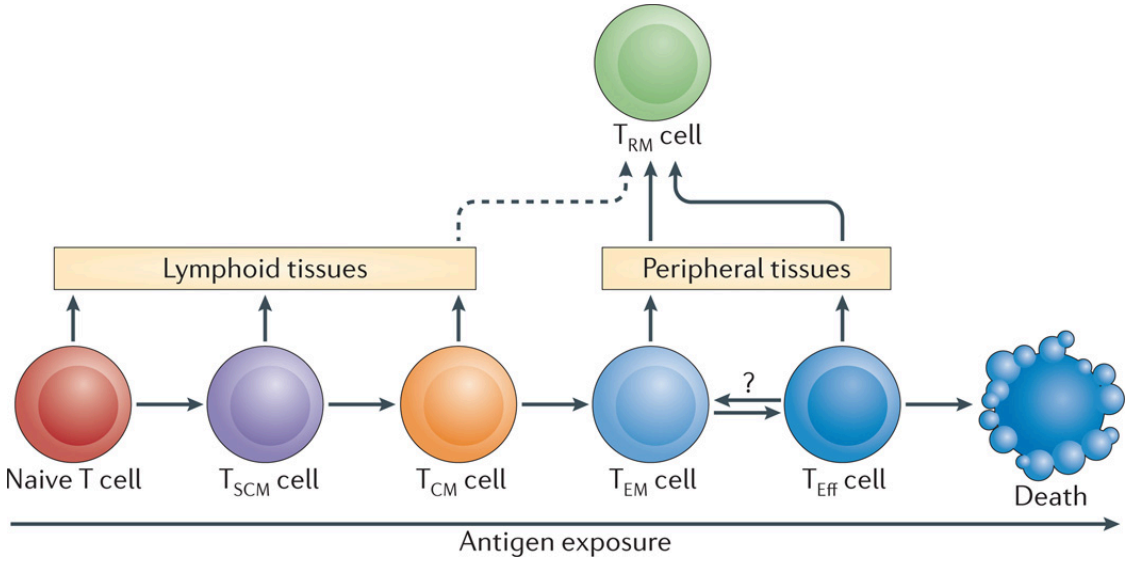

Figure 7 **Model for the generation of human memory T cell subsets**. A schematic model for differentiation of circulating and tissue-resident memory T cell subsets. Progressive differentiation of the three major circulating subsets — stem cell memory T cells (TSCM cells), central memory T cells (TCM cells) and effector memory T cells (TEM cells) — from activated naïve T cells is shown relative to the extent of antigen exposure. Effector T cells (TEff cells) represent terminally differentiated cells, and death is one outcome of increased antigen exposure and proliferation. Naïve, TSCM and TCM cells circulate and and migrate to lymphoid tissue, whereas TEM and TEff cells are the subsets with the capacity to traffic to peripheral tissues. Tissueresident memory T cells (TRM cells) in peripheral tissue sites may derive from either TEM or TEff cells that migrate to these sites via tissue-specific influences. It is possible that TCM cells could develop into TRM cells in lymphoid sites (dotted line). TRM cells in peripheral compartments are likely terminally differentiated since they do not circulate or convert to other memory T cell subsets. From: Human memory T cells: generation, compartmentalization and homeostasis. Farber, Yudanin and Restifo. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology 14, 24–35, doi:10.1038/nri3567, Published online 13 December 2013. Copyright 2014. License #: 3810001060895.

The transcription factors Blimp-1 and Bcl-6 were known to be important drivers of B cell differentiation and memory, however their role in driving T cell effector function and memory induction has only recently been discovered[527–529]. Bcl-6 in particular is needed for CD8+ T_{CM} cells[530,531]. The actions of these two transcription factors cancel each other out, therefore the balance of each determines effector or memory fate in these cells.

The surface molecule phenotype of memory cells differs from that of naïve cells and their functional responses also differ. Murine memory cells express high levels of CD44[496,532] and low low levels of activation markers such as the IL-2 receptor (CD25)[533] and CD69, whereas human memory cells can be identified on the basis of their CD45RA-CD45RO+ phenotype[534].

The $CD127^{HIGH}$ population has been shown in adoptive cell transfer experiments to represent long living T central memory (T_{CM}) cells, especially when combined with $CD62L^{HIGH}$ status[491]. CD62L is also expressed on T_{CM} that reside in secondary lymph tissue awaiting future antigen encounters[495,497]. T_{EM} circulate in the periphery and have immediate effector functions upon encountering antigen, thus they do not express CD62L. CD44 is an indicative marker for T_{EM} as it is expressed at a lower level on naïve T cells compared to memory T cells[496,532]. The combination of CD44 and CD62L is one of the bi-variate analyses used in the identification of CD8+ T_{CM} cells for adoptive cell therapy in cancer[496,521,535].

Naïve and T_{CM} cells express CCR7 and continually recirculate into lymph tissue where they can engage with inbound CCR7+ APC. Activated effector T cells, and T_{EM} cells, are identified by their loss of CCR7 expression, which excludes them from lymph as part of their migration toward peripheral tissue.

Finally, the latest member to join the T cell memory subsets in both humans and mice are CD95+CD122+ T memory stem cells (T_{SCM}). In humans these cells have certain features of naïve T cells, being CD45RA+CD45RO-$CD27^{HI}CD28^{HI}IL7R\alpha^{HI}CD62L^{HI}$ and $CDR7^{HI}$. They are highly proliferative, self-renewing and retain the capacity to differentiate into T_{CM} and T_{EM}, hence their 'stem cell-like' qualities (reviewed in[514]).
Correlations between antigen-experienced T cell phenotype and anti-tumour efficacy have been identified with T_H1 and T_{CM} having positive prognostic indications. Thus in this study, it was important to determine the phenotype of T cell that resulted from presentation of undefined antigen by each type of APC, in terms of surface molecule expression, cytokine production and cytotoxic capability.

1.9.12 NK T cells

Another major T cell subset is that of the natural killer (NK) T cells. Four subsets of Vα14-Jα18 invariant NK T (iNKT) cells have been described[536]. Types I and II are CD1d-restricted, recognizing the glycolipid alpha galactosylceramide (Type I) and an as yet undetermined ligand (Type II). Types III and IV are MHC Class I, and or Class II-restricted and may recognise self-ligands and other also undetermined ligands. Type I iNKT cells are the best characterised. They have the distinctive ability of being able to respond as an innate-like cell with minimal TCR engagement, or as a memory-like cell via their semi-invariant TCR interacting with glycolipids presented on CD1d[537]. During viral infection they can be indirectly activated in the presence of large quantities of pro-inflammatory cytokines, perform functionally in a manner similar to innate natural killer (NK) cells and secrete predominantly T_H1 cytokines[538]. They can also be activated by pathogen-presenting DCs[538,539]. NKT cells play a part in tumour surveillance, though their anti-cancer activities are less well-characterised than those of the CD4+ and CD8+ T cells[537]. Some groups are assessing the potential of NK T cells in immunotherapy for cancer, however the response of NK T cells to undefined TAAs has not been examined in this thesis.

1.10 Immune Surveillance of Altered Cells

Immune cells patrol tissues, continually surveying the environment, recognizing transformed or stressed cells and eliminating them long before they reach clinical detection ([540], reviewed in[541–543]). The immune system's response to cancer maintains a fine line between immune-mediated elimination of transformed cells and suppression of autoimmune reactions. The majority of T cells are educated to ignore self during development. The goal of central tolerance mechanisms is to generate a T cell repertoire that is non-reactive to self antigens and reactive against 'non-self'. While extremely effective, central tolerance is not fail-proof. Self-reactive T cells do enter the periphery where they can cause autoimmune disease under certain circumstances[319,544]. However, peripheral tolerance mechanisms act as a back-stop to control inappropriate immune reactivity in tissues.

The concept of cancer immune surveillance has been broken into three phases: elimination, equilibrium and escape[545] Figure 8. During the elimination phase the

immune system successfully identifies and eliminates altered cells and tumour progression is halted. The unstable nature of transformed cells means that selective pressure from the immune response drives neoantigen development via DNA mutations or alterations of gene expression. Equilibrium is an active phase during which cancerous factors, which avoided elimination, continue to attempt to subvert immune responses. Vulnerable tumour clones are eliminated where possible and the tumour cells continue to evolve. Finally, escape occurs if immune mechanisms are unable to control the malignant changes. Escape variants emerge that have the ability to repel, evade or repress the anti-tumour mechanisms and tumour outgrowth is the result.

1.11 Immune evasion by Tumour Cells

Despite elimination of pre-clinical tumours and immunoediting[542,546,547] tumours that escape continue to display antigens that the immune system can recognise. Thus, while some tumours downregulate antigens targeted by the immune system in an effort to avoid detection, others actively oppose host immune mechanisms, such as the induction of anergy in tumour-specific T cells[548].

Tumours growing in immunologically privileged locations such as the brain or eyes may not be subject to immune surveillance and in some cases tumour stroma has acted similarly, providing a barrier against immune infiltration[68,549,550]. Immune infiltration has also been hindered in cancerous tissue by the down-regulation of adhesion molecules[551,552], furthering immunological ignorance. In an additional example of immunological ignorance tumour cells that have not migrated to the lymph nodes fail to activate lymph node T cells and are ignored[68,553].

The generation of immune-suppressive features by either tumour cells, tumour stroma, or immune or epithelial cells in the tumour microenvironment is another active strategy. High levels of transforming growth factor β (TGF-β) is a key feature in many malignancies. This molecule elicits anti-proliferative effects, as well as inhibiting activation and differentiation of innate and adaptive immune cells[419,554–558], and promoting differentiation of CD4+CD25- cells into CD4+CD25+ TREGs[483]. The angiogenic vascular endothelial growth factor (VEGF) inhibits monocyte

differentiation into DCs[559], and expression of prostaglandins[560,561] by tumour cells has been reported to correlate with reduced tumour control in mice and humans. The pro-tumoural role of IL-10[562] in immune evasion has also been identified via its effects in suppressing macrophages. In concert with IL-10, TGF-β has also been reported to divert the immune response away from anti-tumour T_H1 actions and toward less effective T_H2 humoral responses[563].

Malignant cells may also avoid immune detection due to tolerance mechanisms designed to protect normal tissues from immune-mediated destruction. The lack of costimulatory molecules on MHC-I and MHC-II positive tumours may result in anergic tumour-specific CD4+ and CD8+ T cells[548,564,565], although costimulatory requirements are less stringent for antigen presentation to antigen-experienced T cells in the periphery.

Tumours may also upregulate molecules that allow them to resist immune-mediated killing and they can also co-opt "counter attack" strategies by killing tumour specific CTLs[555]. CD95/CD95L-mediated deletion of TAA-activated T cells via activation induced cell death (AICD) is a result of repeated TCR stimulation with antigen[566], such as may be encountered in the tumour setting[567,568]. FasL-expressing CTLs ligating Fas on target cells, trigger an apoptotic, caspase-mediated death signal cascade[505,506]. In the face of programmed cell death-mediated killing by immune lymphocytes tumours acquire anti-apoptotic capacities such as the overexpression of antiaptoptic proteins $FLIP_{L,S}$, Bcl-2 and survivin, or of serine protease inhibitors that block Granzyme B activity (reviewed in[555]). Conversely tumours can also downregulate proapoptotic molecules and produce soluble decoy receptors for death ligands on CTLs[555]. Many reports demonstrate the existence of CD95-resistant malignancies that express CD95L[569–574] and apoptose TILs in human tumours[570,575]. However other studies have shown that CD95L-expressing tumours are targeted for neutrophil-mediated destruction[576–579]. Thus the effectiveness of this strategy in immune evasion is currently inconclusive.

The perforin-granzyme-mediated killing pathway, described under CD8+ T cell differentiation, is another major mechanism for CD4+ and CD8+ CTLs and may also

be relevant in tumour immune evasion[580–583]. Perforin-deficient mice have demonstrated vulnerability to some tumours induced by direct injection, carcinogen-induction or oncolytic virus generation[584], as well as the spontaneous development of lymphomas[585]. Interestingly, however, one study has shown that Granzymes A and B may not be critical in this process[586]. Evidence for tumours resisting perforin-mediated killing has been observed in myeloid leukemia, though the mechanisms were not studied[587]. In addition, Granzyme B inhibition by serine protease inhibitors has been documented in prostate cancer[588], while impaired perforin binding has been observed on leukaemia cells[589].

1.11.1 T_{REG}s and T Cell Exhaustion

While the concept of T cell exhaustion was originally identified in chronic murine infections it has also been described in cancer patients. Exhausted T cells lose critical functions such as the ability to make IL-2, IFN-γ and TNF-α, which affects their cytotoxic capacity. They show reduced proliferation and deficient memory T cell properties, being unable to renew themselves in the absence of antigen and failing to carry out a strong secondary response if the antigen is re-encountered[590]. Expression of inhibitory receptors such as PD-1, CTLA-4, TIM-3, LAG-3 and others identifies activated effector cells. However their continued expression is an identifying characteristic of exhausted T cells, which have failed to down-regulate these negative immune regulators as memory cells do upon resolution of a threat[591,592].

PD-1 is an important immune-regulatory inhibitory receptor whose expression is low on naïve T cells and upregulated on activated T cells, B cells and myeloid cells[593,594]. Engagement of PD-1 by its ligand programmed death ligand 1 (PD-L1), expressed on APCs, prevents T cell activation to promote tolerance and inhibit autoimmunity. PD-1 promotes programmed cell death (PCD) in antigen-specific T cells in the lymph nodes[595] while simultaneously reducing PCD in T_{REG}s[596]. High levels of PD-1 is a major clinical indicator of exhausted T cells with impaired anti-tumour function[597]. Upregulation of other immunoregulatory molecules such as CTLA-4 also contribute to tumour-specific T cell exhaustion, with co-expression of PD-1 and CTLA-4 corresponding to increased T cell exhaustion. Thus monoclonal antibodies (mABs) against PD-1, and or, CTLA-4, are now used to block the inhibition of tumour-specific T cells in some cancers[597,598].

TILs that co-express PD-1 and T-cell Ig- and mucin-domain–containing molecule–3 (TIM-3) display the most severe exhaustion phenotype and combined targeting of both molecules has been more effective than blocking either one individually in pre-clincical models[599]. More than 300 clinical trials are investigating immune regulators, the majority targeting CTLA-4 and PD-1 and, or, PD-L1, but mABs against killer immunoglobulin-like receptors (KIR), TIM-3, LAG-3 and BTLA are in progress[591,600,601].

1.11.2 Subversion of DCs in Cancer

Understanding how DCs are modified by the tumour-host dynamics is critical for the design of cancer therapies in general and DC-mediated ACT approaches in particular. The earliest reports of DC abnormalities in cancer patients came in the mid 1980s with the observation of dramatically reduced numbers and morphological changes in malignancy-associated Langerhans cells compared to those in benign lesions[602–604]. Experimental evidence eventually accumulated that confirmed direct suppressive effects of tumours on DCs, including IL-10-mediated suppression, defects in expression of various molecules[605], and inhibition of actin polymerization, which limits DC motility and adhesion[606]. The Gabriliovich group reported reduced antigen presentation and T cell proliferation, as well as VEG-F-mediated inhibition of DC maturation[559,607]. This was soon followed with evidence of reduced CD80 and CD86 expression in tumour-associated DCs[608], as well as a report of melanoma-derived factors switching DCs from anti-tumourigenic to tolerogenic[609].

These early reports have been followed up with numerous corroborating studies in which tumours have been demonstrated to inhibit maturation of DCs, which then block antigen-specific T cell responses[610]. Another DC evasion strategy is the impairment of antigen presentation by the downregulation of immunogenic TAAs[611–613] or the evolution of neoantigens[614]. These changes are observed more in metastases than in primary tumours.

Recent work in a mouse model of ovarian cancer showed that DCs responding to tumour-produced molecules lost the ability to stimulate T cell activation[615]. Thus it is possible that ‘exhausted’ T cells in the tumour microenvironment may not necessarily

be exhausted. It may be instead a failure on the part of the DCs, which are responding to the continual presence of transformed cells, to properly activate the T cells.

1.11.3 The Controversial Role of Macrophages in Cancer: Tumour Associated Macrophages (TAMs)

MΦs represent the majority of immune cells in the human body and are highly represented in the tumour microenvironment, sometimes contributing 50% or more of the tumour's mass[616–619]. These MΦs are known as tumour-associated MΦs (TAMs) and are derived from recruited, circulating blood monocytes[620] and locally proliferating MΦs[134,621].

Some studies show MΦs to have a clear anti-tumour benefit[622–625]. MΦs can exhibit direct cytotoxicity of tumour cells, destroy tumour extra-cellular matrix (ECM) and inhibit angiogenesis. They can also present antigen to CTL, thus harnessing the anti-tumour adaptive response. It has been shown, however, that just like DCs, MΦ phenotypes also transition during tumour formation, from M1 during early stage in response to inflammatory stimuli, to M2 in later stages as tumour cells and tumour stroma alter the microenvironment of the MΦs[626]. Thus the functions of TAMs change during tumour growth and tumour progression and analysis of this feature must be taken into account when assigning pro- or anti-tumoural conclusions[627].

In a humanized model Pallasch *et al* give an example of how therapeutic antibodies induce tumour cell death through MΦ activation. In this study they investigated the difference between treatment-resistant and treatment-sensitive leukaemic B cells, which initially respond to treatment with alemtuzumab (anti-CD52 antibody) before developing resistance[624]. Alemtuzumab-mediated cell death was caused by the recruitment of MΦs that phagocytosed the tumour cells. When investigating how to improve the response to alemtuzumab they found a successful synergistic response to alemtuzumab and low dose cyclophosphamide. Cyclophosphamide, but no other alkylating agent, caused the transitory release of stress-related cytokines such as IL-8, VEGF, TNF-α and CCL4 from the malignant cells. These factors activated MΦs, which were able to engulf the tumours.

These results are interesting in light of Asano and colleagues' findings, discussed in the MΦ section, which showed that CD169+CD11c+ MΦs in the border of the cortical sinus and paracortical sinus were able to present dead cell antigen to T cells that had migrated from the T zone to the sinus border[177]. Pallasch and colleagues noted that tumour cells in the bone marrow, a low MΦ region of the body, were refractory to alemtuzumab treatment, while tumour cells in the blood and spleen were highly responsive. This response was abolished after MΦs were depleted with clodronate liposomes. Cyclophosphamide-induced cell death would not only cause an increase in local activation of MΦs in response to stress cytokines, it would also cause increased flow of dead cell antigens into draining LNs, which would be phagocytosed by CD169+CD11c+ MΦs in the sinus, which could then present dead cell antigens to CD8+ T cells. This could help explain Pallasch's compartment-specific findings. However Pallasch *et al* defined MΦs as CD11b+/GR1lo/CD11c–/F4/80+ whereas Asano found that CD169+CD11c- MΦs were not able to stimulate OT-I proliferation. So issues of MΦ definition remain to be clarified in order to rationalize these conclusions, however both studies clearly highlight an important role for MΦs in anti-tumour immunity and treatment.

On the opposing side of the argument, many studies have found MΦs, particularly M2 MΦs, to be distinctly unhelpful[616,628,629]. Several studies have reported that the number of TAMs is associated with poorer outcomes in breast, ovarian and prostate cancers[134,630]. In a murine model of breast cancer, incorrectly polarized MΦs resulted from an EFG-CSF1 paracrine loop between MΦs and cancer cells, which augmented invasion and metastasis[631–633]. Using the murine hepatocellular carcinoma Hepa1-6 model Wang *et al* showed that a change from MHC-II^{HI} to MHC-II^{LOW} in TAMs was associated with tumour progression[634]. This was in concordance with the work of Baumgart in 1998 which had showed that MHC-II^{HI} MΦs were protective and showed a T_H1 bias, while MHC-II^{LO} MΦs promoted a T_H2 response[635]. In other words the loss of antigen-presentation capacity significantly affected the TAMs' ability to halt cancer growth.

Aggressive tumours secrete factors such as macrophage inhibitory factor (MIF), which inhibits the migration of anti-tumour M1 MΦs toward the tumour[636], and IL-10,

which is secreted by M2-type MΦs has been described as a tumour-derived factor involved in converting M1 MΦs to the M2, pro-tumour phenotype[637]. Tumours can attract circulating monocyte-derived MΦs by producing CCL2 and hijack them into pro-tumoural aides, which assist in tumour angiogenesis, ECM degradation and invasion[616]. Figure 8 overviews the multitudinous roles assigned to TAMs in the tumour microenvironment.

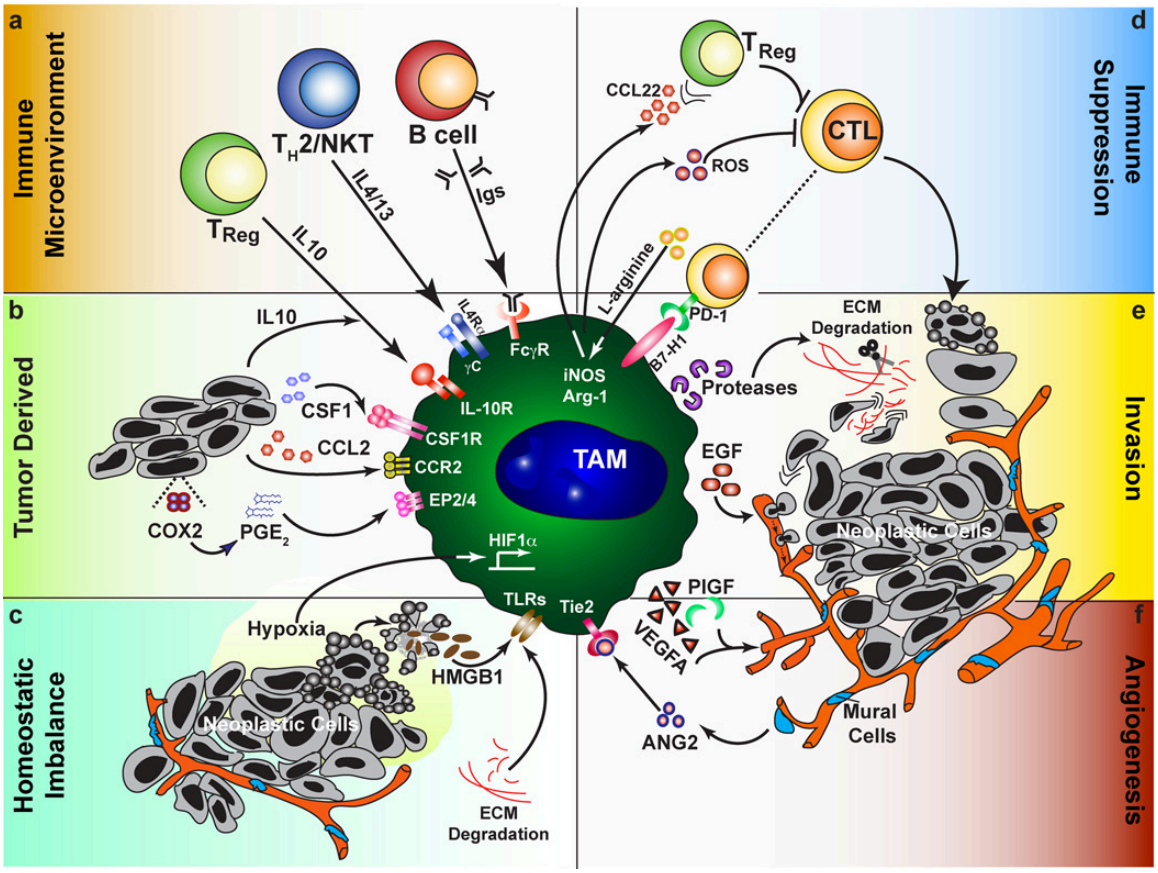

Figure 8 **TAMs as central regulators of the tumour microenvironment**. Factors that promote the polarization of TAMs towards a pro-tumour phenotype (a–c) can be subdivided into those derived from the immune system, actively produced by tumour cells, or resulting from tissue stress. (a) From leukocytes, this includes cytokines and other soluble factors such as immune complexes. (b) Neoplastic cells can produce chemokines that recruit macrophages, including CSF1 and CCL2 depending on the tissue involved, as well as directly producing immunosuppressive molecules such as IL-10 and PGE2. (c) Signs of dysregulated tissues include leaky vasculature, hypoxia, ECM remodeling and cell death. These signals all direct the pro-tumour functions of TAMs (d–f) including immune suppression, tumour cell dissemination, and promoting angiogenesis. (d) Immune suppression can occur through soluble or cell surface mediators, and may be indirect such as through the recruitment of regulatory T cells. (e) Neoplastic cell invasion of ectopic tissue can be promoted through directed release of cytokines such as EGF, or through protease-dependent ECM remodeling that may directly affect neoplastic migration or increase chemoattractant bioavailability. (f) In addition to the interplay of TAMs with endothelial cells through production of VEGFA and other angiogenic factors, subsets of TAMs expressing the Tie2 receptor interact with mural cells/pericytes to regulate vascular structure. Reprinted from Trends in Immunology, Volume 33, Issue 3, Ruffell, Affara & Coussens, **Differential macrophage programming in the tumour microenvironment**, Pages 119–126, Copyright March 2012 with permission from Elsevier. License #: 380622086418 (Ruffell, Affara, & Coussens, 2012).

While much pre-clinical and clinical data corroborate the hypothesis of a tumour-promoting role for MΦs, exceptions do exist. Examples include the loss of tumour control when Kupffer cells were deleted in rat livers[638]. In a model of colon cancer deletion of peritoneal MΦs or Kupffer cells led to highly differentiated tumours and a reduced survival. Interestingly, while the tumours in mice that had a full complement of MΦs displayed hallmarks of malignancy, their tumour loads were lower and survival longer than MΦ-depleted mice[639]. Thus the clear anti- and pro-tumoural roles of MΦs were highlighted in these experiments and point to the need to modify MΦ activities, rather than eliminate them.

A Review by Qian and Pollard stated that "*Analysis of the transcriptome of TAMs derived from studies in mouse models of breast cancer has also provided evidence that an enrichment in macrophage transcripts is predictive of poor prognosis and reduced survival in human breast cancer*"[640]. However, the 2009 study by DeNardo *et al*, showed that care must be taken when drawing direct predictions from mouse models for application to human cancers. That study showed strong differences in the cell populations in human versus mouse breast cancer. In humans CD4+ T cells are the predominant infiltrate, producing IFN-γ at levels 10-fold higher than IL-4 or IL-13. In mice however, the predominant cells in MMTV-PyMT tumours are TAMS and the CD4+ T cells from these tumours show high IL-4, IL-13 and IL-10 with minimal IFN-γ[641]. These cytokines have a very strong influence on MΦ polarization and as such make it difficult to draw correlations between the human and mouse examples. It also highlights the importance of analysing the cytokine setting in which the TAMs are found before considering applications to other tumour types. Pollard, however, objects to DeNardo's assessment of the leukocytic infiltrate in established human tumours as "inflammatory", stating that "*there are few of the hallmarks of inflammation, such as edema, swelling, and fever*"[640]. Clearly there is still much room for discussion on these issues.

Ruffell, Affara and Coussens sum it up nicely when they describe MΦs as having an 'identity crisis'[627]. They point out the difficulty of identifying TAMs due to their lack of lineage-defining markers and their varied marker expression that depends on activation status and the tissue in which the MΦ is found. They additionally highlight

the fact that murine MΦs are typically identified by the expression of F4/80, a molecule not expressed by all MΦ populations and also present on skin-resident Langerhans cells and adipocytic eosinophils. In addition, CD11c, the molecule most commonly used to identify DCs, is constitutively expressed by subsets of tissue-resident MΦs and is induced on other MΦ populations under the kind of inflammatory conditions found in tumour microenvironments. As such F4/80 cannot be said to be the sole identifier of murine MΦs, nor can CD11c alone be used to positively identify DCs.

Positive TAM identification in humans is further clouded since most immunohistochemistry studies rely on the single marker CD68, which is also expressed by fibroblasts in the tumour tissue[627]. This makes accurately assigning functional roles to TAMS very difficult. It may be that roles assigned to TAMS are in fact carried out by other stromal, myeloid or immune cells. Among other suspects, fibrocytes, mesenchymal cells that develop from monocyte precursors, have a dual MΦ/fibroblast phenotype and may be more involved in tumour promotion than is currently appreciated. Further careful dissection of the type of cell carrying out angiogenic, metastatic and immunosuppressive activities is still required for clarification of the situation. The point is not that TAMS do not carry out these activities, but that other cells do also and it is important to accurately identify the causative agent.

An attempt by Galon and colleagues to improve colorectal cancer staging by using large cohort meta-analysis revealed that of the three main APCs, MΦs were at best neutral in terms of prognostic outcome: "*only neutrophils and mast cells … showed a moderate positive impact on patient survival, whereas macrophages and iDCs were slightly (almost but not quite significantly) associated with a bad outcome*"[208]. However it must be noted that this study does not appear to differentiate between M1 and M2 MΦs and a paper published just 11 months earlier by Galon and colleagues specifically identified M1 MΦs as a positive prognostic indicator in colorectal cancer[640].

Continuing to look from the point of view of prognostic indicators, clinical trial results showed that penetration of tumours by CD8+ CTLs[642,643], T_H1 and T_H17 CD4+

T cells, NK cells, DCs and M1 MΦs is an independent predictor of favorable patient outcome. The opposite prognosis is frequently associated with intratumoural infiltration of CD4+CD25+FOXP3+ regulatory T cells, T_H2 CD4+ T cells, myeloid-derived suppressor cells, M2 MΦs and neutrophils. Thus the verdict on MΦs, while currently leaning toward the negative, may still be said to be out.

1.11.4 B Cells in the Tumour Setting

As with DCs and MΦs both pro-tumoural and anti-tumoural B cell correlations have been observed, as well as pertubations in B cell populations in cancer patients[644]. B cell infiltration of squamous cell carcinomas (SCC) of the vulva and head and neck were associated with a worse prognosis and mAB-mediated depletion of B cells improved the response to platinum- and Taxol-based chemotherapies[622]. A review by Gunderson highlights the pro-tumoural activities of B cells and discusses potential ameliorative approaches for cancer therapy[645].

While acknowledging the pro-tumoural role of B cells in certain cancer types, in pre-clinical models of melanoma and breast cancer B cell elimination resulted in the loss of the anti-tumour response *in vivo*[209,446]. In a breast cancer model adoptive transfer of tumour-reactive B cells has been shown to inhibit lung metastases and ignite T cell-mediated tumour regression[213]. In the clinical setting studies have also demonstrated increased survival correlating with B cell infiltration in high grade serous ovarian cancer[646,647]. Further, meta-analysis of immune cell infiltrates has demonstrated a positive correlation between T_H1 cells and B cells in human colorectal tumours[648]. Individual studies have also reported improved clinical outcomes in patients with increased intratumoural or peritumoural CD20+ B cells (either alone, or in combination with other immune cells), including HCC[649], melanoma[650], bladder carcinoma[651,652], prostate carcinoma[653], serous epithelial ovarian carcinoma[646,654], NSCLC[655], HNC[656] and multiple myeloma[657]. Thus, as with DCs and MΦs careful meta analysis of the data is required to refine our knowledge on which APC fosters a favourable outcome in which cancer type.

Using different tumour cell lines modified to express model antigens (secreted OVA and PSA, intracellular GFP and membrane-boundHER-2/neu) Brown *et al* showed in

that absolute numbers of CD4+ and CD8+ T cells increased by about 2-fold in tumour-draining lymph nodes during the anti-tumour response, while B cell numbers increased seven to eight-fold[658]. Assays also showed high levels of anti-tumour antigen antibodies in the sera of mice growing OVA+, PSA+, GFP+ or HER-2/neu tumours. Numbers of tumour antigen-specific B cells also increased. This data highlights the importance of B cells in the anti-tumour response but it must be noted that this work also demonstrated the role of the tumour line itself in the size of the anti-tumour response, in other words, different tumour types will generate differing immune responses. Small cell lung carcinoma-OVA but not B16OVA or B16PSA tumours generated high antigen-specific antibodies. It must be noted that small cell lung carcinoma-OVA is generated in BALB/c mice, while B16OVA are generated in BL/6 mice and strain-specific immune responses have been noted in other studies, albeit MΦ-mediated[153]. However further dissection of these strain-specific differences is possibly warranted.

Using a B cell knock out model Schultz *et al* demonstrated the essential role of B cells in the *in vivo* T cell response to retrovirally induced tumours[659]. Lack of B cells affected both the CD4+ and CD8+ T cells. The reduced CD8+ T cell response could reflect the lack of B cell help to T_H CD4+ T cells, or it could reflect a previously unappreciated role for B cells in the CTL response. Further data by Schultz implied that B cells might be important for priming both T cell subsets. The B cell role in T cell priming was particularly strong in the lymph nodes and may represent an important focus in anti-tumour therapy given that tumours drain specifically to particular LNs as opposed to the spleen where more general lymph filtering occurs. Studies such as the one by Shultz demonstrate the collaborative, systems-biology nature of APCs in the anti-tumour response and emphasise the fact that no one APC alone is responsible for anti-tumour effects. It is for this reason, as well as the limited responses seen in DC ACT, that it is important to assess whether or not a synergistic improvement can be seen when more than one APC is used. The ideal scenario for some cancer types may be therapeutics that drive both a T_H1 response, and an effective antibody response. If, as this thesis hypothesised, the combination of a DC and a B cell drives a superior T cell response, it is possible that this combination could also drive a useful anti-cancer antibody outcome.

When the immune system response to cancer is undermined, cancer outgrowth at the expense of normal tissue function is the result. CTL-resistant tumours develop escape mechanisms, generating an immunosuppressive tumour microenvironment in which CTLs can be shut down by inhibitory tumour mechanisms[541]. Tolerance toward cancer antigens can develop instead of immunogenicity and CTLs can become anergic. That being so cancer immunotherapy seeks to educate T cells and enhance their inherent ability to fight TAAs.

1.12 Cancer Immunotherapy: Augmenting the Inherent Anti-tumour Response

In the late 1800s the physician William Coley inadvertently initiated cancer immunotherapy following his observation of cancer regression in a patient with a concomitant erysipelas infection[660]. Subsequent attempts to treat cancer patients with bacterial infections met with limited success but provided the first hint at immune involvement in cancer control.

The Age of Cancer Immunotherapy arrived in earnest in 2013 with Science Magazine heralding immunotherapy for cancer as "The Breakthrough Of The Year"[661]. The word cure is, understandably, used with caution among oncologists and the goal of many standard of care approaches these days is to prolong survival and augment the quality of remaining life where possible. However, the targeted inclusion of the immune system in the treatment of cancer has brought about a revolutionary approach to cancer care. The observation that patients with certain cancers such as renal cell carcinoma and melanoma sometimes experienced spontaneous remission demonstrated that the immune system could delay and even eradicate malignancies. In addition an increased rate of certain cancers has been documented among immunocompromised patients[662,663]. These observations led to studies of approaches that seek to augment the body's natural immune response to cancer, such as identification of tumour-associated antigens, tumour-produced immune-suppressive factors as well as modifications to immune cells.

Cancer immunotherapy has shown more objective promise in clinical trials than cancer vaccines[664]. A meta-analysis of the response to glioblastoma multiforme

revealed significantly longer times to recurrence in patients who had received immune-based therapies plus chemotherapy compared to those patients who had received immune-based therapy or chemotherapy alone[665]. However complete regression, partial responses, stable disease and progressive disease have been reported in all cohorts of cancer patients treated with immunotherapy, indicating response variation among patients and highlighting the need for ongoing characterization of all immunological aspects these treatments.

In order for the immune system to destroy an established tumour, three conditions must be met: (1) adequate numbers of T cells with high avidity for tumour antigens (2) migration to and infiltration of the tumour stroma, and (3) activation of immune cells at the tumour location to initiate appropriate effector functions (lysis of tumour cells or the secretion of cytokines which can bring about tumour destruction)[440,664]. The success of immunotherapy ultimately depends on the targeted cytotolytic activity of innate natural killer (NK) cells and adaptive cytotoxic T lymphocyte (CTLs) via their production of IFN-γ and Fas/FasL-mediated killing. However there are multiple steps in the development of an anti-tumour response where the immune system can fail, as discussed previously in the section on immune evasion. Thus, there are multiple opportunities along this developmental path for immunotherapy to transform a faulty or faltering immune response.

Figure 10 lists the immunotherapies that are currently available in the USA. Immunotherapy can be broadly divided into passive and active approaches[666]. Passive immunotherapies include tumour-targeting monoclonal antibodies (mABs) such as Keytruda (Pembrolizumab) that binds to PD-1 on the surface of tumours and T cells. Ipilimumab/Yervoy, the FDA-pproved anti-CTLA-4 mAB for advanced melanoma is another example. The use of oncolytic viruses to selectively lyse cancer cells is another passive immunotherapy approach, as is Adoptive Cell Therapy (ACT), which involves adoptively transferring activated lymphocytes back into the patient.

Active immunotherapies include the use of immunostimulatory cytokines such as interleukin-2 (IL-2) or interferons. Inhibition of indoleamine 2,3-dioxigenase 1 (IDO1) is one example of interference with tumour-secreted immunosuppressive

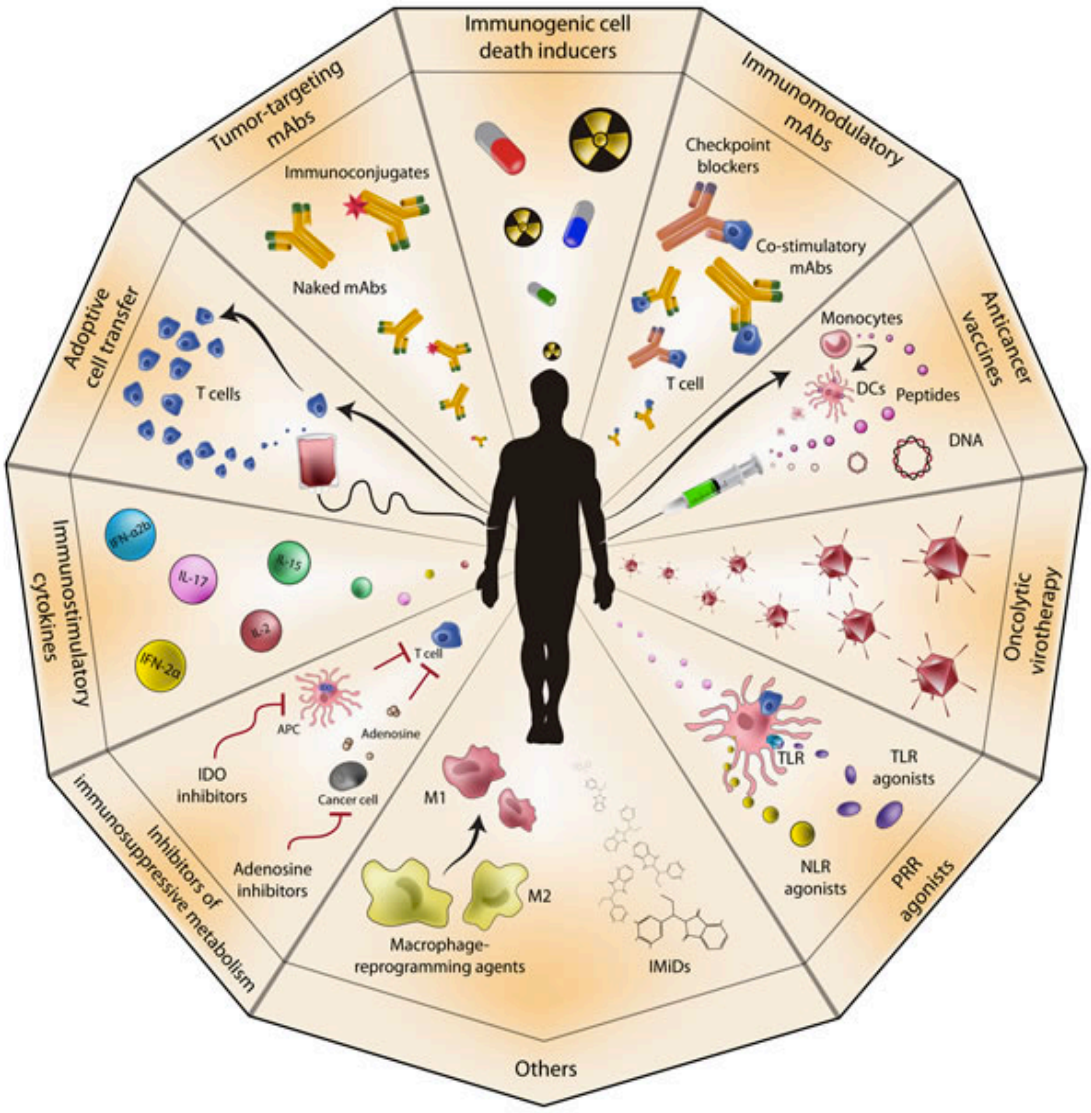

Figure 9 **Anticancer immunotherapy**. Several anticancer immunotherapeutics have been developed during the last three decades, including tumour-targeting and immunomodulatory monoclonal antibodies (mAbs); dendritic cell (DC)-, peptide- and DNA-based anticancer vaccines; oncolytic viruses; pattern recognition receptor (PRR) agonists; immunostimulatory cytokines; immunogenic cell death inducers; inhibitors of immunosuppressive metabolism; and adoptive cell transfer. 1MT, 1-methyltryptophan; APC, antigen-presenting cell; IDO, indoleamine 2,3-dioxigenase; IFN, interferon; IL, interleukin; IMiD, immunomodulatory drug; NLR, NOD-like receptor; TLR, Toll-like receptor. From Classification of Current Anticancer Immunotherapies by Galluzzi et al105 Reprinted under the terms of the Creative Commons Attribution 3.0 License. Reprinted with kind permission of the author Jan 13 2016.

metabolites. There is also the use of pattern recognition receptor (PRR) agonists for their role in augmenting antigen-presenting cell (APC) activation. One example is the use of Imiquimod in basal cell carcinoma to activate toll-like receptor (TLR) 7 on antigen-presenting cells (APC). Finally, agents that provoke immunogenic cell death (ICD), have also been used in active immunotherapy. Doxorubicin, one of the anthracyclines used to treat carcinomas, is just one example of an FDA-approved inducer of ICD. With regards to melanoma specifically, several of these approaches have been used in clinical trials including DC-loaded with melanoma cell lysates[667], the cytokines IL-2[668] and IFN-α[669], adoptive transfer of tumour-infiltrating

lymphocytes (TILs) accompanied by IL-2[670],and the mAB anti-CTLA-4[671], though successful treatment with α-CTLA-4 may be accompanied by immune-mediated vitiligo[672].

During homeostatic environmental sampling healthy tissues avoid phagocytic destruction by their surface expression of CD47, which sends a "Don't Eat Me" signal by binding to SIRPα on the phagocyte surface[673]. Calreticulin, a pro-phagocytic damage-associated molecular pattern (DAMP), can block CD47, permitting removal of cellular remains[674]. The "Don't Eat Me" signal path has been exploited by tumour cells to avoid immune-mediated destruction and attempts have been made to "make MΦs eat cancer" by the use of soluble anti-SIRP-α molecules[52,625]. Thus an understanding of how APCs specifically and non-specifically internalize undefined antigen can lead to improved tactics in the design of tumour immunotherapies.

1.12.1 Adoptive Cell Therapy for Cancer

Adoptive cell therapy (ACT) is a highly individual approach to cancer treatment, which involves removing patient T cells, and or, DCs and activating them *ex vivo* for the treatment of cancer. The patient's immune cells are activated against the patient's tumour outside the body, in some cases adapted to improve their specificity and response to cancer, expanded to large numbers and reinfused back into the patient Figure 11. Prior to reinfusion the patient normally undergoes lymphodepletion, the temporary removal of immune cells by chemotherapy or total body irradiation (TBI)[36,675]. Lymphodepletion eliminates immune-dampening T_{REG}s along with other lymphocytes that would compete for lympho-specific cytokines necessary for survival of the transferred cells[1,36,676,677]. Lymphodepletion also provides better engraftment of transferred T cells, creating an environment that stimulates their proliferation, as well increasing the persistence of infused DCs. ACT with tumour-infiltrating lymphocytes (TILs) has the longest history but this type of 'passive' immunotherapy may not produce long-lived memory T cells, which are necessary to control tumour recurrence.

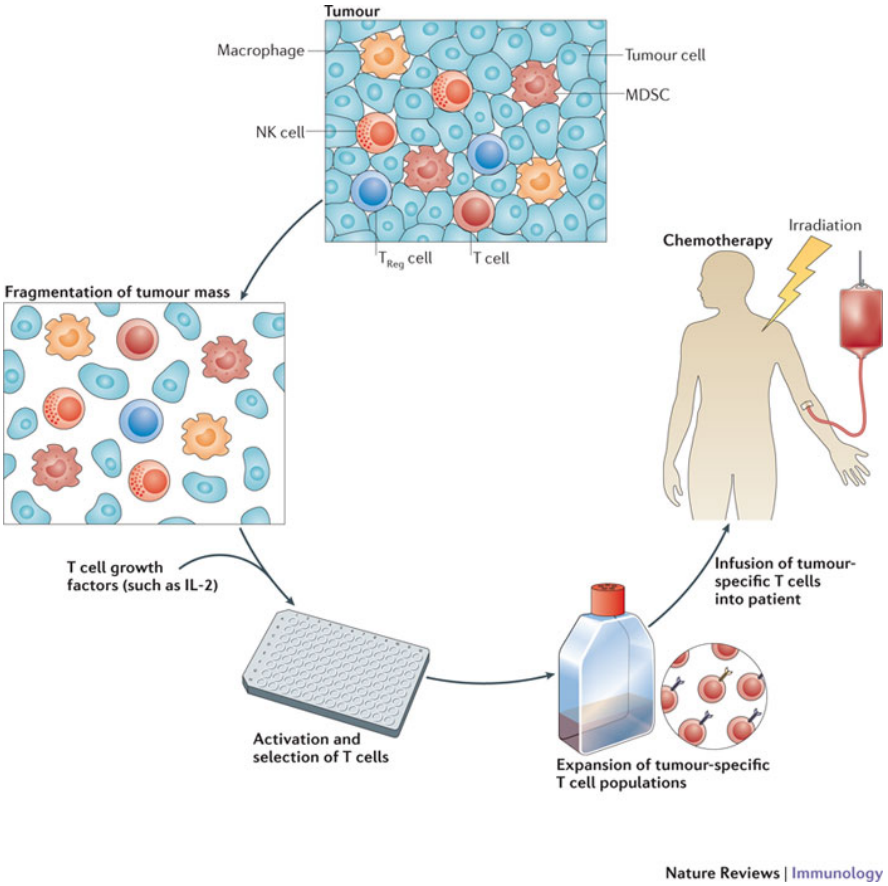

Figure 10 **Tumours are often complex masses containing diverse cell types**. These masses can be surgically resected and fragmented, and the cells can be placed in wells into which a T cell growth factor, such as interleukin-2 (IL-2), is added. T cell populations that have the desired T cell receptor (TCR) specificity can be selected and expanded, and then adoptively transferred into patients with cancer. Prior to this adoptive transfer, hosts can be immunodepleted by either chemotherapy alone or chemotherapy in combination with total-body irradiation. The combination of a lymphodepleting preparative regimen, adoptive cell transfer and a T cell growth factor (such as IL-2) can lead to prolonged tumour eradication in patients with metastatic melanoma. MDSC, myeloid-derived suppressor cell; NK, natural killer; TReg, regulatory T cell[113].

The transfer of tumour-reactive T cells into patients has been applied in different ways: adoptively transferring TILs and high-dose interleukin-2; transducing patient T cells with highly reactive T cell receptors against the patient's main tumour associated antigens (only available if the TAA(s) are known); or T cells genetically engineered to express chimeric antigen receptors (CARs) which essentially fuse a specific monoclonal antibody (immunoglobulin light chain variable fragment) onto the T cell CD3 transmembrane and endodomains yielding a hybrid TCR[678]. CAR T cells comprise an extracellular single chain variable fragment (scFv), hinge and transmembrane regions and intracellular domains that may contain 1, 2 or 3 signaling domains (reviewed in [679]. The scFv is specific for a TAA, while the signaling domains consist of the essential CD3ζ chain and one or two costimulatory domains. Thus, in

the event of cognate antigen encounter *in vivo* the CAR T cell possesses all the necessary requirements for optimal activation.

The most advanced CAR T cell clinical applications target CD19 on B cell malignancies and this approach has shown unprecedented response rates, of up to 94% in acute lymphoblastic leukaemia patients, and striking response rates in Non-Hodgkin lymphoma and chronic lymphocytic leukemia[680–682]. Combinations of the most potent CD4+ and CD8+ effector and memory subsets showed superior efficacy when compared to patients who received random populations of CAR T cells[683]. However, despite these revolutionary successes utility of CAR T cells is limited to cancers in which TAAs have been defined, and to TAAs which are, if not unique to tumours, at least do not provoke unacceptable autoimmunity risks in non-transformed tissues.

Other issues that are yet to be resolved in the use of CAR T cells include improved homing to tumours and in some cases counter-strategies against immunosuppression in solid tumours, such as the induction of anergy in the CAR T cells (reviewed in[679]) and IDO-mediated anti-CD19-CAR T cell inhibition in CLL – although whether this may be circumvented with adjunct use of IDO-inhibitors is the subject of current investigation[684].

T cell therapies using defined tumour antigens has seen encouraging results in melanoma[685,686] and other cancers[681], although T cells specific for TAAs shared with non-transformed tissue have sometimes been shown to induce autoimmune side effects in T cell-mediated ACT[681,682,687–690]. And it must be noted that immunotherapy for melanoma with checkpoint inhibitors is often associated with immune-related adverse events[691]. Nevertheless recent meta-analysis of 5,737 patients showed that the overall cumulative incidence of vitiligo in Stage III-IV melanoma patients receiving immunotherapy was just 3.4%, and that patient group had 2-4 times less risk of disease progression or death[692]. Thus it appears that the risk of autoimmunity is low and may be protective, in the case of melanoma at least. Other studies have shown that autoimmune-mediated pathology does not always result from TIL infusion despite the presence of TCRs specific for identical non-mutated self antigens. These

data suggest exquisite regulation processes that allow the TILs to select between transformed and healthy cells[675,686,693]. One study in mice suggests that genetic predisposition may explain the appearance of autoimmunity in some TAA-loaded DC immunotherapy recipients and not others[694]. However GVHD comparisons between mice and humans receiving genetically altered tumour-specific T cells did not correlate[695] therefore examination of patient genetics is required before genetic susceptibility to autoimmunity in certain human individuals can be confirmed.

Obtaining cells from the patient eliminates issues arising from donor-host mis-matches as in the case of Graft Versus Host Disease (GVHD). Although work with allogeneic ACT has revealed that there may not be an absolute requirement for autologous lymphocytes[696,697]. Allogeneic lymphocytes can be used with minimal GVHD-mediated attack of donated lymphocytes after the anti-tumour response has been initiated[696,698] This is helpful in cases of immune-depleted or immune-exhausted individuals from whom the extraction of sufficiently high numbers of functional immune cells may not be possible.

Another approach to ACT involves activating DC *ex vivo* and adoptively transferring them into the patient to induce or reanimate T cell responses *in vivo*[666,699]. The most common method of DC generation in the clinic is the expansion of precursor cells with GM-CSF and IL-4, due to the relative ease of generation of large cell numbers[700]. However some have questioned whether these DCs are the optimal DCs for use in DC-based ACT and are investigating the possible superiority of peripheral blood monocyte-derived DCs. Many varieties of DC-based approaches are in clinical trials or in clinical use, but broadly speaking they all involve activating patient DCs *ex vivo* against TAAs. TAAs in the form of peptides, lysates, mRNAs or vectors expressing TAAs have all been used.

DCs exposed to biopsy-derived tumour cells, tissues or defined TAAs, are activated by the addition of maturation stimuli including pathogen by-products such as monophosphoryl lipid A (MPL-A), the less toxic, clinical version of lipopolysaccharide (LPS)(reviewed in[701]), immunogenic proteins such as Keyhole Limpet Hemocyanin (KLH), inflammatory cytokines[702,703] or activated cells

expressing the T cell co-stimulatory molecule CD40L. Once activated these TAA-loaded DCs have the capacity to generate TAA-specific effector CTLs *in vivo*, which can cause cancer regression and the generation of memory T cells thus preventing tumour recurrence.

A variation on the DC ACT theme involves co-culturing the tumour-activated DCs with the patients' own CD8+ T cells. The T cells that respond to TAAs are selected for further stimulation and these tumour-specific CTLs are returned to the patient. These CTLs are specific for TAAs that may be rare, or alternatively highly expressed on cancer cells, and are often not unique to cancer tissue.

The combination of ACT and lymphodepletion with the T cell stimulatory cytokine IL-2 has brought about persistent tumour removal in several cancer types, including metastatic melanoma[686,704,705]. However, the use of IL-2 has also been shown to result in T cell exhaustion and poor memory T cell induction in patients (reviewed in[706]) and despite the patient's T cell population being highly enriched for TAA-specific T cells tumours often persist, or recur (reviewed in[26]).

DC ACT has been gradually established over the past twenty-something years with much *in vitro* work showing activation of T cells[707] and several clinical studies showing that DC-based interventions can increase overall survival for some patients[7,697,704,708–714]. A highlight of this work came in 2010 with FDA approval of Provenge® (sipuleucel-T), the first DC-based cancer vaccine for prostate cancer[715]. While DC are undoubtedly the single most effective APC, they do not work alone *in vivo* and the field would benefit from an assessment of the how other APC enhance the DC-T cell activation process.

1.12.2 DCs in Cancer Immunotherapy

It has been demonstrated, both *in vitro* and in ACT in mice, that DCs pulsed with tumour lysates, synthetic tumour-derived MHC-I-restricted peptides or tumour-cell-derived RNA all generate CTL-mediated anti-tumour responses[713,716–721]. Similarly clinical trials of DCs treated with peptides, tumour lysates or viral vectors that encode specific antigens have demonstrated objective anti-tumour responses[714,722]. *Ex vivo* DC pulsed with autologous tumour antigens have been reported to induce tumour-specific

responses in cancer patients[723,724] and numerous clinical trials have been undertaken to investigate the ability of DC exposed to whole tumour lysate to provoke curative T cell responses against cancer[397,697,709,712,713,725–730]. However, while an immunological response, as measured by anti-tumour T cell activation, can be detected in DC vaccination strategy evaluations, a divergence remains between the immune response and ultimate clinical outcomes[664].

Recent work by Engelhardt *et al* indicates that CD11c+ DCs present in the tumour margin, can be seen to couple with CD45.1+ cells of haematopoietic origin in the tumour micro-zone and are the cells that best present tumour antigen to these cells[731]. This study points toward DCs being the primary APC in the tumour microenvironment, however much remains to be fully clarified about the cells observed. Fairly 'broadly applicable' cell surface markers were used for cell identification and it remains to be confirmed whether or not the prime cells were M1-type tumour associated macrophages or DCs.

In two tumour models, TS/A mammary carcinoma and MC-105 sarcoma, BALB/c or C57BL/6 mice immunized with tumour lysate-pulsed DCs developed significantly fewer pulmonary metastases than mice immunized with irradiated tumour cells[732]. Compared to unimmunized mice, subcutaneous immunization with lysate-loaded DC, DC pulsed with apoptotic tumour cells or DC co-cultured with live tumour cells all yielded significantly fewer lung metastases. These data indicated that DCs loaded with undefined tumour antigens are able to generate effective anti-tumour T cell responses. Two further studies have also demonstrated the effectiveness of DCs loaded with undefined TAAs. In one, co-administration of the TLR-9 agonist CpG with tumour lysate in a murine model of glioma brought about increased total T cells and activated DCs in draining lymph nodes; two times greater survival; up to 55% tumour-free mice; and significantly greater IFN-γ production and tumour cell lysis by splenocytes[733]. The other study also utilized the immune-enhancing effects of CpG but used a different approach. Pulsing lymphoma cells with CpG for 24 hours prior to co-culture with DC led to the CD4+ T cell-mediated eradication of large, established tumours[734].

Pulsing *ex vivo* DCs with autologous tumour lysate has also been used in various clinical trials. In double-negative breast cancer this approach saw increased circulating NK and CD8+ IFN-γ+ T cells along with delayed-type hypersensitivity and T_H1 cytokine production, however there was no overall survival benefit compared to patients not receiving the vaccine[735].

Human studies with tumour lysate have all used CD14+ monocyte-derived DCs[172,397,398,707,711–713,726,728–730,735–754] and work is ongoing to perfect the parameters for GMP-grade production of autologous, tumour-lysate-pulsed dendritic cells[755]. To our knowledge only DCs have been used as APCs in clinical trials of ACT for cancer, with or without tumour lysate as the antigen source, and DC-mediated ACT has had some very encouraging, but limited successes[713,756,757]. The benefits of the use of tumour lysate as a source of TAA will be discussed further in a later section.

Due to their potent antigen presenting capability DCs are by far the most commonly used in clinical trials, however they are not immune to modulation by growing tumours[615]. There is evidence for DC cancer vaccines inducing good immune responses and clinical benefit has been gained in some individuals[711,725,758], however widespread, consistent results remain elusive. This highlights the importance of this study in ascertaining whether or not benefit can be gained by including other APCs in the presentation of undefined tumour antigens to T cells. It was thought until quite recently that DCs were the only APC capable of priming a naïve T cell. However it is now well established that appropriately activated B cells[238,243,250,759–761] and MΦs[42,145,167,177,178,180,181,312,762] can also act as effective APC to both CD4+ and CD8+ T cells. There is precedent for the use of both MΦs and B cells[293,763] as lysate-loaded APCs in the anti-cancer setting.

1.12.3 Macrophages in Cancer Immunotherapy

Under resting conditions BMMΦs produce high levels of suppressive cytokines (IL-10 and TGF-β)[764] in line with their homeostatic anti-inflammatory role in tissues, therefore polarisation toward M1 is desirable clinically for use in anti-tumour ACT. They are highly responsive to their milieu and modify their functions accordingly. Coussens *et al* suggest that re-polarization, rather than elimination, of MΦs is probably the way forward for using MΦs in anti-tumour therapies[627].

While most immunotherapies have been directed at adaptive immune responses, MΦ-mediated innate-directed treatments are also entering the immunotherapeutic arena[765–767]. Clinical use of MΦs has focused on their tumouricidal activity with the use of IFN-γ-activated "killer MΦs", not their APC capacity[180]. However, MΦ phagocytosis has also been implicated as an important mechanism of action in mAB-mediated immunotherapies, leading to exploration of approaches for enhancing MΦ responses to mABs[767].

In 2011 Beatty and colleagues reported on a clinical trial in which anti-CD40 mABs plus gemcitabine chemotherapy were tested in a small cohort of pancreatic ductal adenocarcinoma (PDA) patients[768]. The CD40 mABs were used for their T cell-stimulating properties and tumour regressions were observed in some patients. In testing the mechanism of action in a mouse model they unexpectedly discovered that tumour regression required MΦs, but not T cells or gemcitabine. CD40-activated MΦs rapidly infiltrated tumours, lysed tumour cells and mediated tumour stroma destruction, demonstrating MΦ-mediated, T cell-independent elimination of these tumours.

In another example of immunotherapy harnessing the direct cancer-fighting ability of MΦs in conjunction with mABs, Weiskopf and colleagues used MΦ recognition of CD47 to ameliorate tumour escape mechanisms[625]. CD47 on healthy cells is recognised by MΦs via the SIRPα receptor. Tumours escape MΦ-mediated immune destruction by expression of CD47. Weiskopf *et al* created SIRPα variants, high-affinity CD47 antagonists and used them in combination with the anti-tumour mABs rituximab in lymphoma, or trastuzumab in breast cancer, to effectively treat several mouse tumour models. These studies highlight, amongst other things, the direct roles of MΦs in the anti-tumour response, and the possibilities of harnessing those capabilities in cancer immunotherapy.

In a review Kershaw and Smyth note that the work by Weiskopf *et al* suggests that MΦs anticancer potential may have been underestimated[769]. They reason that mABs against the T cell-regulatory molecules cytotoxic T lymphocyte-associated antigen 4 (CTLA4) and programed death 1 (PD-1) combined with the likes of variant SIRPα

monomers to block the 'Don't Eat Me' signal to MΦs could potentially revive both T cell and MΦ anti-cancer responses. In light of these many potential cancer-fighting properties of MΦs we wished to compare their ability to activate T cells against lysate antigens.

1.12.4 B Cells in Cancer Immunotherapy

Because the use of DCs as the sole APC in cancer immunotherapy has yielded disappointingly low and inconsistent results some investigators are now pursuing the therapeutic potential of B cells as an alternative autologous APC[213,293,770–772]. Advantages over DC include larger cell numbers from smaller volumes of peripheral blood, a significant consideration for sick patients. Autologous B cells are available in vastly higher numbers than DCs and easily further expanded *in vitro* with soluble CD40 ligand to generate oligoclonal populations[294,772].

In the pre-trial phase *ex vivo* expansion of CD40-stimulated B cells loaded with melanoma cells, cell lysates or peptides resulted in activation and expansion of antigen-specific naïve and memory CD4+ and CD8+ T cells[293,294]. Interestingly antigen acquisition was via non-specific uptake mechanisms and was not BCR-mediated. Nonetheless the high levels of antigen combined with CD40 signaling yielded sufficient costimulatory action for T cell priming.

Two further pre-clinical studies have also demonstrated that antigen uptake via the BCR was dispensable in the induction of T cell immunity both *in vitro* and *in vivo* avoiding the requirement for antigen specific B cell expansion. In one study B cells were used to deliver viral antigen to T cells via mRNA electroporation[773]. In the other anti-CD19 mABs were used to specifically target B cells as APCs[259]. This feature of B cell antigen presentation raises the interesting possibility of B cells being extremely useful in the presentation of undefined TAAs to T cells.

In an example of B cell-mediated immunotherapy, the Herrera group have reported data from ongoing clinical trials of antibody-inducing vaccines that high tumour-specific IgG and IgM titres were generated in 80% of patients and that survival was significantly improved with these vaccines. The induced ABs were specific for the patient's tumour cells and were responsible for the direct killing of the tumour cells. Notably it was the cytotoxic ability of the patient sera, not the AB titre *per se*, that

corresponded with increased survival. (Herrera, unpublished data from ongoing clinical trial, Cancer Immunotherapy Consortium conference, Aug 2012; [311]).

In the literature we found that two other groups have looked at B cell presentation of tumour lysate [293,763] and demonstrated successful CD4+ T cell activation by lysate-pulsed B cells. We are not aware, of any studies examining B cell activation of CD8+ T cells with tumour lysate.

1.13 Tumour lysate As a Source of Tumour Antigen

In early phase cancer clinical trials researchers and clinicians face an uphill battle. Patients in early phase clinical trials are often those for whom gold-standard levels of care options have been exhausted. They have received the best treatments that current medical knowledge allows and those treatments have failed. Consequently these patients are ill, possibly immune-suppressed, and have established tumours, which have immune avoidance mechanisms in place to switch off immunotherapy-supplied T cells that enter the tumour microenvironment. In this scenario it is a wonder that any patient has benefited from immunotherapy – and yet some do. And it may be that immunotherapy with tumour lysate, with its broad repertoire of TAAs, that offers hope for preventing future tumour escape.

Many TAAs recognised by T cells have been characterised. Some TAAs are unique to tumour tissue and are the result of mutations of critical genes in cell proliferation, differentiation and death pathways. Mutations in β catenin[774], cdk4[775], mutated p53[776] and RAS–RAF–MEK–ERK–MAP kinase signaling[777] are some examples. Other TAAs are shared with healthy tissues and include cancer testes antigens (MAGE, BAGE, GAGE[778]), differentiation antigens (MART-1/Melan-A, tyrosinase, gp100[779]) and overexpressed, non-mutated antigens (wild type p53[776], Her2/neu[780]). Antigens derived from carcinogenic viruses, such as the human papilloma virus responsible for cervical cancer in women, are also able to be recognised by T cells[781]. APCs located in the blood, spleen, LNs and tissues can be exposed to disseminated TAAs in the form of soluble proteins, RNA, DNA, immune complexes, apoptotic debris and necrosis, from tumour tissue that has broken down under cell death or stress processes[782].

However, despite melanoma and numerous other cancers having various known TAAs that can be recognised by T cells[783–785], limited success has been seen with the use of molecular vaccines based on individual cancer proteins, peptides or nucleic sequences. A 2008 meta-analysis of hundreds of clinical trials of many different cancer types showed that greater clinical success has been obtained using whole tumour cells or their extracts than defined antigen approaches[786]. For these reasons the use of tumour lysate (TL) has been investigated in pre-clinical models and used in clinical trials as a source of the full repertoire of TAAs.

However, live tumour cells are poorly immunogenic and actively produce immunosuppressive factors such as vascular endothelial growth factor (VEGF) that suppresses DC differentiation and maturation[787], Fas-ligand that induces T cell programmed cell death[788] and MIC ligands that block NKG2D-facilitated destruction by immune cells[789]. Galectin-1[113] and indoleamine 2,3-dioxygenase[790] prevent T cell activation while IL-10[791] and TGF-β[792] have additional immunopathology-repressing effects on DC and T cell functions that impede an anti-tumour response.

Thus TL contains all possible immunogenic antigens in the patient's tumour, while at the same time containing tolerising and immunosuppressive, properties due to it being prepared from self-tissue. These properties make it exquisitely suited to activating a patient's immune system against their TAAs, however the self-antigens among the transformed targets render it a problematic immune stimulator. Overcoming the immune-inhibition mechanisms, which serve to protect against anti-self responses, that are utilized by tumours to evade destruction by the immune system is one of the major hurdles of immunotherapy in general, and of the use of TL in particular.

Many examples exist in the literature of the successful use of TL both in pre-clinical models and in clinical trials[709,711,728,733,739,745,793–795]. From a clinical perspective there are several clear benefits to using autologous whole tumour lysates. In the first instance no patients are ineligible for antigen-presenting cell-whole tumour lysate therapy as there is no requirement to select patients on the basis of their HLA-A2 status. Furthermore, TL potentially offers the full spectrum of TAAs to both CD4+ and CD8+ T cell types, generating a more robust primary immune response, as well as

lessening the possibility of tumour escape. 'Help' from CD4+ T cells fosters long-term CD8+ T cell memory, giving them the ability to expand should they ever re-encounter that antigen, essential preventing recurrence of the cancer[796–798].

However, perhaps the most compelling evidence for the continued investigation of TL's clinical utility comes from the clinical outcomes of peptide-pulsed versus whole tumour cell-pulsed vaccinations, which show that more patients experienced objective clinical responses to whole tumour or tumour extracts used as antigens (including DC loaded with tumour extracts, modified tumour cells or tumour RNA) than to specific tumour antigens (synthetic peptides or proteins and viral or plasmid vectors encoding peptides or proteins)[786].

In this thesis MHC class I and II-restricted presentation and cross-presentation of whole OVA protein, OVA peptides and OVA-secreting B16.OVA melanocyte lysate was assessed using transgenic mice bearing T cell clones with TCR specific for H-2K(b)/$OVA_{323-339}$ and H-2K(b)/$OVA_{257-264}$ (SIINFEKL) epitopes. The TCR of OT-I CD8+ mice respond exclusively to the SIINFEKL peptide, while the TCR of OT-II CD4+ mice respond only to $OVA_{323-339}$, thus allowing the generation of high numbers of responding T cells and tracking of CD4+- and CD8+-mediated responses. The B16 murine melanoma model is widely used and established as invaluable in the development of effectual ACT approaches for melanoma patients[799]. As a non- or low-immunogenic tumour[799] B16 melanoma provides one of the more rigorous litmus tests for evaluating an immunotherapy procedure. Murine DCs loaded with apoptotic B16 melanoma cells have been shown to induce anti-tumour immunity[173]. In those studies both CD4+ and CD8+ T cells were activated and the anti-tumour response required both cells.

Targeting a genetic mutation in a tumour, such as the ALK mutation in some neuroblastomas, is another approach to individualized treatment. But the only a small percentage of tumours are caused by a single, or few, genetic mutations, and most show genetic evolution and plasticity during their development. Melanoma's inflammation-induced reversible dedifferentiation is just one example[800]. The results of a large genomic study of the childhood cancer neuroblastoma indicated that the

likelihood of finding common genetic anomalies appears to be quite low[801]. While confirming ALK mutations in approximately 10% of patients, and finding single digit percentages in a few other gene mutations, they found few of the expected recurrent gene mutations that would identify potential neuroblastoma targets[801,802]. The challenge with defining immunogenic targets by gene sequencing alone is that you are pursuing a moving target. They concluded that tumour targets must be thought of as dynamic, not static targets due to their DNA instability. Each tumour will be at a different stage of development in each patient and the knowledge of when to target each genetic change as they arrive would take decades of study, if it could ever be attained.

By contrast, cell therapy targeted against known and unknown TAAs with TL ignores genetic changes that have occurred, or may yet occur, and treats the current state of the tumour. Enhancing the patients' own APC and T cell responses to that current state, should, in theory, generate an anti-tumour response. Speed is critical when trying to eliminate rapidly changing tumour cells. The cells must be prepared within as short a time frame as possible – hours to days where possible, not weeks or months.

Finally, Cornforth makes the point that lysates from patient tumours may contain cancer stem cells, which could account for their effectiveness at preventing recurrence, since the 'source' cell antigens are also identified and destroyed[415].

1.13.1 Which Form of Cell Death is More Immunogenic?

Polly Matzinger introduced the "Danger Model", a paradigm shift suggesting that immunity is not just activated against foreign, non-self entities but also distressed, damaged, destroyed and dead cells of the organism[78,803]. Under this paradigm the encounter by immune cells of intracellular components in the extracellular milieu is what alerts the immune system to "danger". Items such as nucleic acids, lipids, HMGB1 protein, ATP, uric acid and other intracellular components are only released under conditions of anomalous cell death[804]. These trauma-induced intracellular components can, in addition to bacterial and viral moieties, bind to TLRs and activate inflammatory responses[95]. Identification of which components of endangered cells engage with the immune system has been the focus of many studies of discrimination between forms of cell death[804–806]. A large study showed that TLRs are not only used

for PAMPs but also for stress-signals such as HMBG1 released from apoptotic tumour cells dying after chemotherapy or radiotherapy. The mutational loss of TLR4 in breast cancer patients abrogated the anti-tumour response[12].

Tumour cells can be treated by non-lethal UVB irradiation, gamma irradiation, oxidation, heat treatment and freeze-thaw lysis to achieve apoptotic or necrotic cell death or senescence (permanent arrest of the cell cycle in G1 phase) as required. There has been debate in the literature over which form of cell death is more immunostimulatory. Apoptotic cells can release TLR4-binding HMGB1[807]. Additionally intracellular calreticulin appears on the apoptotic cell's surface and binds to the CRT receptor on the surface of MΦs and DC leading to receptor-mediated phagocytosis[14], DC activation and a CTL response. Phosphatidylserine (PS) is another normally intracellular molecule that 'flips' to the external cell surface of apoptotic cells and encounters the PS receptor on DC leading to the initiation of cross-presentation of TAAs by the DC[808–810]. Work by Asano showed that apoptotic, x-ray irradiated OVA-transfected EG7 T cell thymoma cells presented by CD169+CD11c+ MΦs in the boundary between the sinus and the T cell zone were able to stimulate OT-I T cell proliferation and cytokine production[177].

However the majority consensus appeared to be that necrotic cell death is the more immunogenic form of cell death, primarily owing to the release of inflammation-inducing DAMPs[811]. Necrotic cells release heat shock proteins (HSP) such as HSP70 and 90 and HMGB1, all of which interact with toll-like receptor 4 (TLR4) on DC triggering antigen processing and presentation[12,812]. Inhibition of fusion between the phagosome and the lysosome means these potential TAA are not degraded but traffic to the antigen presentation compartment[794]. Glucose-regulated protein 170 (GRP170), an endoplasmic reticulum (ER) chaperone related to HSPs, is able to interact with scavenger receptor A on DC and increase tumour cell immunogenicity[813]. Uric acid, a by-product of purine metabolism, is also released by necrotic cells and induces DC maturation[814,815]. Necrotic cells are able to induce incomplete DC maturation in the absence of maturation stimuli[816] most likely as a response to the profusion of HSPs released by dead cells[172,753]. Necrotic cell lysate can be generated by repeated freeze-thaw cycles and produces a substance comprising all cell components from the

demolished plasma membrane, cell RNA and DNA and organelles such as the mitochondria.

Obeid *et al* made a shift in this thought paradigm with the discovery that calreticulin relocation from the cytosol to the cell surface dictated the immunoreactivity of the cell as opposed to the necrotic or apoptotic status of the cell[14], pointing to the fact that the style of death is probably of less importance than which immunosuppressive or immunoreactive molecules are upregulated or released in response to the death process. Yatim *et al* may have concluded the argument with their recent report that cross-priming of CD8+ T cells depends on receptor-interacting protein kinase-1 (RIPK1) dimerization and nuclear factor κB (NF-κB)–induced transcription within dying cells, which can be upregulated by both apoptotic and necrotic cells[817]. Nonetheless, while neither CRT, nor RIPK1 expression was analysed, Chiang's work with TL has shown that necrosis of tumour cells can be most efficiently achieved by oxidation of the tumour cells with hypochlorous acid (HOCl) prior to lysis[739,741] and that oxidized lysate stimulates an enhanced immune response compared to freeze-thaw lysis.

Debate continues over whether necrotic or apoptotic lysate is more immunogenic, but both have been shown to have comparable ability to mature DCs and to generate an anti-tumour immune response when presented by DCs *in vivo*. We had observed T cell responses to soluble freeze-thaw lysate in previous work but we were interested to compare the response with a potentially more immunogenic lysate.

1.13.2 Improving the Immunogenicity of Tumour Lysate

Various approaches to TL have been used in pre-clinical models and in the clinic, including necrotic TL, apoptotic tumour cells, oxidised whole tumour cell lysates, DC-tumour fusion vaccines, DCs pulsed with whole tumour RNA (reviewed in [794]).

Supernatants (SN) of freeze-thawed TL, such as was used in this study, have also been used in clinical trials as a source of tumour-derived antigens. These supernatants mainly consist of soluble tumour proteins, which are taken up by DC via non-specific macro-pinocytosis[818], processed and presented on MHC class II molecules to CD4+ T cells. However this non-receptor-mediated uptake of antigen does result in

significantly less cross-presentation of soluble ovalbumin than cell-associated OVA protein[819]. In addition, SNs are also likely to contain tumour-produced immunosuppressive factors alongside the cross-presentation-enhancing HSPs, HMGB1, uric acid and pro-inflammatory cytokines.

Debate also continues over whether or not TL is immunogenic at all, with some researchers reporting freeze-thaw-derived lysates as being inhibitory to DCs[175,820] and others observing the opposite[397,821]. Of critical importance in this debate is the method of TL preparation, which determines the way in which the tumour cells die. It is believed that cells releasing 'stress' factors, or 'danger' signals upon death are able to generate a strong immune response whereas cells quietly and neatly undergoing apoptosis do not tend to elicit this same response. Hatfield has demonstrated the partial reversal of lysate-induced DC suppression *in vitro* by inducing tumour cell stress prior to lysis. Only DCs pulsed with stressed cell lysates provided protection against tumour challenge[175].

HOCl oxidation of cells prior to lysis has also been demonstrated to enhance TL immunogenicity[740,741,822]. After comparing different cell death methods Chiang found that induction of oxidative death prior to lysis was a superior method for increasing the immunogenicity of ovarian cancer lysate[398,739–741,823]. Chiang cites three possible mechanisms why oxidized lysate is more immunogenic than non-oxidised. The deamination of serines converts side chains to aldehydes, which are more immunogenic[824]; the unraveling of protein structure to reveal hidden epitopes; and scavenger receptor recognition of oxidized lipids. However Prokopowicz and colleagues reported that modification of the N-linked carbohydrate side chain, not the formation of aldehydes or chloramines, was responsible for enhanced presentation of HOCl-modified OVA[825]. Increased efficiency of uptake of HOCl-OVA was receptor-mediated and they are investigating whether the scavenger receptor lectin-like oxidized low-density lipoprotein receptor (LOX-1) is the target. Targeting glycoprotein antigens to scavenger receptors on the APCs surface helps explain the improved immunogenicity of HOCl-lysate.

Given that TL is often suppressive, using APCs with TL enables researchers to improve TL immunogenicity by influencing DC behavior in the course of *ex vivo* pulsing. Interferons, Toll-like receptor agonists and p38 mitogen-activated protein kinase (MAPK) inhibitors can all be used to influence the milieu of the DCs, leading to DC differentiation that steers the polarization of T cells into CTL and T_H17 cells as opposed to T_{REG}[826]. Indeed initial mouse vaccine research indicated that murine bone-marrow-derived DC pulsed with whole TL could provoke a strong anti-tumour response and cytolytic activity *in vitro* and *in vivo*[827]. Inducing DC maturation with maturation stimuli such as TNF-α, IL-1β, IFN-γ, prostaglandin E2 and R848 (a TLR8 agonist) can improve DC capacity to present TAA[703] and the effectiveness of clinical-grade DC-based vaccines should be greatly enhanced once the ideal combination of maturation compounds is outlined[828].

Lysates have shown varying immunogenic responses *in vitro*, however both apoptotic and necrotically prepared cells have demonstrated anti-tumour responses when presented by DCs *in vivo*. The first clinical trial for ovarian cancer using oxidized whole tumour lysate and DCs was begun in 2012[745]**.** A combinatorial approach was used that included intravenous bevacizumab to inhibit tumour-induced angiogenesis, and oral cyclophosphamide to inhibit T_{REG}s, lymphodepletion and vaccine-primed T cells. Initial results published in 2013 showed that of the initial six patients four had achieved clinical responses, including one complete response[728]. The trial is ongoing with further modifications to optimise outcomes.

1.14 Significance: Defined Tumour Antigens and DCs Are Not Enough

Mature DCs have been demonstrated to provide protective anti-tumour immunity, and the majority of the immunological community believes that a protective T-cell-based anti-tumour response is contingent upon fully-functioning DCs. However, despite some dramatic successes, clinical results in DC ACT remain disappointing for reasons that are not yet completely understood. In spite of the DC's impressive antigen presentation credentials various factors do limit their clinical use, namely their scarcity in peripheral blood (<1% of total leukocytes)[829], inefficient migration to secondary lymph tissue after administration, their destruction by antigen-specific T_{EM} cells en route to lymph tissue, lack of proliferation, and their relatively short T cell

presentation time *in vivo*. Hence, the promising pre-clinical studies have not resulted in further FDA-approved DC-based immunotherapies beyond the 2010 approval of Provenge for prostate cancer[830,831].

Thus the best APC, or combination of APCs, for tumour immunotherapy is yet to be defined. Published work describes DCs as the foremost APCs but clearly shows that B cells and MΦs also have important antigen-presentation abilities, which differ according to the particular immune setting. MΦ behaviour can be manipulated toward the desired effector functions while B cells can be easily expanded and migrate to LNs avoiding destruction by antigen-specific T cells[283,294,832,833]. Both MΦs and B cells have been explored as alternatives to DCs for cell-based therapies[176,181].

Importantly, while many studies have looked at non-naturally occurring virally transduced or genetically engineered antigens few studies have assessed cross-presentation of naturally occurring antigens processed from apoptotic/necrotic tumour cells. Given that the promise of DC ACT has failed to deliver robust clinical results this work seeks to identify whether we have overlooked something in the choice of DC as the singular APC for ACT.

Ralph Steinman, in commenting on limitations in the use of defined tumour peptides for DC-based immunotherapy states: "*Cancer cells are much too formidable to combat with limited weaponry*"[699]. This sentiment seems appropriate not just for the use of a broad range of TAAs, as are found in tumour lysate, but also for the examination of a wider range of APCs. The success of ACT depends, in part, on being able to generate sufficient numbers of patient cells to carry out the procedure. Deriving adequate numbers of DCs from cancer patients for generating DCs for ACT is difficult, even more so in the case of peripheral blood DCs to which the ACT community may be turning[700,834], therefore being able to combine patient DCs and B cells and, or, MΦs would help reduce this rate-limiting step in the procedure. To help delineate a more efficient APC therapeutic strategy this study considered the efficiency of T cell responses to lysate TAAs presented by DCs compared with MΦs, B cells and combinations thereof. In the same way that combination immunotherapies

are the treatment *du jour* for cancer, we believe that combinations of APC should be given more attention in ACT.

1.15 Hypothesis and Aims:

The over arching goal of this research project was the improvement of adoptive T cell therapy for cancer, which was addressed by asking these questions:

1. Does oxidised lysate result in an enhanced T cell response compared to soluble freeze-thaw lysate?
2. Does a combination of APCs enhance T cell responses to lysate when compared against using DCs alone?
3. Are both CD4+ and CD8+ T cells activated into effector cells when primed with tumour lysate(s) by GMDC or a combination of APCs?
4. What is the cytolytic capacity of lysate-primed CD4+ and CD8+ T cells *in vitro* and *in vivo*?

We hypothesised that priming T cells against tumours using tumour lysate-derived antigens would be enhanced by the combination of a DC and a MΦ, or a DC and a B cell, or a B cell and a MΦ, or all three APCs combined.

This hypothesis was tested through the following objectives:

1. Preparation of soluble fraction freeze-thaw lysate and oxidised whole freeze-thaw lysate.
2. Analysis and comparison of the three APCs' response to loading with soluble and HOCl-oxidised lysates.
3. Examination of the proliferation response and cytokine profile of T cells primed with the various lysate-loaded APC(s).
4. Identification of which APC(s) stimulated the greatest cytotoxic T cell response.

Figure 12 outlines the basic experimental approach to these hypotheses. Chapter 3 comprises the results of soluble and oxidised lysate preparation, optimising APC culture conditions and the response of APCs to loading with lysates. Chapter 4 presents the results of the T cell phenotype response to being primed with lysate-

loaded APCs. Chapter 5 encompasses the T cell functional response of *in vivo* cytotoxicity post priming with oxidised lysate-loaded APCs.

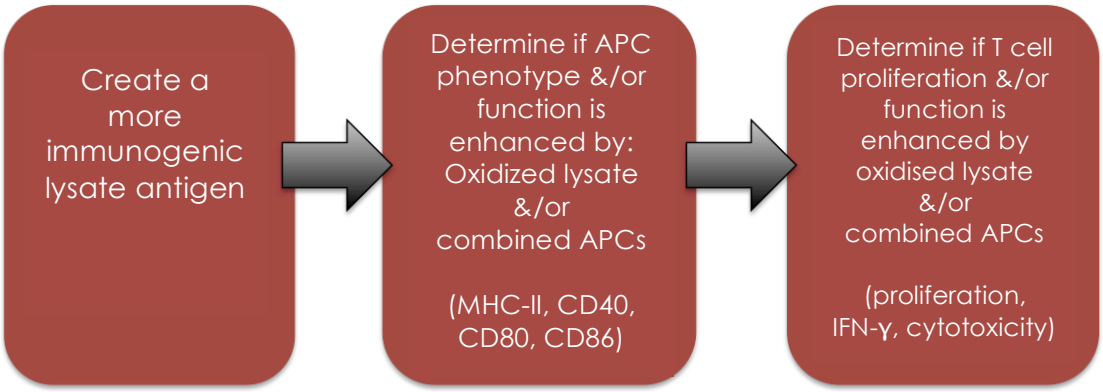

Figure 11 Flow Chart outline of experimental approach.

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2 Materials & Methods

"Cancer cells are much too formidable to combat with limited weaponry"

Steinman & Dhodapkar 2001[1]

2.1 Culture of B16.OVA Tumour Cells

B16-F0 is a murine melanoma cell line of C57BL/6 origin and H-2^b haplotype. B16.OVA cells are derived from B16.F0 but have been stably transfected with chicken ovalbumin (OVA) cDNA and secrete the OVA protein, which was used as a model tumour antigen in this study. These cells originated from cells that were transferred from the Malaghan Institute of Medical Research, Wellington, New Zealand on March 5, 2008. The cells were tested to confirm they were free of *Mycoplasma* (Appendix 1, Supplementary Figure 1).

Vials of B16.OVA melanoma cells were removed from liquid nitrogen storage and thawed quickly in a 37°C water bath until a small piece of ice remained in the vial. In a laminar flow hood Dulbecco's Phosphate Buffered Saline (DPBS) (Appendix 2) with 5% Foetal Bovine Serum (FBS) (DPBS5) was immediately added to the thawed cells and the cells centrifuged at 300 x *g* for 5 minutes at 4°C. This wash step removes the freezing mix that contains the cryoprotectant dimethyl sulfoxide (DMSO) added to prevent cell death during freezing. The supernatant was discarded and the cells resuspended in 5 mL Roswell Park Memorial Institute Medium1640 supplemented with 10% FBS (RPMI10; Appendix 2) and 1% Geneticin® (Geneticin® Selective Antibiotic (G418 Sulfate) (50 mg/mL); Life Technologies; Cat: 10131035) for plasmid antibiotic selection. The addition of Geneticin® creates a selective medium that selects for cells expressing the plasmid responsible for production of ovalbumin (OVA) protein. The cells were added into an additional 5 mL RPMI10 in a BD Falcon tissue culture flask (BD Biosciences) and the resuspended cells pipetted into the flask. Cells were incubated at 37°C with 5% CO_2. Cells were grown to approximately 80% confluence prior to being split into two (or more) tissue culture flasks for further expansion of cell numbers. B16.OVA cell growth was tested in complete Dulbecco's Modified Eagle Medium (cDMEM) and complete Iscove's Modified Dulbecco's Medium (cIMDM) but enhanced cell growth was achieved in RPMI10.

Cells were passaged by pouring off non-adherent cells into a 50 mL Falcon tube, rinsing the flask with DPBS and adding enough warm DPBS with 0.02% EDTA to cover the surface of the flask. EDTA is used for the dissociation of attachment-dependent cell lines from plastic ware. EDTA is an ion-chelating agent that deprives cells of calcium, which is critical for cell adhesion. The use of EDTA avoids the non-specific proteolysis of proteins, including the critical protein OVA, inherent in the use of trypsinisation methods of cell release. Previous work in our laboratory and others[2] has shown that this method yields more consistent lysates than trypsinisation. Cell death is consistently minimal with this method. The flask was incubated at room temperature for 5-10 minutes until adherent cells lifted off into suspension when the flask was banged twice, gently but firmly, against a hand. Using a 10 mL pipette on the fastest setting the flask surface was flushed approximately 10 times and the adherent cell suspension added to the non-adherent cells. The flask was rinsed once with DPBS and the cell suspension centrifuged at 250 x g for 7 minutes at 20°C. The supernatant was discarded and the cells resuspended in RPMI10+Geneticin (1%). The cells were divided equally between two, or more, fresh flasks and media added up to the appropriate volume. The cells were incubated at 37°C with 5% CO_2 until they reached approximately 80% confluence.

Once the desired volume of cells was reached (up to 10 x 175cm^2 flasks) cells in active growth (log) phase, approximately 80% confluent, were treated with 5 μg/mL Brefeldin-A (BrefA)(Sigma) for 5 hours to allow OVA protein to accumulate inside the cells prior to harvest.

Flow Cytometry confirmed negligible MHC-I-SIINFEKL expression by the B16.OVA cells prior to incubation in Brefeldin A. Brefeldin A redistributes intracellularly produced proteins from the cis/medial Golgi complex to the endoplasmic reticulum, trapping proteins that are normally secreted inside the cells. Thus the cell lysates were also assayed by Western Blot (WB), which confirmed the presence of OVA (Chapter 3, Figures 2 and 3s).

2.2 Polymerase Chain Reaction (PCR) Testing of *Mycoplasma*

B16.OVA cells were washed twice in DPBS and resuspended in DPBS for DNA extraction. Cells were stored at -20°C prior to DNA extraction. DNA was extracted using Purelink DNA minikit from Invitrogen (Cat: K1820-01) according to manufacturer's instructions. DNA concentration was measured by Nanodrop and diluted to 10 ng/mL in miliQ (MQ) water. Ten µL was added to PCR reaction for a total of 100 ng. One ng of *Mycoplasma* DNA was used as a positive control. The primers used amplified DNA from highly conserved regions of the *16SrRNA* gene, which do not detect eukaryotic DNA or bacteria closely related to *Mycoplasmas*.

PCR PROTOCOL

Reaction Mix	Volume (µL)
MQ water	6.26
10X PCR buffer	2
50 mM $MgCl^2$ (1.85 mM final)	0.74
10 mM dNTPs (0.2 mM final)	0.4
10 µM fwd primer (0.1 mM final)	0.2
10 µM rev primer (0.1 mM final)	0.2
Platinum Taq	0.2
Sample	10
Total	20

PCR Cycle Conditions

94°C	90s
94°C	30s
70-60°C	30s (touch down, 1°C per 2 cycles for first 20 cycles, last 20 remain at 60°C)
72°C	45s
Repeat 40 cycles	
72°C	4 mins

The following day the PCR DNA was run on a 2% agarose gel for 30 mins at 97 volts. No *Mycoplasma* DNA was observed in B16.OVA cell samples (Appendix 1, Supplementary Data Figure 1).

2.3 Preparation of Soluble Fraction Tumour Lysate (s-L)

We tested the soluble fraction of freeze-thaw-generated B16.OVA and the whole portion of HOCl-oxidised B16.OVA. Tumour lysate (TL) preparation was carried out under sterile conditions, in a laminar flow hood, 5 hours after the addition of 5 μg/mL BrefA to the B16.OVA tumour cells. We used Brefeldin A at 5 μg/mL as we reasoned based on previous data that this concentration should be sufficient to yield the desired effect, while minimising unwanted side effects such as changes to the cell structure (Golgi, ER, actin filaments and others) and reduction in protein synthesis[3]. Cells were harvested using a 0.02% solution of EDTA in Dulbecco's Phosphate-Buffered Saline (DPBS).

Complete EDTA-free protease inhibitor (PI) tablets (Roche Diagnostics Ltd, Mt Wellington, Auckland, NZ) were dissolved in DPBS (no Ca^{++}, no Mg^{++}). (1 tablet in 50 mL DPBS as per manufacturer's instructions). Bref-A-treated B16.OVA cells were resuspended in the protease inhibitor cocktail at 2.4 x 10^7 cells/mL before being subjected to six repeat cycles of freeze-thaw lysis (dry-ice and methanol or isopropanol bath for 15 minutes followed by 37°C water bath for 5 minutes). The protease inhibitor solution was important for minimizing protein degradation by proteases released during lysis and subsequent storage. The resultant lysate was centrifuged at 1700 x *g* for 10 minutes to generate a soluble fraction and an insoluble pellet. The bulk of the soluble fraction was removed and the tube re-spun for a further 10 minutes to extract as much soluble fraction as possible. The soluble fraction was dispensed into aliquots and stored at -80°C.

Total protein concentration was ascertained by either BCA Assay or spectrophotometer (Nanodrop, Thermo Fisher) where 1 A280 = 1 mg/mL. The standard curve of a BCA assay generated a total protein concentration of 13.72 mg/mL (10.48 – 16.97). Nanodrop protein concentration averaged 11.34 (9.38 – 13.29) (Appendix 1, Supplementary Data Table 1). Western Blots revealed two bands in the 40 and 45 kDa range as expected for OVA proteins (Chapter 5, Figure 63).

2.4 Preparation of Oxidised lysate (ox-L)

Cells destined for oxidized lysate (ox-L) were treated prior to lysis with hypochlorous acid (HOCl) according to the method of SK-OV-3 cell oxidation described by Chiang

et al[3]. A 90 μM HOCl solution was prepared by diluting the stock sodium hypochlorite solution (NaOCl, reagent grade, available chlorine 10-15%; Sigma Aldrich Corp.) with DPBS and adding immediately to B16.OVA cells (final cell density 1 x 10^6/mL). The oxidizing cell suspension was incubated for 1 hour at 37°C, 5% CO_2, with gentle agitation every 15 - 30 minutes to induce oxidation-dependent tumor cell death. Tumor cells were washed twice in DPBS to remove all NaOCl and resuspended in protease inhibitor cocktail prior to 6 cycles of freeze-thaw lysis (dry-ice and methanol or isopropanol bath for 30 minutes followed by 37°C water bath for 5 minutes). Cells were lysed to complete fragmentation, determined by Trypan Blue staining, prior to aliquotting and storage at -80°C.

2.5 Measurement of stock HOCl concentration

Chiang et al oxidised ovarian cancer at 60 μM hypochlorous acid (HOCl), therefore it was necessary to determine the concentration of HOCl in the stock sodium hypochlorite reagent (NaClO; 12-15% solution; ECP Lab Chem ltd, Birkenhead, Auckland, NZ). HOCl concentration was calculated using the following protocol. A calibrated pH meter was used to test the pH of the stock NaClO solution, which was found to be 11.6. Sodium hydroxide (NaOH) was added to the NaClO to pH12 and absorbance measured at 292 nm on a Spectronic Genesys2 spectrophotometer (Thermo Fisher Scientific, USA).

The absorbance of the undiluted stock NaClO averaged 3.16 (range: 3.007 - 3.275) and that of the pH12 NaClO averaged = 3.25 (Range: 3.209 – 3.247). Similar absorbance results were obtained by Nanodrop (ND1000, Thermo Fisher, USA). Undiluted stock NaClO absorbance measured 0.315 (at 1 mm therefore x 10 = 3.15). NaClO at pH12 measured 0.37 (at 1 mm therefore x 10 = 3.7).

The following formula was used to measure the concentration:

- [concentration] = Abs_{252nm}/ε

ε = extinction coefficient = 350 $M^{-1}cm^{-1}$

- $A = \varepsilon c l$

Absorbance = extinction coefficient x concentration of the substance being measured x path length (cuvette) Therefore c = A/(ε x l)

C = 3.16/(350 M^{-1} x 10 cm^{-1}) = 9.03 x 10^{-4} M (0.903 mM or 903 μM)

Therefore the following calculation was used to determine the volume of stock NaClO to add to the cell suspensions:

(90 μM/903 μM) x 2000 μL = 199 μL

The following table was used when adding NaClO to the cell suspensions

HOCl concentration (μM)	Cells (μL)	2 Molar NaClO solution (903 μM HOCl) (μL)	DPBS (μL)	TOTAL VOLUME
140	200	310	1490	2000
130	200	288	1512	2000
120	200	266	1534	2000
110	200	244	1556	2000
100	200	221	1578.5	2000
90	200	199	1601	2000
80	200	177	1623	2000
70	200	155	1645	2000
60	200	133	1667	2000
50	200	111	1689	2000
40	200	89	1711	2000
30	200	66	1734	2000
20	200	44	1756	2000
10	200	22	1778	2000
0	200	0	1800	2000

2.6 Western Blot

B16.OVA tumour cells (+/- oxidation treatment) were washed twice with DPBS, lysed on ice in RIPA Lysis Buffer (1 Unit of Complete Protease Inhibitor (CPI), 1 μM of Sodium Orthovanodate (NaO), and 1mM of Phenyl Methane Sulfonyl Fluoride (PMSF) in Radio-Immuno-Precipitation Assay (RIPA) buffer. RIPA buffer consists of 50 mM of Tris Cl (pH = 8), 150 mM NaCl, 1% Nonyl Phenoxypolyethoxylethanol 40 (NP-40), 0.5% Sodium Deoxycholate, 0.1% SDS in milliQ water). The protein lysate was centrifuged at 1,300*g* for 20 minutes at 4°C, the pellet discarded and lysate aliquotted and stored at -80°C.

Laemmli buffer (240 mM Tris, 6.9% SDS, 40% Glycerol, 20% β – mercaptoethanol, and 0.02% bromophenol blue) was added to each protein sample to make a final volume of 20 μL with MilliQ water. Protein samples were denatured in a heating block at 99 °C for 10 minutes, and immediately transferred to ice to maintain denaturation. A total of 1x 10^6 tumour cells per sample was resolved on Bolt 4-12% Bis-Tris Plus pre-cast electrophoresis gels (Novex by Life Technologies, Carlsbad, CA, USA) and transferred to iBlot nitrocellulose Gel Transfer Stack membranes (Novex by Life Technologies, Carlsbad, CA, USA). The membrane was blocked with 5% skim milk powder in TBST solution (1 hr at room temperature or overnight at 4°C). The membrane was probed with optimally titrated anti-OVA (gift from Professor Vernon Ward, Department of Microbiology and Immunology, Otago University, Dunedin). Whole OVA protein was used as a positive control. Optimally titrated anti-Mouse IgG (Fab Specific)-Peroxidase (Sigma Aldrich, St Louis, MO, USA) was used as the 2° antibody and the Amersham Biosciences Enhanced Chemi-Luminescent (ECL; Thermo Scientific) substrate used to detect the presence of OVA protein.

2.7 APC Preparation and Activation

2.7.1 Mice

Specific pathogen-free male and female C57BL/6 and OT transgenic mice were housed in and obtained from the Hercus Taieri Resource Unit, Hercus Building, University of Otago, Dunedin, New Zealand. Experiments were carried out under the approval of the Animal Ethics Committee, University of Otago, Dunedin, New Zealand. Tissue retrieval was carried out under AEC# 37/13 and 09/14. *In vivo* studies were conducted under AEC #59/15.

2.7.2 Dendritic Cells

The tibiae and femurs of six to twelve week old C57BL/6 mice were isolated asceptically and placed in DPBS (Gibco, Paisley, Scotland). The prepared bones were washed in cold 70% ethanol for two minutes and rinsed twice in cold DPBS. The ends of the bones were removed and the bone marrow flushed into a sieve with a 25-gauge needle and 10 mL syringe filled with cold DPBS. The cell suspension was collected in a 50 mL Falcon tube (BD BioSciences) and centrifuged for 7 minutes at 300x g at 4°C. The supernatant was poured off, the cells resuspended by gentle pipetting and 2

mL of warm ACK RBC lysis buffer (Appendix 2, Recipes) added for 3 minutes to lyse red blood cells (RBCs). Residual cells were washed in DPBS and resuspended in 5 mL IMDM (Gibco) supplemented with 5% FBS and 20 ng/mL Granulocyte Macrophage Colony Stimulating Factor (GM-CSF; R&D Systems, Minneapolis, MN, USA) (IMDM5+GM) for counting. IMDM5+GM was added to six well plates and 2.5 x 10^6 cells plated, to a total volume of 4 mL per well. The cells were incubated at 37°C plus 5% CO_2 for four days. On day four approximately 50% of the culture medium was removed and replaced with a corresponding volume of fresh, warm medium. On day six cells were harvested, washed with DPBS, resuspended at 1 x 10^6 c/mL and the appropriate number of cells plated in 12, 24 or 96 well plates for pulsing with antigen. In 72 hour proliferation assays a total of 5.0 x 10^4 APCs were plated with 5.0 x 10^5 T cells. In double combination wells 2.5 x 10^4 of each APC was plated. In triple combination wells 1.25 x 10^4 APCs was plated. In this way the number of APCs remained the same i.e. 10:1 ratio to T cells, with the proportion of APCs in each well being equally divided. In 10-day expansion studies 1.0 x 10^5 APCs were plated with 1 x 10^6 T cells.

Immature DCs (iDC) were pulsed with lysate or whole OVA protein antigen overnight prior to the addition of splenocytes. Peptide antigens were added at least 4 hours prior to the addition of splenocytes.

Antigens used were:

- Whole OVA protein (Sigma): 50 μg/mL
- Soluble TL: the soluble fraction of 2.4 x 10^7 lysed cells per mL used at a ratio of 6:1, 3:1 or 1:1 lysed tumor cells per APC
- Oxidised TL: the whole portion of 2.4 x 10^7 lysed oxidized cells per mL used at a ratio of 6:1 or 1:1 lysed tumor cells per APC
- The OT-I-specific peptide $OVA_{257-264}$, (SIINFEKL; Mimotopes, Clayton, VIC, Australia) (2 μg/mL)
- The OT-II-specific peptide $OVA_{323-339}$ (ISQAVHAAHAEINEAGR; Mimotopes, Clayton, VIC, Australia) (2 μg/mL)
- The irrelevant melanoma peptide gp100: melanocyte-associated gp100; amino acid sequence: NH2-ALNFPGSQK-COOH; reconstituted in miliQ water. A melanoma-associated melanocyte differentiation antigen used as a negative

control against non-specific responses (2 μg/mL); Mimotopes, Clayton, VIC, Australia; cat: 100509

Lipopolysaccharide (LPS; 1 μg/mL) and the unmethylated oligodeoxynucleotide CpG (1896) (Geneworks) (0.3 μg/mL) were used to activate the APCs. These immune stimulators were variously added either at the same time as the lysates (overnight loading and activation) or after overnight lysate loading, 6 hours prior to the addition of T cells.

Phenotype analysis was conducted by Flow Cytometry and the cell preparation protocol is detailed under Flow Cytometry: T cell Phenotype and Proliferation Response.

Table 1 lists the surface markers that were used to investigate phenotypic differences between the cells grown in M-CSF and GM-CSF +/- IL-3. The markers Ly6C, Ly6G and CD11b were eliminated early on as being unhelpful for distinguishing between DC and MΦ as these markers were expressed to a high degree on both cell populations. The markers commonly used to identify DCs and MΦs are usually expressed by both cells and also on other cell types. Often the level of expression, high versus intermediate or low, is the only distinguishing feature of a given molecule. Thus careful phenotyping is required to analyse the particular capabilities of each cell population.

The antibody panels shown in Table 2 were chosen for on going comparison. These panels provided a range of molecules that are common to both DC and MΦ. Variations in expression levels of the molecules allowed differences between GMDC, IL4DC and MΦ to be distinguished, indicative of differing functions and capabilities.

Table 1 Surface markers investigated for phenotyping DCs and MΦs

Molecule	Brief description & reason for inclusion in panel
MHC-II	Molecule for presentation of exogenous antigen. To identify ability to present peptides to T cells
CD11c	Transmembrane integrin for adherence to endothelium & phagocytosis. Highly expressed on DC subtypes and also on MΦs.
F4/80	One of a family of tissue adhesion GPCRs. Extensively used MΦ marker. Expressed on many, but not all, murine MΦs
CD169	Sialoadhesin, a cell adhesion molecule. Binds sialic acids. CD169+ MΦs noted to be responsible for cross-presentation of dead cell antigen to CD8+ T cells in vivo[4]
CD40	APC costimulatory molecule. Binds CD154 (CD40L) on T cells. Necessary for APC activation & subsequent T cell activation. Identifies APC costimulatory capacity.
CD80	Costimulation molecule for T cell activation and survival. Binds to CD28 and CTLA4 on T cells. Identifies APC costimulatory capacity.
CD86	Costimulation molecule for T cell activation and survival. Binds to CD28 and CTLA4 on T cells. Works in tandem with CD80. Identifies APC costimulatory capacity.
CD115 (CSF1R)	Colony stimulating Factor 1 receptor. CSF1R controls MΦ production, differentiation and function. Present at high levels on MΦs and at low to intermediate levels on DCs
Ly6C	Enriched on monocytes. Useful when used with LY6G to determine relative amounts of granulocytes and monocytes or MΦs.
Ly6G (Gr-1)	Enriched on granulocytes. Useful when used with LY6C to determine relative amounts of granulocytes and monocytes or MΦs.
CD135 (Flt3)	Flt3L receptor. Important in haematopoietic stem cell and progenitor cell development. Present on common lymphoid progenitors and multipotent progenitors but not haematopoietic stem cells. Has been reported to be required for DC development and to be a differentiating marker between DCs and MΦs at the gene level.
CD206	Mannose receptor. Primarily present on MΦs and immature DCs. Role in antigen uptake and presentation.
CD45R (B220)	Subset of CD45 isoforms. Predominantly expressed on B cells but also to a lower degree on plasmacytoid DC.
CD8	Co-receptor for the TCR. Binds MHC-I. Also expressed on subsets of DCs. CD8+ and CD8- DCs reported to have different roles in anti-tumour response.
CD11b (Mac-1)	Macrophage-1 antigen. Involved in phagocytosis, cell-mediated cytotoxicity, chemotaxis and activation. Expressed on monocytes, granulocytes, MΦs and NK cells.

Table 2 Panels of Antibodies to Surface Markers Used To Phenotype DCs and MΦs

	Panel 1	Panel 2	Panel 3	Panel 4
FL1	MHC-II-FITC 1/800 (0.125 μL)	MHC-II-FITC 1/800 (0.125 μL)	MHC-II-FITC 1/200 (0.125 μL)	MHC-II-FITC 1/200 (0.125 μL)
FL2	CD135 (Flt3)-PE 1/200 (0.5 μL)	F4/80-PE 1/200 (0.5 μL)	CD169-PE 1/200 (0.5 μL)	CD115-PE (BL) 1/200 (0.5 μL)
FL4	B220 (CD45R) PerCP-Cy5.5 1/400 (0.25 μL) pDC marker			MHC-I PerCPCy5.5 1/800 (0.125 uL)
FL5	CD8-PE-Cy7 1/400 (0.25 μL)	CD8-PE-Cy7 1/400 (0.25 μL)	CD8-PE-Cy7 1/400 (0.25 μL)	CD8-PE-Cy7 1/400 (0.25 μL)
FL6			CD40-APC 1/160 (0.625 μL)	
FL8	L/D NIR*	L/D NIR*	L/D NIR*	L/D NIR*
FL9	CD11c-BV421 1/160 (0.625 μL)	CD206-BV421 1/333 (0.3 μL)	CD80-BV421 1/200 (0.5 μL)	CD11c-BV421 1/160 (0.625 μL)

*L/D NIR: Dead Cell Discrimination Dye Live/Dead Near InfraRed, Life Technologies

2.7.3 Macrophages

Bone marrow progenitor cells were prepared in the identical manner to bone marrow derived DCs as described above. In M1 MΦ experiments residual cells were washed in DPBS5 and resuspended in IMDM (Gibco) supplemented with 10% FBS and 5 ng/mL Granulocyte Macrophage Colony Stimulating Factor (GM-CSF; R&D Systems, Minneapolis, MN, USA) (IMDM10+GM). In M2 MΦ experiments the cells were resuspended in IMDM+10% FBS+1-100 ng/mL macrophage colony-stimulating factor (M-CSF).

Cells were re-suspended at 1 x 10^6 cells/mL in IMDM10+GM-CSF or M-CSF, placed in a T75 tissue culture flask and incubated overnight at 37°C plus 5% CO_2. The following day interleukin-3 (IL-3) (5 ng/mL) was added to the cells in the flask, and, to minimize contaminating fibroblasts[5], nonadherent bone marrow cells were transferred to sterile, non-tissue culture-coated 94 mm bacteriological petri dishes (5 mL per dish) and returned to the incubator (37°C plus 5% CO_2). Cells were observed each day. On days 4 and day 8 the petri dishes were tilted and the dish gently rinsed once in the aspirated medium to remove non-adherent cells. Five mL of fresh warm medium was replaced into the dishes. On D10 non-adherent cells were aspirated by 10 mL pipette and discarded and the adherent cells used for experiments.

The M1 MΦs used in this study were never loosely adherent, in fact adherence was one feature that was used to distinguish between MΦs and DCs. Significant time was spent ascertaining the best method for gently removing them from non-tissue culture-treated plastic ware. Studies by La Flamme *et al* indicated that cold PBS was used for BMMΦ harvest[6,7] therefore in early experiments ice-cold DPBS (5 mL) was added to each dish and the dishes refrigerated for 5 minutes. The cell solution was then flushed vigorously with a 10 mL pipette on the highest speed and the released cells in solution aspirated into a 50 mL Falcon tube. This refrigeration-flushing procedure was repeated two or three more times until few cells were left on the plates. In our hands the cold PBS method took inordinate amounts of time and was less effective than a 0.02% solution of EDTA in DPBS.

Optimization of MΦ culture methodology revealed that culture on non-tissue culture-coated bacteriological petri dishes and incubation in 0.02% EDTA for 5 minutes prior to harvest allowed much easier release of the MΦs. EDTA is an ion chelator that causes the cells to downregulate their adherence molecules by calcium deprivation[8]. For this harvest method to work optimally the medium must be discarded and the dishes rinsed once in the EDTA solution to minimise remaining medium prior to starting the 5-minute incubation period. Following incubation the M1 MΦs released easily from the dishes with gentle flushing by 10 mL pipette.

Released cells were centrifuged (7 minutes, 300 *g*, 4°C), resuspended in IMDM10+GM-CSF/M-CSF at 1 x 10^6 c/mL and plated in 12, 24 or 96 well plates for pulsing with antigen. Cell numbers were the same as those described for DCs. The MΦs were activated with LPS&CpG and pulsed with identical antigens at the concentrations described for DCs.

2.7.4 B cells

The spleens of six to twelve week old C57BL/6 mice were isolated asceptically and placed in Dulbecco's Phosphate Buffered Saline (DPBS; Gibco, Paisley, Scotland). In a laminar flow hood on an ice tray the spleen was placed inside a 70 μM cell filter sitting in a petri dish of DPBS. The ice tray was used to keep cell temperatures as low as possible to maximise murine cell viability. BD Biosciences recommend that murine cells be kept between 4-8°C during preparations while human cell viability is better

preserved at room temperature. The plunger from a sterile syringe was used to gently press the spleen through the sieve. Cell aggregates were dissociated by flushing the cell suspension through the sieve with a 10 mL pipette. The cell suspension was collected in a 50 mL Falcon tube (BD BioSciences) and centrifuged for 7 minutes at 300 x g at 4°C. The supernatant was poured off, the cells resuspended by gentle pipetting and 5 mL of warm ACK RBC lysis buffer (Appendix 2, Recipes) was added for 3 minutes to lyse red blood cells (RBCs). Residual cells were washed in DPBS and resuspended in 90 µL of AutoMACS® buffer (Appendix 2, Recipes) per 10^7 cells. Anti-CD43 (Ly-48) MicroBeads (Miltenyi Biotec GmbH, Germany), which are present on nearly all leukocytes except immature and resting mature B cells, were vortexed and 10 µL of beads were added per 90 µL of cell suspension. The bead/cell suspension was briefly vortexed and incubated at 4°C for 15 minutes. Cells were washed with AutoMACS buffer (1-2 mL per 10^7 cells), centrifuged (300 *g*, 5 minutes, 4°C) and resuspended in 500 µL of buffer per 10^8 cells. At this point cells were passed through a large cell column on an AutoMACS Pro Separator (Miltenyi Biotec GmbH, Germany) 'DepleteS' programme. The negative fraction was collected, resuspended in B cell medium (Iscove's Modified Dulbecco's Medium plus 10% FBS; IMDM10) and counted. Aliquots of pre-sorted cells along with the positive and negative fractions were stained for the B cell markers B220 (CD45R), CD19, the pan-T cell marker CD3 and the DC marker CD11c to check for purity and DC or T cell contamination. Spleen-derived B cells were pulsed with whole protein overnight and peptide antigen for four hours prior to the addition of splenocytes. The antigens used and their dosages were identical to those described for DCs.

Table 3 Antibody Panel Used for Staining AutoMACS-Isolated B cells

	Panel
FL1	
FL2	CD19 PE 1/200 0.5 µL
FL3	CD3 PE-CF594 1/200 0.5 µL
FL4	CD45R (B220) PerCPCy5.5 1/400 0.25 µL
FL5	
FL6	CD11c APC 1/400 0.25 µL
FL8	
FL9	FVS450*

*FVS450: Dead Cell Exclusion Dye Fixable Viability Stain 450, BD Biosciences

2.8 T cells

2.8.1 Isolation of T cells

The spleens of six to twelve week old OT-I and OT-II mice were isolated asceptically and placed in Dulbecco's Phosphate Buffered Saline (DPBS; Gibco, Paisley, Scotland). In a laminar flow hood, on an ice tray, spleens were placed inside a 70 μM cell filter sitting in a petri dish of DPBS. The plunger from a sterile syringe was used to gently mince the spleens through the sieve. Cell aggregates were dissociated by flushing the cell suspension through the sieve with a 10 mL pipette. The cell suspension was collected in a 50 mL Falcon tube (BD BioSciences) and centrifuged for 7 minutes at 300 x g at 4°C. The supernatant was poured off, the cells resuspended by gentle pipetting and 5 mL of warm ACK RBC lysis buffer (Appendix 2, Recipes) added for 3 minutes to lyse red blood cells (RBCs). Residual cells were washed in DPBS and resuspended in 40 μL of AutoMACS® buffer (Appendix 2, Recipes) per 10^7 cells.

The anti-CD4 and anti-CD8 negative selection kits (Miltenyi Biotec GmbH, Germany), consist of a cocktail of biotin-conjugated monoclonal antibodies against CD8a (for isolating CD4 T cells) or CD4 (for selecting CD8 T cells), CD11b, CD11c, CD19, CD25, CD45R (B220), CD49b (DX5), CD105, anti-MHC Class II, Ter-119, and TCRγ/δ. The antibody cocktail was vortexed and 10 μL of antibodies were added per 10^7 cells. The antibody-labeled cell suspension was briefly vortexed and incubated at 4°C for 5 minutes. AutoMACS buffer was added (30 μL per 10^7 cells), and anti-biotin beads (20 μL per 10^7 cells). The bead/cell suspension was incubated at 4°C for 10 minutes, topped up with AutoMACS buffer to 500 μL per 10^8 cells (if necessary). At this point cells were passed through a large cell column on an AutoMACS Pro Separator (Miltenyi Biotec GmbH, Germany) 'DepleteS' programme. The negative fraction was collected, resuspended in T cell medium (Advanced DMEM/F12+5% FBS, penicillin and streptomycin (P/S; 2 U/mL), β-Mercaptoethanol (2Me; 1%), GlutaMax (1%), HEPES Buffer(1%), and IL-7 (5 ng/mL) and counted.

In 10 day Prime-Boost experiments additional cytokines and growth factors were added as needed for T cell growth, differentiation and survival. These included IL-2

(for the first 24-48 hours only; 2 ng/mL); IL-7, IL-15 and IL-21 all at 100 ng/mL; and 5 ng/mL GM-CSF.

GM-CSF was included in the T cell medium for several reasons. GM-CSF-differentiated DCs become reliant on GM-CSF and die when GM-CSF is removed from the medium therefore we reasoned that retaining low levels of GM-CSF would provide critical DC support to ensure good presentation to T cells. In addition increased Type 1-skewing DC content and activity has been observed following local and systemic GM-CSF administration, which supports a role for GM-CSF as an immune stimulant and vaccine adjuvant in cancer patients. GM-CSF has shown clinical activity as an immune stimulant in tumor cell and dendritic cell vaccines and other clinical cancer treatments[9–15], and may increase antibody-dependent cellular cytotoxicity. It has been noted that the successful use of myeloid-acting cytokines to enhance anti-tumor responses may require the use of GM-CSF in combination with cytotoxic or other targeted therapies[16].

The negative fractions were stained for the surface molecules CD3, CD8 or CD4, CD28, CD27, PD-1, CD44, CD122, CD127, CD62L, CD157 (CTLA-4) and CCR7 to assess the naïve T cell status. Table 4 shows the antibody panels used to identify T cell phenotypes.

Table 4 Antibody Panels Used To Assess T Cell Phenotype

CHANNEL	PANEL 1	PANEL 2	PANEL 3
FL1	CD28-FITC 1/200 0.5 µL	CD44-FITC 1/800 0.125 µL (BD)	CD3-FITC 1/400 0.25 µL
FL2	CD27-PE 1/200 0.25 µL	CD122-PE 1/400 0.25 µL	CCR7-PE 1/400 0.25 µL
FL3			
FL4	PD-1 PerCPCy5.5 1/400 0.25 µL	CD127 PerCPCy5.5 1/400 0.25 µL	
FL5			
FL6	CD3-APC 1/400 0.25 µL	C62L-APC 1/400 0.25 µL	CD152-APC 1/200 0.55 µL
FL7			
FL8	CD8 APC-H71/400 0.25 µL CD4 APC-H7 1/800 0.125 µL	CD8 APC-H71/400 0.25 µL CD4 APC-H7 1/800 0.125 µL	CD8 APC-H71/400 0.25 µL CD4 APC-H7 1/800 0.125 µL
FL9	FVS450	FVS450	FVS450
FL10			

2.8.2 VPD450 Labeling

In initial proliferation assays Violet Proliferation Dye 450 (VPD450; BD Biosciences, San Jose, CA, USA) was used to identify proliferating T cells in lieu of the more widely used Carboxyfluorescein succinimidyl ester (CFSE). The emission wavelength of VPD450 is located at the left end of the emission spectrum (450 nm), enabling more flexibility when choosing remaining fluorophores for the antibody panel. CFSE with its broad emission spectrum peaking around 517 nm is more restrictive, sitting entirely within the FITC channel and overlapping significantly with many mid-range fluorophores.

Freshly isolated CD4+ and CD8+ T cells were resuspended for proliferation dye labelling in DPBS with no FBS. Fluorescent proliferation dye labelling took place in a laminar flow hood with the lights off. We confirmed that the optimal dose of Violet Proliferation Dye 450 (VPD450, BD Biosciences, NJ, USA) was 1 μM VPD450 added undiluted to cells in suspension at between 1 x 10^7 to 2 x 10^7 cells/mL. The dye binds to plastic therefore dilution in DPBS prior to adding to cells is not recommended unless used at a higher concentration such as 10 μM (BD BioSciences Technical Bulletin Jan 2012). One μL of undiluted 1 mM VPD450 stock solution was added per 1 mL of cell suspension for a final VPD450 concentration of 1 μM. The cells were immediately vortexed briefly, wrapped in tin foil and incubated at room temperature on a ground rotator for 5 - 10 minutes. The reaction was quenched by diluting the cells in DPBS (9 x the volume of the cell suspension). Cells were centrifuged (300*g*, 7 mins, 4°C), resuspended in 10 mL medium+10% FBS and centrifuged again (300*g*, 7 mins, 4°C). Cells were resuspended in T cell medium and counted. Cell concentrations were adjusted as appropriate in T cell medium and added to lysate-loaded APCs.

We noted that VPD450-labeled cells had a 'shoulder' of apparent proliferation on untreated cells (Figure 12), which did not correlate with what was observed under the microscope. Elia *et al*[17] showed that under optimal conditions processing cells by Flow Cytometry on Day 0 of VPD labelling generates two peaks in both human peripheral blood mononuclear cells (PBMCs) and mouse splenocytes (Figure 13). At this time point no proliferation has occurred and thus the double peaks are an artefact of VPD labelling. In a similar manner, our VPD-labelled splenocytes did not return a

sharp single peak in untreated cells, as is observed in CFSE labelled cells, making identification of true proliferation difficult. However, by taking a Day 0 sample we could verify that the splenocytes also showed two peaks at this time point and thus true proliferation could be determined. Examples of gating strategies are found in Chapter 4, Figure 38 and Appendix 1, Supplementary Figure 16.

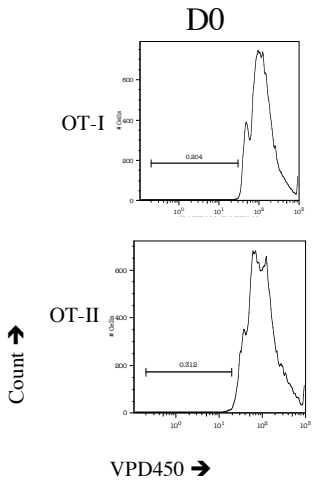


Figure 12 **VPD450-labelled splenocytes display false proliferation peak.** Freshly isolated OT-I and OT-II splenocytes were sorted for CD4+ and CD8+ T cells by antibody and magnetic bead labelling as described above. Cells were labelled with 1 μM VPD450 and analysed within hours on a Gallios Flow Cytometer. Data was analysed and graphed in Flow Jo Version 9. Representative data from twenty independent experiments.

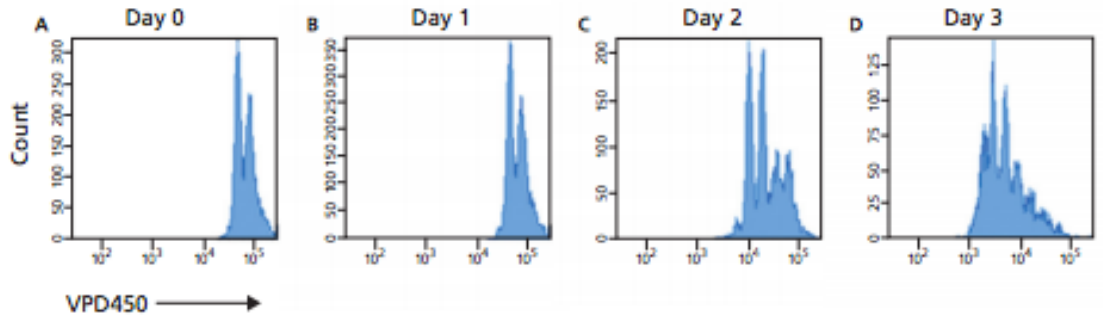


Figure 13 **VPD450-labelled mouse splenocytes have double peak on Day 0.** Mouse splenocytes were enriched for CD4+ cells using a positive panning selection technique. The enriched CD4+ cells were loaded with 1 μM of VPD450, then stimulated with antiCD3ε + CD28 (2 μg/mL) antibodies for three days. Cells were harvested at each day of culture including an aliquot for a day 0 time point to determine how the cell population loaded with the VPD450 dye. A = day 0 load, B = day 1 time point, C = day 2 time point, and D = day 3 time point. The cell population did not load as a single peak at day 0 (A). If this control was not present, it would be assumed that the cells had completed one round of division at day 1 (B). At day 2 (C), the cell population started to divide, which is evident by the three additional peaks, and at day 3 (D), the cell population had completed five rounds of division. The day 0 load is very important for recording the baseline VPD450 load. Reproduced with permission from BD Biosciences Technical Bulletin, Jan 2012.

2.8.3 CFSE Labeling of T cells

Owing to the Day 0 double peak issue with VPD450 we elected to use carboxyfluorescein succinimidyl ester (CFSE; Life Technologies, Vybrant CFDA SE Cell Tracer Kit, V12883, Thermo Fisher, MA, USA) in later proliferation experiments. Working in a hood with the light off we resuspended cells at 5 x 10^7 cells/mL. A 10 μM (2X) CFSE solution was prepared by adding 1 μL of 10 mM stock to 1 mL DPBS. This solution was added to an equal volume of cell suspension resulting in a final concentration of 5 μM CFSE. The tube was immediately vortexed briefly, wrapped in foil and incubated for 5 minutes at room temperature on a ground rotator to ensure even staining. The cells were washed twice by diluting in ten volumes of room temperature DPBS+5% FBS followed by sedimentation by centrifugation (300g, 5 min, 20°C). Labelled cells were resuspended in T cell medium (Advanced DMEM+5% FBS+HEPES (1%)+GlutaMax (1%) +/- IL-2 (5 ng/mL) +/- α-CD28 (2 μg/mL) +/- IL-7, IL-15, IL-21 (all 100 ng/mL) at 10 – 20 x 10^6 cells/mL for plating with APCs.

2.8.4 T Cell-APC Co-Cultures

At least four hours after the addition of peptide antigen OT-I and OT-II splenocytes dyed with violet proliferation dye 450 (VPD450) or CFSE were added to the lysate-, OVA- or peptide-loaded APCs at a ratio of 10 T cells to 1 APC. In 72-hour proliferation assays 5.0 x 10^4 single APCs were cultured with 0.5 x 10^6 T cells. In double APC combinations 2.5 x 10^4 of each APC were added to each well for antigen loading. In triple APC combination wells, 1.67 x 10^4 of each APC were added to each well for antigen loading. This was achieved by adding 100 μL of an individual APC, 50 μL per two APCs or 34 μL per three APC cell suspensions to each well.

The APCs and T cells were co-cultured for 72 hours and the CD3+CD8+ or CD3+CD4+ T cell proliferation response measured by Flow Cytometry. Co-culture supernatants were also harvested at the 24, 48 and 72-hour time points for cytokine analysis by Enzyme Linked Immunosorbent Assay (ELISA) (IFN-γ, TNF-α, IL-2 and IL-12). Some co-culture supernatants were also assayed by Legendplex Flow Cytometric Bead array (BioLegend, San Diego, CA, USA).

In 10-day Prime-Boost Assays 5.0 x 10^4 APCs and 0.5 x 10^6 T cells or 1.0 x 10^5 APCs and 1 x 10^6 T cells were added to each starting co-culture. Cells were fed with fresh media and cytokines no less than every 2 days, as described in Section 2.8.1.

2.9 Flow Cytometry: Cell Phenotype and Proliferation Analysis

Early experiments of T cell proliferation and membrane or intracellular expression of molecules were analysed on an LSR Fortessa (BD Immunocytometry Systems, San Jose, CA, USA), equipped with 405 nm, 488 nm, 561 nm and 640 nm excitation lasers located in the Flow Facility of the Department of Microbiology and Immunology, Otago University, Dunedin New Zealand.

However the vast majority of experiments were processed on a Gallios instrument (Beckman Coulter) containing 405 nm, 488 nm and 640 nm excitation lasers located in the Pathology Department of the Dunedin School of Medicine, Otago University, Dunedin New Zealand. Data were analysed using FlowJo software versions 9 and 10 (TreeStar, Ashland, OR, USA).

Antibodies were purchased from Becton Dickinson (BD) Biosciences (Franklin Lakes, NJ, USA) and BioLegend (San Diego, CA, USA). Unstained, single-fluorophore-stained cells and Fluorescence Minus One (FMO) controls were used to determine negative and positive populations.

Cells were washed once in room temperature PBS prior to viability staining in live cell exclusion dye (room temperature, 15 mins). Cells were then washed with ice cold FACS Staining Buffer (DPBS, 2% fetal bovine serum and 0.1% sodium azide), pelleted, vortexed prior to the addition of optimally titrated antibodies to the residual liquid (80-100 μL). The antibodies used for surface staining of DCs and MΦs are listed in Table 2. The antibodies used for surface staining of B cells are listed in Table 3. The antibodies used for surface staining of T cells are listed in Table 4.

After 15 minutes incubation at 4°C in the dark, cells were washed twice in FACS Buffer, fixed with 4% paraformaldehyde, stored at 4°C and usually analyzed the following day, or within a week at the most. Alternatively, for intracellular staining, following surface staining cells were fixed and permeabilised (FoxP3 Fix/Perm Buffer

set, BioLegend, San Diego, CA, USA) and incubated with intracellular mABs for 20-30 minutes at 4°C in the dark. Cells were stored at 4°C and analysed the next day. The number of events acquired was 3×10^4 to 1×10^5 .

Identification of responding T cells was achieved by labelling APC-splenocyte co-cultures with antibodies to the pan-T cell marker CD3, CD4 (in OT-II splenocytes) and CD8 (in OT-I splenocytes). T cell responses were assessed by analysing proliferation as determined using Flow Cytometry of VPD450- (BD) or CFSE- (Life Technologies) stained T cells.

Cells were stained with the antibodies shown in Table 5 as appropriate. Dilutions were used to ascertain the optimal AB titration, as is common practice in many labs. Thus, while AB concentrations are not stated, the company that supplied the AB, along with the catalogue number and clone have been supplied, therefore the concentration may be calculated by future investigators.

Table 5 Titrations of fluorophore-conjugated antibodies used in Flow Cytometric analysis of antigen-specific T-cell proliferation, phenotype functionality and memory.

ANTIBODY	FLUOROPHORE	TITRATION	CHANNEL	VOLTAGE	GAIN
CD3	FITC	1/400	FL1	395	1.0
CD8α	PE-CY7	1/400	FL5	625	2.0
CD8α	APC	1/200	FL6	475	2.0
CD8α	APC-H7	1/400	FL8	450	2.0
CD4	PE-Cy7	1/800	FL5	625	2.0
CD4	APC	1/800	FL6	475	2.0
CD4	APC-H7	1/800	FL8	450	2.0
CD28	FITC	1/100	FL1	395	1.0
CD44	FITC	1/100	FL1	395	1.0
CD27	PE	1/200	FL2	375	2.0
CD122	PE	1/200	FL2	375	2.0
CCR7	PE	1/400	PE	375	2.0
PD-1	PerCPCy5.5	1/400	FL4	550	2.0
CD127	PerCPCy5.5	1/400	FL4	550	2.0
CD69	APC	1/200	FL6	475	2.0
CD62L	APC	1/200	FL6	475	2.0
CD152	APC	1/200	FL6	475	2.0
Viability	FVS450	1 μL/mL	FL9	300	1.0

Figure 14 shows the gating strategies used in DC-T cell proliferation experiments.

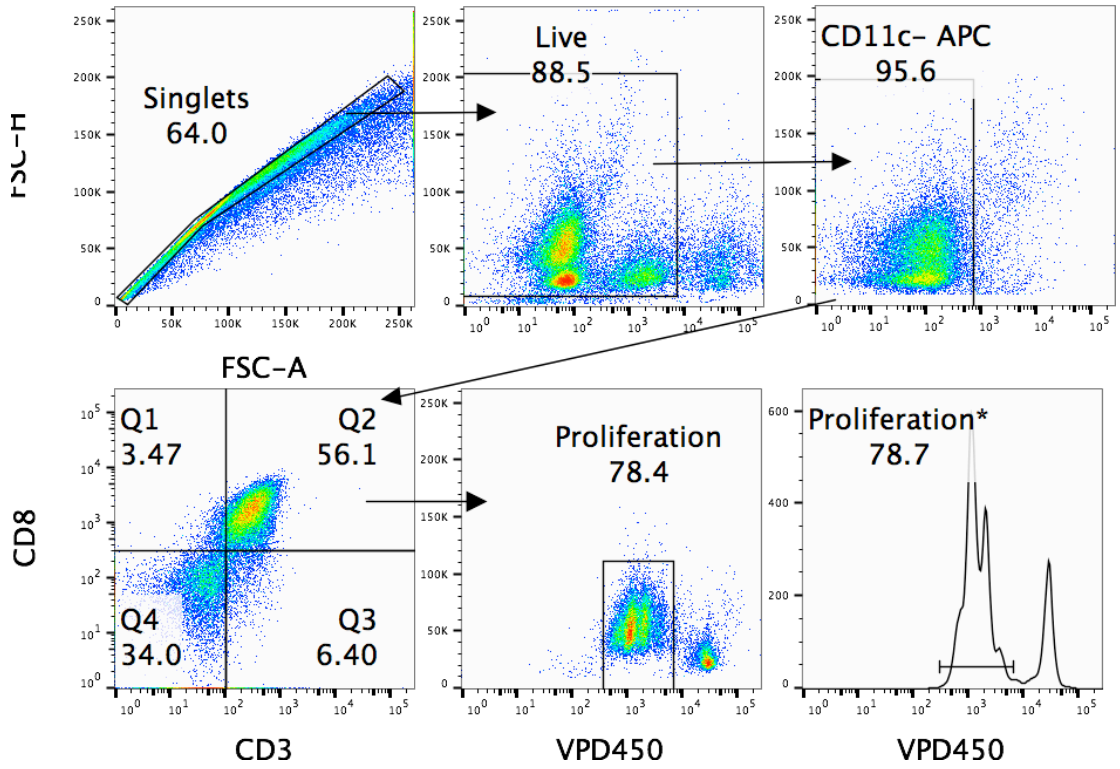


Figure 14 **Gating strategy used in DC-T cell proliferation co-cultures.** Day 6 bone marrow-derived GMDCs were prepared as previously described. GMDCs were loaded overnight with lysates (1:1 tumour cell:APC) or whole OVA protein (50 μ g/mL). The following morning the irrelevant melanoma peptide gp100, SIINFEKL peptide or $OVA_{323-339}$ peptide (all 2 μ g/mL) were added at least 4 hours prior to the addition of VPD450-labelled OT-I or OT-II splenocytes. 72 hours later cells were harvested, stained with dead cell exclusion dye, and labelled with mABs against CD11c (Dump channel), CD3, CD8α and collected by LSR Fortessa or Gallios Flow Cytometer. Data was analysed on FlowJo Version 9 or X. Proliferation was expressed as percentage of cells that had reduced fluorescence compared with untreated cells. Sample data of numerous independent experiments.

Figure 15 shows the gating strategies used in MΦ-T cell proliferation experiments. Doublets were first gated out off the forward scatter height (FSC-H) versus side scatter area (SSC-A) plot. Dump channels were used to gate out dead cells (viability dye positive cells) as well as any APCs (CD11c and CD19 in DC and B cell co-cultures respectively) that may have been harvested along with the T cells. Due to the highly adherent nature of MΦs gating out CD11c+ cells was unnecessary in MΦ-T cell co-cultures. Finally the cells in the CD3+CD8+, or CD3+CD4+ double positive quadrants were selected and analysed for VPD+ or CFSE+ proliferation. CD19+ B cell and DC gating strategies are shown in Appendix 1, Supplementary Figure 16 and Chapter 3, Figure 25.

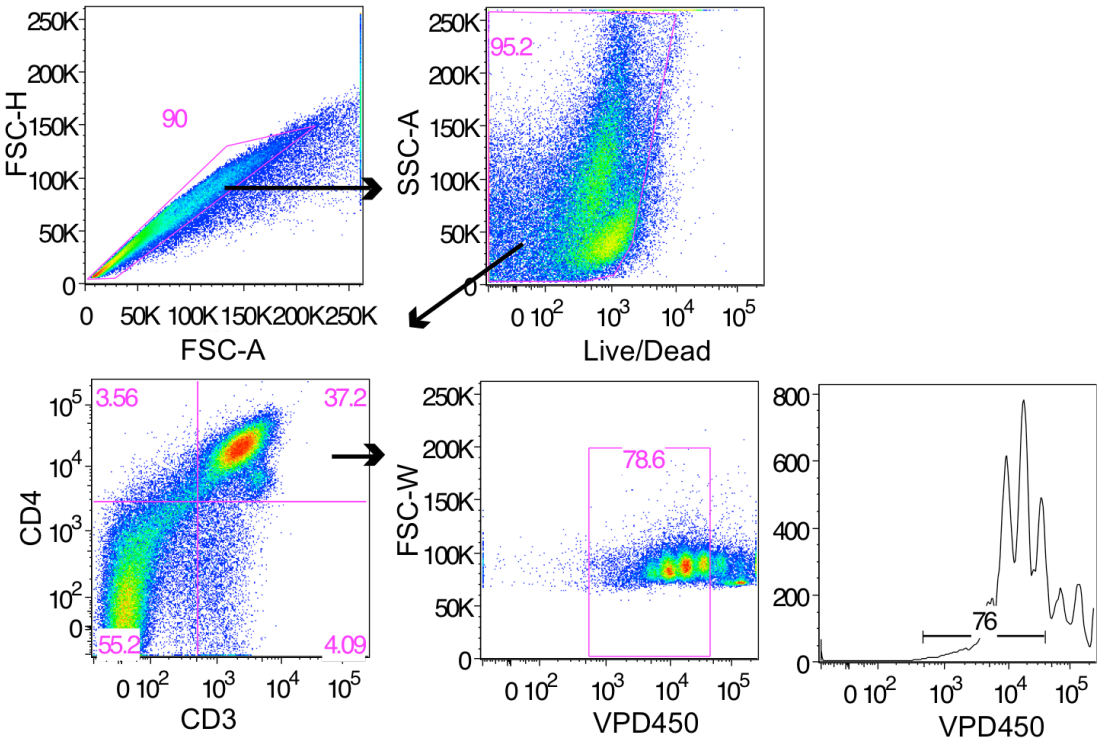


Figure 15 **Gating strategy used in macrophage-T cell proliferation co-cultures**. Day10 bone marrow derived MΦs were prepared as previously described. MΦs were loaded overnight with lysates (1:1 tumour cell:APC) or whole OVA protein (50 μg/mL). The following morning the irrelevant melanoma peptide gp100, SIINFEKL peptide or $OVA_{323-339}$ peptide (all 2 μg/mL) were added at least 4 hours prior to the addition of VPD450-labeled OT-I or OT-II splenocytes. 72 hours later cells were harvested, stained with dead cell exclusion dye, and labelled with mABs against CD3, CD8α/CD4 and collected by LSR Fortessa or Gallios Flow Cytometer. Data was analysed on FlowJo Version 9 or X. Proliferation was expressed as percentage of cells that had reduced fluorescence compared with untreated cells. Sample data of numerous independent experiments.

Table 5 lists information on fluorophore-conjugated antibodies used in various experiments in this thesis. Details of individual staining panels are provided in figure legends or in associated supplementary figures. Fluorescence Minus One (FMO) controls were used for gating analyses to identify issues of spectral overlap. Compensation was calculated using unstained and single colour stained cells or CompBeads. Post-acquisition compensation matrices were calculated and applied and data analysis performed using FlowJo version 9.4.10 and version X (Tree Star, Ashland, OR, USA). Gates were set by comparing unstained cells, single colour stained cells and fully-stained cell populations.

Table 6 Fluorophore-Conjugated Antibody Information

ANTIBODY SPECIFICITY	FLUOROPHORE	PURPOSE	SUPPLIER	Catalogue Number	Concentration (μg/mL)
Dendritic Cells & Macrophages					
Anti-mouse I-A/I-E (MHC-II) Clone: M5/114.15.2	FITC	Activation	BioLegend	107606	0.5
Hamster anti-mouse CD11c Clone: HL3	FITC	Phenotype	BD Biosciences, Pharmingen	561045	0.5
Anti-mouse CD135 Clone A2F10	PE	Phenotype	BioLegend	135306	0.2
Anti-mouse CD169 (Siglec-1) Clone: 3D6.112	PE	Phenotype	BioLegend	142403	0.2
Anti-mouse F4/80 Clone: BM8	PE	Phenotype	BioLegend	1123110	0.2
Anti-mouse CD115 Clone: 53-6.7	PE	Phenotype	BioLegend	135505	0.2
Rat Anti-Mouse CD8α Clone: 53-6.7	PE-CY7	Phenotype	BD Biosciences, Pharmingen	552877	0.2
Anti-mouse CD86 Clone: GL-1	PE-Cy7	Activation	BioLegend	105014	0.2
Anti-mouse CD11c Clone: N418	APC	Phenotype	BioLegend	117310	0.2
Anti-mouse CD40 Clone: 3/23	APC	Activation	BioLegend	124612	0.2
Anti-mouse F4/80 Clone: BM8	APC	Phenotype	BioLegend	123115	0.2
Anti-mouse CD11c Clone: N418	BV421	Phenotype	BioLegend	117343	0.2
Anti-mouse CD80 Clone: 16-10A1	BV421	Activation	BioLegend	104725	0.1
Anti-mouse CD206 Clone: 068C2	BV421	Phenotype	BioLegend	141717	0.1
T cells					
Anti-mouse CD3 Clone: 17A2	FITC	Phenotype	BioLegend	100204	0.5
Anti-mouse CD28 Clone: E18	FITC	Naive	BioLegend	122008	0.5
Anti-mouse CD44 Clone: IM7	FITC	Memory	BioLegend	1003006	0.5
Rat anti-mouse CD44 Clone: IM7	FITC	Memory	BD Biosciences, Pharmingen	553133	0.5
Rat anti-mouse CD27 Clone: LG.3A10	PE	Memory	BD Biosciences, Pharmingen	558754	0.2
Rat anti-mouse CD122 Clone: TM-β1	PE	Memory	BD Biosciences, Pharmingen	55362	0.2
Rat anti-mouse CCR7 Clone: 4B12	PE	Memory	BD Biosciences, Pharmingen	560682	0.2
Hamster Anti-Mouse CD3e; Clone: 145-2C11	PE-CF594	Phenotype	BD Biosciences, Pharmingen	562286	0.2
Anti-mouse CD127 Clone: SB/199	PerCPCy5.5	Memory	BioLegend	121113	0.2
Anti-mouse PD-1 Clone: 29F.1A12	PerCPCy5.5	Exhaustion	BioLegend	135208	0.2
Rat Anti-Mouse CD8α Clone: 53-6.7	PE-CY7	Phenotype	BD Biosciences, Pharmingen	552877	0.2
Rat Anti-Mouse CD4 Clone: RM4-5	PE-Cy7	Phenotype	BD Biosciences, Pharmingen	552775	0.2
Anti-mouse CD3	APC	Phenotype	BioLegend	100236	0.2

Clone: 17A2					
Anti-mouse CD62L Clone: MEL14	APC	Naïve/Memory	BioLegend	104411	0.2
Anti-mouse CD152 (CTLA-4) Clone:	APC	Activation	BioLegend	106310	0.2
Anti-mouse CD4 Clone: 6K1.5	APC-H7	Phenotype	BD Biosciences	560181	0.2
Anti-mouse CD8 Clone: 53-6.7	APC-H7	Phenotype	BD Biosciences	560182	0.2
Anti-mouse IL-10 Clone: JES5-16E3	BV-421	T_H2	BioLegend	505021	0.2
Rat anti-mouse IFN-γ Clone: XMG1.2	AF700	T_H1	BD Biosciences	557998	0.2
Cell Division	Violet Cell proliferation Dye (VPD450)	Proliferation	BD Horizon™	562158	n/a
Cell Division	Vybrant® CFDA SE Cell Tracer Kit (CFSE)	Proliferation	Life Technologies	V12883	n/a

B Cells					
Rat anti-mouse/human CD45R/B220 Clone: RA3-6B2	FITC	Phenotype	BD Biosciences, Pharmingen	561877	0.5
Anti-mouse CD19 Clone: 6D5	PE	Phenotype	BioLegend	115507	0.2
Anti-mouse/human CD45R/B220 Clone: RA3-6B2	PerCP/Cy5.5	Phenotype	BioLegend	103236	0.2
Anti-mouse/human CD11c Clone: N418	APC	Isolation Purity	BioLegend	117310	0.2
Hamster Anti-Mouse CD3e; Clone: 145-2C11	PE-CF594	Isolation Purity	BD Biosciences, Pharmingen	562286	0.2

CELL VIABILITY				
LIVE/DEAD® Fixable Yellow Dead Cell Stain Kit	Yellow fluorescent reactive dye	Dead Cell Exclusion	Invitrogen Molecular Probes	L-34959
LIVE/DEAD® Fixable Red Dead Cell Stain Kit	Near IR red-fluorescent reactive dye	Dead Cell Exclusion	Invitrogen Molecular Probes	L-23102
LIVE/DEAD® Fixable Violet Dead Cell Stain Kit	Violet fluorescent reactive dye	Dead Cell Exclusion	Invitrogen Molecular Probes	L-34955
Sytox Green Nucleic Acid Stain	Green fluorescent nuclear and chromosome stain	Dead Cell Exclusion	Molecular Probes, Life Technologies, Thermo Fisher Scientific	S7020
Fixable Viability Stain 450 (FVS450)	Violet fluorescent reactive dye	Dead Cell Exclusion	BD Horizon™	562247

Figure 16 shows a Fluorescence Minus One (FMO) layout for the panel used for T cell phenotyping. Three panels were used with the same fluorophores attached to mABs specific for different molecules. This FMO was used to identify any issues of spectral overlap within the panel. This FMO identified that PE was contributing significantly into the FITC channel and that the PerCPCy5.5 signal was not strong enough. After voltages were adjusted these issues were eliminated (see Figure 17).

Figure shows the compensation matrix for the corrected panel along with the unstained and singly-stained cells used to generate the compensation matrix. Table 6 shows the voltages used in these experiments.

Finally, intracellular staining for IDO was carried out on lysate-loaded APCs. APCs were prepared as previously described and loaded with lysates+LPS&CpG overnight. Twenty-four hours later cells Brefeldin A was added to the cells for 3 hours prior to harvest. Cells were centrifuged in plates (3 mins, 300 x *g*, 4°C), the media aspirated and cells incubated in Accutase Cell Detachment Solution for 10 minutes (Sigma Aldrich). Cells were centrifuged in plates (3 mins, 300 x *g*, 4°C), the Accutase discarded and the cells resuspended in DPBS for transfer to FACS tubes. Cells were centrifuged (3 mins, 300 x *g*, 4°C), the excess DPBS discarded and the cells stained with FVS450 dead cell exclusion dye (BD; 1 µL/mL, 15 mins, 4°C). Cells were washed in FACS buffer and stained for surface CD11c-FITC (15 mins, 4°C). Cells were washed twice in FACS buffer and fixed with FoxP3 Fix/Perm buffer (BioLegend) (500 µL 1X Fix Buffer per tube, 20 mins, 4°C). Cells were washed once with FACS buffer and permeabilised with 1 X FoxP3 Perm Buffer (BioLegend) (500 µL per tube; 15 mins, 4°C). Permeabilisation of cells renders cells more buoyant so post-permeabilisation wash step centrifugations were at 700 x *g* (3 mins, 4°C). Following a single centrifugation cells were resuspended in 1 X FoxP3 Perm Buffer and the intracellular mAB (anti-IDO1 Antibody conjugated to Alexa Fluor® 647; BioLegend; 654003) was added to the residual cell suspension in the tube i.e. 80-100 µL (30 mins, 4°C). Following incubation 500 µL of FACS buffer was added to each tube and the cells washed twice (700 x *g*, 3 mins, 4°C). Stained cells were resuspended in 100 µL of FACS buffer for acquisition on a Gallios Flow Cytometer.

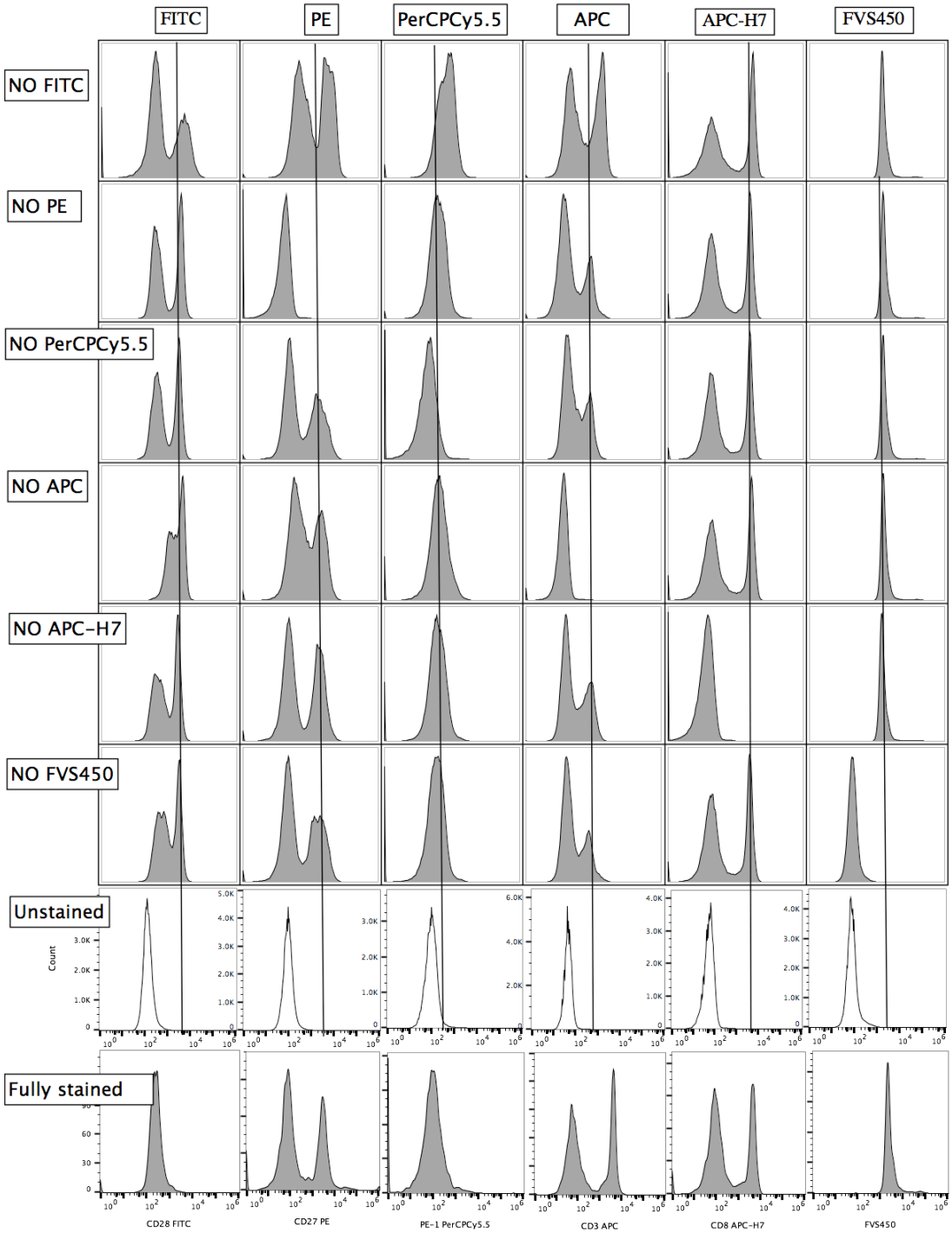
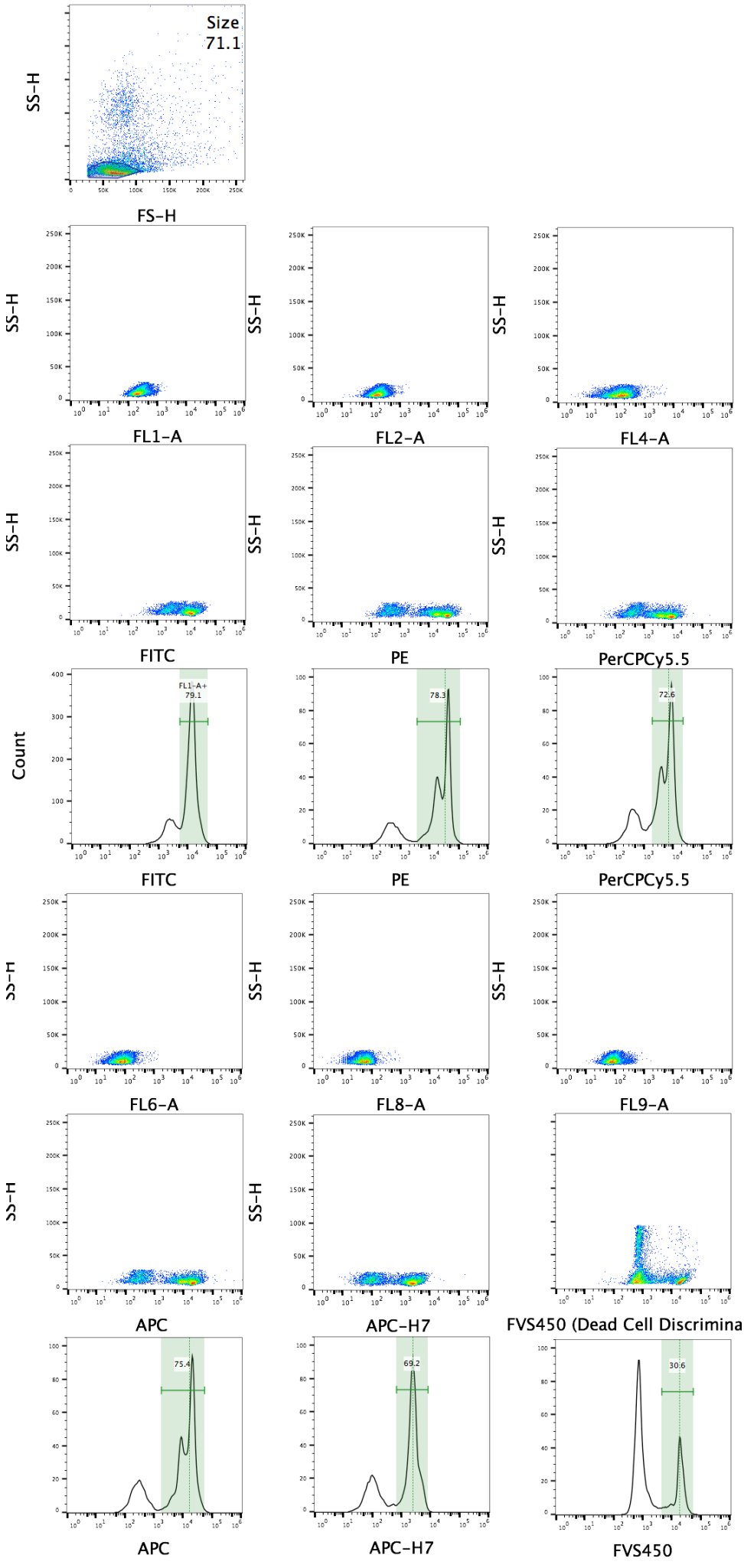
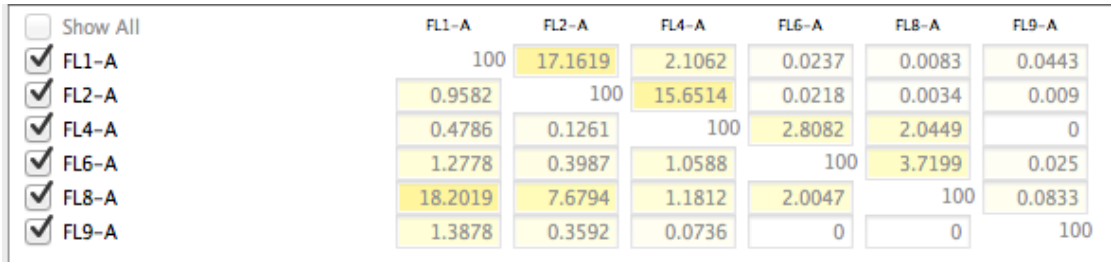


Figure 16 **Fluorescence Minus One (FMO) for T cell phenotype panel.** T cells were isolated from OT mice by magnetic bead separation as previously described. 0.5 x 10^6 T cells were labeled with Fixable Viability Stain 450 (FVS450) followed by surface staining for FITC, PE, PerCPCy5.5, APC, APC-H7, minus one mAB-conjugated fluorophore per sample. Cells were fixed in 4% paraformaldehyde, stored overnight at 8°C and acquired the following day on a Gallios Flow Cytometer. 100,000 events were collected per sample. Data was analysed and graphed in FlowJo Version X.


Size
71.1
SS-H
FS-H
FL1-A
FL2-A
FL4-A
FITC
PE
PerCPCy5.5
Count
FL1-A+
79.1
78.3
72.6
FL6-A
FL8-A
FL9-A
APC
APC-H7
FVS450 (Dead Cell Discrimina
75.4
69.2
30.6
FVS450



Show All	FL1-A	FL2-A	FL4-A	FL6-A	FL8-A	FL9-A
FL1-A	100	17.1619	2.1062	0.0237	0.0083	0.0443
FL2-A	0.9582	100	15.6514	0.0218	0.0034	0.009
FL4-A	0.4786	0.1261	100	2.8082	2.0449	0
FL6-A	1.2778	0.3987	1.0588	100	3.7199	0.025
FL8-A	18.2019	7.6794	1.1812	2.0047	100	0.0833
FL9-A	1.3878	0.3592	0.0736	0	0	100

Figure 17 **Compensation matrix and unstained and single stained controls used to set compensation in T cell phenotyping panels.** T cells were isolated from OT mice by magnetic bead separation as previously described. 0.5 x 10^6 T cells were labeled with Fixable Viability Stain 450 (FVS450) followed by surface staining for FITC, PE, PerCPCy5.5, APC and APC-H7. Cells were fixed in 4% paraformaldehyde, stored overnight at 8°C and acquired the following day on a Gallios Flow Cytometer. 100,000 events were collected per sample. Data was analysed and graphed in FlowJo Version X.

Table 7 Voltages used in T cell phenotyping panel

CHANNEL	FLUOROPHORE	VOLTAGE	GAIN
FSC		250	5.0
SSC		300	10.0
FL1	FITC	395	1.0
FL2	PE	375	2.0
FL4	PerCPCy5.5	550	2.0
FL6	APC	475	2.0
FL8	APC-H7	450	2.0
FL9	FVS450	300	1.0

2.10 Cytokine Measurement: ELISA

Enzyme-linked immunosorbent assay (ELISA) was utilised to assess T cell functionality. Levels of the cytokines interferon gamma (IFN-γ), tumor necrosing factor alpha (TNF-α), interleukin-10 (IL-10) and interleukin-12 (IL-12) were measured in lysate-loaded APC or T cell-APC co-culture supernatants (cell-conditioned media) after 24 – 72 hours, as appropriate.

The amount of IL-12, IL-10, IFN-γ or TNF-α was assayed by ELISA using Nunc-Immuno Maxisorp 96 well plates (Thermo Fisher Scientific, Denmark), purified anti-mouse IL-12, IFN-γ, TNF-α and IL-10 (all from BioLegend); recombinant mouse IL-12, IFN-γ, TNF-α and IL-10; and biotinylated anti-mouse IL-12, IFN-γ, TNF-α and IL-10 (all from BioLegend). The ELISA antibody pairs are listed in Table 8.

Table 8 ELISA Antibody Pairs

Purified Cytokine Standard	Primary Antibody	Secondary Antibody	Supplier	Catalogue Number
IFN-γ	Purified anti-mouse IFN-γ		BD Biosciences	551216
		Biotin anti-mouse	BD Biosciences	55410
TNF-α	Purified anti-mouse TNF-α; clone: MP6-XT22		BioLegend	506302
		Biotin anti-mouse TNF-α; clone: MP6-XT22	BioLegend	506312
IL-10	Purified anti-mouse IL-10; clone: JES5-16E3		BioLegend	505002
		Biotin anti-mouse IL-10; clone: JES5-16E3	BioLegend	504906
IL-12	Purified anti-mouse IL-12 p70		BD Biosciences	554658
		Biotin anti-mouse IL-12 p70/p40	BD Biosciences	554476
Streptavidin-HRP			BioLegend	405210

Plates were coated with 50 µL of purified cytokine diluted in the appropriate coating buffer (Appendix 2, Recipes). Plates were incubated for 2 hrs at 37°C or overnight at 4°C. Plates were washed six times in freshly made wash buffer (Appendix 2, Recipes), excess liquid removed by tapping upside down on paper towels, and blocked with 200 µL blocking buffer (Appendix 2, Recipes), for 1 hour at 37°C. Plates were washed six times and blotted. Standard curves were generated on each plate by doubling dilutions of 100 µL of purified cytokine standards. Standards and samples were plated in duplicate and incubated for 2 hrs at 37°C or overnight at 4°C. Plates were washed six times and blotted. The appropriate biotinylated rat anti-mouse antibody was diluted in blocking buffer, 100 µL added to the plates, and incubated at 37°C for no longer than 30 minutes. Plates were washed six times and blotted. Streptavidin-HRP (BioLegend) was diluted 1/3000 in blocking buffer. 100 µL was added to each well and the plates incubated at 37°C for 20 minutes. Plates were washed six times and blotted. 3,3',5,5'-tetramethylbenzidine (TMB) soluble substrate (Novex, Life Technologies, Cat # 00-2023) was added to each well. TMB yields a blue color when enzymatically digesting HRP. With the addition of sulfuric acid the reaction is stopped and the color changes to yellow with maximum absorbance at 450 nm. 10N H2SO4 (FIXANAL, Fluka Analytical, Sigma-Aldrich, Chemie GmbH, Steinheim, Germany; cat: 38294; sulfuric acid 0.5 mol) was diluted 1/10 with dH2O

and 100 µL of 1N H2SO4 added to each well. Absorbance at 450 nm was read within 30 minutes on a BioTek Synergy 2 Microplate Reader (Thermo Fisher Scientific).

2.11 T cell *In Vitro* Cytotoxicity: VITAL Assay

The VITAL assay was developed by Hermans *et al*[18] as a fluorometric alternative to the use of radioactive isotopes for the measurement of cytotoxic capability against target cells. Survival of fluorescent targets can be measured as a readout of an effector cells' killing ability.

Effector cells were generated as described above by co-culturing T cells with lysate-loaded APCs for 72 hours. B16.OVA melanoma target cells were labelled with CFSE as described above under CFSE labelling of T cells. CFSE-labelled melanoma cells were added to 96 well flat-bottom plates (2 e 5 cells/well) and incubated for 2 hours at 37°C+5% CO_2 to allow a monolayer to form.

Effector T cells were added in different effector to target ratios, in triplicate. Cells were incubated for 6 – 8 hours at 37°C+5% CO_2. Following incubation cells were harvested and propidium iodide (PI; 5 µg/mL) added to each tube. Cells were collected by Flow Cytometry, analysed by gating on CFSE+PI+ cells and calculating the survival of target cells relative to antigen-negative controls. The percentage of specific lysis was calculated as follows:

1. Adjusted % survival = 100 x % survival/Mean % survival in absence of effectors
2. % specific lysis = (100 – adjusted % survival)

We were concerned that due to the large number of samples the use of PI may yield a falsely high level of cytotoxicity due to the length of time cells remained in PI prior to processing. We therefore modified this protocol by staining cells with a live/dead discrimination dye followed by fixing the cells in 2-4% paraformaldehyde prior to harvesting into tubes for FACS analysis. This allowed us to harvest cells and store them overnight at 4°C for processing the next day rather than analysing them immediately as is required for PI staining (as per R&D Systems "Flow Cytometry Protocol for Analysis of Cell Viability using Propidium Iodide").

2.12 *In Vivo* Cytotoxicity

These experiments were approved by the Animal Ethics Committee, University of Otago and conducted under Animal Ethics approval number AEC59/15. Figure 18 outlines the timeline of these assays. APCs and T cells were generated as previously described in this chapter. GMDCs +/- B cells were loaded with lysate and activated with LPS and CpG. The following day CD4+ OT-II and CD8+ OT-I T cells were isolated and equal numbers of CD4+ and CD8+ T cells added to the lysate-loaded APCs at a ratio of 10 T cells to 1 APC. At Day 3 or 4, effector T cells were harvested, washed twice in sterile DPBS, counted and resuspended at 1 x 10^7 cells/mL. Effector cells were adoptively transferred into C57BL/6 female mice via i.v. injection into the tail vein (200 µL per mouse).

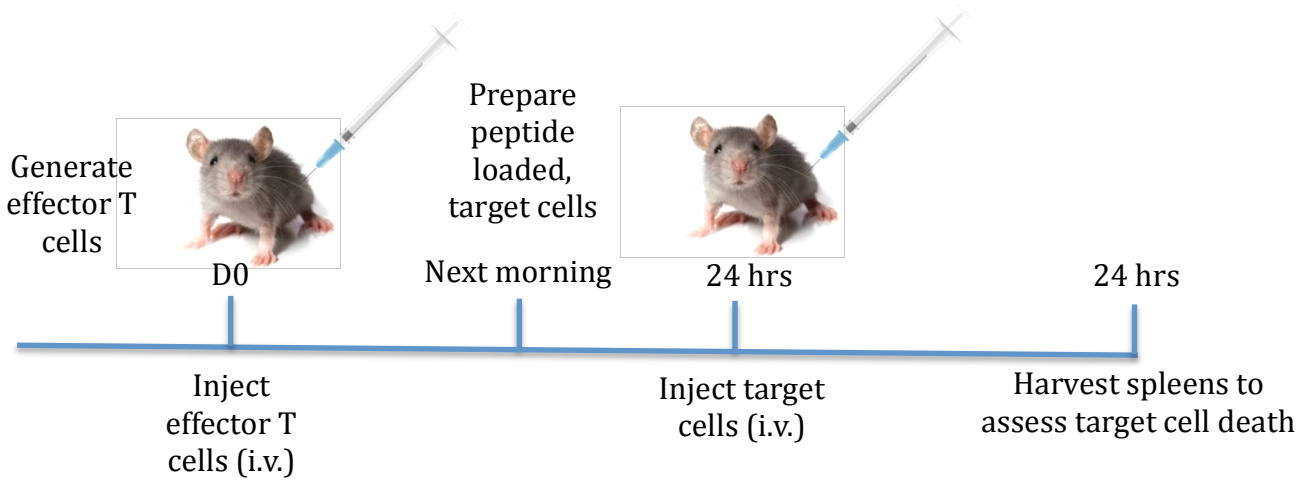


Figure 18 **Schematic of *in vivo* cytotoxicity assay timeline.** GMDC and B cells were prepared as previously described loaded with lysates+LPS&CpG and co-cultured with T cells to generate effector cells. 2 x 10^6 effectors per mouse were i.v. injected in 200 µL of PBS. The following morning peptide loaded and unpulsed target cells and were prepared and injected into the effector-bearing mice (3.3 x 10^6 per target type per mouse). 24-hours later mice were sacrificed and splenocytes prepared into single cell suspensions. Cells were stained with dead cell exclusion dye, fixed in 4% PFA and stored overnight at 4°C. 1 x 10^6 cells per mouse were acquired the following day on a Gallios Flow Cytometer and the data analysed to assess target cell killing.

The following morning target cells were prepared. Splenocytes isolated from C57BL/6 mice were divided into three tubes and pulsed with 1 µg/ml $OVA_{257-264}$ (SIINFEKL) peptide or 5 µg/ml $OVA_{323-339}$ peptide, or left unpulsed as a control against non-specific killing. Peptide loaded cells were incubated at 37°C for 3 hours, washed three times in DPBS and the concentrations adjusted to 5 x 10^7 c/mL for CFSE and VPD450 labeling. SIINFEKL pulsed cells were stained with 25 µM CFSE ($CFSE^{HI}$) and $OVA_{323-339}$ peptide-pulsed cells were stained with 2.5 µM CFSE ($CFSE^{LO}$) to allow identification of which targets were killed. Unpulsed cells were

stained with 10 μM VPD450. CFSE and VPD450 labeling was carried out as described previously in this chapter.

Dye-stained cell concentrations were adjusted to 50 x 10^6 c/mL in DPBS. Equal volumes of the 50 x 10^6 c/mL target cell types (unpulsed, SIIN-pulsed & $OVA_{323-339}$-pulsed) were mixed together and 200 μL (10 e 6 target cells; 3.33 x 10^6 per target type per mouse) was drawn into sterile syringes and injected into the tail vein of recipient mice.

Mice were sacrificed 17-24 hrs after adoptive transfer of target cells. Spleens were isolated and splenocyte single cell suspensions prepared as described previously in this chapter for OT splenocytes. Splenocytes were labelled with the dead cell exclusion dye Live/Dead Near InfraRed (Life Technologies), fixed with 4% paraformaldehyde, stored overnight at 4°C and analysed the following day on a Gallios Flow Cytometer (Beckman Coulter). Table 9 lists the dyes and mABs used to label cells in these assays. Figure 62 in Chapter 5 shows the gating strategy and sample data from these experiments.

The percentage lysis of target cells was calculated using the formula:

$$\% \text{ Lysis} = 100 - (\text{number of peptide-pulsed} \div \text{number of unpulsed})_{,}$$

Table 9 Dyes and monoclonal antibodies used in *in vivo* cytotoxicity assays

	In Vivo Cytotoxicity Panel
FL1	$CFSE^{HI}$ and $CFSE^{LO}$
FL2	PE CD19, CD11c (Dump Channel) 1/400 0.25 uL
FL3	
FL4	PerCPCy5.5 CD3 1/400 0.25 uL
FL5	
FL6	APC CD8 1/400 0.25 uL or APC CD4 1/800 0.125 uL
FL7	
FL8	Live/Dead Near Infrared
FL9	$VPD450^{unpulsed\ cell}$
FL10	

2.13 *Ex Vivo* Proliferation and Cytokine Analysis

Wild type C57BL/6 mice were injected with 50,000 B16.OVA cells and sacrificed when tumours reached 150 mm^2. Tumour draining lymph nodes were excised and, in a laminar flow hood, gently pressed through a 70 μM cell sieve to create a single cell

suspension. Cells were washed twice in DPBS (300 x *g*, 5 mins, 4°C) and resuspended in freezing mix (50% medium, 40% FBS, 10% DMSO). Single cell suspensions were place in pre-cooled "Frosty Boys" and cryopreserved in liquid nitrogen for future use. Cells were retrieved from liquid nitrogen, quickly thawed in a 37°C water bath until a small lump of ice remained in the vial. Cells were washed twice in room temperature fresh media, plated in flasks and allowed to rest overnight. The following day tumour dLN cells were added to lysate-loaded APCs at a ratio of 10:1, +/- α-PD-1 and α-CTLA-4 as indicated in the appropriate figure legends. Cells were cultured for 10 days and fed no less than every other day. The media included IL-2 (2 ng/mL); 24-48 hours only); IL-7, IL-15 and IL-21 (all 100 ng/mL). Anti-CD28 was also included in the medium in an attempt to augment the tumour-specific T cells' fold expansion. Cell conditioned media were harvested, stored at -20° and analysed by anti-IFN-γ and anti-IL-12 ELISA.

2.14 Statistical Analyses

Analyses were performed and graphs created in Graph Pad Prism (GraphPad Software Inc, San Diego, CA). A significance level of 0.05 or less was considered statistically significant.

The Mann-Whitney U test was used for comparisons of two groups. The Kruskal-Wallis non-parametric comparison of location was applied for comparisons of three or more groups. This test was chosen based on the assumption that data was not likely to be normally distributed considering the group sizes. The measures were calculated as independent not paired since some samples had different numbers of data points. Dunn's test for multiple comparisons, with Bonferroni correction, was applied *post-hoc* to the rank sums to calculate differences between the groups. The Bonferoni adjustment is conservative so statistically significant results noted ought to be robust.

Differences between treatments for *in vitro* and *in vivo* cytotoxicity assays were analysed by a biostatistician using negative binomial regression, which accounts for an over-dispersed Poisson distribution. The model included terms for the groups and adjusted for the number of cells collected. No adjustment was made for multiple comparisons, however all p values were <0.0001 indicating that the results are highly unlikely to be due to chance.

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3 Generation of Tumour Lysate & the APC Response

3.1 Delivering Antigen to the Immune System: DC, MΦ and B cell Style

3.1.2.1 Dendritic Cells

The DC used in most, if not all, murine studies using tumour lysate have been GM-CSF-differentiated myeloid-type DCs[1–3]. However, another method for generating DCs *in vitro* involves culturing the bone marrow-derived precursor cells with FMS-like tyrosine kinase 3 ligand (Flt3L) to generate a lymphoid-like DC[4,5]. Flt3L binds to its receptor Flt3 on monocytes and DCs and has been shown to preferentially expand the CD8α+ DC population and enhance the T cell response in the *Plasmodium* infection setting[6]. While CD8α+ DCs have been shown to preferentially cross-present antigen, Flt3L-generated DCs were not used in this study as previous work has demonstrated their tolerogenic nature[7] as well as their inferior nature in the *in vivo* anti-tumour setting[4,5,8,9]. Moreover, it has been noted that more Flt3L-derived cells were required to elicit the same anti-tumour results *in vivo* as were observed with fewer GM-CSF-derived cells[4,5]. More evidence for the choice of GM-CSF-generated DCs for this current study comes from work showing that *in vitro* differentiation of DCs in Flt3L resulted in cells resembling the comparatively tolerogenic lymphoid-resident DCs obtained under non-inflammatory, homeostatic conditions. This contrasted with the more immunogenic phenotype and function of GM-CSF–differentiated cells[9]. Examination of the signaling pathways of GM-CSF and Flt3L reveals that the tolerogenic nature of Flt3L is explained by its activation of STAT3, which inhibits NFκB and MHC-II and upregulates production of the inhibitory molecule IDO[7]. GM-CSF, by contrast, activates canonical NF-κB signalling, and STAT5 as well as STAT3. This signalling combination overrides STAT3-mdiated inhibition resulting in an immunogenic DC[7]. The elaboration of distinct murine dendritic cells by GM-CSF versus Flt3 ligand has ramifications for inflammation and trafficking with GM-CSF–driven DC development *in vivo* driving the production of strongly immunogenic DCs that promote T_H1 immune responses. In a murine model

of cancer, both Flt3L and GM-CSF administration gave rise to DCs, but GM-CSF resulted in an enhanced anti-tumour response[8].

The majority of pre-clinical and clinical studies use GM-CSF-differentiated bone-marrow precursor cells or monocyte-derived DCs respectively. IL-4 is required when culturing human DCs to prevent their return to a CD14+ monocyte[10]. Some evidence exists showing that adding IL-4 to murine DC cultures increases their immunostimulatory properties[11]. It has been shown that under serum-free growth conditions murine BMDCs differentiated with GM-CSF +/- IL-4 have similar macropinocytosis and mannose receptor-mediated uptake of FITC-albumin and FITC-dextran particles respectively[11]. In that work IL-4+ and IL-4- GMDCs showed similar IL-12 production in response to LPS stimulation. However the GMDCs differentiated in the absence of IL-4 showed suboptimal levels of the presentation molecule MHC-II and costimulatory molecules CD40 and CD80. In contrast the GMDCs cultured in the presence of IL-4 showed stronger stimulation of allogeneic splenocytes in a mixed lymphocyte reaction (MLR) and of naïve, antigen-specific OT-II transgenic T cells when loaded with the Class II ovalbumin peptide ($OVA_{323-339}$) or with whole OVA protein.

We are not aware of any studies using Flt3L-differentiated DCs and tumour lysate, however for the reasons described we used the GM-CSF-stimulated DCs whose characteristics resemble *in vivo* inflammatory, monocyte-derived, myeloid DCs. We required an immunogenic DC that could efficiently take up lysate, present lysate-derived peptides on MHC-II, cross-present peptides on MHC-I, secrete IL-12, upregulate costimulatory molecules and effectively stimulate antigen-specific T cells. Therefore we chose to use the GM-CSF-derived DCs and evaluated the importance of IL-4 by comparing the response of GMDC and IL4DCs to soluble lysate and oxidised lysate.

3.1.2.2 Macrophages

Many studies demonstrate the potential utility of MΦs in the response to undefined tumour antigens and we hypothesized that MΦs may cooperate with DCs and B cells in priming anti-tumour T cells, whether directly, or indirectly via their DC or B cell interactions.

Subcapsular sinus (SCS) MΦs access lymphatic antigens by extending processes into the sinuses. Elegant multiphoton intravital microscopy of murine LNs identified intricate MΦ-B cell-DC interactions in antigen presentation activities[12–15]. SCS MΦs acquired antigen in sinus-extending processes and translocated it to their follicularly-located tail regions where specific antigen was passed to B cells via their BCRs and non-specific antigen immune complexes were recognised via the B cells' complement receptors. In these studies antigen retention by MΦs, as opposed to antigen degradation, greatly enhanced B cell activation. B cells internalized these antigens and migrated into the follicle where antigen was transferred to fDCs, or migrated to the B cell-T cell border to present antigen to T cells. This *in vivo* evidence demonstrates extensive antigen acquisition/presentation interactions between these cells, with SCS MΦs playing a vital role in the entry of native antigen to B cell follicles. Work from a different group has also demonstrated transfer of BCR-captured antigen from B cells to MΦs[16].

We are only aware of one pre-clinical study that has utilized TL-loaded MΦs as the APC[17]. MΦs transduced to produce M-CSF and IFN-γ displayed increased MHC-II, CD80 and ICAM-1; increased antigen presentation and cytotoxicity; and increased production of tumour necrosis factor (TNF), nitric oxide (NO) and IL-1. Taking these MΦs *in vivo* yielded increased splenic CTL IFN-γ and TNF production after ACT of the transduced MΦs and elicited anti-tumour effects in murine pulmonary metastatic melanoma. This study argued that improperly activated MΦs may give misleading results and demonstrated that MΦs engineered to secrete M-CSF and IFN-γ, which had been pulsed with freeze-thaw generated TL were able to generate a strong anti-tumour response[17], namely secretion of IL-2 by T cells, specific cytotoxicity and reduced metastases in tumour-bearing mice. Given that MΦs work alongside DC during both T cell activation and maintenance we wished to investigate their role in presenting undefined antigens either alone or in combination with DCs and/or B cells.

3.1.2.3 B Cells

Lanzavecchia was the first to report that B cells are able to act as effective APCs, efficiently taking up and presenting MHC-restricted antigen[18]. Many subsequent studies have shown that activated B cells act as efficient APCs[16,19–41] and have demonstrated their role in the priming and restimulation of CTL[21,42–46]. B cells have

been demonstrated to play a vital role in anti-tumour responses. Therefore spleen-derived B cells were also assessed as individual APCs and in combination with GMDCs and M1 MΦs.

While unactivated B cells are poor antigen presenters, or tolerogenic, the B cell role as cross-presenters to CD8+ CTLs is a fact that is not widely appreciated[47]. The use of EBV-transformed B cells to present human melanoma-derived antigen was reported by Topalian *et al* in 1994[48] and suggested the idea of using B cells in anti-tumour therapy. The focus in the ensuing two decades was on the exciting possibilities raised by using DCs in ACT. However subsequent limitations in the use of DCs as APCs for ACT, combined with further understanding of B cell antigen presentation capacities have sparked renewed investigations of the use of B cells as APCs pre-clinically and in the clinic. B cells loaded with melanoma lysate have been successfully used to examine murine and human T cell responses. Murine studies demonstrated effective generation of IFN-γ-producing CD4+ T cells by lysate-loaded B cells, and these B cell-primed T cells conferred protection after melanoma rechallenge[32]. In humans activated melanoma-lysate-loaded B cells also generated tyrosinase-specific CD4+ T cells[36]. Therefore precedent exists for the use of B cells as presenters of undefined antigens to T cells in both mice and humans and we wished to investigate whether lysate-loaded B cells in combination with DCs, MΦs, or both, could stimulate an improved T cell response.

3.1.3 Phenotype Analysis of DC and Macrophage Populations

During this study no definitive panels of markers existed that were in common practice for defining different DC and MΦ populations – or if these panels did exist they were not widely advertised. It is our observation that different groups use different combinations of surface markers and this can make judgments about cell type imprecise at best - as in the case of defining cells as MΦs or DCs solely on the basis of F4/80 or CD11c expression respectively - and inaccurate at worst.

This study aimed to compare two distinct GMDC and MΦ cell populations, therefore in order to determine phenotypic differences a robust panel of markers needed to be designed. Based on the literature we designed and tested a range of markers to identify differences between the populations. The surface markers that were used to

investigate phenotypic differences between the cells grown in M-CSF and GM-CSF +/- IL-3 are listed in Table 2 of Materials and Methods.

MHC-II along with the canonical costimulation markers CD40 and CD80 were used to assess the immature status of the DC and MΦ prior to lysate loading and the activation response post lysate loading. These markers gave us an indication of the antigen presentation potential of the APCs.

The transmembrane integrin CD11c has historically been used as a single identifying marker for DCs. However, this molecule is only found on certain DC subsets and is also expressed to a significant degree on MΦs. In this study it was used to identify which type of DC and MΦ were being generated. CD135 is a lymphoid DC marker and was also used to help identify the type of DC generated.

The tissue adhesion G-protein coupled receptor (GPCR) F4/80 is expressed on many, but not all murine MΦs, and thus also gives insight into which type of MΦ is being generated. F4/80 has been used as a classical MΦ marker, however it is also expressed on subsets of DCs. The combination of both F4/80 and CD11c as a bare minimum is more helpful in assigning a more DC- or MΦ-like status to GM-CSF-stimulated cells.

The colony stimulating factor 1 receptor (CSF1R), also called CD115, is expressed at high levels on bone marrow MΦs[49] and at low to intermediate levels on DCs, making it another useful distinguishing marker.

The mannose receptor CD206 is known to be expressed on MΦ and immature DCs and we were interested to see if our cells expressed this marker as it could be useful for analysis of lysate uptake mechanisms. We were also interested in the CD169 status of these cells since studies have identified them as important in presenting dead cell antigen to T cells in vivo[50,51].

Table 1 (Materials and Methods) lists all the molecules that were investigated during the optimization phase. All GMDCs and MΦ cultures were compared to identify key

differences between the two cell populations and ensure consistency of cell phenotypes. The antibody panels shown in Table 2 (Materials and Methods) were chosen for on going comparison. These panels provided a range of molecules that are common to both DC and MΦ. Variations in expression levels of the molecules allowed us to distinguish differences between the three cell types (GMDC, IL4DC and MΦ), indicative of differing functions and capabilities.

We generated two types of DC and two types of MΦs and compared their phenotypes and their responses to the two lysates in terms of their ability to upregulate presentation and costimulation machinery, and to secrete IL-12. We are not aware of any other studies combining APCs in anti-cancer treatments. However one study has used DCs, MΦs and B cells in the analysis of adjuvant-stimulated intracellular signal pathways[52] for improving vaccine development and other groups have looked at antigen transfer between MΦs and DCs and B cells[16,40,53].

3.1.4 Tumour Lysate: A Source of Undefined Tumour Antigens

ACT for cancer can be generated against defined or undefined antigens in the forms of known tumour associated proteins, peptides or lysates. In the ideal scenario a patient's tumour antigens are defined. However, for the majority of cancers, this is not the case, therefore particular antigens cannot be targeted with therapy. Thus oncologists initially utilize more generalized systemic treatment approaches such as chemotherapy and radiotherapy. This fact, as well as the issues with the use of single or multiple peptides, was the key reason for the use of TL in this study. Since lysate comprises the total contents of the neoplastic cell, it potentially represents an ideal source of TAA, including immunodominant antigens. APC processing of TL leads to presentation of CD4+ and CD8+ peptide sequences, thus covering both T helper and CTL activation. In addition, since the lysate is sourced from the patient's own tumour, HLA haplotyping is irrelevant.

A further reason for the choice of TL as the source of antigen is the highly heterogeneous nature of tumour tissue.[54–58]. This is a critical issue, as vaccinating against a single immunodominant antigen will only target one area of the tumour, potentially allowing other areas of the tumour to continue to grow unchecked. TL has

been used in pre-clinical and clinical studies and has been demonstrated to be a safe, well-tolerated and effective source of tumour antigens[59–66].

The obvious drawback to using TL is that TAA can also be self-antigens and tumours can hijack immune tolerance mechanisms to escape immune control. While some TAAs may be mutated, most of the antigens in TL are normal tissue antigens and so tolerance must be overcome in order to mediate cancer regression. This is particularly so in the case of non-mutated self-antigens that are simply overexpressed on tumours as compared to healthy tissue.

The corollary to breaking tolerance is the risk of autoimmune disease. Numerous studies with TAA-specific T cell transfer have shown autoimmune side effects in both mice and humans (reviewed in[67]). By contrast fewer studies in mice have reported severe, fatal autoimmune responses when using DCs loaded with tumour antigens that are shared with normal tissue[68,69]. One recent mouse study showed that TL-loaded DCs, which induced tumour control after ACT, stimulated increased levels of autoantibodies but that this was transient and did not lead to overt symptoms[70]. The autoimmune response was required for the anti-tumour response, but did not lead to outright autoimmune pathology. This phenomenon of biochemical autoimmunity in the absence of clinical symptoms has also been observed in melanoma patients (reviewed in[71]). In clinical trials to date many autoimmune reactions to self-antigens in cancer immunotherapy have been minor, self-limiting and without chronic sequelae, for example vitiligo and uveitis caused by deletion of healthy melanocytes along with the cancerous cells in melanoma after treatment with TILs[72–75]. In the case of melanoma at least, these autoimmune reactions appear to be necessary for induction of a robust anti-cancer response[73,76,77]. However there are exceptions and serious side-effects of severe uveitis with visual impairment and enterocolitis with bowel perforation requiring colectomy have also been reported (reviewed in[78]). Cancer immunotherapy clinicians are aware of autoimmune risks inherent in treatment with TAAs and are continually refining their approaches to ameliorate undesirable adverse events, while maximizing anti-tumour efficacy.

While acknowledging the issue of autoimmune reactions, we wished to increase the immunogenicity of the tumour lysate because in spite of specific TAAs being recognizable by the immune system, TLs can be immunosuppressive. Thus there was a need to make the lysate TAAs more immunogenic. It has been noted that cells that are stressed prior to lysis yield a more immunostimulatory lysate[2,79,80]. We therefore experimented with making TL more immunogenic in order to overcome normal tolerance-associated immune suppression since other studies have demonstrated poor DC response to freeze-thaw lysate[2]. Comparative studies by Chiang *et al* demonstrated that ovarian cancer cells damaged by oxidation created a lysate that was superior at activating DCs and T cells than lysates prepared from heat-treated, freeze-thawed or UVB-irradiated cells[61,81,82]. Based on these studies the APCs' responses to the soluble fraction of freeze-thaw-generated lysate and the whole portion of oxidised cell lysate were compared.

We wished to determine whether lysate antigens presented by various APCs could activate CD4+, or CD8+ T cells, or both to induce an anti-tumour response. For this reason we used B16.OVA melanoma cells as the source of our tumour lysate. While OVA is a foreign, and therefore imitation, cancer antigen, this model allows the generation of high numbers of CD4+ and CD8+ T cells making it a good model to use as a starting point for further studies using actual tumour antigens.

3.1.6 Functional Analysis of Lysate-Loaded APCs

In addition to analysis of upregulation of MHC and costimulatory molecules we assessed the production of IL-12 by the APCs in response to lysate. IL-12, produced by NK cells[83], B cells[84], DCs[85] and MΦs[86] is the major factor in the induction of IFN-γ production by T cells[85,87] and is a key driver in the differentiation of CD4+ T cells into T_H1 cells[85]. Therefore, the APCs' output of IL-12 in response to TL was assessed as an indication of their potential to drive a T_H1 response.

3.2 Objectives

The aim of the studies summarised in this chapter was to assess the response of the three APCs to two types of tumour lysate. We aimed to create a form of tumour lysate that would yield an enhanced APC presentation profile. We also aimed to identify which culture conditions would generate the best APC(s) for presenting TL to CD4+ and CD8+ T cells.

We hypothesized that of the three APCs GMDC and M1 MΦ would be the most efficient at taking up TL and would respond best to tumour lysate through their upregulation of MHC Class II and costimulatory molecules CD40 and CD80 as well as their production of IL-12.

To address the hypothesis the following objectives were undertaken:

1. Preparation of OVA-positive soluble fraction freeze-thaw lysate (soluble lysate) and HOCl-oxidised whole freeze-thaw lysate (oxidised lysate)
2. Optimisation of culture conditions for generation of bone marrow-derived DC and MΦ
3. Assessment of activation and cytokine responses of GMDC, MΦ and B cells to soluble and oxidised lysates as an indicator of the potential for each lysate-APC combination to stimulate T cells

3.3 Results

3.3.1 Generation of B16.OVA Tumour Lysate

We aimed to produce a lysate that would overcome suppression via increased danger or stress signals, and, ultimately, induce maximal T cell responses. Following on from previous work *Mycoplasma*-free (Appendix 1, Supplementary Figure 1), OVA-secreting B16.OVA cells were used as the lysate source. Since OVA is a secreted protein cells were pre-incubated in the protein transport inhibitor Brefeldin A prior to freeze-thaw lysis to ensure adequate levels of OVA in the lysate.

Soluble OVA protein is located in the soluble fraction of the lysate making this fraction the immunostimulatory portion of the lysate. Brefeldin A, impedes the dissemination of newly synthesized MHC class I molecules from the endoplasmic reticulum (ER) to the *trans*-Golgi network[88]. Based on previous data[88] it was assumed that no loss of Brefeldin A's effects should occur during the 1 hour oxidation treatment. Comparison of the T cell proliferation response to B16.OVA cells that were incubated in Brefeldin A for 1 hour and 5 hours prior to lysis is discussed in Appendix 1, Supplementary Data 8.17.

B16 cells have very low levels of MHC-I expression[89] and OVA is a secreted protein, therefore we did not expect high expression of the $OVA_{257-264}$ peptide (hereafter referred to as SIINFEKL) bound to MHC class I on the cell surface (MHC-I-SIIN). As expected, after incubation with the protein transport inhibitor Brefeldin A the cells expressed very low levels of MHC-I-SIIN (Figure 19) (average 7%; range 2 – 13 in four independent experiments). For comparison B16F10 cells, which do not express OVA, were also assayed for MHC-I-SIIN expression, which was absent (Appendix 1, Supplementary Figure 2).

The presence of MHC-I-SIIN on the cell surface prior to lysis was negligible and therefore not useful as an indicator of the presence of OVA. Therefore the resulting lysates were assayed by Western Blot (WB) to confirm that the OVA protein had been retained through the oxidation and freeze-thaw procedures. Lysates containing 20, 40 and 60 μg of protein were assayed. Western Blots revealed two bands in the 40 and 45 kDa range as expected for OVA protein (Figure 20), with the size of the bands

increasing in a concentration-dependent manner, indicative of increasing amounts of OVA.

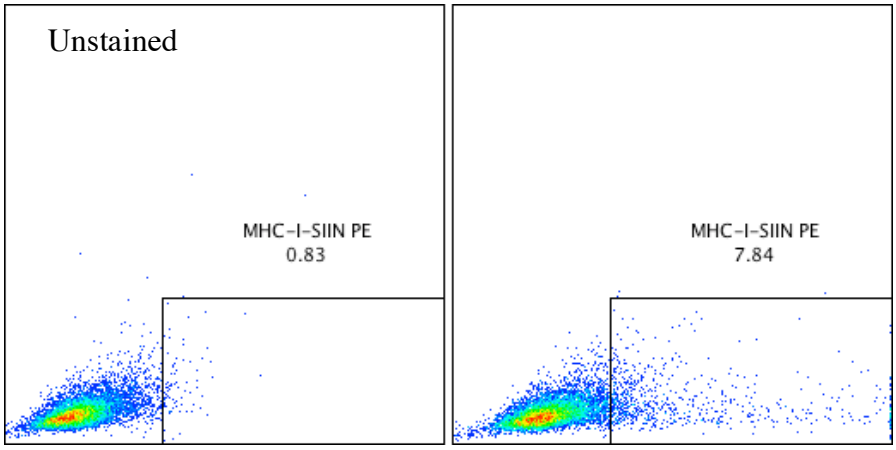


Figure 19 **Very little SIINFEKL peptide in the context of MHC-I is expressed on the surface of B16.OVA cells**. B16.OVA cells were cultured as described in Materials & Methods. Following 5 hours incubation with Brefeldin A 5 x 10^5 cells were stained with antibodies to H-2Kb bound to SIINFEKL conjugated to PE. Cells were fixed with 2% paraformaldehyde, stored overnight at 4°C and acquired the following day on a Gallios Flow Cytometer. Representative dot plots of. B16.OVA cells after Brefeldin A treatment. Representative data from three independent experiments in which results were very similar.

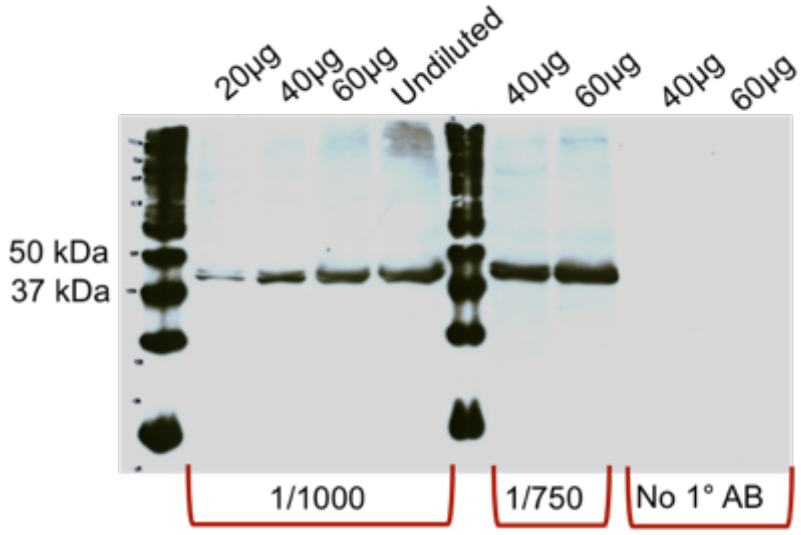


Figure 20 **Protein blot showing expression of model tumour antigen OVA in soluble lysate of B16.OVA cells after death by freeze-thaw lysis**. 1 x 10^6 B16.OVA cells were lysed in sample buffer. 20, 40 and 60 µg of protein were resolved on BOLT 4-12% Bis-Tris Plus Pre-Cast gradient gels and transferred to nitrocellulose membranes with the iBlot Transfer system. OVA expression was detected with anti-OVA monoclonal antibody. The membrane was probed with secondary HRP-conjugated anti-mouse IgG followed by SuperSignal WestPico Chemiluminescent Substrate. The film was developed after a 5 minute exposure and scanned using Canon Scan software. 45 kDa bands are indicative of OVA protein. 1/1000 and 1/750 refers to the dilution of the primary antibody. Data shown are representative of three independent experiments.

Previous studies have demonstrated superior immunogenicity of HOCl-oxidised freeze-thaw lysate[61,79,82]. Therefore we compared the APC response to both the soluble fraction of freeze-thaw lysate (hereafter referred to as soluble lysate and abbreviated as s-L) and to HOCl-oxidised whole freeze-thaw lysate (hereafter referred to as oxidised lysate and abbreviated as ox-L). Oxidation of the B16.OVA cells was achieved using sodium hypochlorite prior to six rounds of freeze thaw lysis. We assayed the oxidised lysate by Western Blot for the presence of OVA protein in order to verify that oxidation of the cells had not altered the OVA protein. Once again concentration-dependent levels of OVA protein were detected in the oxidised lysate (Figure 21). However in contrast to the clean result seen with the soluble lysate in the oxidised lysate blots lower molecular weight bands were also observed.

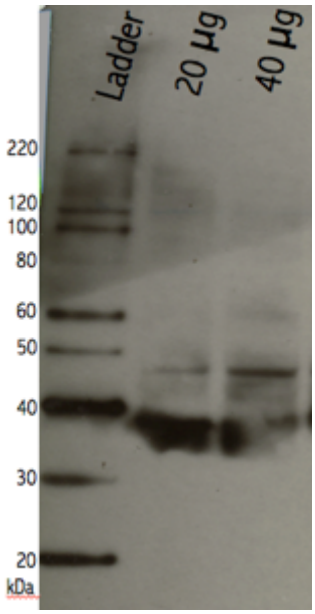


Figure 21 **Protein blot showing expression of model tumour antigen OVA in lysate of B16.OVA cells is retained after death by oxidation followed by freeze-thaw lysis**. 1 x 10^6 oxidised B16.OVA cells were lysed in sample buffer. 20 and 40 µg of protein were resolved on BOLT 4-12% Bis-Tris Plus Pre-Cast gradient gels and transferred to nitrocellulose membranes with the iBlot Transfer system. OVA expression was detected with anti-OVA monoclonal antibody. The membrane was probed with secondary HRP-conjugated anti-mouse IgG followed by SuperSignal WestPico Chemiluminescent Substrate. The film was developed after a 1 minute exposure and scanned using Canon Scan software. 45 kDa bands are indicative of OVA protein. The numbers on the left denote the migration of the molecular weight markers. Data shown are representative of three independent experiments.

3.3.2 Cell Types Differ in Their Resistance to Oxidation-Induced Cell Death

Chiang *et al* showed that incubating cells in 60 μM HOCl achieved 95% cell death of SK-OV-3 human ovarian cancer cells[79,82]; therefore we used the same HOCl concentration. However, incubating B16.OVA cells in 60 μM HOCl resulted in only 57% cell death (46 – 73) (Figure 22). This variation in HOCl doses prompted evaluation of other cell lines to validate our B16.OVA result. For a direct comparison with B16.OVA, three more melanoma cell lines were chosen: B16gp33, B16F10 and Mel888 (Figure 23). In addition, we compared three non-melanoma cell lines to ascertain if differences in oxidation were cell type-specific. Thus we also compared the colon carcinoma cell line MC38, the human monocytic leukaemia cell line THP-1 and the murine lung epithelial tumour cell line TC1. The different cell lines each required a different amount of HOCl to achieve 95% cell death (Figure 22 and Appendix 1 Supplementary Fig. 4). The melanoma and THP-1 cell lines were more sensitive to death by oxidation than TC-1 or MC38 cells. With 90 μM HOCl we saw a range of 80 to 96% death of melanoma and THP-1cells. By contrast TC-1 and MC38 cell lines required greater than 90 μM to achieve 95% cell death. These data showed that the cell types differed in their sensitivity to death by oxidation and therefore the 90 μM HOCl concentration was used in the B16.OVA oxidation.

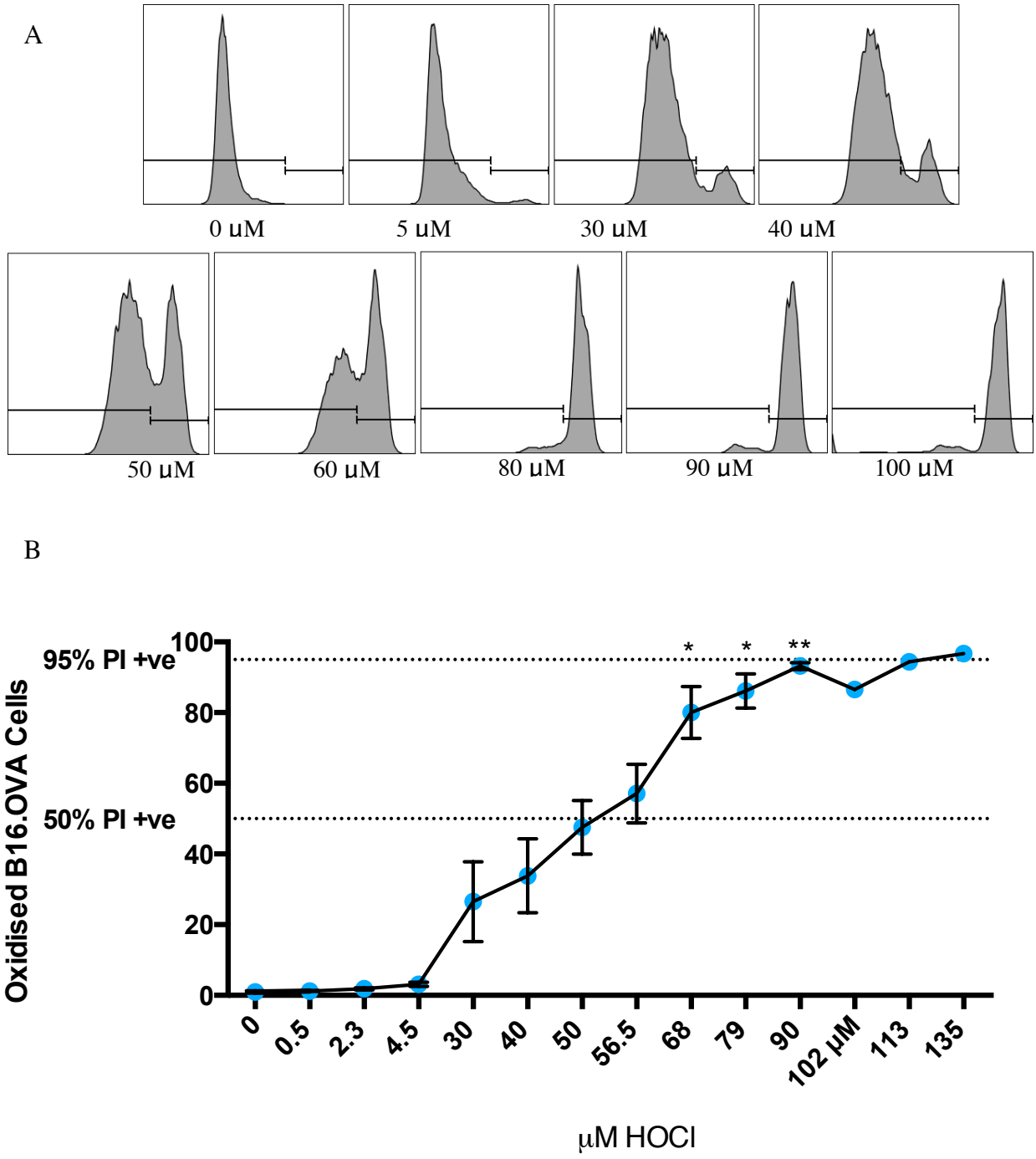


Figure 22 **Concentration-dependent oxidation-induced death of B16.OVA cells**. B16.OVA melanoma cells were cultured as described in Materials and Methods. Cells were incubated with different concentrations of HOCl as shown on the x axis for 1 h at 37°C. Cells were washed, stained with Propidium Iodide (5 µg/mL) and acquired by a Gallios Flow Cytometer within an hour. Data was analysed by FlowJo V9 or V10. The percentage of highly PI positive cells corresponded to the percentage of dead cells.. **A**) Representative data from three independent experiments. **B**) The percentage of highly PI positive cells corresponded to the percentage of dead cells and was plotted against HOCl concentration. Summary data of 3 independent experiments. Statistically significant differences were calculated by Kruskal-Wallis test followed by Dunn's post test with Bonferroni correction. compared to untreated * $p<0.05$; ** $p<0.01$. Error bars = mean ± s.e.m. of 3 independent experiments.

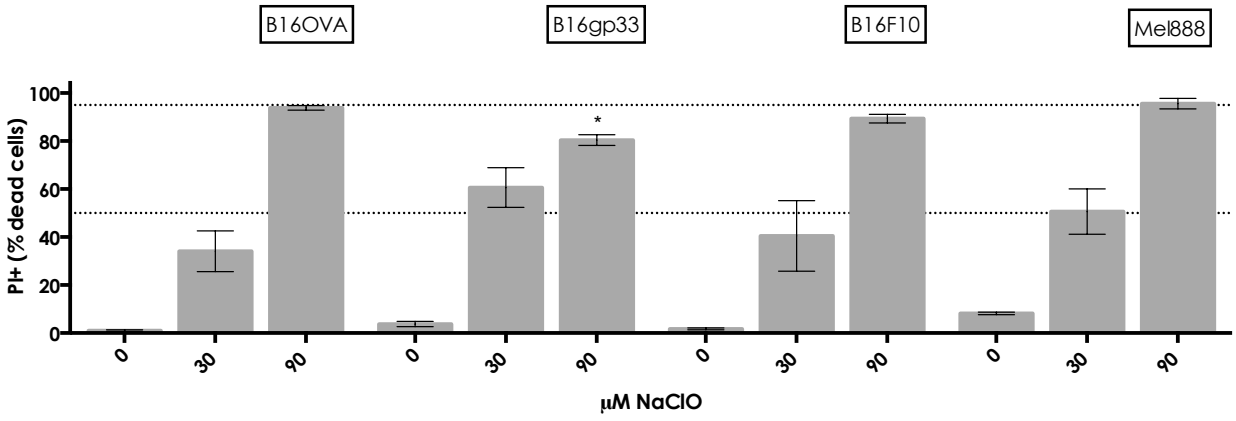


Figure 23 **Concentration-dependent induction of death in four melanoma cell lines by HOCl oxidation**. B16.OVA, B16.gp33, B16.F10 and Mel888 melanoma cells were cultured as described for B16OVA tumour cells in Materials and Methods. Geneticin was only added to B16.OVA cells. Harvested cells were incubated in different concentrations of HOCl as shown on the x axis for 1 hr at 37°C. Cells were washed, stained with Propidium Iodide (5 µg/mL) and acquired by a Gallios Flow Cytometer within an hour. Data was analysed by FlowJo V9 or V10. The percentage of highly PI positive cells corresponded to the percentage of dead cells. Statistically significant differences were calculated by Kruskal-Wallis test followed by Dunn's post test with Bonferroni correction. Error bars = mean ± s.e.m. of 3 independent experiments.

3.3.3 Optimising Dendritic Cell and Macrophage Culture Conditions

To compare the immune response to the lysates, two types of dendritic cells and two types of macrophages were generated from bone marrow-derived precursors. Different culture conditions for APC generation were assessed (Figure 24). Bone-marrow precursor cells differentiated with GM-CSF were designated GMDC. Those differentiated with GM-CSF+IL-4 were designated IL4DC. Cells differentiated with GM-CSF+IL-3 were designated M1 MΦs. For comparison we cultured bone marrow cells in macrophage colony-stimulating factor (M-CSF), and these were designated M2 MΦs. Given that this study aimed to compare the ability of different cell types to act as APCs for tumour cell lysate it was important to verify that three different cell types were being generated. Therefore the morphology and molecular phenotype of these cells was carefully and continuously monitored.

GM-CSF and M-CSF respectively are known M1 and M2 MΦ-inducing stimulants[90], with GM-CSF driving an M1 inflammatory MΦ phenotype and M-CSF promoting an anti-inflammatory or homeostatic M2 MΦ response[91]. For simplicity MHC-II, CD11c, F4/80, CD40 and CD80 are shown in Figure 24. CD169 and CD206 were also assessed but no differences between culture conditions were observed for these molecules (data not shown). As expected, differentiating bone marrow precursor cells in M-CSF yielded a distinct M2 MΦ phenotype (Figure 24) with low expression of

MHC-II (6%; 4 – 7), CD11c (17%; 12 – 26) and F4/80 (7%; 3 – 14) (Figure 24). The full Flow Cytometric gating strategy is shown in Figure 25, using sample GMDC data. Sample M1 MΦ data is shown in Appendix 1, Supplementary Figure 6.

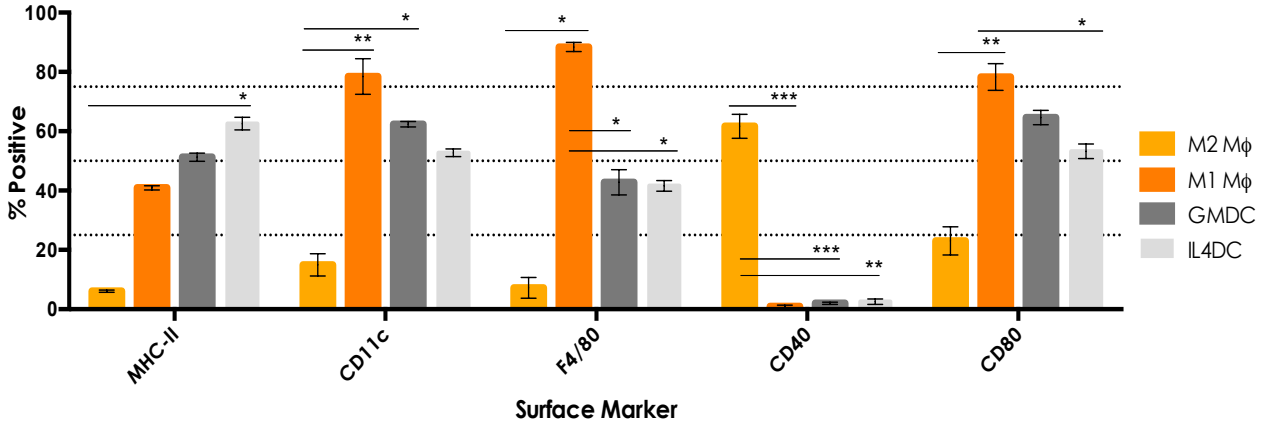


Figure 24 **M2 MΦs, M1 MΦs, GMDCs and IL4DC display different phenotypes.** Bone marrow-derived precursor cells were cultured in IMDM+10 ng/mL M-CSF and 10% FCS (M2 MΦ), IMDM + 5 ng/mL GM-CSF+5 ng/mL IL-3 and 10% FCS (M1 MΦ), IMDM + 20 ng/mL GM-CSF and 5% FCS (GMDC) or IMDM + 20 ng/mL GM-CSF+10 ng/mL IL-4 and 5% FCS (IL4DC). Day 10 MΦs and Day 6 DC were harvested as described in Materials & Methods, stained with Live/Dead NIR dead cell exclusion dye and labeled with mABs against DC and MΦ surface markers: MHC-II, CD11c, CD169, F4/80, CD40 and CD80, as per Table 2 in Materials & Methods. Cells were fixed in 4% paraformaldehyde, stored overnight at 4°C and acquired the following day on a Gallios Flow Cytometer. Data was analysed on FlowJo V9 or VX and graphed in Prism. Summary data of 3 (M2 MΦ) and 11 (M1 MΦ, GMDC, IL4DC) separate experiments is shown. Statistically significant differences were calculated by Kruskal-Wallis test followed by Dunn's post test with Bonferroni correction. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Error bars = mean +/- s.e.m.

Cells cultured in M-CSF yielded markedly lower levels of MHC-II than those grown in GM-CSF+/-IL-3 or IL-4 (M2 MΦs: 6%; M1 MΦs: 42% ns; GMDC: 52% ns; IL-4DC: 62% $p<0.05$) (Figure 24).

A similar result was observed with the CD11c molecule with 17% of M2 MΦs positive for this marker compared with 78% of M1 MΦs ($p<0.001$), 62% of GMDC ($p<0.05$), and 53% of IL4DC (ns).

CD40 expression was unexpectedly high on M2 MΦs (62%) and unexpectedly low on M1 MΦs (1%), GMDC (2%) and IL4DC (2.5%).

In line with expected results M2 MΦs displayed significantly lower percentages of CD80+ cells compared to M1 MΦs (78%, $p<0.01$), GMDC 65% (ns) and IL4DC (53% $p<0.05$).

Finally, F4/80 expression was also very low on M-CSF-differentiated M2 MΦs: 7% compared with 88% on GM-CSF+IL-3-differentiated M1 MΦs, 43% of GMDCs and 41% of IL4DCs ($p<0.05$).

Having identified that GM-CSF+IL-3 yielded the required MΦs we went on to identify the optimal media for these DCs and MΦs. Various media had been noted in the literature, with one study reporting that IMDM and RPMI had differential impacts on the levels of MHC Class II molecules, TLRs and scavenger receptors on DCs owing to differing amounts of aryl hydrocarbon receptors in these two media[92]. We therefore cultured these cells in two different media and on three different types of plastic ware and compared the resulting phenotypes.

GMDC grown in RPMI and IMDM showed no difference between any surface markers tested (Figure 26). Culturing M1 MΦs on bacteriological or tissue culture plates did not alter expression of any of the markers tested, however harvesting these cells was significantly easier when grown on bacteriological plates. The M1 MΦ showed no differences in expression of MHC-II, CD11c, Ly6C, CD169, CD40, CD11b or CD135 when grown in RPMI or IMDM. M1 MΦs grown in RPMI on tissue culture plates, however, showed a strong, though not statistically significant, down-regulation of F4/80: 75% in IMDM versus 41% in RPMI; ($p<0.3$) (Appendix 1, Supplementary Data, Figure 25). A strong, though not statistically significant, down-regulation of F4/80 in RPMI was also observed on the bacteriological dishes: 82% in IMDM versus 4% in RPMI ($p=0.3$). (Figure 27) Because of the down-regulation of this canonical MΦ marker that was observed in M1 MΦs cultured in RPMI we used IMDM as the culture medium for both GMDC and M1 MΦs in all future experiments.

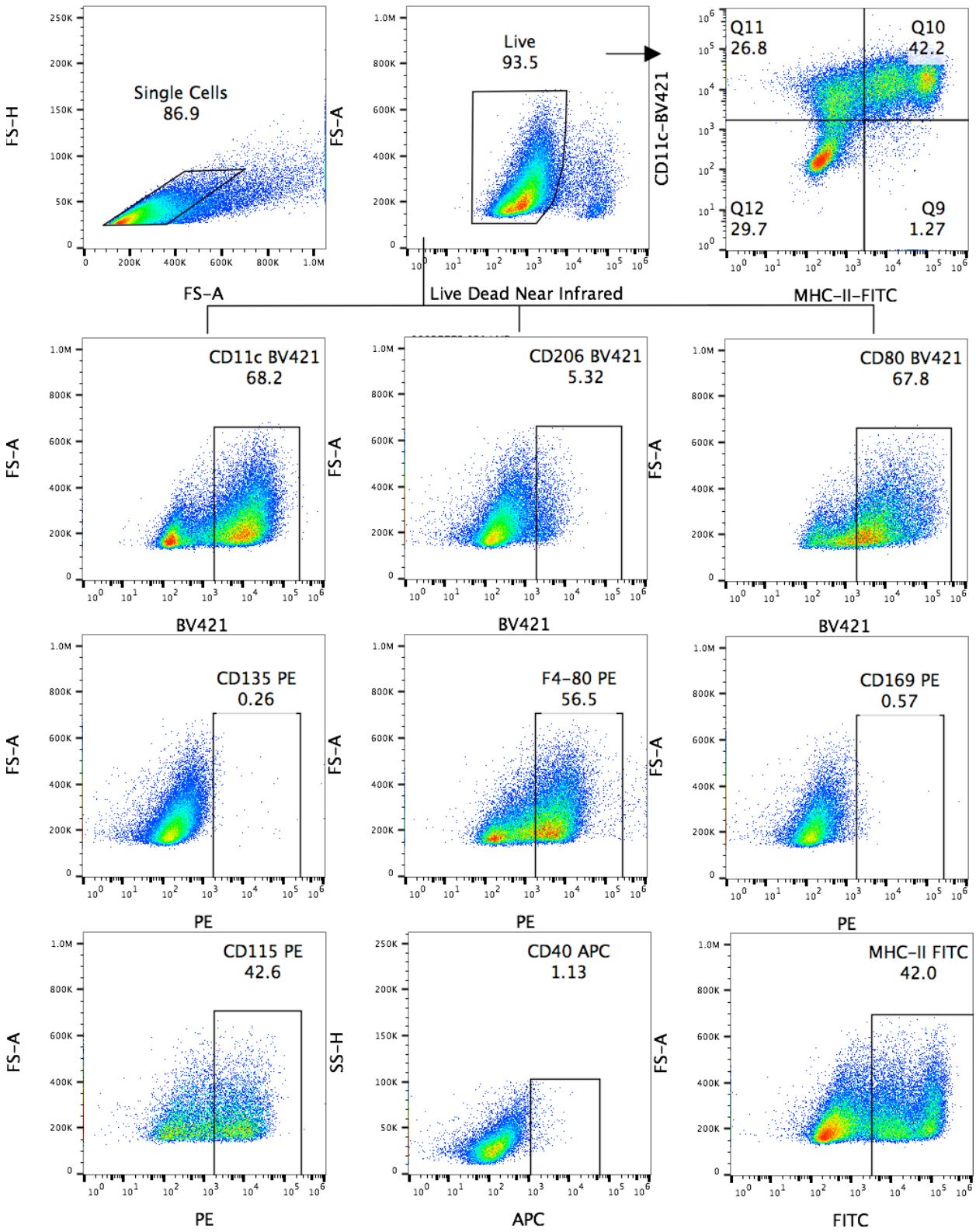


Figure 25 **Gating strategy used in DC MΦ phenotype analysis**. D6 GMDC, D6 IL4DC and D10 MΦ were stained with Live/Dead exclusion dye (Live Dead Near Infrared)) and Fc receptor block followed by surface molecule staining with the following monoclonal antibodies: MHC-II-FITC, CD11c-BV421, CD135-PE, CD169-PE, CD115-PE (BL), F4/80-PE, CD206-BV421 (BL), CD40-APC, CD80-BV421 (BL). Antibody-stained cells were washed twice and fixed in 4% paraformaldehyde. Cells were stored overnight at 4°C and analysed the next day on a Gallios Flow Cytometer. 5 x 10^4 - 1 x 10^5 cells were collected in each sample wherever possible. The gating strategy consisted of doublet exclusion followed by dead cell exclusion and then gating on the cells of interest using unstained cells and single stained cells as negative gating controls. Representative data of GMDC staining. BL: BioLegend; BD: BD Biosciences.

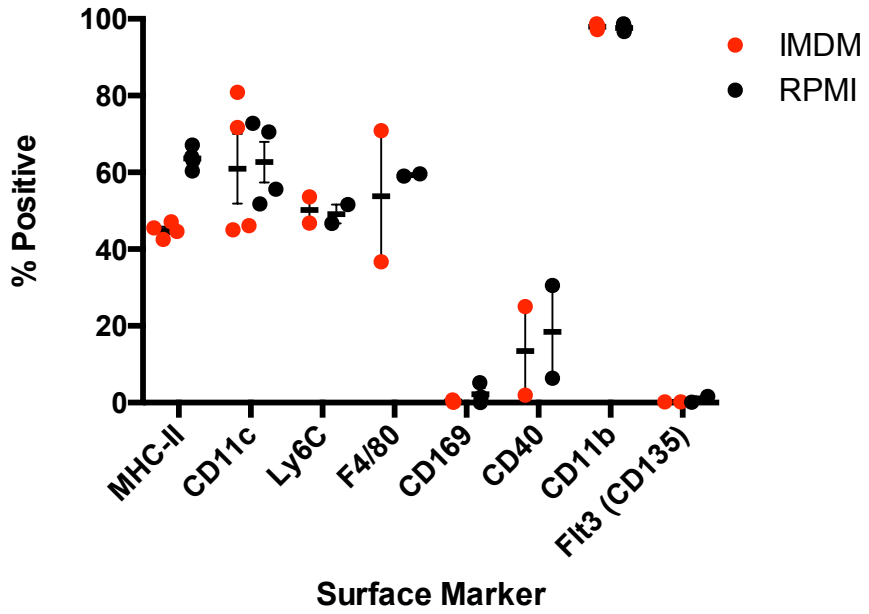


Figure 26 **GMDC display the same phenotype irrespective of RPMI or IMDM culture media**. Bone marrow-derived precursor cells were prepared as described in Materials & Methods and cultured in RPMI or IMDM (+ 20 ng/mL GM-CSF and 5% FCS). On Day 6 cells were harvested, stained with Live/Dead NIR dead cell exclusion dye and labeled with mABs against the surface markers MHC-II, CD11c, Ly6C, F4/80, CD169, CD40, CD11b and Flt3 (CD135) as per Table 2 in Materials & Methods. Cells were fixed with 4% paraformaldehyde, stored overnight at 4°C and acquired the following day on a Gallios Flow Cytometer. Data was analysed on FlowJo Version 9 or X and graphed in Prism. Statistically significant differences were calculated using a Mann-Whitney U test. Error bars = mean +/- s.e.m of two independent experiments.

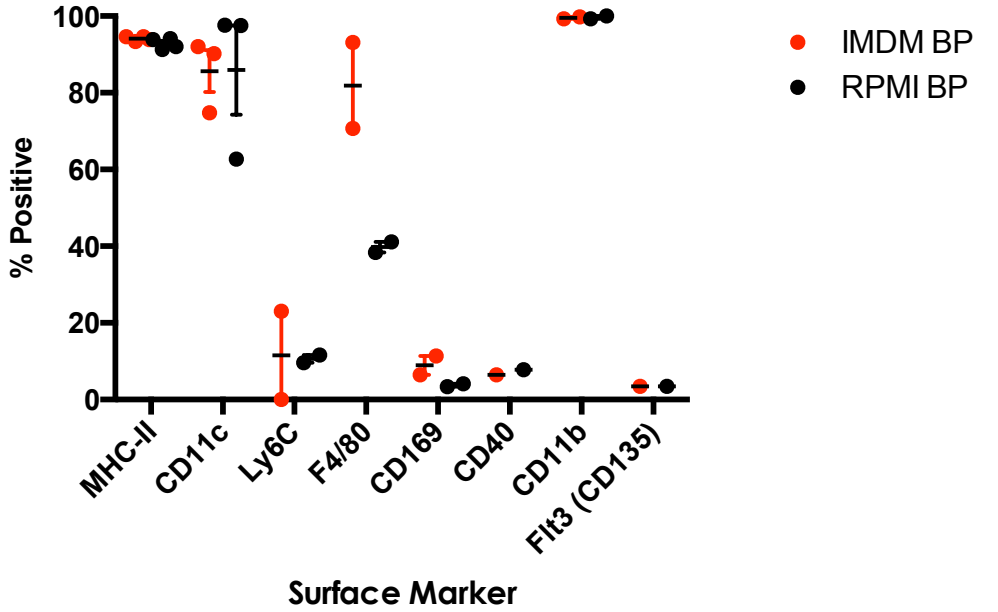


Figure 27 **Comparison of M1 macrophages cultured in IMDM versus RPMI**. Bone marrow precursor cells were prepared as described in Materials & Methods were cultured in RPMI or IMDM (+ 5 ng/mL GM-CSF, 5 ng/mL IL-3 and 10% FCS) on bacteriological (non-tissue culture-treated) dishes (BP). On Day 10 M1 MΦs were harvested as described in Materials & Methods, stained with Live/Dead NIR dead cell exclusion dye and labeled with mABs against MHC-II, CD11c, Ly6C, F4/80, CD169, CD40, CD11b and Flt3 (CD135) as per Table 2 in Materials & Methods. Cells were fixed with 4% paraformaldehyde, stored overnight at 4°C and acquired the following day on a Gallios Flow Cytometer. Data was analysed on FlowJo Version X and graphed in Prism. Statistically significant differences were calculated using a Mann-Whitney U test. Summary data of two independent experiments. CD40 and Flt3 were only measured in the second experiment. Error bars = mean +/- s.e.m.

3.3.4 Comparison of Unactivated GMDC, IL4DC and MΦ Morphology & Phenotypes

Having identified the optimal medium for GMDCs and M1 MΦs, we compared GMDCs cultured with and without the cytokine interleukin-4 (IL-4). The three cell culture conditions generated three distinct cell populations, hereafter referred to as GMDC, IL4DC and MΦ. These three cell populations differed in morphology (Appendix 1, Supplementary Figure 5), phenotypic characteristics (Figure 28) and in their surface molecule (Figure 30) and IL-12 (Figure 35) response to tumour lysate antigens.

3.3.4.1 GMDC, IL4DC and MΦ Morphologies

The GMDC and IL4DC cultures appeared the most similar under the microscope, however a noticeable difference between them was the appearance in the IL4DC cultures of a much higher proportion of large (≈ 36 μM), round, clear cells that dotted the dishes (Appendix 1, Supplementary Figure 5). In the GMDC cultures these cells were present in much lower percentages (experimenter observation; data not quantified). We included anti-B220 in the DC and MΦ panels to ascertain whether or not these cells could be plasmacytoid DCs but the percentage of B220+ cells was extremely low indicating very low pDC percentages in the cultures (Data lost – sample images of GMDC and IL4DC can be seen in Appendix 1, Supplementary Figure 5). GMDC and IL4DC cultures had high numbers of semi-adherent and floating cells above a background of adherent and semi-adherent cells.

On Day 0 the cells in the MΦ cultures are round and non-adherent or minimally adherent. By Day 3 adherent cells emerge in clusters that are much smaller than DC clusters, and begin to show MΦ morphology. By Day 10 there was a confluent, or near-confluent, layer of MΦs on the bacteriological dishes. The cells in the MΦ cultures were much larger than those in the DC cultures (diameters of 10 μM and above and elongations of up to 86 μM compared with diameters of 4-10 μM in the GMDC and IL4DC cultures). The MΦs were fewer in number and resolutely adherent with very few non-adherent cells. In contrast to the two DC cultures MΦs could not be harvested by pipetting but required growth in non-tissue culture-treated dishes and incubation in EDTA solution before they could be harvested by repeated flushing with a 10 mL pipette.

3.3.4.2 GMDC, IL4DC and MΦ Surface Phenotypes

In addition to observing morphological variation the three cell populations were analysed by Flow Cytometry for the presence of DC and MΦ surface markers. Figure 25 shows the gating strategy used in GMDC surface marker phenotype analysis. An example of MΦ gating is found in Appendix 1, Supplementary Figure 6.

Staining a mixed population of DCs and MΦs for use as single stain controls proved unsatisfactory for use in compensation, as did using one set of voltages for both cell types. This is due to DCs and MΦs differing in size and granularity and thus autofluorescence. Accordingly a set of single stain control cells was prepared for each cell type and the voltages adjusted for each cell type to ensure accurate compensation and optimal voltages. This method allows for direct comparison of the percentage of cells positive for a given fluorophore within a population, and for comparison of mean or median fluorescence intensity (MFI) between untreated and treatment groups of the same cell type. However MFI between the DCs and MΦs could not be directly compared due to the lack of an internal standard control.

Figure 28 shows the summary data of phenotyping analysis of GMDCs, IL4DCs and M1 MΦs. Contrary to expectations CD11c expression was higher on MΦs than GMDC or IL4DC. In subsequent experiments the percentage of GMDC positive for CD11c remained consistently around 60%. No difference in CD11c expression was observed between GMDC and IL4DC. By contrast CD11c expression on M1 MΦs remained consistently higher than either GMDC or IL4DC (84%; 62 – 96; $p < 0.01$).

No difference was identified in the percentage of GMDC and M1 MΦ positive for MHC-II (49% and 43% respectively). The percentage of IL4DC positive for MHC-II was significantly higher on 69%. Analysis of the median fluorescence intensity of MHC-II was not informative due to bimodal distribution in the GMDC population

CD169 was not expressed on any of these APCs (Appendix 1, Supplementary Figure 5). CD40 expression was also consistently negligible across the three unstimulated APCs.

In contrast to the low levels of CD40 CD80 was constitutively expressed at intermediate to high levels across the three cell types. No difference was seen in CD80 expression between GMDC and IL4DC (66% and 53% respectively) but CD80 was consistently significantly higher on M1 MΦs (82%; 60 – 95; $p<0.01$).

As was the case with MHC-II, CD11c and CD80 F4/80 also distinguished the two populations by the level of expression. The two DC populations expressed F4/80 at intermediate levels (51% on GMDC and 42% on IL4DC) while the MΦs expressed it to a significantly higher degree (91%($p<0.001$).

None of the three cell types showed expression of the Flt-3 ligand receptor, CD135 (Appendix 1, Supplementary Figure 5). GMDCs, IL4DCs and MΦs all showed low levels of CD8α (5%, 7% and 10% respectively) with no significant difference detected.

MΦs and GMDCs showed intermediate positivity for CD115 (CSFR1) (62% and 49% respectively). IL4DCs expressed CD115 at significantly lower levels (14%) however this difference was only statistically significant between IL4DCs and MΦs ($p<0.01$). The mannose receptor CD206 appeared at low levels on the GMDC and IL4DC (< 5%) (Appendix 1, Supplementary Figure 5). On MΦs the expression of CD206 varied widely between 22.5 and 62.3%, but expression was significantly higher than either GMDC or IL4DC ($p<0.05$).

Finally, CD11b, the so-called macrophage-1 antigen (Mac-1) proved unhelpful as a distinguishing marker as it was expressed to very high levels on both GMDC and MΦs (data not shown). CD11b expression on IL4DC was not assessed as we had discounted it as a potential distinguishing marker prior to comparing GMDC and IL4DC.

Thus these data showed by morphology and molecular phenotype that the three culture conditions generated three distinct populations of cells whose response to adjuvants and lysates could now be compared.

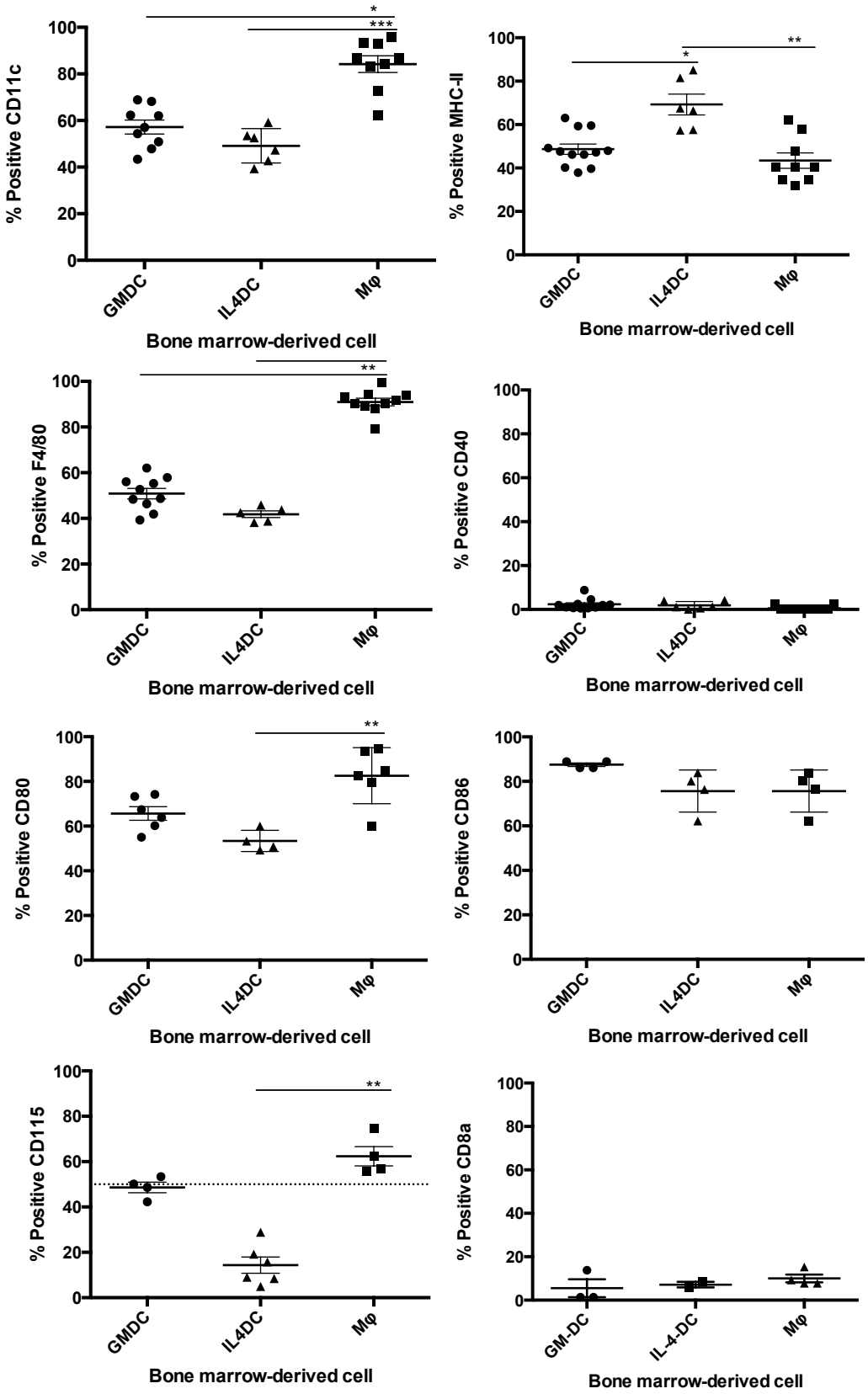


Figure 28 **Variations in GMDC, IL4DC and MΦ surface marker phenotypes**. Bone marrow-derived precursor cells were cultured for 10 days in GM-CSF (5 ng/mL)+IL-3 (5 ng/mL)+FCS10% (MΦ); 6 days in GM-CSF (20 ng/mL)+FCS (5%) (GMDC); or 6 days in GM-CSF (20 ng/mL)+IL-4 (10 ng/mL)+FCS (5%) (IL4DC). Cells were harvested and 0.5-1 x 10^6 cells were stained with dead cell exclusion dye and labeled with fluorophore-conjugated antibodies against a range of common DC and MΦ identifying markers: CD11c, MHC-II, F4/80, CD115, CD135, CD8α; as well as the costimulatory markers CD40, and CD80). Cells were fixed with 4% paraformaldehyde, stored overnight at 4°C and collected the following day on a Gallios Flow Cytometer. Data were analysed on FlowJo Version X and graphed in Prism. Summary data showing the mean (± s.e.m) of ten independent experiments. Each dot represents one experimental replicate. These molecules were measured on IL4DCs in 4 experiments. CD86, CD115 and CD8α were measured on all cells in 4 experiments. Statistically significant differences were calculated by Kruskal-Wallis test followed by Dunn's post test with Bonferroni correction. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

3.3.5 B cell Isolation

B cells were isolated from the spleens of C57BL/6 mice by magnetic bead selection as discussed in Materials & Methods. Isolated cells were stained for the common B cell markers anti-CD19 and anti-B220, as well as the DC/MΦ and T cell markers CD11c and CD3 to verify the effectiveness of the sorting procedure. CD19CD45 double positive cell purity consistently averaged 95.4% (93.2 – 98%). Figure 29 shows the gating strategy and representative data from one B cell isolation procedure. Previous work in our lab had ascertained the optimal B cell culture conditions therefore further optimization was not required.

3.3.6 APC Responses to Lysates & Activation Stimuli

Once the growth conditions had been optimised and having demonstrated, based on morphology and phenotype, that different cell populations could be cultured and isolated, we then compared the APC response to loading with tumour lysate and immune stimulants. Previous work in our lab had shown that soluble lysate was able to induce upregulation of MHC-II and costimulatory markers on DC[93]. However, antigen presented by immature APCs often induces immunological tolerance[94–96], therefore we wished to add appropriate immune stimuli to enhance APC activation. Certain antigen uptake capacities in immature DC are greater than those of activated DC, therefore we tested the response of immature APC loaded with lysate alone (Figure 30). We also compared the APC response to a range of immune activators (LPS&CpG, LPS&IFN-γ and CpG&IFN-γ; Appendix 1, Supplementary Figure 8) and the response to loading the APCs with lysate and activators (Figure 32).

3.3.6.1. GMDC But Not M1 MΦ Upregulate CD40 In Response to Oxidised Lysate

No statistically significant differences in the percentage of GMDC, IL4DC or M1 MΦs positive for MHC-II, were observed in response to soluble or oxidised lysate (Figure 30). Likewise, neither lysate had any impact on the median fluorescence intensity (MFI) of MHC-II of these cells (Data not shown).

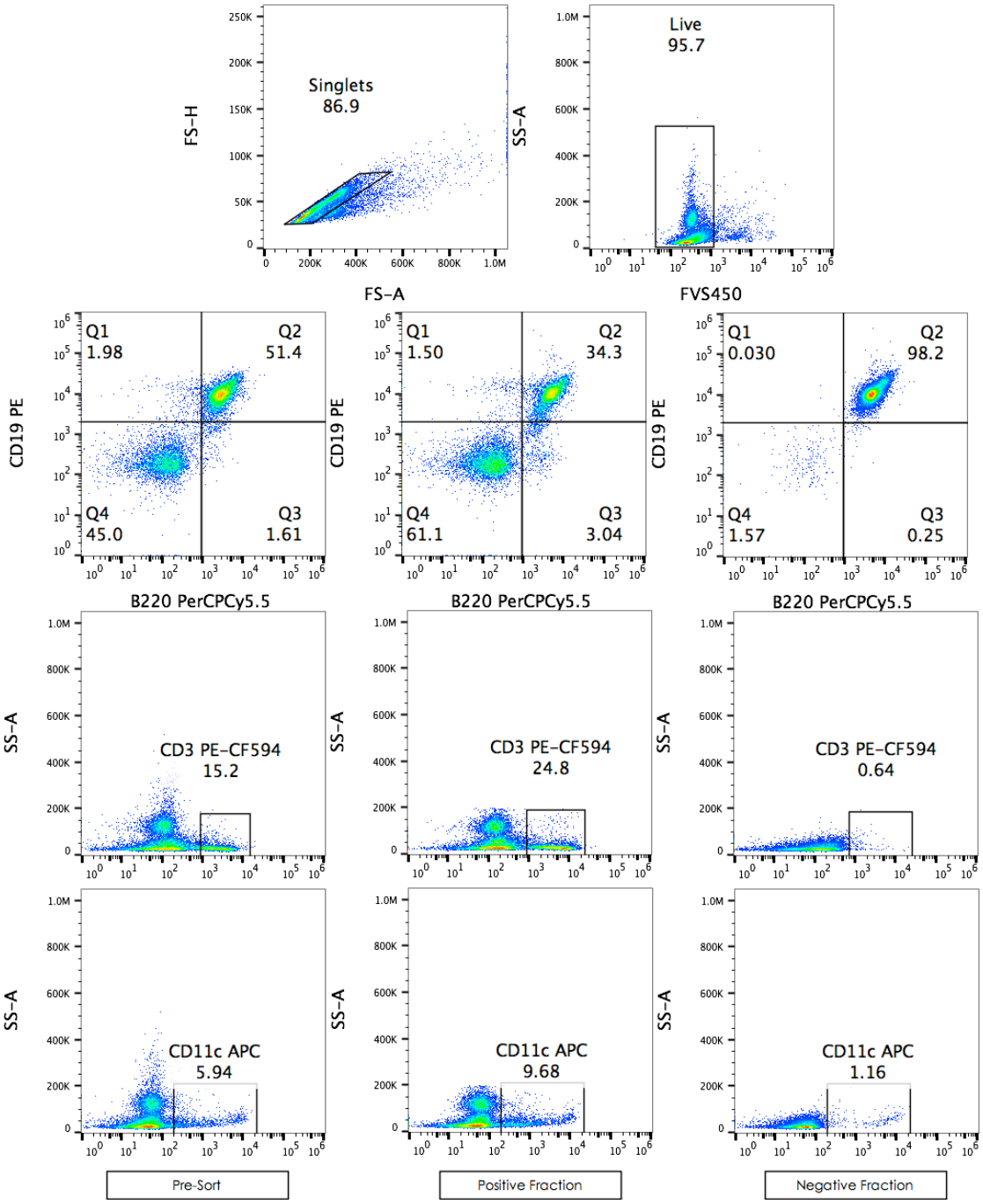


Figure 29 **C57BL/6 splenocytes labeled with anti-CD43 magnetic beads yield up to 98% pure B cell populations**. Splenocytes from WT C57BL/6 mice were lysed with RBC lysis buffer and incubated with anti-CD43 magnetic beads as per the manufacturer's instructions (described in Materials and Methods). Cells were subjected to a negative selection protocol on an AutoMACS Pro and the resulting populations stained with Lived/dead exclusion Dye (FVS450). Cells were incubated with Fc receptor block prior to incubation with monoclonal antibodies against CD11c (APC), CD3 (PE-CF594), CD19 (PE) and B220 (PerCPCy5.5). Cells were fixed with 4% PFA and stored overnight at 4°C. Cells were processed the following day on a Gallios Flow Cytometer and the data analysed on FlowJo Version 10 or Flow Jo 2. Representative Flow Cytometric dot plots from multiple experiments.

The percentage of GMDC that was positive for CD40 was significantly upregulated only in response to oxidised lysate (53%; $p<0.05$ compared to UT). No statistically significant increases in CD40 were observed in either IL4DCs or M1 MΦs in response to either lysate when measured by either Mann Whitney U or Kruskal-Wallis followed by Dunn's post test.

With regards to CD80, this molecule was highly expressed on all three APCs, and no increases were seen in response to lysates. However, the median fluorescence intensity of CD80 increased significantly on both IL4DCs ($p<0.01$) and M1 MΦs ($P<0.05$) compared to UT cells (Appendix 1, Supplementary Figure 11).

Finally, CD86 was also highly expressed on UT cells and no increases were seen in response to the lysates.

Taken together these data demonstrated few, slight variations in the individual APCs' response to the lysates in terms of their stimulation of peptide presentation and co-stimulation capacities.

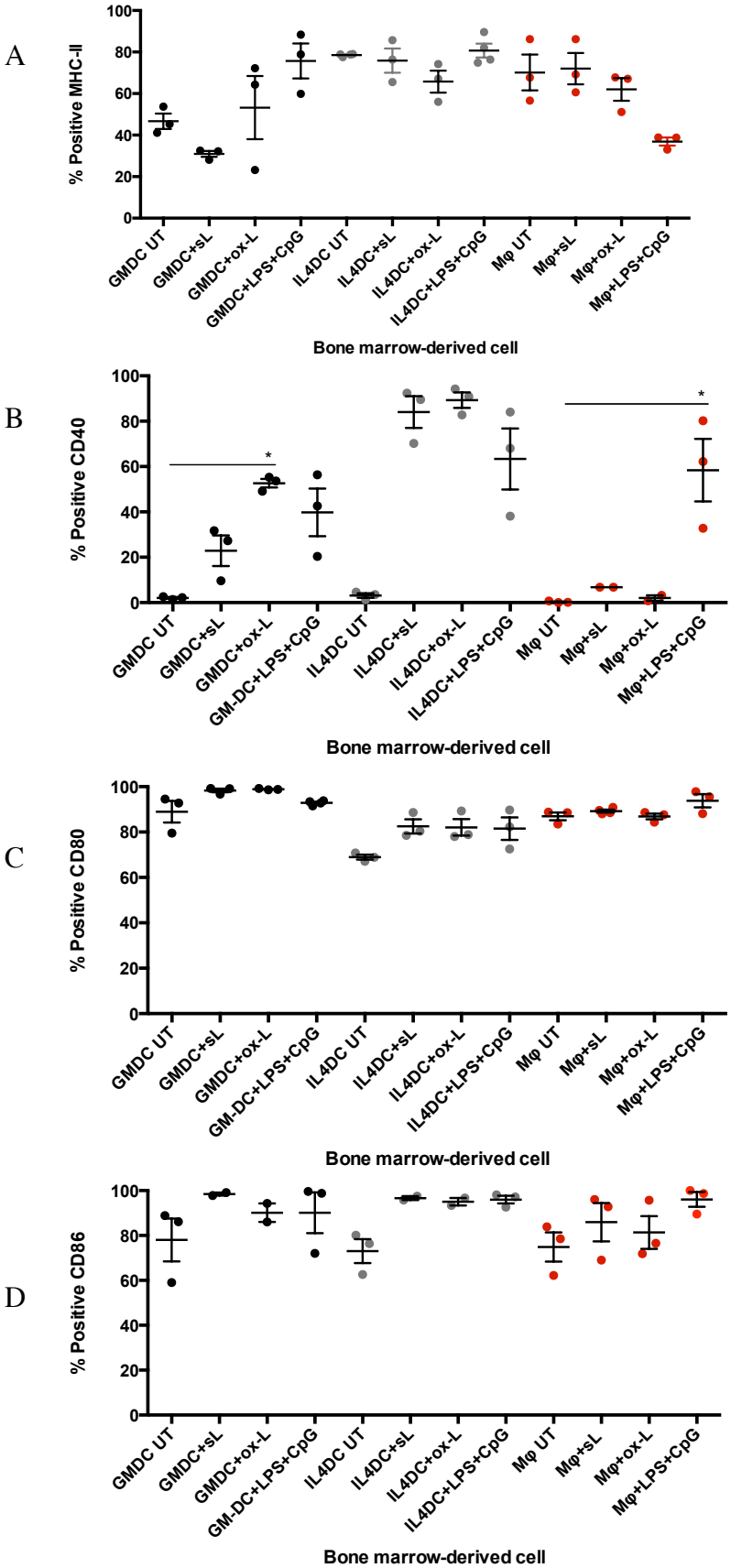


Figure 30 **Unactivated GMDC, IL4DC and MΦ vary in their responses to soluble and oxidised lysates**. Bone marrow-derived precursor cells were cultured for 10 days in GM-CSF (5 ng/mL)+IL-3 (5 ng/mL)+FCS10% (**MΦ**); 6 days in GM-CSF (20 ng/mL)+FCS (5%) (**GMDC**); or 6 days in GM-CSF (20 ng/mL)+IL-4 (10 ng/mL)+FCS (5%) (**IL4DC**). APCs were resuspended at 0.5 x 10^6 cells/mL and 1 x 10^5 cells pulsed overnight with s-L and Ox-L (1:1 ratio tumour cell:APC) or LPS&CpG as a biological control. The following day cells were harvested and stained for dead cell exclusion dye. Cells were labeled with mABs against **A**) MHC-II, B) CD40, **C**) **CD8** D) CD86, fixed in 4% paraformaldehyde and stored overnight at 4°C. Cells were acquired the following day on a Gallios Flow Cytometerthe data analysed on FlowJo Version 10 and graphed in Prism. Summary data of 3 independent experiments in which the means of the 3 experiments were compared. Statistically significant differences were calculated by Kruskal-Wallis test followed by Dunn's post test with Bonferroni correction. * $p<0.05$. Error bars = mean ± s.e.m. UT: untreated; sL: soluble lysate; ox-L: oxidised lysate.

3.3.6.2 B cells Upregulate CD86 In Response to Oxidised Lysate

The percentage of B cells positive for MHC-II did not increase in response to overnight loading with soluble or oxidised lysate (Figure 31). However the percentage of B cells positive for CD86 increased from 76% (UT) 95% in response to oxidised lysate (p<0.05) (Figure 31). By contrast neither CD40 nor CD80 percent positivity, nor MFI, increased in response to either lysate (Appendix 1, Supplementary Fig 12).

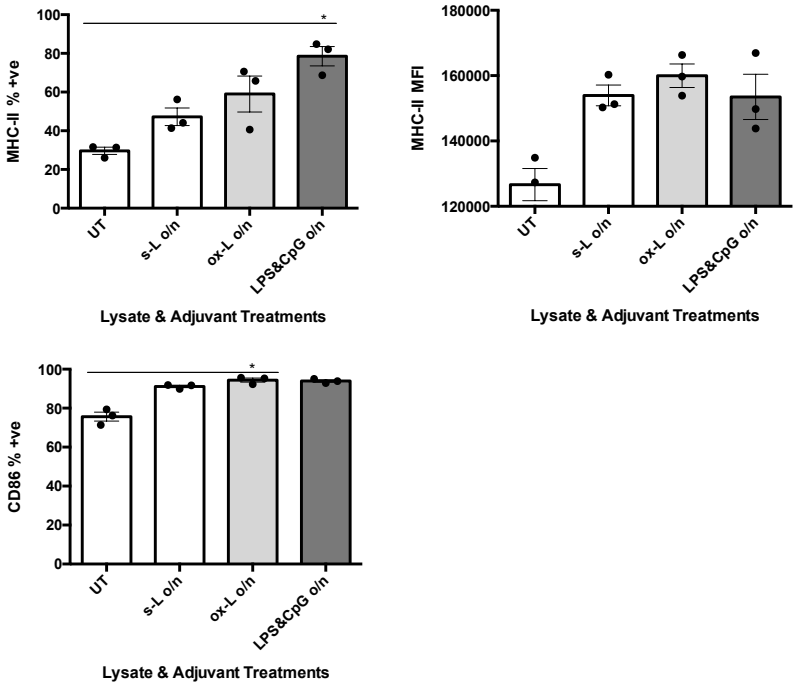


Figure 31 **The percentage of B Cells positive for MHC-II increases in response to LPS&CpG but not soluble or oxidised lysate, and CD86 expression increases on B cells exposed to oxidised lysate**. Freshly isolated splenocytes were sorted for CD43 negative cells by magnetic cell sorting (AutoMACS Pro). 1 x 10^5 B cells were pulsed overnight with s-L and Ox-L (1:1 ratio tumour cell:APC) or LPS&CpG as a biological control. The following day cells were harvested and stained for dead cell exclusion dye. Cells were labeled with mABs against MHC-II, CD40, CD80 and CD86, fixed in 4% paraformaldehyde and stored overnight at 4°C. Cells were acquired the following day on a Gallios Flow Cytometer, the data analysed on FlowJo 2 and graphed in Prism Summary data of 3 independent experiments in which the means were compared. Statistically significant differences were calculated by Kruskal-Wallis test followed by Dunn's post test with Bonferroni correction. * p<0.05.. Error bars = mean ± s.e.m. UT: untreated; o/n: overnight.

3.3.6.3 APC Responses to Lysates Plus Activation Stimuli

Freeze-thaw tumour lysate-loaded human DC, matured with IFN-γ and LPS, have been shown to express high levels of costimulatory molecules and to produce high levels of IL-12[81]. Therefore the APC responses to loading with tumour lysate and our chosen activation stimuli were also compared. Tumour lysate has been variously used by different groups at ratios of between 1:1 and up to 10:1 (tumour cell:DC). Earlier experiments were carried out using 10 μL of lysate, which equated to a 3:1 ratio. For reasons of attempting to reproduce their results as well as clinical applicability in later experiments we later elected to use the 1:1 ratio used by Chiang *et al* in their clinical trials of ovarian tumour lysate[61].

In the experiments described in this section GMDC, IL4DC and MΦ were pulsed overnight with soluble lysate and oxidised lysate at a ratio of 1 lysed tumour cell to 1 APC plus LPS+CpG. Differences in the exogenous presentation molecule MHC-II and the co-stimulation markers CD40, CD80 and CD86 were analysed by Flow Cytometry (Figure 32). Appendix 1, Supplementary Table 2 shows the antibody panels used in these experiments.

3.3.6.4 No Further Increase in GMDC Upregulation of CD40 Is Observed In Response to the Addition of LPS&CpG

The percentage of GMDC and IL4DC positive for MHC-II showed no statistically significant increase over untreated cells when pulsed with soluble lysate+LPS&CpG or oxidised lysate+LPS&CpG (Figure 33A). While the percentage of M1 MΦs positive for MHC-II was approximately halved compared to UT (70%) on exposure to soluble lysate+LPS+CpG (35%), oxidised lysate+LPS&CpG (37%) and LPS&CpG alone (37%), these differences were not statistically significant.

If normal Gaussian distributions are assumed then significant increases in the percentage of GMDC and IL4DC positive for CD40 were observed in response to oxidised lysate+LPS&CpG (Figure 33B). However, normal distribution was considered unlikely due to group sizes. For this reason a Kruskal-Wallis non-parametric comparison of location was used, followed by Dunn's post test with Bonferroni correction, was applied for statistical significance. Under these

assumptions of abnormal distribution CD40 only increased on GMDC in response to oxidised lysate+LPS&CpG (53%; $p<0.05$).

CD80 and CD86 were both highly expressed on all three APCs and no increases were observed in either percent positivity or median fluorescence intensity in response to lysates and activators (Figure 33C, D).

3.3.6.5 B Cells Upregulate MHC-II In Response to LPS&CpG But Not Lysates

Previous work had demonstrated that unactivated B cells were not able to stimulate a T cell response (Chapter 4, Figure 45) and that the B cell response to soluble lysate plus CpG alone did not greatly enhance the T cell response (Appendix 1, Supplementary Figure 22). Therefore three different B cell activation scenarios were compared to assess the B cell response to lysate and LPS&CpG. The aim of this experiment was to assess whether loading B cells with lysates after activating with LPS&CpG yielded a more favourable B cell phenotype for T cell presentation and activation. B cells were either loaded overnight with lysate and LPS&CpG, or loaded overnight with LPS&CpG followed by 6 hours of lysate loading, or loaded overnight with lysate followed by 6 hours of LPS& CpG stimulation (Figure 33).

While marked increases in the percentage of cells positive for MHC-II were recorded, once again, under the assumptions of Kruskal-Wallis test (that does not require the data to follow a normal distribution), *post hoc* Dunn's test revealed that these increases were not statistically significant. Nonetheless, loading B cells with LPS&CpG overnight prior to loading with s-L resulted in a significant increase in MHC-II over cells that were loaded with s-L overnight and stimulated with LPS&CpG the following day (86% and 24% respectively; $p<0.05$). An identical, though not statistically significant, trend was observed for oxidised lysate. There was no difference between B cells loaded with lysate only.

In a similar manner the percentage of B cells positive for CD86 increased from 76% (UT) to 97% in response to overnight pulsing with LPS&CpG followed by loading with soluble lysate ($p<0.05$). These results demonstrated that LPS&CpG were not required for the increase in CD86 in response to soluble lysate. The trend was again

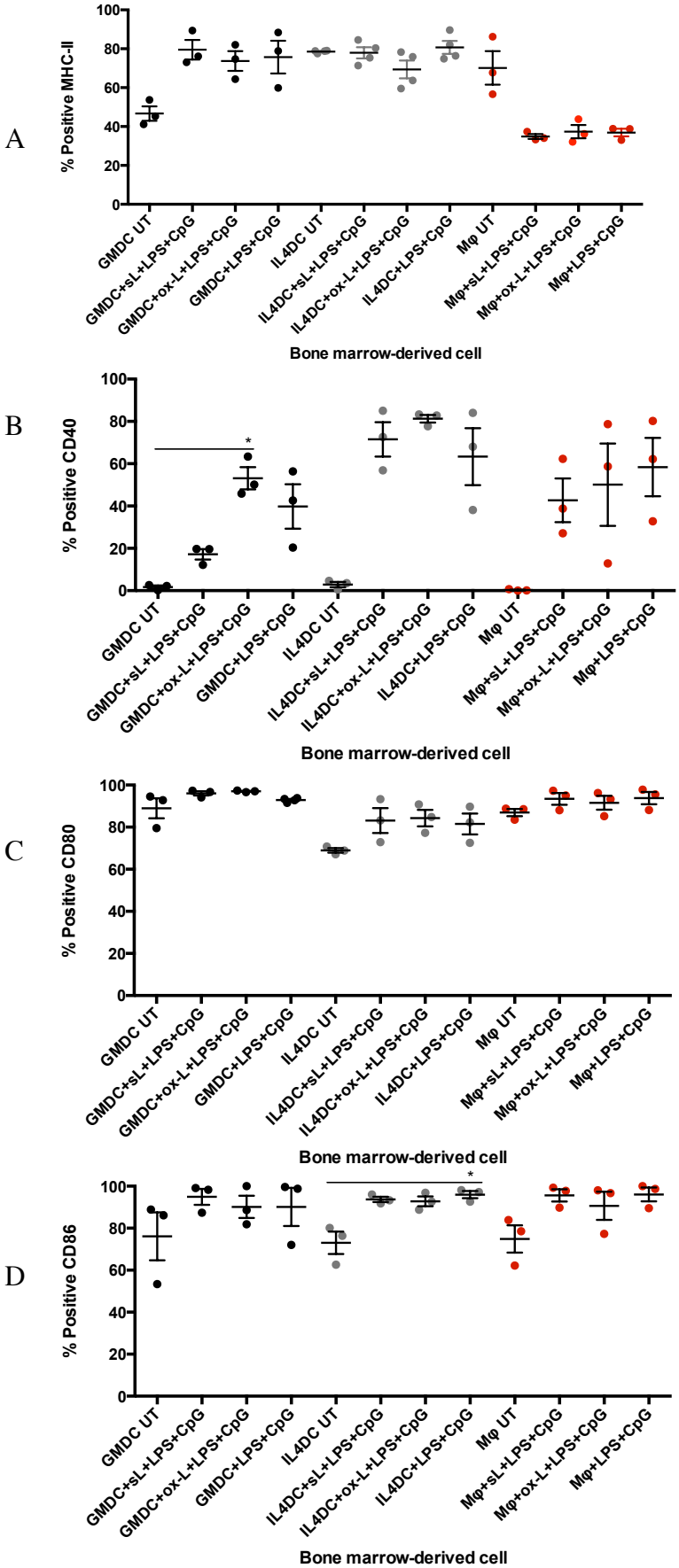


Figure 32 **GMDC, IL4DC and MΦ vary in their activation response to s-L and ox-L plus LPS+CpG**. Bone marrow-derived precursor cells were cultured as described previously. APCs were resuspended at 0.5 x 10^6 cells/mL and 1 x 10^5 cells pulsed overnight with s-L and Ox-L (1:1 ratio tumour cell:APC) +/- LPS&CpG as per the x axes. The following day cells were harvested and stained for dead cell exclusion dye. Cells were labeled with mABs against **A**) MHC-II, B) CD40, **C**) **CD8** D) CD86, fixed in 4% paraformaldehyde and stored overnight at 4°C. Cells were acquired the following day on a Gallios Flow Cytometer, the data analysed on FlowJo Version 10 and graphed in Prism. Summary data of 3 independent experiments in which the means of the 3 experiments were compared. Statistically significant differences were calculated by Kruskal-Wallis test followed by Dunn's post test with Bonferroni correction. * $p<0.05$. Error bars = mean ± s.e.m. UT: untreated; sL: soluble lysate; ox-L: oxidised lysate.

identical, but not statistically significant for oxidised lysate. Though this is likely due to the conservative Bonferroni adjustment.

In contrast to MHC-II and CD86 neither CD40 nor CD80 percent positivity, nor MFI, increased in response to any lysate plus activation stimuli scenario (Appendix 1, Supplementary Figure 12).

These data showed that for GMDC, IL4DC and B cells the addition of LPS&CpG stimulated a trend toward upregulated MHC-II in response to both lysates. However the LPS&CpG was not required to stimulate upregulation of CD40 in response to oxidised lysate on GMDC or IL4DC. Likewise, for B cells oxidised lysate alone stimulated upregulation of CD86 without the addition of LPS&CpG. Similarly for M1 MΦs the addition of LPS&CpG stimulated no change in phenotype in response to the lysates. Taken together these data demonstrated differences in the individual APCs' responses to the lysates. Soluble lysate and oxidised lysate differed in their ability to stimulate the APCs' antigen presentation and co-stimulation machinery and oxidised lysate alone was able to differentially induce upregulation of two key costimulatory factors: CD40 in GMDCs and CD86 in B cells.

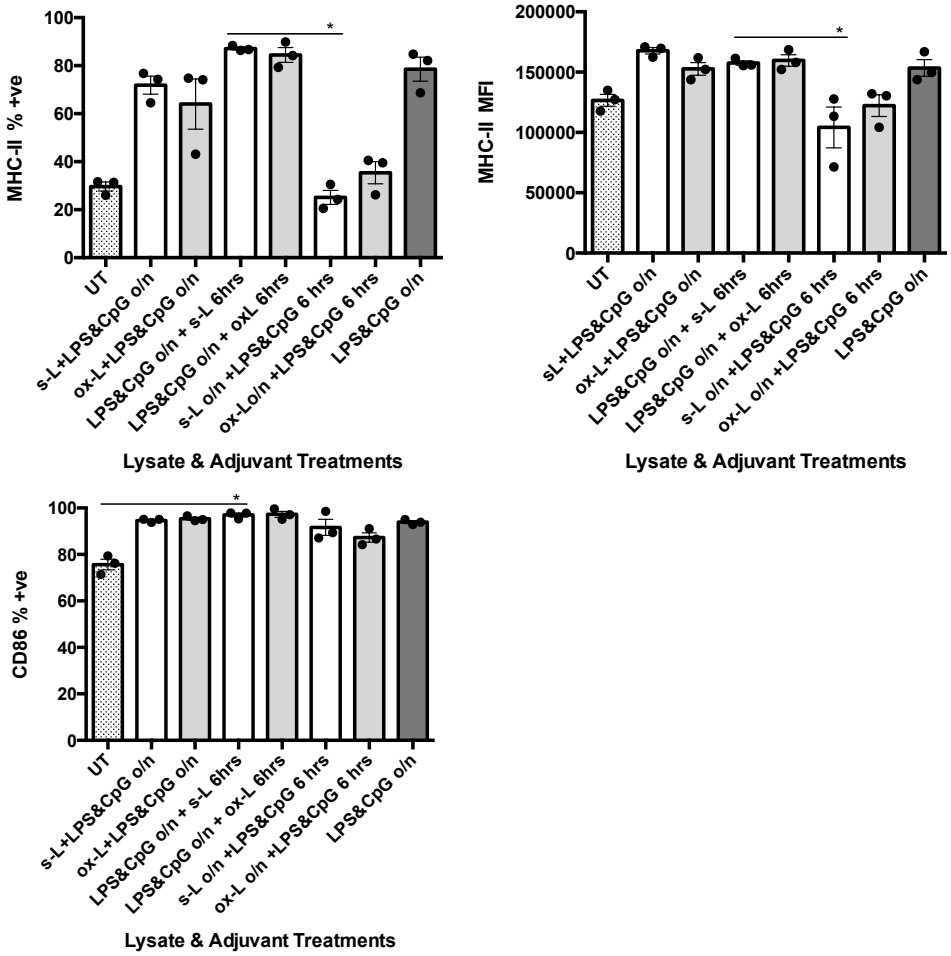


Figure 33 **The B Cell MHC-II and CD86 response to various s-L and ox-L +/- LPS&CpG loading approaches**. Freshly isolated splenocytes were sorted for CD43 negative cells by magnetic cell sorting (AutoMACS Pro,). B cells were pulsed overnight with s-L and ox-L (1:1 ratio tumour cell:APC) +/- the immune stimuli LPS (1 μg/mL) & CpG (0.3 μg/mL) as indicated on the x axes. The following day cells were harvested, stained with live/dead exclusion dye and mABs against the surface markers MHC-II, CD40 and CD80. Cells were fixed with 4% paraformaldehyde, stored overnight at 4°C and acquired by a Gallios Flow Cytometer the next day. Data were analysed on FlowJo 2 and graphed in Prism. Summary data showing results of 3 independent experiments. Statistically significant differences were calculated by Kruskal-Wallis test followed by Dunn's post test with Bonferroni correction. * $p<0.05$. Error bars = mean ± s.e.m. UT: untreated; o/n: overnight.

3.3.7 APC Viability Varies in Response to Lysates

Previous reports have noted that tumour lysate was not toxic to DCs[2], however we noted differences in the ability of soluble lysate to induce APC death (Figure 34). GMDC activated with LPS+CpG and loaded with oxidised lysate were highly susceptible (39% viability compared with 8% in UT; $p<0.05$).

Overall there was a general trend toward reduced viability in all conditions in the IL4DC with oxidised lysate-loaded cells showing only 22% viability after 24 hours compared with 74% in the untreated cells ($p<0.01$).

M1 MΦs retained excellent viability irrespective of lysate loading or LPS&CpG stimulation. We examined whether a soluble MΦ-secreted factor may have an effect on DC viability. GMDC, IL4DC MΦ were incubated overnight with lysate at a 1:1 ratio. The next day MΦ conditioned medium (MCM) was added to the wells of the GMDC and IL4DC and these cells were incubated for a further 24 hours and analysed by Flow Cytometry. No significant increases in viability were observed in the GMDC and IL4DC groups in the presence of MCM (data not shown).

Reduced viability is not unexpected in UT B cells since approximately 50% of unstimulated B cells in culture will die every 24 hours. Low B cell viability was unchanged with the addition of lysate, unless activation occurred prior to lysate loading. Significantly improved viability was observed in B cells activated with LPS&CpG overnight followed by lysate loading. LPS&CpG-activated B cell viability was 61% after loading with soluble lysate compared to 15% for B cells loaded overnight with lysate followed by the addition of LPS&CpG ($p<0.05$). The trend was identical for oxidised lysate (72% and 19% respectively; $p<0.05$)

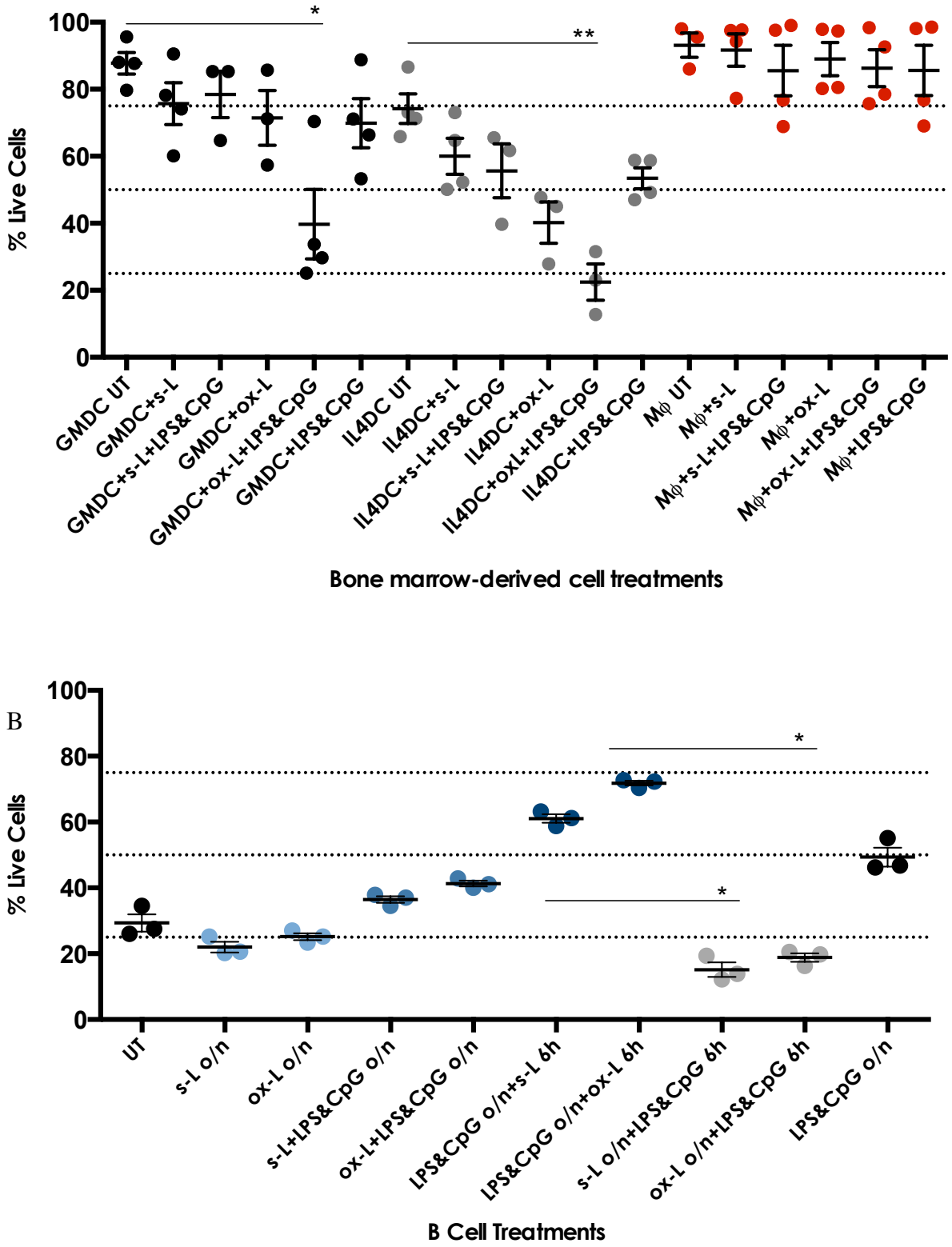


Figure 34 **GMDC, IL4DC, MΦ and B cell viability varies in response to soluble and oxidised lysates +LPS&CpG**. Bone marrow-derived precursor cells and B cells were prepared as previously described. 1 x 10^5 DCs and M**Φs** were pulsed overnight with s-L and ox-L (1:1 ratio tumour cell:APC) and LPS+CpG. 1 x 10^5 B cells were activated with LPS&CpG and loaded with lysate(s) as per the x axis in B. After 24 hours cells were harvested, stained with live/dead exclusion dye and mABs against surface markers. Cells were fixed with 4% paraformaldehyde, stored overnight at 4°C and acquired by Gallios Flow Cytometer the next day. Data were analysed on FlowJo 2 and graphed in Prism. Summary data showing results of 3 (B cells) and 4 (DCs & MΦs) independent experiments. Statistically significant differences were calculated by Kruskal-Wallis test followed by Dunn's post test with Bonferroni correction. * p<0.05; ** p<0.01. Error bars = mean ± s.e.m. UT: untreated; o/n: overnight.

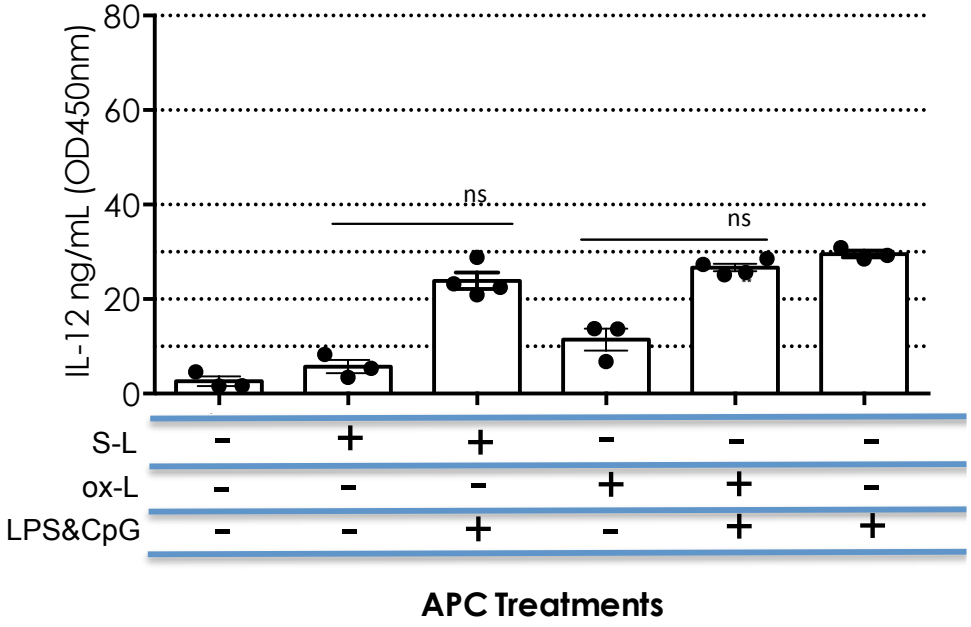
3.3.8 The Addition of LPS&CpG Yields Increased IL-12 Over Lysates Alone

The APC populations were compared for their ability to produce IL-12 in response to the two tumour lysates (Figure 35). After 24 hours exposure to soluble and oxidised lysate in the presence or absence of LPS&CpG cell conditioned media were collected and stored at -20°C for analysis by anti-IL-12 ELISA.

GMDC, M1 MΦs (Figure 35) and IL4DC (Appendix 1, Supplementary Figure 13) all produced small amounts of IL-12 in response to soluble lysate (6 ng/mL, 10 ng/mL and 2 ng/mL respectively). All three APCs also produced IL-12 in response to HOCl-wFTL, however GMDC and M1 MΦs produced more IL-12 (11 ng/mL and 8 ng/mL respectively) than IL4DC (4 ng/mL; $p<0.05$).

Taken individually there was no difference in IL-12 production between soluble and oxidised lysate-loaded APCs, irrespective of whether the APCs were stimulated with LPS&CpG. However, stimulating the cells with LPS&CpG during lysate loading resulted in strong trends toward increased IL-12 for GMDC, and a statistically significant increase for M1 MΦs when analysed by Mann Whitney U test between the two groups (for example GMDC+s-L and GMDC+s-L+LPS&CpG). No improvement in IL-12 response over GMDCs was observed in IL4DCs and these results can be seen in Appendix 1, Supplementary Figure 13. The difference in IL-12 response between GMDC and M1 MΦs was notable (s-L+LPS&CpG 24 ng/mL and 46 ng/mL respectively; ox-L+LPS&CpG: 26 ng/mL and 45 ng/mL respectively) but not statistically significant ($p=0.0571$; Mann Whitney U test).

A



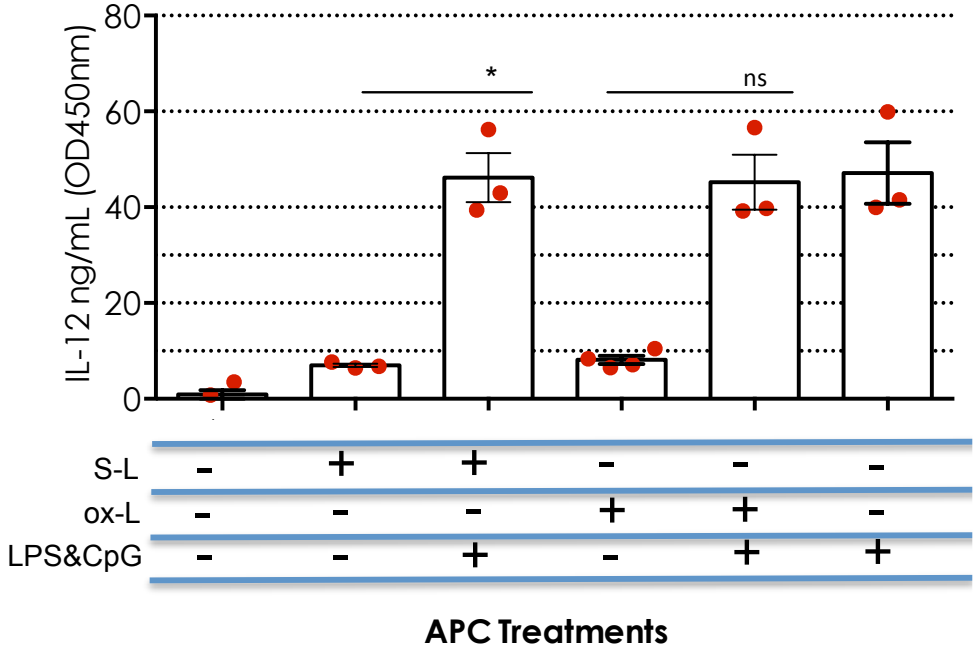


Figure 35 **Stimulation of GMDCs and M1 MΦs with LPS&CpG during lysate loading yields enhanced IL-12 production over lysates alone and the M1 MΦ IL-12 response to lysate is greater than that of GMDC**. Bone marrow-derived precursor cells were prepared as described previously. 50,000 APCs were pulsed overnight with s-L and ox-L (1:1 ratio tumour cell:APC) +/- LPS+CpG as indicated on the x axis. After 24 hours cell conditioned media (supernatants) were collected and stored at -20°C. Supernatants were analysed by anti-IL-12 ELISA. Summary data of at three or four independent experiments showing IL-12 production after 24- hours exposure to s-L and ox-L +/- LPS+CpG. Statistically significant differences were calculated by Mann Whitney U test. * $p<0.05$. . Error bars = mean ± s.e.m of the combined independent experiments. **A**) GMDC; **B**) M1 MΦs

3.4 Discussion

3.4.1 Oxidation Can Increase the Immunogenicity of Tumour Lysate

The use of TL represents a clinical method for accessing undefined TAAs in solid tumours, which may be inaccessible to patient immune cells, whether due to lack of infiltration or active tumour inhibition mechanisms. Different methods of tumour lysate preparation have been described in the literature, including heat treatment[97–100], gamma irradiation[2,101], ultraviolet irradiation (UVC)[102], *in situ* ablation[103,104] and oxidation[61,79,80,82,105–108]. Oxidation was chosen over irradiation, as access to an irradiator was difficult. Heat treatment was rejected since Chiang *et al* have shown that T cells primed with DC loaded with heat killed SK-OV-3 ovarian cancer cells did not mount an immune response against the antigens they were testing[82]. In addition data from another study also argued that heat shock proteins (HSP) *per se* were not responsible for an improved APC response to lysate prepared from heat-stressed cells[2]. We prepared the soluble fraction of freeze-thaw lysate (soluble lysate) and the entire contents of HOCl-oxidised lysate (oxidised lysate) and compared the response of APCs to both of these lysates.

Lysate contains all possible immunogenic antigens in the patient's tumour, while at the same time containing highly immunosuppressive and immune-tolerising properties, primarily due to being self-tissue-derived. The outcome of antigen presentation is directly controlled by pathogen-related and cell death-related signals, and indirectly controlled by molecules and cytokines generated during tissue infection or damage that direct the immune response. At the start of this study there was still uncertainty over which cell death conditions were immunogenic and which are immunologically ignored[109] and a 2014 review noted that cell-death-associated immunomodulators of DC function were still in urgent need of further characterisation[110]. However a recent publication has reported that inflammatory substances such as DAMPs are insufficient to elicit cross-priming of CD8+ T cells. The key requirement for robust cross-priming by DCs is for receptor interacting protein kinase-1 (RIPK-1) signalling and NFκB transduction in the dying cells, irrespective whether the cells are dying by apoptosis or necrosis[111]. This information on how the nature of the cell death impacts on the function of the lysate-pulsed APCs would have been useful for manipulating the cells used for generating tumour lysate

in this study, however at the time of creating oxidised lysate this information was not known.

OVA protein, the model tumour antigen in this system, is not a natural TAA and is immunogenic therefore there are pros and cons to using this system. The use of OVA with transgenic T cells specific for OVA peptides allows the generation of large numbers of T cells as well as the identification of whether CD4+ or CD8+ T cells, or both, are contributing to the anti-tumour response to undefined TAAs. OVA was detected in Western blots of both soluble lysate and oxidised lysate however bands below the OVA molecular weight of 45 kDa were detected in the oxidised lysate. These lower molecular weight products may be a result of proteolytic degradation. Despite resuspending the oxidised cells in protease inhibitors after oxidation and immediately prior to freeze thaw lysis, it is possible that degradation of proteins occured prior to extraction. Other researchers have noted that even with protease inhibitors, using ice cold solutions and working on ice smaller bands are still sometimes observed. Proteins can degrade before, during, and after extraction. Some peptide bonds can be more prone to hydrolysis than others giving rise to specific lower molecular weight proteolytic degradation products during boiling with reducing or denaturing agents. Amino acids are neutralized by sodium hypochlorite to form water and salt and this loss of hydroxyl ions is accompanied by a drop in pH. Hypochlorous acid, which is present in sodium hypochlorite solution, acts as a solvent when it comes into contact with organic tissue, releasing chlorine that, when combined with protein amino groups, forms chloramines. The combination of hypochlorous acid ($HOCl^-$) and hypochlorite ions (OCl^-) lead to amino acid degradation and hydrolysis[112]. Thus it is likely that pre-treatment of the B16.OVA cells with NaClO rendered the proteins more susceptible to hydrolysis during subsequent Western Blot preparation, resulting in the lower weight molecular bands observed in the Western Blots.

3.4.2 Cell Lines' Response To Oxidation-Induced Death

It was noted during B16.OVA oxidation experiments that 100% cell death could not be achieved. The killing ability of hypochlorous acid always plateaued leaving a small proportion of cells (≈ 2-5%) alive (Figure 22). We remain curious to know whether

these cells would be more immunostimulatory than cells that were sensitive to death by oxidation. However culturing these oxidation-resistant cells and generating lysate from them to compare the T cell response against soluble lysate and oxidised lysates remains to be done.

We were concerned that oxidation may require changing to a cell line that expressed membrane-bound OVA to avoid loss of soluble OVA during oxidation as NaOCl treatment may generate necrotic cells with porous membranes. We assumed that the holes in the membranes could allow OVA that accumulated by treatment with the transport inhibitor Brefeldin A to leak out and be lost during subsequent wash steps to remove the NaOCl. However this did not appear to be the case as anti-OVA Western Blots demonstrated the presence of OVA in the oxidised lysates as well as the soluble fraction lysates (Figure 20, Figure 21).

Small but significant differences were noted in the cytotoxic capacity of HOCl among the seven cell lines tested. These differences may reflect where these cells are located *in vivo*. For example the murine lung epithelial tumour cell line TC1 showed the greatest resistance to death by oxidation, with only 50% of cells dead in 90 μM HOCl (Appendix 1, Supplementary Figure 4). This result is biologically plausible since the lungs are exposed to the highest concentration of oxygen in the body and thus these cells may have evolved mechanisms of dealing with oxidative stress, such as a high capacity to neutralise reactive oxygen species (ROS). In contrast 90 μM HOCl killed 95% of THP-1, a human monocytic leukaemia cell line, and 90% of MC-38, a colon carcinoma cell line MC38. In the blood, the site of monocytic leukaemic cells, and in the glandular, mucus-secreting structures of the gut where adenocarcinomas such as MC-38 form, oxygen levels are tightly controlled within narrow ranges[113]. The cells of the gastrointestinal tract are well endowed with superoxide disumutase activity to neutralise free-radicals generated by food and pathogen-mediated inflammation[114,115]. Thus it would appear that, for the MC-38 cells at least, the increased sensitivity of these cells to death by oxidation might not result from a reduced capacity to adapt to high levels of ROS.

3.4.3 Generation of GMDCs & Macrophages

DCs and MΦs were generated using the technique of isolating murine bone marrow precursor cells and culturing them with GM-CSF that has been common practice in labs worldwide for decades since Inaba and Steinman first published this protocol[116]. There are two main methods commonly employed to generate what are currently known as bone marrow-derived DCs (BMDC) and two methods for generating either M1 or M2 bone marrow-derived MΦs (BMDM). In the longest used method so-called BMDC are generated with high-dose GM-CSF+/-IL-4, which gives rise to a heterogeneous cell population of adherent, semi-adherent and non-adherent myeloid-like cells. BMDM are cultured in GM-CSF+IL-3 or M-CSF to give rise to M1 or M2 MΦs respectively. Both the BMDC and M1 BMDM cell populations display all the typical monocyte-derived MΦ markers F4/80, CD11b, Ly6C, Ly6G, MHC-II, CD11c, CD80 and CD40, albeit to varying degrees.

MΦs are found in virtually every tissue type where they can limit infections early in the disease process and can initiate specific immune responses. The heterogenic nature of MΦs may be the reason for their remarkably plastic and wide-ranging involvement in innate and adaptive immune responses. However it also makes studying them difficult, and makes phenotyping BM-derived MΦs important so that we know which MΦ type we are studying. Any given myeloid cell population shares overlapping features of both DCs and macrophages making it difficult to assign them to one or other cell type. If one takes the argument that all GM-CSF-derived cells are MΦs[117], then this thesis lends weight to the idea that MΦs are in fact more effective in the anti-tumour response than previously thought.

M1-type bone marrow-derived MΦ are typically generated with low-dose GM-CSF and a high percentage of FCS (at least 10%) that induces a more MΦ-like phenotype to the cells due to as yet unidentified factors in the FCS[118]. IL-3 may or may not be included. According to Hume IL-3 and GM-CSF are not additive in culture, however we were unable to find any reference to murine studies that have analysed this. The phenotype of MΦs cultured +/- IL-3 was not compared in this study.

Several reports have highlighted that the GM-CSF BMDC-differentiation method generates a heterogeneous population of granulocytes, dendritic cells and MΦs[11,119,120]. As such, instead of comparing DCs, MΦs and B cells, or a combination thereof, it could be argued that this thesis has in fact compared two different populations of BM-derived APCs, each generated by different methods and displaying different phenotypes, both under the microscope and on the Flow Cytometer. However, irrespective of the name given to each population of cells, this thesis has demonstrated that BM-derived cells grown in GM-CSF+5% FCS or GM-CSF+IL-4+5% FCS or GM-CSF+IL-3+10% FCS show differential antigen presentation capacity and costimulation responses to lysate loading.

A statement from a recent review highlights that there are strong opinions on both sides of the DC MΦ divide but makes the important point, which this thesis and the bulk of current literature supports, that GM-CSF-differentiated cells are important tools that can be harnessed in the anti-cancer response: "*Csf-2 (GM-CSF) is a cytokine that is critical for promoting the differentiation of mouse and human hematopoietic progenitors and monocytes into cells that resemble mouse splenic cDCs … and remains to date a key cytokine for generating DC-based vaccines for clinical use*"[121].

Whether they are called DCs or MΦs or APCs, the bone marrow-derived APCs generated by GM-CSF are an immunogenic APC. It is for this reason that GM-CSF is in current clinical use in a number of treatment approaches, such as increasing the DC yield and reconstitution during haematopoietic stem cell (HSC) transplant[122]; most, if not all, *ex vivo* DC generation and tumour antigen loading protocols[123]; GM-CSF-secreting viral vectors for cancer that increase GM-CSF levels at the tumour site[124]; and finally as a vaccine adjuvant in immune-compromised Hepatitis B patients as well as Hepatitis C, HIV, fungal and mycobacterial treatments[125]. GM-CSF activates the MEK/ERK, PI3K/PKB,NFκB and STAT5 pathways that increase DC proliferation, survival, differentiation and immunogenicity. Flt3L, on the other hand, induces STAT3 signalling leading to downregulation of NFκB, MHC class II and upregulation of IDO, all of which produce tolerogenic DC functions[7].

Hume makes that point that "*Whether or not tissue macrophages derive from definitive progenitors in the marrow, and must transit through a blood monocyte precursor, it is possible to isolate committed progenitor cells from bone marrow that express receptors for hematopoietic growth factors, and to use those growth factors to generate relatively pure populations of macrophages (in CSF1), classical DC (in FLT3L), or macrophages with APC activity (in GM-CSF; otherwise called bone-marrow-derived DC)*"[126]. In other words Hume would argue that this study compared two variations on the MΦ theme as opposed to a DC and a MΦ. Hume would also argue that what this thesis refers to as GMDCs should more accurately be called antigen-presenting MΦs (APMΦ). Irrespective of the name given to these cells, this study aimed to define which of the three APCs would yield a phenotype that was most favourable for T cell presentation and activation.

3.4.4 Unactivated GMDC, IL4DC & Macrophage Morphology & Phenotypes

This is not the first study to compare cells generated from bone marrow with different cytokine combinations. Masurier *et al* compared human cells grown in GM-CSF, GM-CSF+Flt3L and GM-CSF+IL-4 and found that the MHC-II^{HI} cells with typical myeloid DC morphology and immunophenotype (CD11c^{+} 33D1^{+} DEC-205^{+} F4/80^{+}), were the most effective in tumour trials[120]. Campisano also examined heterogenous GM-CSF-stimulated murine cultures. They found that the addition of IL-4 induced DC maturation while impairing granulocyte and monocyte development[119]. The CD11c^{+} and CD11c^{-} fractions had similar MHC-II and CD80 levels, and similar phagocytic and endocytic capacities, however only the CD11c+ fraction when loaded with apoptotic/necrotic lysate induced protection in mice. Finally, another recent study has also highlighted the heterogeneic nature of GM-CSF-differentiated bone marrow populations. They compared the loosely adherent and non-adherent populations and found differences in MHC-II and CD11c expression indicative of DCs and MΦs[127]. Their bi-variate analysis of MHC-II versus CD11c expression revealed similar MHC-II CD11c profiles in their non-adherent cells to our GMDCs while the MHC-II CD11c profile of their loosely adherent cells was similar to our M1 MΦs.

The differences in the levels of MHC-II on M1 MΦs in Figures 25, 28 and 29 may be attributed to different MΦ culture methodologies employed between these two experiments. In the former the key difference between GMDC and M1 MΦ culture conditions were the variation in GM-CSF and FCS concentrations and the addition of IL-3 to the MΦ cultures. In the latter experiment, in the interests of methodological refinement to ensure the generation of two different cell populations, differentiating MΦ cultures were fed differently to those in Figure 27. On days four and seven non-adherent cells were removed by very gentle flushing of the plate, discarding of culture medium and replacement of fresh medium. This resulted in a greater proportion of large, adherent MΦ-like cells, whereas not removing the non-adherent cells resulted in a population that contained a greater proportion of cells that were more similar to those in the GMDC cultures. While CD11c expression in the MΦ populations was unaltered, MHC-II expression was greatly reduced to levels that would be expected on unactivated MΦs.

Direct comparison of MΦ versus DC median fluorescence could not be drawn due to the difference in MΦ auto fluorescence compared to DCs and the requirement for different voltages during acquisition by Flow Cytometry. Comparison of MHC-II density on the surface of these cells could be used as a mechanistic explanation for differences in T cell response initiated by MΦs and DCs. This may require the use of an internal standard measure, such as fluorescent beads, against which changes in median fluorescence intensity could be normalized. Since cells and not fluorescent beads were used for single stain controls in the majority of experiments, this standard measure is not available but could be included in future investigations.

The GMDCs generated in this study were CD8α- and therefore myeloid-like DCs. It has been reported that cross-presenting CD8α+ lymphoid DCs are responsible for potent anti-tumour responses[128] however CD8α- myeloid DCs engineered to secrete GM-CSF have also been shown to have potent anti-tumour activity[8]. Thus CD8α positivity or negativity *per se* may be irrelevant in the anti-tumour response.

The phenotypes generated in this study highlight the misnomer of CD11c+ cells being called DCs. We saw higher CD11c on our MΦs than our DCs and an abundance of

expression of CD11c on many MΦs as well as activated NK cells has been previously noted.

Our M1 MΦ displayed all the same markers as the GM-CSF-derived GMDC, with only variations in expression levels of some markers to distinguish the two cell populations (Figure 28). By contrast M2-like MΦ, generated with M-CSF, showed distinct marker expression levels from those of M1 MΦ and GMDC (Figure 24). The differences between the M-CSF- and GM-CSF-stimulated MΦs pointed to functional differences between the two MΦ types. These functional differences have been shown, for example, in a study that found that protective or immunostimulatory MΦs expressed high levels of MHC class II and drove a T_H1 bias, unlike MHC-II^{LO} MΦs, which predisposed to a T_H2 outcome[129].

Monocytes and eosinophils express low to intermediate levels of F4/80. F4/80 is commonly used as the canonical MΦ marker but levels of this marker vary by tissue and it is expressed on other cells. We saw F4/80 downregulation in RPMI medium. This alteration in cell phenotype would suggest a change of function, and highlights the importance of optimizing the growth conditions to generate the particular MΦ of interest. The fact that F4/80 is not present on various MΦ populations in different tissues, and expressed, albeit to a lower level on certain DC subpopulations, as well as on eosinophils, highlights the need for caution when using it as a sole MΦ-identifying marker[204].

Merad *et al* state in their 2013 review that Flt3L/CD135 expression is an "excellent marker" for discriminating cDCs from MΦs[121] but point out that collagenase digestion affects Flt3L expression. When raising cDCs from BM precursors collagenase is not used, thus this issue is only relevant when analysing non-lymphoid cDCs in tissue. However, if the GM-CSF-derived cells are Flt3L negative, then by Merad's argument they must be called MΦs and not DCs.

The gene transcription analysis used in Merad's review[121] also puts subcapsular MΦs inside cDC functional territory (Figure 36), which also argues for their antigen-

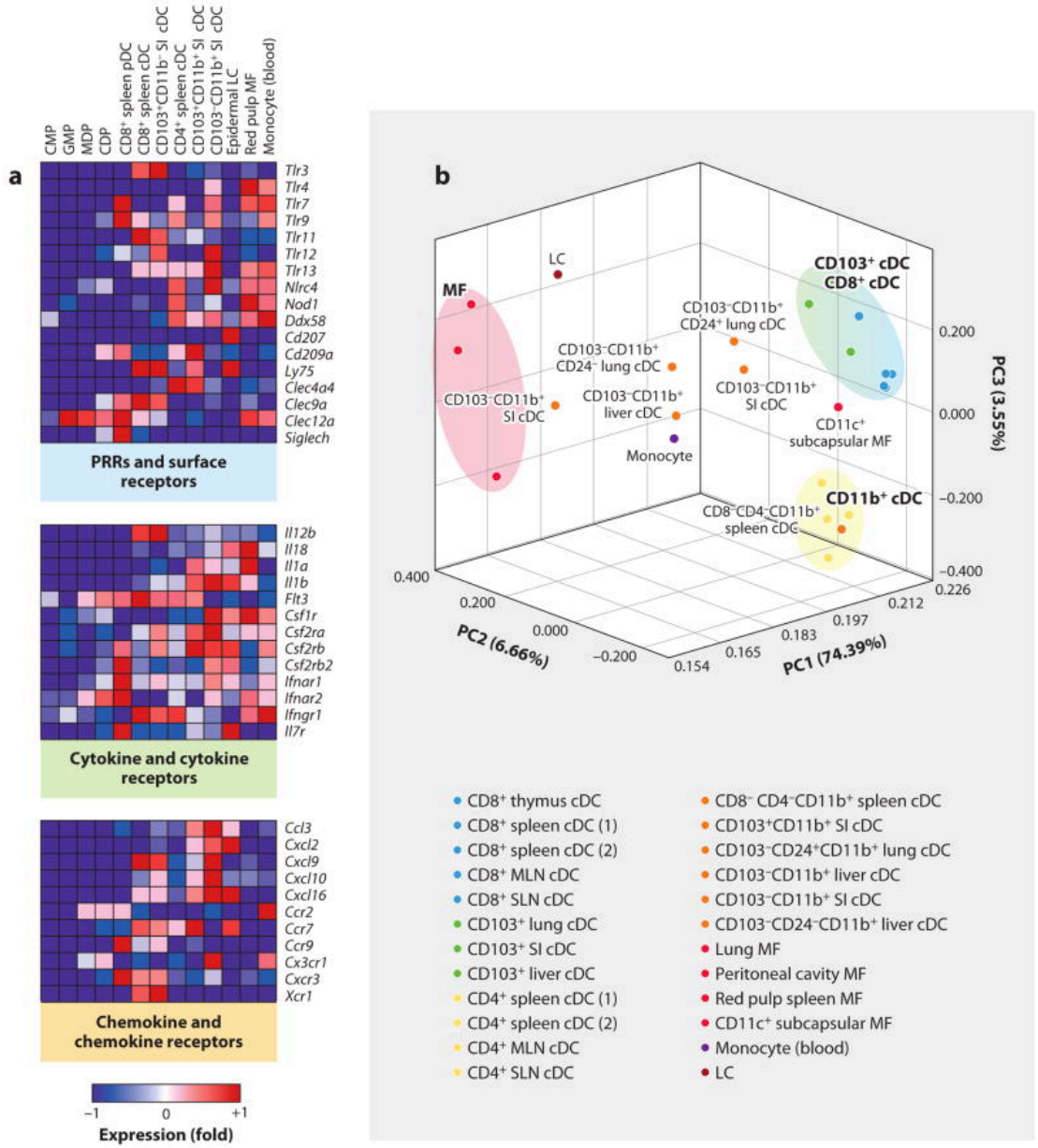


Figure 36 **Heat map representation of transcripts differentially expressed in progenitor and differentiated DCs**. (a) Heat map representation of pathogen-recognition receptors (PRRs) and antigen receptors, cytokine and cytokine receptors, and chemokines and chemokine receptors in common myeloid progenitors (CMPs), granulocyte macrophage progenitors (GMPs), macrophage DC progenitors (MDPs), common DC progenitors (CDPs), and $CD8^+$ spleen pDCs, $CD8^+$ spleen cDCs, $CD103^+CD11b^-$ lamina propria cDCs, $CD4^+$ spleen cDCs, $CD103^+CD11b^+$ lamina propria DCs, $CD103^-$ $CD11b^+$ lamina propria cDCs, epidermal Langerhans cells (LCs), red pulp macrophages (MFs), and blood monocytes. Red represents high and blue represents low relative expression. (*b*) Principal components analysis (PCA) of 15% of the most variable transcripts expressed by lymphoid tissue $CD8^+$ cDCs, lymphoid tissue $CD8^-$ cDCs, nonlymphoid tissue $CD103^+$ cDCs, nonlymphoid tissue $CD11b^+$ cDCs, epidermal LCs, monocytes, and MF populations provides a visual representation of the heterogeneity of the mononuclear phagocytic lineage. cDC and MF populations cluster distinctly on opposite sides of the PCA, whereas the $CD11b^+$ cDC distribution throughout the PCA suggests that these cells are more heterogeneous. Intriguingly, the CD11c subcapsular MF population clusters near DCs, suggesting that this population is more closely related to DCs than to macrophages (which is further suggested by the expression of zbtb46). (Additional abbreviations used in figure: MLN, mesenteric lymph node; SI, small intestine; SLN, skin-draining lymph node.) Figure published in The Dendritic Cell Lineage: Ontogeny and Function of Dendritic Cells and their Subsets in the Steady State and the Inflamed Setting[193]. Used with permission of the author, Miriam Merad.

presentation ability. "*The CD11b$^+$ cDC subset consists of a mixture of tissue cDCs and macrophages, which contributed to the confusion that still exists on the exact contribution of DCs and macrophages to tissue immunity*"[121]. This sentence from the Merad review implies that this current study worked with a mix of cDCs and MΦs and as such can not assign causality to either. However, once again, the purpose of this thesis was not to assign names to cell populations but to compare the functional abilities of the three populations.

We were particularly interested in CD169 after reading that CD169+ MΦs were responsible for cross-presentation of dead-cell associated antigens *in vivo*[50]. However none of our *in vitro*-generated cell populations were positive for this molecule (Figure 28). Therefore there is no evidence that CD169 played a role in cross-presentation by these cells.

By contrast the Colony stimulating Factor 1 receptor (CD115) was another marker, in conjunction with MHC-II, which proved somewhat useful as a DC-MΦ distinguishing marker (Figure 28). MΦs and GMDCs showed varying levels of intermediate expression. IL4DCs expressed this marker at levels significantly lower than MΦs. However, a *Csf1r*-EGFP transgene has been used as a marker for all tissue macrophages[130], and Helft *et* al recently described CD115 as being present on MΦs and absent on DCs[127]. Therefore this data would also argue for describing both of these cell populations as variations on the MΦ theme, rather than distinct DC and MΦ populations. The phenotype profiles of the two populations suggest that our GMDCs contain a greater proportion of DCs and our M1 MΦs contain a lesser proportion of DCs.

The mannose receptor CD206 appeared at surprisingly low levels on the GMDC and IL4DC (< 5%) (Figure10). On MΦs the expression of CD206 varied between 22.5 and 62.3%, significantly higher than either DC. However higher levels of the mannose receptor have been observed to be associated with M2 MΦs[131] therefore it is possible that high levels of CD206 were not necessarily desirable.

CD40 was also unexpectedly absent on GMDC. This key co-stimulatory molecule is usually expressed to some degree on immature GMDC, though not on M1 MΦs[127] and suggests a reduced T cell stimulatory capacity in these cells.

3.4.5 GMDC, IL4DC and M1 MΦ Activation Response to Soluble and Oxidised Lysates

Previous work has demonstrated activation of DCs in response to human tumour lysate[132,133]. Gallucci *et al* showed that signals from stressed or necrotically dying but not healthy or apoptotically dying cells activated DCs in the absence of foreign signals[134]. However it has also been shown that high numbers of apoptotic cells can stimulate DC maturation and presentation of endogenous antigen from apoptotic cells, even without accompanying "danger" signals[211]. Others have also reported DC cross-priming and activation of CTLs with apoptotic human cells[135,136] Thus controversy exists over the ability of apoptotic cells and cell lysates to activate APCs with some groups reporting DC inhibition in the presence of lysates[2]. Although as mentioned in the opening chapter of this discussion, RIPK-1 signalling and NFκB transduction have recently been identified as the key components in immunogenic cell death[111].

GMDCs, IL4DCs and MΦ were compared for upregulation of MHC Class II and the co-stimulatory markers CD40,CD80 and CD86 (Figure 30) in response to oxidised and soluble lysates. In line with Hatfield *et al*[2] who found that the soluble fraction of B16.OVA freeze-thaw lysates inhibited the DC response we found no upregulation of MHC-II, CD80 or CD86 on DCs or M1 MΦs in response to soluble or oxidised lysates (Figure 30). By contrast an increase in CD40 was observed on oxidised lysate-loaded GMDCs. There were some differences in methodology with Hatfield *et al* using a ratio of 3 tumour cells to 1 DC whereas our ratio was 1:1 (although Hatfield *et al* tested a range of ratios, 1:1 – 10:1 and found the same response). In addition Hatfield *et al* incubated their DCs with lysate for 8 hrs prior to the addition of the TLR agonists LPS 100 ng/mL; OK-432 10 μg/mL; poly I:C 100 μg/mL and CpG 2 μg/mL to mature the DCs. We added the LPS&CpG at the same time as the lysate.

Hatfield *et al* found that stressing cells with radiation or heat shock prior to freeze-thaw lysis significantly reduced lysate-induced inhibition of MHC-II as well as IL-12

production in LPS-treated DCs[2]. Their data showed that that induction of heat shock proteins (HSP), particularly HSP70, in the cells prior to lysate preparation resulted in less suppression of DC function. While we found no increase in MHC-II, CD80 or CD86 in DCs loaded with oxidised lysate, we did find that that pre-incubation of tumour cells in sodium hypochlorite increased the percentage of APCs positive for CD40 ($p<0.05$). HSP levels in the lysate were not assessed and therefore it is unknown whether HSPs played a role in upregulation of CD40 on the DCs by the oxidised lysate.

IL4DC showed no statistically significant upregulation of MHC-II, CD40, CD80 or CD86 in response to either lysate, with or without immune stimulants. Furthermore IL4DCs viability was lower than that of GMDC or MΦs when pulsed with soluble lysate and significantly so when pulsed with oxidised lysate (Figure 34). As such these surface molecule data may be biased due to the low viability of samples, but biased or not, IL4DC do not appear useful as lysate-presenting cells because of their low viability in the presence of the lysates.

Filaci and colleagues found that the DNA of apoptotic cells bound to HLA Class II molecules on human MΦs and this resulted in inhibited antigen presentation and down-regulation of costimulatory molecules, as well as increased production of anti-inflammatory TGF-β by the MΦs[137]. When DCs and MΦ were co-cultured the DCs showed reduced antigen presentation capacity, reduced costimulatory capacity and reduced TNF-α. Their summation of the situation was that the MΦs were behaving in a self-tolerising manner, in other words skewing toward an M2 response typical of homeostasis.

Thus, in our hands the superiority of oxidised tumour lysate over soluble freeze-thaw lysate depended on which APC was processing the lysate. Taken together these data showed that GMDC were more likely than MΦs to promote a T cell response against undefined lysate antigens.

3.4.6 B Cell Activation Response to Soluble and Oxidised Lysates

Activated B cells pulsed with freeze-thaw lysates have been successfully used to present undefined antigen to murine and human CD4+ T cells[32,36]. In our hands B cells only upregulated MHC-II in response to LPS&CpG whereas CD86 was upregulated in response to oxidised lysate (Figure 31).

A statistically significant increase in MHC-II was recorded when B cells were activated overnight with LPS&CpG and lysate loading occurred the following morning (Figure 33). No difference was observed between B cells loaded with lysates in this manner and B cells loaded overnight with LPS&CpG. When compared to the lysate-loaded B cells these data suggest that the LPS&CpG was important in the observed B cell MHC-II responses.

By contrast, the statistically significant increase in CD86 observed in oxidised lysate-loaded B cells, did not increase in the presence of LPS&CpG suggesting that factors in the lysate stimulated this costimulatory molecule whereas the LPS&CpG activated the MHC-II presentation machinery.

3.4.7 APC IL-12 Response to Soluble & Oxidised Lysates

IL-12 secreted by activated DCs after CD40 ligation provides crucial cytokine information that drives T_H1 differentiation[138], CTL effector function[139] and the development of T cell memory[140]. Therefore we were interested in the production of IL-12 by lysate-loaded APCs as an indicator of their capacity to stimulate IFN-γ production and promote T_H1 skewing of the immune response to undefined antigens.

GMDCs, M1 MΦs and IL4DCs all produced IL-12 in response to both soluble lysate and oxidised lysate (Figure 35) and the IL-12 production was significantly higher when the immune stimulants LPS&CpG were added to the APCs at the time of lysate loading. LPS&CpG were added in an attempt to improve the ability of the APCs to present lysate antigens to T cells. However comparison of the response to lysates alone versus lysates+LPS&CpG demonstrated that LPS&CpG was not required for the observed upregulation of CD40 on DCs, or for the upregulation of CD86 on B cells. The addition of LPS&CpG only enhanced MHC-II expression on GMDC.

However, despite the lack of consistent MHC-II and costimulatory molecule upregulation across the APCs in response to the addition of LPS&CpG to the lysates, a strong trend toward increases IL-12 production (p=0.06) was observed when LPS&CpG was added to both soluble and oxidised lysate. This increase was statistically significant in the M1 MΦ+s-L+LPS&CpG group compared to soluble lysate alone.

We were surprised to see strong IL-12 production by the M1 MΦs in response to activation and lysate loading, given their lacklustre MHC-II, CD40, CD80 and CD86 responses compared to the DCs. However, as with the GMDCs and IL4-DCs the M1 MΦ-produced IL-12 was not greater than that induced by LPS&CpG alone. Therefore the increase was due to the effect of the activation stimulants and not the lysate.

The B cell conditioned media was inadvertently discarded therefore no IL-12 response by B cells was evaluated. These experiments were carried out after the MΦ+B cell combination had been excluded as a viable option therefore no MΦ+B cell data exists for IL-12 production.

The combinations of GMDC+B cell and IL4DC+B cell were analysed and GMDC+B cell, but not IL4DC+B cell, stimulated greater IL-12 production than GMDC alone. In contrast to all other APCs loaded with lysate+LPS&CpG, in which there was no increase in IL-12 over that induced by LPS&CpG, the GMDC+B cell combination elevated IL-12 to levels higher than LPS&CpG alone. This suggested a synergistic effect generated by the interactions of these two APCs.

3.4.8 APC Viability Response to Lysates

In concordance with previous work that found that lysate was not toxic to DCs[2] we noticed only small reductions in viability in GMDC loaded with soluble lysate +/- stimulants and in GMDC loaded with oxidised lysate. However we did observe a significant drop in viability of GMDC loaded with oxidised lysate+LPS&CpG.

IL4DC viability was not as robust in the presence of either lysate. Only 60% of IL4DC remained alive after overnight pulse with soluble lysate and 56% were viable after overnight pulse with soluble lysate+LPS&CpG. IL4DC fared even worse when loaded with oxidised lysate and oxidised lysate+LPS&CpG. For this reason we terminated the comparative experiments on these DCs.

Tumour cells treated with HOCl prior to lysis were washed three times in DPBS therefore the likelihood of enough HOCl remaining in the lysates to cause APC cell death seems low. Also M1 MΦs retained excellent viability irrespective of lysate loading or LPS&CpG stimulation, and thus HOCl as a contributing factor in APC death seems further unlikely.

Incubation of GMDC and IL4DC for a further 24 hours after the addition of MΦ conditioned cell medium (MCM) resulted in no statistically significant improvement in viability (data not shown). However this experiment would have been improved if the MCM had been added at the start of the lysate pulsing period, rather than after 24 hours of lysate pulsing.

3.4.9 Concluding Remarks

In this study we investigated the capacity of three APC cell types to present tumour antigens using a model system in which tumour cells have been engineered to express ovalbumin. OVA is not a tumour antigen, however it is frequently used as a model system for for assessing MHC Class II-mediated CD4+ and MHC Class I-mediated CD8+ T cell responses. We assessed the potential for antigen presentation and costimulation of T cells by mouse GMDC, IL-4DC, M1 MΦ and B cells after loading with B16.OVA tumour lysates.

We hypothesized that, on exposure to lysates, GMDCs and M1 MΦs would display superior upregulation of MHC-II, costimulatory molecules and IL-12 compared to B cells. However, following oxidised lysate exposure GMDC, but not IL4DC or M1 MΦs, upregulated CD40, while B cells upregulated CD86. On exposure to LPS&CpG, though not lysates, GMDC and B cells upregulated MHC-II. These data indicated that GMDC and B cells, in concert with LPS&CpG, could potentially present undefined lysate-derived tumour antigen to CD4+ T cells, and provide

costimulatory signals for T cell activation. M1 MΦ, by contrast, appear unable to present lysate-derived tumour antigen to CD4+ T cells or to provide appropriate costimulation. IL4DC, by virtue of their low viability when pulsed with lysates, also appeared less useful as APCs in the lysate setting.

These data demonstrated by morphology, surface molecule phenotype and IL-12 response that the three culture conditions generated three distinct populations of cells whose response to lysate differed in key aspects of presentation and APC and T cell activation capacity. *In vivo,* cells of the mononuclear phagocyte system (MPS) encounter a diversity of growth factors, including CSF-1/IL34, GM-CSF, IL-4, IFN-γ, and Flt3L amongst others. Each factor influences the resultant MPS cell phenotype. Progenitor cells cultured in one of these individual growth factors most likely have no corresponding cell *in vivo*, and there are no data that distinguishes a growth factor that explicitly promotes distinct DCs nor excludes them from the MPS[117]. The name assigned to the cells in this study may be technically erroneous, however the data supports the conclusion that bone marrow-derived precursors stimulated with GM-CSF alone, which may be more accurately called MΦs, perform better when presenting undefined lysate antigens than those differentiated with GM-CSF and IL-4.

Finally GMDC and M1 MΦs both demonstrated enhanced IL-12 responses to both forms of lysate when LPS&CpG was included during antigen loading. Taken together these data suggest variances in processing of the undefined TAAs in TL by the APCs and point to varying T cell stimulatory capacities. We next compared their ability, individually and in combination, to stimulate CD4+ and CD8+ T cell responses to tumour-associated and self antigens found in the two lysates.

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4 The T Cell Response To Priming by Tumour Lysate-Loaded Dendritic Cells, B Cells, Macrophages & Combinations Thereof

4.1 Introduction

4.1.1 Activating T Cells With Lysate-Loaded APCs in Cancer Therapy

In studies using tumour lysate as the source of tumour antigen to activate T cells the APC used has primarily been a DC[1–43], with few studies utilizing MΦs[44] or B cells[45,46]. Tumour lysate-loaded DCs have been used in clinical trials for treatment of melanoma[6,20,21,29,30,47], breast cancer[5,32,48], glioblastoma multiforme[49,50], renal cell carcinoma[48], fibrosarcoma[39], cutaneous T cell lymphoma[18], prostate cancer[34], gynaecological[51], bone and soft tissue malignancies[41], and multiple myeloma[52], demonstrating the broad applicability of this approach to cancer in general. All of these lysate-loaded DC studies, both human and murine, showed an induction of immunogenic T cell responses. These variously included T cell proliferation; production of IFN-γ and other T_H1 cytokines; circulating antigen-specific T cells; *in vitro* and *in vivo* cytotoxicity; control of primary tumours and metastases; tumour-specific T cell responses; desirable T cell phenotypes; delayed type hypersensitivity (DTH) reaction against cancer cell lysates; and CD8+ cytotoxic T lymphocyte (CTL) influx at the tumour site. These responses demonstrate the immunogenic potential of lysate-primed T cells.

Lysates consist of large clusters of undefined proteins and MΦs or B cells may be able to acquire these proteins more effectively than DCs via their scavenging, BCR-mediated or BCR-independent uptake mechanisms. They may also serve as transfer agents passing lysate antigens to DCs for presentation to T cells[53–56]. We hypothesized that combining DCs with MΦs, and or B cells, may yield a more robust T cell response against tumours via a systems biology approach of cooperating APCs. In this chapter we evaluated the T cell response evoked by priming with the lysate-pulsed APCs over a range of time points.

4.1.2 The T cell Differentiation & Effector Phenotype Response To Lysate-Loaded APCs

T cell stimulation can be triggered by antigen-dependent and antigen-independent[57–59] means, the latter being triggered by cytokines. Analysis of cytokines produced by the cells also assists with identification of the type of T cell produced by the treatment. Cytokines secreted by T helper cells that stimulate APCs include IL-2 and IFN-γ by T_H1 cells and IL-4, IL-5, IL-6, IL-10, IL-13 by T_H2 cells (Reviewed in[60]). The cytokine profile (IFN- γ , TNF-α and IL-10) of T cells primed with DCs, MΦs, B cells and combinations of these three APCs was assessed as an indication of their skewing toward T_H1 and CTL effector functions.

Analysis of the T cell phenotype response to treatment is critical for determining why a treatment was succesful or why the treatment failed.We examined the naïve, T effector (T_{EFF}), T effector memory (T_{EM}), and T central memory (T_{CM}) status of the naïve and lysate-experienced CD4+ and CD8+ T cells. We assessed the surface markers CD27, CD28, PD-1, CD44, CD122, CD127, CD62L and CCR7 to compare the phenotypes of T cells primed by each of the lysate-loaded APCs or APC combinations.

Many groups look at the production of IL-2 as an indicator of T cell activation. IL-2 binds to activated T cells and the complex is rapidly internalised, resulting in a short signal time. Therefore there is little point staining for this cytokine unless cells are harvested at very early time points. More importantly, T cell proliferation by definition would suggest IL-2 production therefore assessing IL-2 production seemed tautologous in these experiments.

4.1.2.1 IL-12

IL-12 forms part of the Signal 3 cytokine information secreted by DC[61], IFN-γ-primed MΦs[62] and B cells[63–65] after CD40 ligation. IL-12 drives T_H1 differentiation[66], CTL effector function[67] and the development of T cell memory[68]. IL-12 potently induces IFN-γ production in T cells[63] and inhibits IL-4, thereby skewing the immune response away from a Type 2 response and promoting the T_H1 profile[61].

In situations of high antigen T cell activation can occur in the absence of Signal 3, but in situations of low antigen the contribution of adjuvant or IL-12 assistance is more critical. It has been noted that the "… *presence or absence of the third signal appears to be a critical variable in determining whether stimulation by antigen results in tolerance versus development of effector function and establishment of a responsive memory population*"[67c].

Optimal secretion of IL-12 is another commonly used indicator of successful T cell activation conditions[4] therefore we evaluated whether co-cultures of lysate-loaded APCs and T cells contained high levels of IL-12.

4.1.2.2 IFN-γ

The ability of T cells to secrete IFN-γ and demonstrate tumour cytotoxicity *in vitro* are two measures applied to the selection of cells for ACT for patients with solid tumours[69–71]. However retrospective analysis of ACT in melanoma patients has identified a negative correlation with *in vivo* effectiveness and *in vitro* TA-specific IFN-γ production and cytolysis[69,71–73](Figure 37). Cells expressing high levels of CD62L correlated with enhanced patient responses after adoptive transfer. Thus it is the combination of phenotype and effector cytokine function, which must be carefully analysed when assessing the T cell response to priming with undefined antigen.

The ratio of T_H1 to T_H2 cytokines induced by treatment, and induction of IFN-γ+ T cells in particular, is currently one of the most commonly used clinical indicators of a patient's tumour-specific immune response to cancer immunotherapy[5,6,24,31,74,75], therefore we wished to verify that IFN-γ was being produced in the undefined TAA-primed T cell co-cultures.

4.1.2.3 TNF-α

TNF-α is produced by monocytic lineage cells, primarily MΦs, which are also sensitive to TNF-α's effects (reviewed in[76] and[77]). Trauma or exposure to LPS stimulates production of high levels of TNF-α and it has been shown to be strongly pro-inflammatory with a central role in several inflammatory disease processes (reviewed in[76] and[77]).

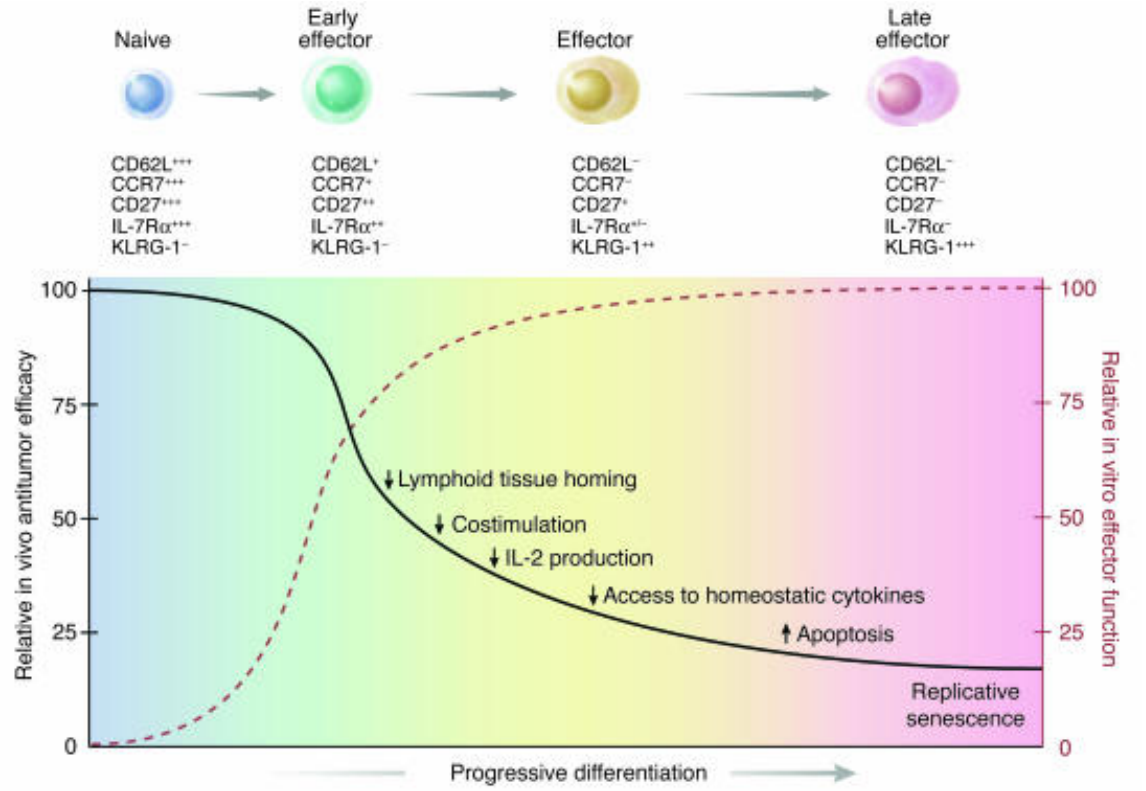

Figure 37 **A schematic illustration summarizing results indicating the inverse relationship of in vitro and in vivo effector functions of adoptively transferred naive and effector T cell subsets**. After primary antigen stimulation, naive CD8+ T cells proliferate and progressively differentiate into terminally differentiated effector T cells. Phenotypic and functional changes characterize the differentiation process. The gradual acquisition of complete effector functions (dashed red line) is associated with the progressive inability of T cells to cause tumour regression upon adoptive transfer (black line). Such mechanisms initially involve the downregulation of lymphoid-homing and costimulatory molecules, which results in a poor in vivo activation of T cells. Other mechanisms occur later and include the inability to produce IL-2 and access homeostatic cytokines, the imbalance of proapoptotic and anti-apoptotic signals, and the acquisition of a state of replicative senescence. +, low expression; ++, intermediate expression; +++, high expression. Figure taken from Acquisition of full effector function in vitro paradoxically impairs the in vivo antitumour efficacy of adoptively transferred CD8+ T cells. Gattinoni et al. J Clin Invest. 2005 Jun 1; 115(6): 1616–1626. doi: 10.1172/JCI24480. Used with kind permission of N. Restifo email correspondance 28.5.15.

TNF-α functions via activation of pathways that lead to three distinct cellular outcomes: survival and proliferation, pro-inflammatory gene transcription and cell death (reviewed in[78]). TNF-α has been shown to cooperate with IFN-γ to mediate tumour rejection[79,80]. Cellular growth is suppressed in tumour cells engineered to secrete high levels of TNF-α, even in the absence of T, B and NK cells (reviewed in [81]). The pleiotropic nature of TNF-α is highlighted by the anti-tumoural[82–84] and pro-tumoural[85–92] effects that have been extensively described, and by the fact that anti-TNF-α therapy has been implicated in the development of skin cancer[93] and lymphoma[94] .

Some recent insight into TNF-α's seemingly opposing roles has been gained with a study that suggests the membrane-bound TNF-α may have a protective role in cancer

while the soluble form may be responsible for pro-tumoural effects[95]. Therefore we wished to assess the level of soluble TNF-α in the conditioned cell media of our lysate-primed T cells.

4.1.2.4 IL-10

IL-10 is a well-characterised immunoregulatory cytokine that is important in maintenance of peripheral tolerance via its induction of T_{REG}s[96] and its skewing of the immune response toward a T_H2 outcome[97]. Tumour-derived IL-10, amongst other factors, can inhibit effective maturation of DC by suppressing their upregulation of MHC and co-stimulatory molecules, as well as IL-12 production[98]. MΦ-produced IL-10 has been shown to block CD8+ T cell-dependent responses to chemotherapy by suppressing IL-12 expression in intratumoural dendritic cells[99].

However, the role of IL-10 in the anti-tumour response is not clear-cut. Some studies demonstrated clear pro-tumoural associations in melanoma and other cancers[100–105]. Other studies have reported IL-10's anti-tumoural benefits, such as inhibition of tumour stroma formation[81] and tumour-toxic NO production[106], and identified IL-10 as a potentially positive prognostic factor[107,108]. For these reasons it was important to characterize the IL-10 response in lysate-primed T cells to verify if lysate skewed CD4+ T cells toward a T_H1 or T_H2 response, or may be responsible for any inhibition of T cell response observed.

4.1.3 Development of Memory Phenotype After Priming By Lysate-Loaded APCs

T_{EM} cells are found primarily in peripheral tissues where they deliver immediate protection upon antigen exposure through, for example, the prompt production of effector and inflammatory cytokines. T_{CM}, on the other hand, reside primarily in secondary lymphoid organs, and elicit recall reactions to previously encountered antigens. Even though T_{CM} lack immediate effector functions, they proliferate and differentiate quickly into effector T cells following antigen re-exposure. The molecules we examined are summarised in Table Form in Table 10.

Table 10 **Summary of expression and function of T cell molecules examined**

Surface Molecule	Expression and Function
CD27	Expressed on all naïve CD4+ T cells & approximately 80% of memory T cells. Downregulated on murine CD8+ T cells after antigenic stimulation – presence & absence used to identify memory T cells and effector T cells respectively [110,111]. CD27+CD8+ T cell persistence highly correlated with anti-tumour responses in ACT for metastatic melanoma[112,113]
CD28	Usually constitutively expressed on naïve, non-antigen-experienced T cells[114–117] Binds costimulatory markers CD80 (B7.1) and CD86 (B7.2)[118] on APCs. Ligation of CD28 and TCR delivers potent signals for naïve T cell activation and survival[119–122], proliferation and cytokine production - especially IL-2[121] and IL-6[123] Antigen presentation without CD28-mediated costimulation leads to T cell anergy[124,125]
CD44	Indicative marker for effector memory T cells (T_{EM}). Expressed at lower level on naïve T cells compared to memory T cells[126,127]
CD62L	Highly expressed on naïve T cells, allowing these cells access to secondary lymph tissue where they can encounter antigen. Shed from the cell membrane after antigen encounter[127,128] allowing exit from lymph tissue. Also expressed T_{CM} residing in secondary lymph tissue awaiting future antigen encounters[128,129]. T_{EM} circulating in the periphery have immediate effector functions upon encountering antigen, thus they do not express CD62L. The combination of CD62L, which is discarded after antigen encounter, and CD44, which is expressed at high levels after T cell activation, is used to identify T_{CM} cells[127]
CD122	β chain of the trimeric IL-2 receptor (IL-2R). IL-2 binding IL-2R promotes differentiation of T cells into effector T cells and memory T cells, as well as being required for memory cell maintenance[130,131]. CD122-negative cells display unresponsiveness to IL-2, indicative of reduced long-term persistence *in vivo*
CD127	Also known as IL-7 receptor α (IL-7Rα). Expressed by most resting T cells, downregulated following T cell activation. Long-living T cells characterized by constitutive CD127 expression[132]. CD127-negative cells display unresponsiveness to IL-7 indicative of reduced long-term persistence *in vivo*. Combination of CD122 and CD127 are used to assess survival potential and responsiveness of cells to homeostatic cytokine signalling *in vivo*. $CD127^{HIGH}$ population shown in ACT experiments to represent long living T central memory (T_{CM}) cells, especially when combined with $CD62L^{HIGH}$ status[133]
PD-1	Important immune-regulatory inhibitory receptor expressed on activated T cells, B cells and myeloid cells[134,135]. Low expression on naïve T cells. Expression upregulated on activated T cells[135,136]. Promotes programmed cell death (PCD) in antigen-specific T cells in LNs[136] while simultaneously reducing PCD in T_{REG}s[137]. High expression indicative of exhausted T cells with impaired anti-tumour function[138]

Taken together the combination of surface molecules expressed, and cytokines produced, by T cells allows us to assess their functional response to priming with lysate-derived undefined antigen and their potential utility in ACT.

We examined the memory phenotype transition in naïve CD4+ and CD8+ T cells and lysate-primed T cells. We wished to identify whether lysate-primed T cells would

adopt the T_{CM} phenotype that has been shown to be more effective on a per cell basis than T_{EM} in ACT for cancer[109].

4.2 Objectives

The aim of the studies summarised in this chapter was to assess the T cell response to being primed and/or restimulated by a lysate-loaded GMDC, M1 MΦ, B cell, or combination of these APCs. We hypothesized that the combination of two or more APCs would enhance the T cell response leading to stronger T cell proliferation, cytokine production and cytotoxic capacity.

To address this hypothesis the following objectives were undertaken:

1. Determine the strongest *in vitro* T cell responses to B16.OVA lysate antigens when presented to ovalbumin transgenic (OT)-II (CD4+) and OT-I (CD8+) T cells by lysate-loaded GMDC, M1 MΦ, B cells and combinations of these three APCs.

   This was accomplished via:

   1. Proliferation assays
   2. Cytokine analysis of IFN-γ, TNF-α, IL-12 and IL-10
   3. Phenotype analysis

2. Analysis of fold expansion and T cell phenotype yielded by priming naïve T cells with one antigen presenting cell and restimulating those antigen-experienced T cells with the same antigen presenting cell or with a different antigen presenting cell

4.3 Results

4.3.1 Tumour Lysate Dose Titrations

Our first aim was to determine the ability of the different lysate-loaded APC to stimulate CD4+ or CD8+ T cell proliferation. Isolated T cells were stained with the proliferation dyes Violet Proliferation Dye 450 (VPD450) or carboxyfluorescein succinimidyl ester (CFSE) and cultured with lysate-loaded APCs. We first confirmed that 72 hours was the optimal time point for assessing proliferation (See Appendix 1, Supplementary Figure 17) and thereafter conducted proliferation assays to analyse differences in T cell proliferation generated by each of the lysate-loaded APCs.

Previous work in our lab had shown by thymidine uptake assay that DCs pulsed with soluble lysate were able to induce CD8+ T cell proliferation[139]. Initially we wished to ascertain an optimal T cell stimulatory dose of tumour lysate therefore T cells were primed with DCs loaded with different volumes of lysate equating to different ratios of tumour cells to APC. We compared the T cell response to different concentrations of soluble fraction of freeze-thaw lysate (Figure 38) and to the whole portion of freeze-thaw lysate (data not shown). Figure 39 shows the Gating Strategy used to assess the proliferation response to OVA by Violet Proliferation Dye 450 (VPD450) stained CD8+ T cells.

CD8+ T cells responded differently to DCs loaded with different volumes of soluble lysate. Five microlitre volumes were insufficient to stimulate CD8+ T cell proliferation whereas 20 and 50 μL appeared to have an inhibitory effect on T cell proliferation. Ten microlitres of soluble lysate produced from B16.OVA cells that had been incubated in Brefeldin A for 5 hours prior to freeze-thaw lysis generated an average of 16% proliferation in five experiments and was chosen as the T cell-stimulatory volume for initial ongoing experiments. Ten microlitres equated to the contents of 240,000 lysed tumour cells, or a ratio of 6 tumour cells per DC. The whole portion freeze-thaw lysate yielded no proliferation response. These experiments were conducted prior to the oxidised lysate experiments. Later experiments that compared soluble and oxidised lysate used the oxidised lysate at the same volumes (APC:tumour cell ratios) as that of the soluble lysate.

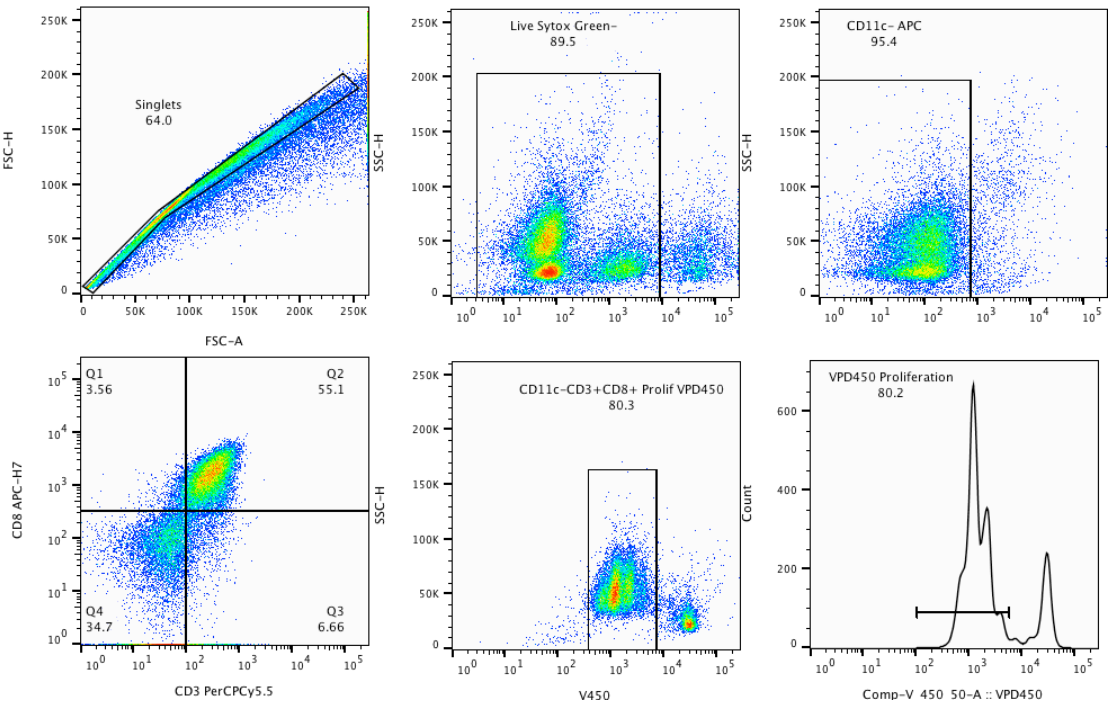

Figure 38 **Gating strategy for flow cytometric analysis of APC stimulated T cell proliferation assays.** 5 x 10^4 Day 6 GMDC were prepared as previously described. 5 x 10^4 DCs were pulsed overnight with soluble lysate (1 tumour cell:1APC) or OVA protein (50 μg/mL). The following morning gp100 and SIINFEKL peptides (2 μg/mL) were added to the relevant wells 4 hours prior to the addition of Violet Proliferation Dye 450-labelled, magnetic bead-isolated CD4+ or CD8+ T cells (10 T cells:1 APC). 72 hours later cells were stained with dead cell discrimination dye and labeled with mABs against CD11c, CD3 and CD8. Cells were fixed in 4% paraformaldehyde, stored overnight at 4°C and collected the following day on an LSR Fortessa or a Gallios Flow Cytometer. Flow data was analysed and graphed on FlowJo software V9 or VX. Representative data of the DC-stimulated CD8+ T cell response to OVA from approximately 30 experiments.

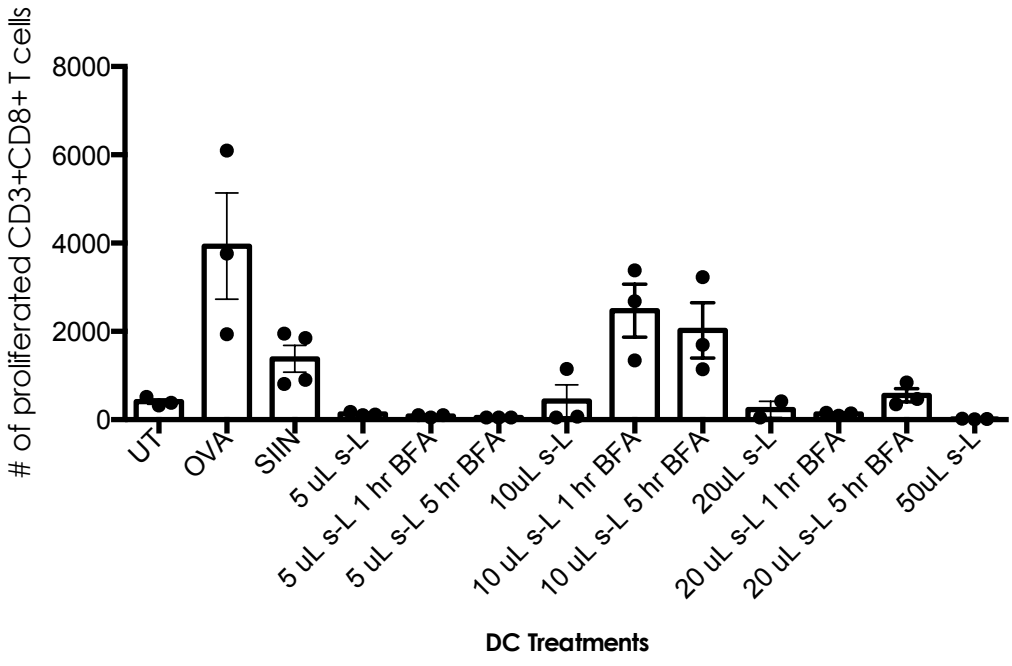

Figure 39 **GMDCs loaded with 10 μL of soluble fraction freeze-thaw lysate stimulate CD8+ T cell proliferation**. Bone marrow-derived precursor cells were incubated for 6 days in GM-CSF (20 ng/mL)+FCS (5%) (GMDC) 4.0 x 10^4 unactivated APCs were pulsed overnight with the soluble fraction of freeze-thaw lysate (s-L) (6:1 ratio tumour cell:APC) or whole OVA protein (50 µg/mL). The following morning 2 µg/mL SIIN peptide was added for at least 4 hours prior to the addition of VPD450-stained OT-I splenocytes (10 T cell:1 APC). After 72 hours cells were stained with dead cell exclusion dye, labeled with mABs to surface markers and fixed in 2% paraformaldehyde. Cells were stored overnight at 4°C and acquired the following day on an LSR Fortessa Flow Cytometer. Data was analysed on FlowJo Version 9 and graphed in Prism. Summary data of T cell proliferation after 72 hours exposure to s-L-loaded DC. Error bars = mean ± s.e.m. of 3 independent experiments plated in triplicate.

4.3.2 Activating Stimuli Concentration Titrations

An experiment was conducted to identify whether any improvements could be observed in the T cell response to APCs loaded with OVA protein and activated with various APC-activating stimuli. No difference was observed between doses or between the groups (LPS+IFN-γ, LPS+CpG, IFN-γ+CpG) (data not shown) therefore we continued with the LPS+CpG combination due to its MHC-II-enhancing effect on the B cells and its IL-12-inducing effect on GMDC and M1 MΦs.

4.3.3 T Cell Proliferation Response To Priming by Lysate-Loaded APCs

The clonal expansion of antigen-specific T cells is the outcome of effective peptide presentation and costimulation by APC. Thus proliferation is one key indicator of T cell responsiveness to antigen. Class I and Class II-restricted presentation of peptides from B16.OVA melanoma cell lysate was assessed via *in vitro* T cell proliferation assays of CD8+ and CD4+ T cells respectively. We assessed the proliferation response to the soluble fraction of freeze-thaw lysate and the entire portion of oxidized whole freeze thaw lysate .

Initial experiments utilised GMDC loaded with soluble lysate to prime CD4+ and CD8+ T cells. Immature Day 6 GMDCs were pulsed overnight with lysate at a ratio of 6 lysed tumour cells to 1 DC. The following day unsorted OT-I or OT-II splenocytes, or magnetic bead isolated CD4+ or CD8+ T cells were stained with proliferation dye and added at a ratio of 10 T cells to 1 DC. The cells were harvested after 72 hours of culture as this was the time point in which we saw the greatest proliferation when compared with 24 or 48 hours (Appendix 1, Supplementary Figure 19).

In preliminary experiments we used unsorted splenocytes in an attempt to get closer to the systems biology nature of the *in vivo* situation, and we compared these responses to those of sorted T cells. No differences were observed in the T cell responses between unsorted OT-I and OT-II splenocytes and isolated CD4+ and CD8+ T cells, therefore we continued with isolated CD4+ and CD8+ T cells for ongoing experiments.

4.3.3.1 DC Priming of T lymphocytes

We began by examining the T cell response to undefined tumour antigens in soluble lysate presented by unactivated GMDC. Figure 3 shows the gating strategy used to identify lysate-responsive T cells and Figure 40 shows summary data of these results.

In T cells primed with GMDC no response was observed to the irrelevant melanoma peptide gp100 in either OT-I or OT-II splenocytes (Figure 40). The functional control samples stimulated 62% CD4+ and 49% CD8+ T cell proliferation in response to whole OVA protein. The proliferation response to SIINFEKL peptide by CD8+ T cells was lower on 27%, while CD4+ T cell proliferation was 61% in response to the $OVA_{323-337}$ peptide. No difference was observed between the responses to OVA and $OVA_{323-337}$ peptide by CD4+ T cells. GMDC loaded with soluble lysate stimulated 19% CD8+ proliferation. This proliferation was not statistically significant when analysed by Kruskal-Wallis non-parametric comparison of location followed by a Dunn's post hoc test with Bonferroni correction for multiple comparisons. However a Mann Whitney U test comparing the UT and lysate-treated groups was statistically significant ($p<0.01$) CD4+ T cells did not proliferate in response to soluble lysate-loaded GMDCs.

Having noticed the low response to SIINFEKL peptide we compared the GMDC-primed T cell response to the SIINFEKL in our laboratory and the SIINFEKL of three other independent laboratories. There was no concentration-response to different doses of SIINFEKL (2, 4, 8, 10 μg/mL; data not shown) and no differences in the response to SIINFEKL from the four different sources indicating that the source of the low response did not lie in our batch of SIINFEKL (Appendix 1, Supplementary Figure 20).

Later experiments with LPS&CpG-activated APCs appeared to stimulate less CD8+ T cell proliferation than unactivated APCs (Appendix 1, Supplementary Figure 23). However the two conditions (+/-LPS&CpG) were not compared side by side in the same experiment so the reduced proliferation was not attributed to the use of the LPS&CpG at the time and these APC stimulants were used throughout the remainder of experiments.

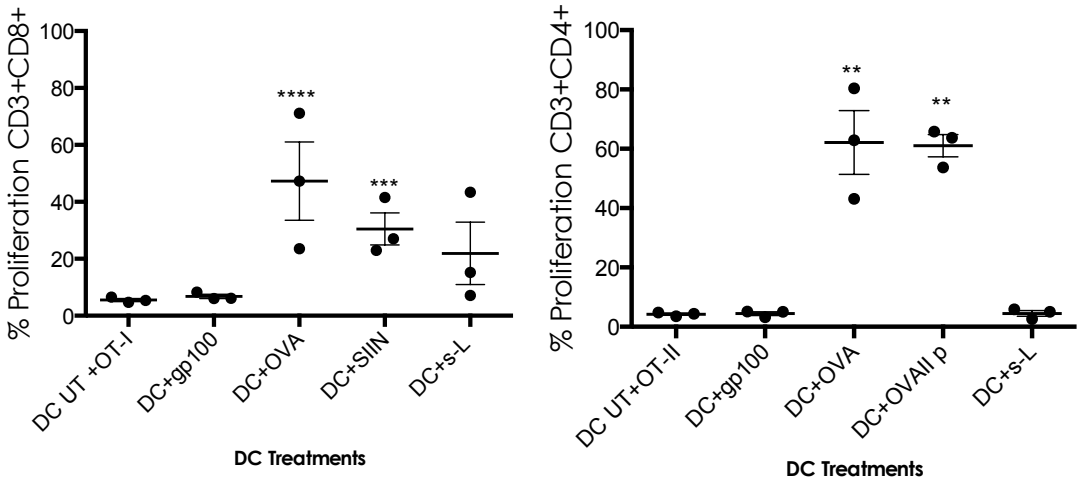

Figure 40 **GMDCs do not induce CD8+ or CD4+ T cell proliferation in response to soluble freeze-thaw lysate.** Day 6 GMDC were prepared as described previously. 5 x 10^4 GMDCs were pulsed overnight with lysate (6:1 tumour cell:APC) or OVA protein (50 μ g/mL). The following morning the irrelevant melanoma peptide gp100 and $OVA_{257-264}$ peptide (SIINFEKL) or $OVA_{329-337}$ peptide (2 μg/mL).\ were added 4 hours prior to the addition of 0.5 x 10^6 Violet Proliferation Dye 450-labelled CD4+ or CD8+ T cells. 72 hours later cells were stained with dead cell discrimination dye and labeled with mABs against CD11c, CD3 and CD8. Cells were fixed in 2% paraformaldehyde, stored overnight at 4°C and analysed the following day on an LSR Fortessa Flow Cytometer. Flow data was analysed on FlowJo software V9 and graphed in Prism. Summary data of the means of 3 independent experiments plated in triplicate. Statistically significant differences were assessed by Kruskal-Wallis test followed by Dunn's post test with Bonferroni correction. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. Error bars = mean ± s.e.m.

4.3.3.2 M1 MΦ Priming of T Lymphocytes

MΦs are known to have excellent scavenging capacity, which we hypothesised may be useful in acquiring lysate antigens for presentation to T cells. Unactivated M1 MΦs pulsed overnight with whole OVA protein generated an increase in proliferation, however this was not statistically significant by Kruskal-Wallis or Mann-Whitney U tests. Likewise the proliferation observed in response to the OVA323-339 peptide was also not statistically significant when analysed by the same tests. Finally, neither unactivated M1 MΦs pulsed overnight with soluble lysate (Figure 41), nor M1 MΦs pulsed overnight with soluble lysate+LPS+CpG (data not shown) were able to stimulate either CD4+ or CD8+ T cell proliferation.

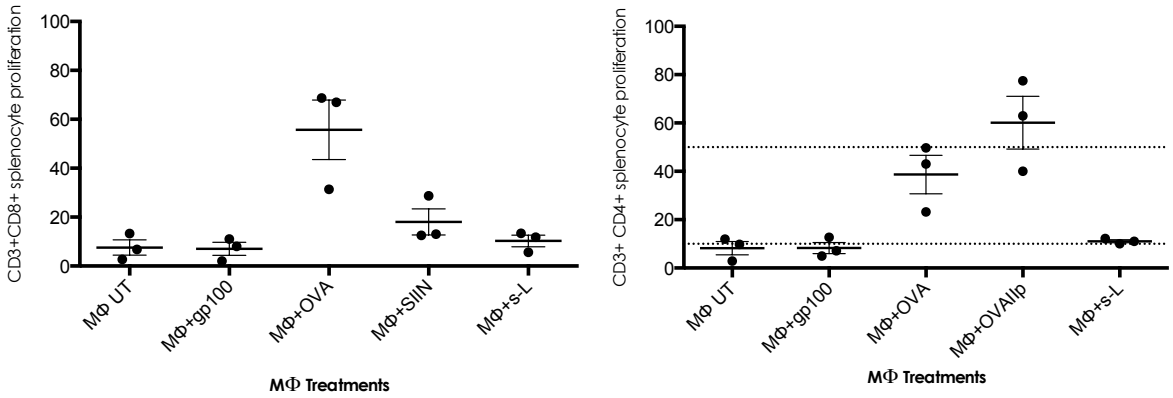

Figure 41 **M1 MΦs pulsed overnight with soluble lysate do not induce CD4+ or CD8+ T cell proliferation**. Day 10 M1 MΦs were prepared as previously described. 5 x 10^4 M1 MΦs were pulsed overnight with lysate (6:1 tumour cell:APC) or OVA protein (50 μg/mL). The following morning the irrelevant melanoma peptide gp100 and $OVA_{257-264}$ peptide (SIINFEKL) or $OVA_{329-337}$ peptide were added 4 hours prior to the addition of 0.5 x 10^6 Violet Proliferation Dye 450-labelled OT-I or OT-II splenocytes. 72 hours later cells were stained with dead cell discrimination dye and labeled with mABs against CD11c, CD3 and CD8. Cells were fixed in 2% paraformaldehyde and stored overnight at 4°C and analysed the following day on an LSR Fortessa Flow Cytometer. Flow data was analysed on FlowJo software V9 and graphed in Prism. Summary data of the means of 3 independent experiments plated in triplicate. Statistically differences were assessed by Kruskal-Wallis test followed by Dunn's post test with Bonferroni correction. Error bars = mean ± s.e.m.

4.3.3.3 B cell Priming of T lymphocytes

Activated B cells have been shown to be effective antigen presenting cells. Lysate-loaded activated B cells have been used in at least two studies[45,46], therefore we compared the ability of lysate-loaded B cells to stimulate T cell proliferation with that of GMDCs and MΦs. B cells were isolated from C57BL/6 mice by magnetic assisted cell sorting, pulsed with lysates overnight and co-cultured with proliferation dye-labeled OT-I or OT-II splenocytes for 72 hours. We compared unstimulated and CpG-activated lysate-loaded B cell stimulation of T cells. No significant improvements in T cell proliferation were observed in CpG-activated B cells (Appendix 1, Supplementary Figure 22) therefore unactivated B cells were used in these initial experiments.

Figure 42 shows summary data of the T cell proliferation response to unactivated soluble lysate-loaded B cell antigen presentation. B cells stimulated 80% CD8+ ($p<0.05$) and 66% CD4+ (ns) T cell proliferation in response to whole OVA protein. Likewise SIINFEKL and $OVA_{323-339}$ peptides yielded strong T cell responses - 94% and 93% respectively. However soluble lysate presented by unactivated B cells was unable to stimulate CD4+ or CD8+ T cells to proliferate. The ability of B cells to prime T cells with oxidised lysate was also assessed in two priming experiments. B cells activated overnight with oxidised lysate+LPS&CpG were unable to stimulate T

cell proliferation (data not shown) and so we discontinued assessing them as individual APCs.

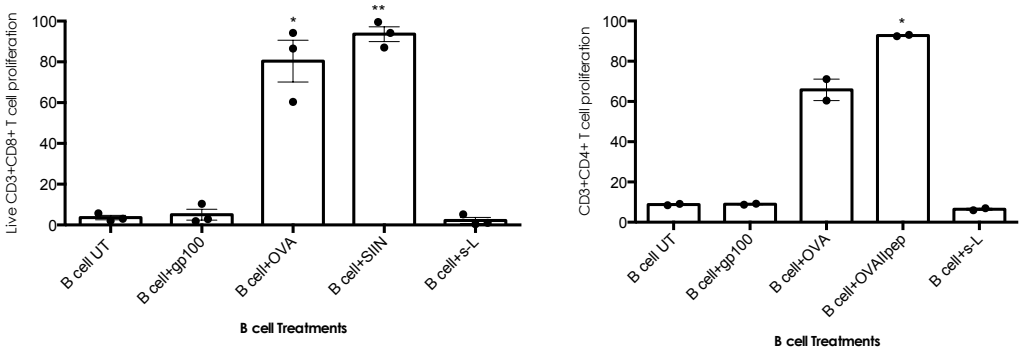

Figure 42 **Unactivated B cells do not induce CD4+ or CD8+ T cell proliferation in response to soluble freeze-thaw lysate**. B cells were isolated from spleens of C57/BL6 mice by anti-CD43 magnetic bead negative selection on an AutoMACS Pro. . Cells in the negative fraction were pulsed overnight with lysate (6:1 tumour cell:APC) or OVA protein (50 μ g/mL). The following morning the irrelevant melanoma peptide gp100 and $OVA_{257-264}$ peptide (SIINFEKL) or $OVA_{329-337}$ peptide were added 4 hours prior to the addition of 0.5 x 10^6 Violet Proliferation Dye 450-labelled OT-I or OT-II splenocytes. 72 hours later cells were stained with dead cell discrimination dye and labeled with mABs against CD19, CD3 and CD8. Cells were fixed in 2% paraformaldehyde, stored overnight at 4°C and analysed the following day on an LSR Fortessa (BD Biosciences) or a Gallios (Beckman Coulter) Flow Cytometer. Flow data was analysed on FlowJo software V9 and graphed in Prism. Summary data of 2 (CD4) and 3 (CD8) independent experiments carried out in triplicate. Statistically differences were assessed by Kruskal-Wallis test followed by Dunn's post test with Bonferroni correction. CD8+: the difference between OVA and UT was only significant without the adjustment for multiple comparisons. CD4+: the difference between OVA and UT was not significant with or without the adjustment for multiple comparisons * $p<0.05$; ** $p<0.01$. Error bars = mean ± s.e.m.

4.3.3.4 T Cell Priming by Combined Unactivated APCs Loaded With Tumour Lysate Antigens

Having assessed the ability of lysate-loaded GMDC, M1 MΦs and B cells to prime CD4+ and CD8+ T cells we now compared combinations of these three APCs. Figure 44 shows the gating strategy and sample raw data of the T cell response to soluble and oxidised lysate. Figure 43 shows summary data of the proliferation response by CD4+ T cells. Consistent with earlier results (Figure 40) CD4+ T cells did not proliferate in response to priming with lysate by any individual APC, nor did they proliferate when primed with any combination of APCs.

Figure 46 shows summary data of the proliferation response by CD8+ T cells in response to soluble lysate when presented by unactivated APCs. Each of the APC combinations yielded an increased number of CD8+ T cells over priming by an individual APC, however there was wide variability in the responses. The only

statistically significant increases were achieved in the B cell+M1 MΦ and triple combination groups when analysed by Kruskal-Wallis followed by a Dunn's post test without Bonferroni adjustment for multiple comparisons. Thus the results of these experiments were inconclusive.

In an effort to increase the low percentage of proliferation in response to lysate the selected immune stimulants LPS&CpG were added to the APCs at the time of lysate loading and the T cell proliferation response to both soluble and oxidized lysate was compared. No difference was recorded in the CD4+ T cell proliferation response to oxidised lysate compared to soluble lysate (figure 46) after two experiments and so, in light of previous similar CD4+ results this was not repeated a third time.

When the combination of LPS&CpG-activated GMDC+B cells was used to present oxidised lysate antigens CD8+ T cell proliferation averaged 29% compared to 11% in three independent experiments (Figure 46). However this proliferation was only statistically significant compared to UT groups (p<0.05). In addition the viability of T cells primed with oxidised lysate was markedly improved in nearly all priming conditions compared to T cells primed with soluble lysate. For these two reasons we continued to evaluate the usefulness of oxidised lysate.

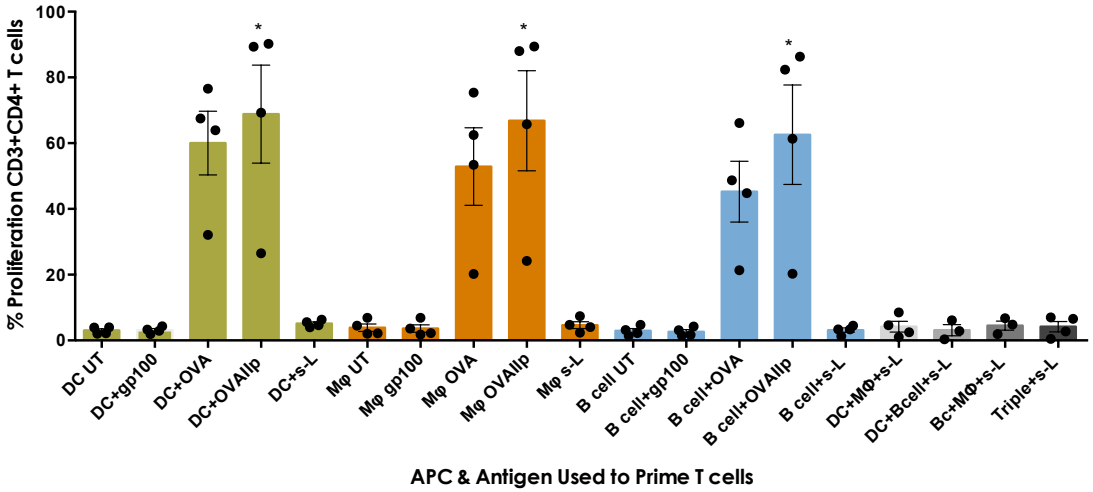

Figure 43 **Combining GMDC, M1 MΦs and B cells has no effect on the response of CD4+ T cells to priming with soluble lysate**. 5 x 10^4 Day 6 GMDC, Day10 M1 MΦs and freshly isolated splenic B cells, or combinations thereof, were pulsed overnight with whole OVA protein (50 μ g/mL) and B16.OVA TL (1:1 ratio tumor cell to APC). The total number of APCs plated was the same in each well. In double APC combination wells the ratios were 50:50. In triple combination wells the ratio was 1/3 of each APC. The following morning, the irrelevant melanoma peptide gp100 (2 μ g/mL) and SIIN (2 μ g/mL) were added 4 hours prior to the addition of 0.5 x 10^6 VPD450-labeled CD4+ T cells (10:1 ratio, T cell to APC). APCs and T cells were co-cultured for 72 hours, stained with dead cell exclusion dye, labeled with surface antibodies against CD11c, CD19 (dump), CD3 and CD4 or CD8. Cells were fixed in 4% paraformaldehyde, stored overnight at 4°C and collected the following day on a Gallios Flow Cytometer. Data was analysed on FlowJo version X and graphed in Prism. Summary data of 3 experiments plated in duplicate or triplicate. Statistically significant differences were assessed by Kruskal-Wallis followed by unadjusted Dunn's test* $p < 0.05$. Error bars = mean +/- s.e.m.

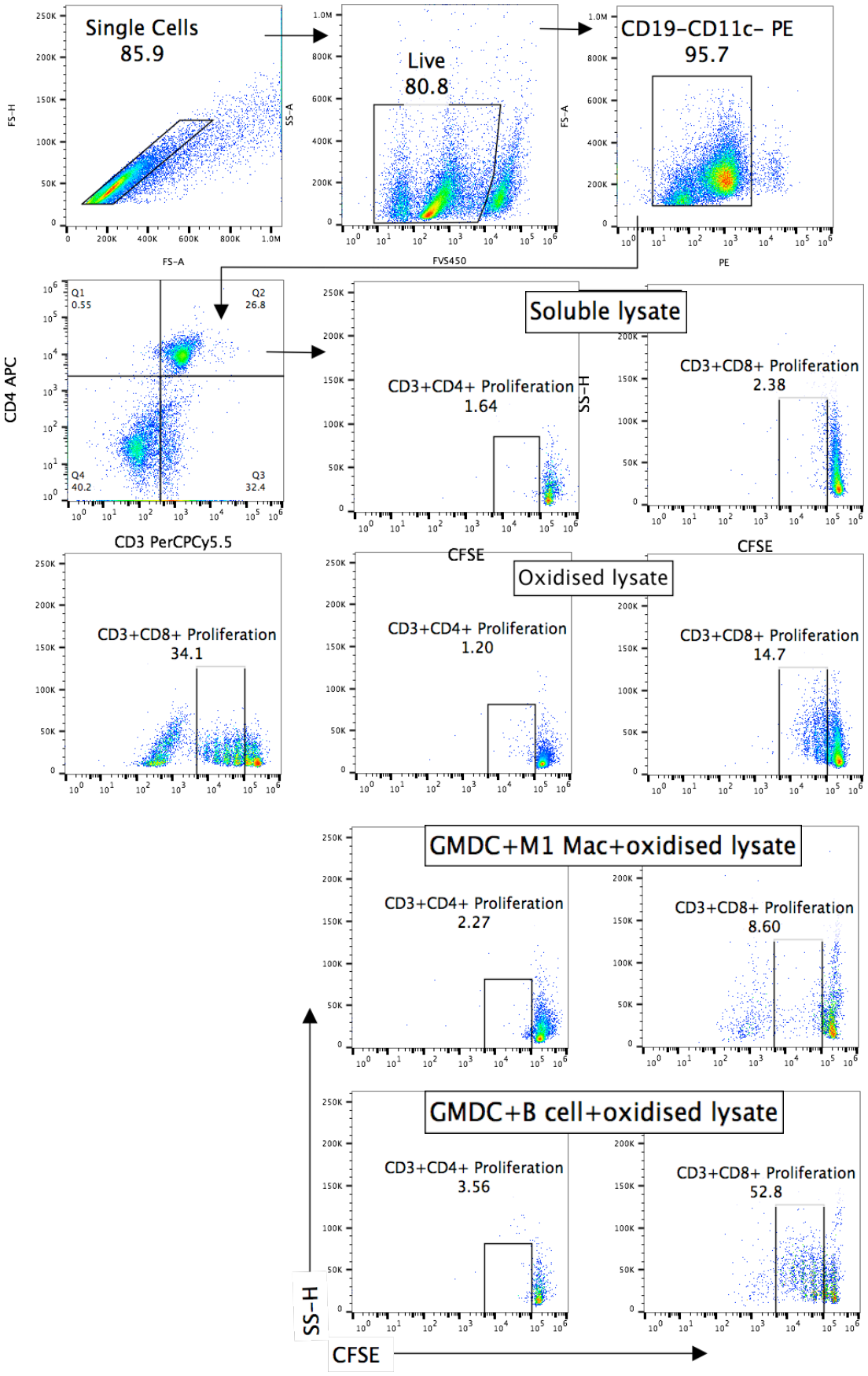

Figure 44 **Sample raw data of CD4+, CD8+ and CD4+CD8 T cell response to priming by GMDC, GMDC+MΦ and GMD+B cell loaded with soluble or oxidised lysate**. Day 6 C57/BL6 BMDC, Day10 M1 MΦs and freshly isolated splenic B cells, or combinations thereof, were prepared as described previously. 5.0 x 10^4 APCs in varying ratios (double combination: 50:50; triple combination: 1/3 each) pulsed overnight with whole OVA protein (50 μ g/mL) and B16.OVA TL (1:1 ratio tumor cell to APC) +/- LPS (1 µg/mL) and CpG (0.3 µg/mL). The following morning, VPD450- or CFSE-labeled 0.5 x 10^6 CD4+ or CD8+ T cells, or 0.25 x 10^6 of each, were added (10:1 ratio, T cell to APC). APCs and T cells were co-cultured for 72 hours, stained with dead cell exclusion dye, labeled with surface antibodies against CD11c, CD19 (dump), CD3 and CD4 or CD8. Cells were fixed in 4% paraformaldehyde, and stored overnight at 4°C. 5.0 x 10^4 cells per sample were acquired the following day on a Gallios Flow Cytometer. Data was analysed on FlowJo Version X. A dump channel for CD19 + and CD11c+ cells was included to preclude the possibility of B cell proliferation. The B cell+MΦ and Triple combinations were eliminated and so are not shown. Representative data from one experiment is shown. The GMDC+M1 MΦ and GMDC+B cell response to oxidised lysate only is shown.

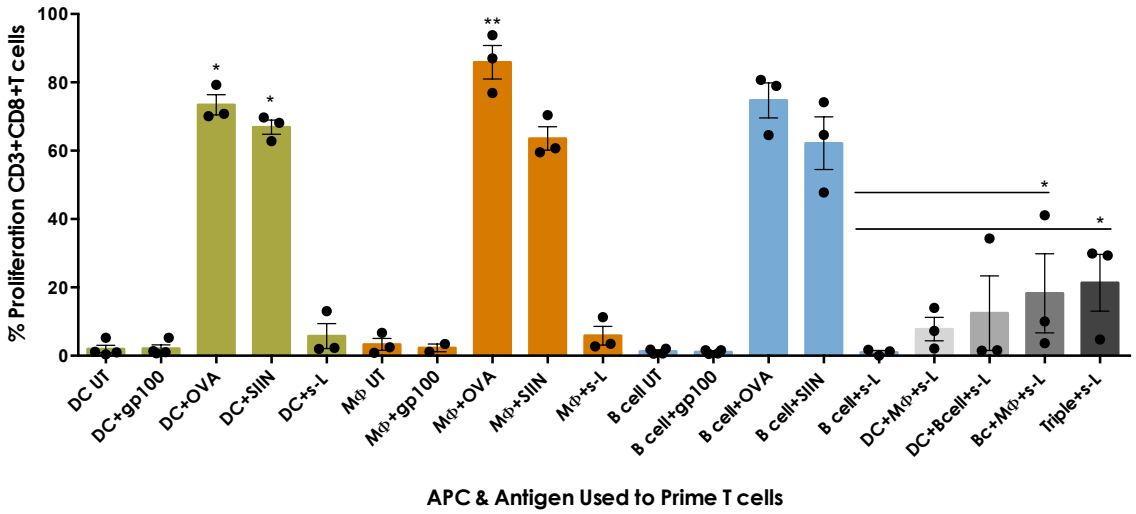

Figure 45 **The combination of GMDC+B cell, GMDC+M1 MΦ or GMDC+B cell+M1 MΦ stimulates a non-statistically significant trend toward increased proliferation of CD8+ T cells over that stimulated by GMDC, M1 MΦ or B cells alone**. 5. 5 x 10^4 Day 6 GMDC, Day10 M1 MΦs and freshly isolated splenic B cells, or combinations thereof, were pulsed overnight with whole OVA protein (50 μg/mL) and B16.OVA TL (1:1 ratio tumor cell to APC). The total number of APCs plated was the same in each well. In double APC combination wells the ratios were 50:50. In triple combination wells the ratio was 1/3 of each APC. The following morning, the irrelevant melanoma peptide gp100 (2 μg/mL) and SIIN (2 μg/mL) were added 4 hours prior to the addition of 0.5 x 10^6 VPD450-labeled CD8+ T cells (10:1 ratio, T cell to APC). APCs and T cells were co-cultured for 72 hours, stained with dead cell exclusion dye, labeled with surface antibodies against CD11c, CD19 (dump), CD3 and CD4 or CD8. Cells were fixed in 4% paraformaldehyde, stored overnight at 4°C and collected the following day on a Gallios Flow Cytometer. Data was analysed on FlowJo version X and graphed in Prism. Summary data of 3 experiments plated in duplicate or triplicate. Statistically significant differences were assessed by Kruskal-Wallis test followed by unadjusted Dunn's test* $p < 0.05$; ** $p < 0.01$. Error bars = mean +/- s.e.m.

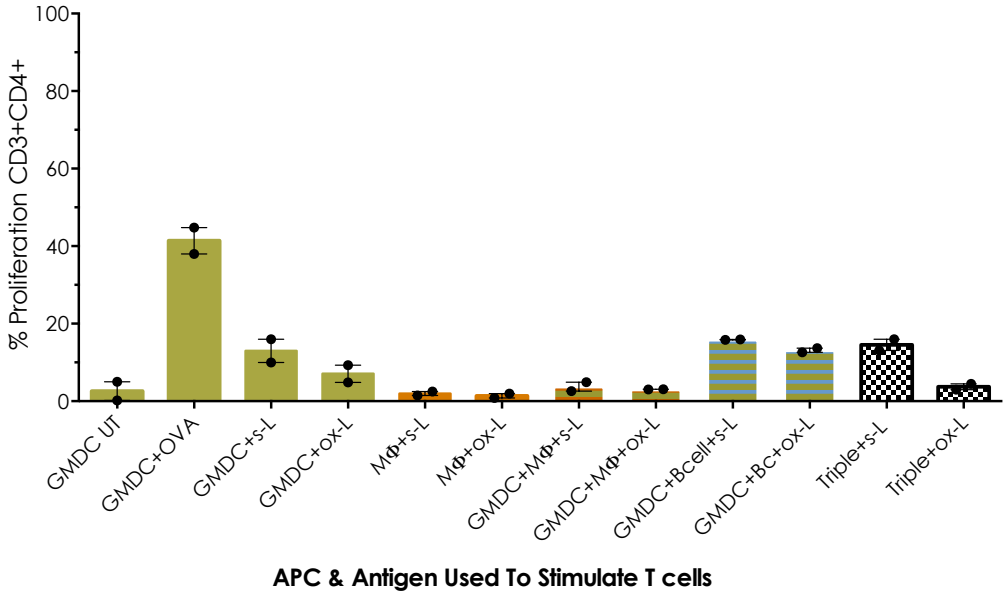

Figure 46 **Irrespective of whether it is presented by GMDC, M1 MΦ, B cell or combinations thereof, oxidised lysate does not stimulate more CD4+ T cell proliferation than soluble freeze-thaw lysate**. 5 x 10^4 Day 6 GMDC, Day10 M1 MΦs and freshly isolated splenic B cells, or combinations thereof, were pulsed overnight with whole OVA protein (50 μg/mL) and B16.OVA TL (1:1 ratio tumor cell to APC). The total number of APCs plated was the same in each well. In double APC combination wells the ratios were 50:50. In triple combination wells the ratio was 1/3 of each APC. LPS (1 µg/mL) and CpG (0.3 µg/mL) were added at the same time as the lysates. The following morning, the irrelevant melanoma peptide gp100 (2 μg/mL) and SIIN (2 μg/mL) were added 4 hours prior to the addition of 0.5 x 10^6 VPD450-labeled CD4+ T cells (10:1 ratio, T cell to APC). APCs and T cells were co-cultured for 72 hours, stained with dead cell exclusion dye, labeled with surface antibodies against CD11c, CD19 (dump), CD3 and CD4 or CD8. Cells were fixed in 4% paraformaldehyde, stored overnight at 4°C and collected the following day on a Gallios Flow Cytometer. Data was analysed on FlowJo version X and graphed in Prism. Summary data of 2 experiments plated in duplicate. Statistically significant differences were not assessed as only 2 replicates were conducted. Error bars = mean +/- s.e.m.

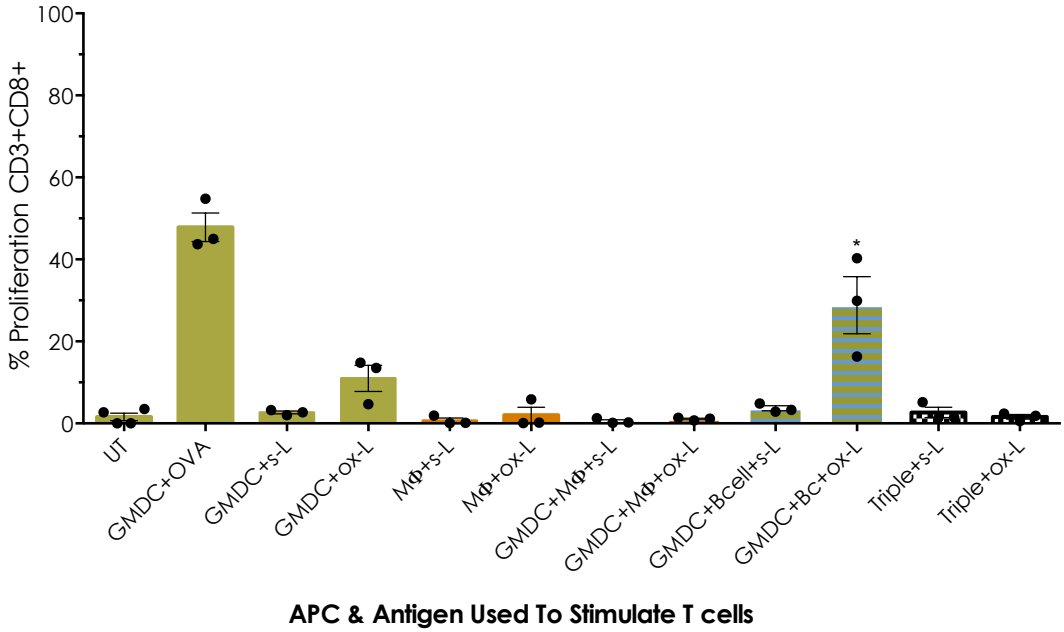

Figure 47 **Oxidised lysate only stimulates more CD8+ T Cell Proliferation than soluble freeze-thaw lysate when presented by GMDC+B cell**. 5 x 10^4 Day 6 GMDC, Day10 M1 MΦs and freshly isolated splenic B cells, or combinations thereof, were pulsed overnight with whole OVA protein (50 μ g/mL) and B16.OVA TL (1:1 ratio tumor cell to APC). The total number of APCs plated was the same in each well. In double APC combination wells the ratios were 50:50. In triple combination wells the ratio was 1/3 of each APC. LPS (1 μg/mL) and CpG (0.3 μg/mL) were added at the same time as the lysates. The following morning, the irrelevant melanoma peptide gp100 (2 μ g/mL) and SIIN (2 μ g/mL) were added 4 hours prior to the addition of 0.5 x 10^6 VPD450-labeled CD4+ T cells (10:1 ratio, T cell to APC). APCs and T cells were co-cultured for 72 hours, stained with dead cell exclusion dye, labeled with surface antibodies against CD11c, CD19 (dump channel), CD3 and CD8. Cells were fixed in 4% paraformaldehyde, stored overnight at 4°C and collected the following day on a Gallios Flow Cytometer. Data was analysed on FlowJo version X and graphed in Prism. Summary data of 3 independent experiments. Statistically significant results were calculated by Kruskal-Wallis test followed by unadjusted Dunn's test. * $p < 0.05$. Error bars = mean +/- s.e.m.

4.3.4 T Cell Cytokine Production in Response To Priming by Lysate-Loaded APCs

T cell proliferation assays assess only T cell clonal expansion and give no indication of effector functional capacity. Since T cell proliferation is not always accompanied by effector functions[140] functional assays such as cytokine production and cytotoxic capability are normally conducted alongside proliferation assays.

4.3.4.1 IFN-γ Response to Soluble and Oxidised Lysates

Lysate-loaded GMDCs, M1 MΦs and B cells, and combinations thereof, were compared for their ability to stimulate IFN-γ production in APC-CD4+ and APC-CD8+ T cell co-cultures. We tested cytokine production in both unactivated and LPS&CpG-stimulated samples. In CD4+ co-cultures unactivated lysate-loaded APCs, or APC combinations, stimulated essentially no IFN- γ (Figure 48).

In CD4+ T cell-APC co-cultures where the APCs had been activated with LPS&CpG the amount of IFN-γ produced was much higher than in unactivated samples (Figure 49). GMDC loaded with soluble lysate+LPS&CpG stimulated production of 35 ng/mL IFN- γ , and GMDC loaded with oxidised lysate +LPS&CpG stimulated 29 ng/mL. The DC+B cell combination yielded equivalent levels of IFN-γ (34 ng/mL in response to soluble lysate +LPS&CpG and 32 ng/mL in response to oxidised lysate +LPS&CpG). M1 MΦs, and the DC+MΦ and triple combinations stimulated essentially no IFN- γ when loaded with either soluble or oxidised lyate +LPS&CpG.. Thus in CD4+ T cell samples the GMDC+B cell combination yielded no advantage in IFN- γ production over GMDC alone.

In APC-CD8+ T cell co-cultures none of the unactivated individual APCs, or APC combinations stimulated production of statistically significant levels of IFN- γ (Figure 50).

As with the CD4+ T cells, the use of LPS&CpG along with the lysates stimulated greatly increased production of IFN- γ in CD8+ T cell-APC co-cultures (Figure 51), though these levels were not greater than those observed in CD4+ cultures. CD8+ T cells primed with GMDC loaded with soluble lysate+LPS&CpG or oxidised lysate+LPS&CpG induced production of 34 ng/mL and 39 ng/mL IFN-γ respectively, compared with 0.34 ng/mL in untreated samples. As with CD4+ T cells IFN-γ production in cultures of CD8+ T cells primed by M1 MΦs or the DC+MΦ or triple combinations loaded with either soluble or oxidised lysate +LPS&CpG was markedly lower than that stimulated by the GMDC or GMDC+B cell combinations. The DC+B cell combination again yielded the most IFN-γ in response to both soluble lysate+LPS&CpG (40 ng/mL) and oxidised lysate +LPS&CpG (44 ng/mL. However these increases were not statistically significant compared to GMDC when analysed by Kruskal-Wallis followed by unadjusted Dunn's test. The only significant differences were between GMDC or GMDC+B cell lysate-treated groups and UT cells.

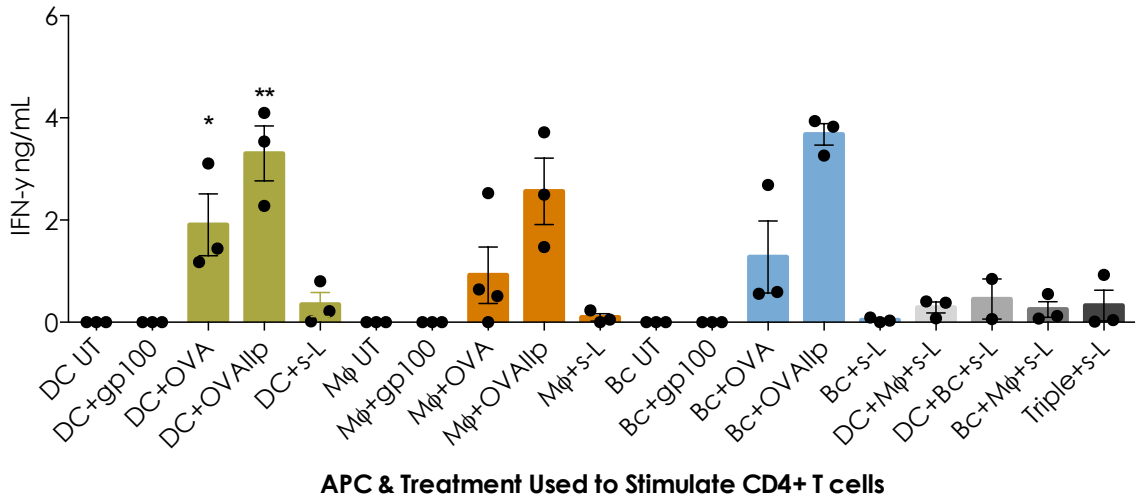

Figure 48 **A No increase in IFN-γ production is observed when CD4+ T cells are primed with combinations of unadjuvanted soluble lysate-loaded GMDC, M1 MΦ and B cells**. 5 x 10^4 Day 6 GMDC, Day10 M1 MΦs and freshly isolated splenic B cells, or combinations thereof, were pulsed overnight with whole OVA protein (50 μg/mL) and B16.OVA TL (1:1 ratio tumor cell to APC). The total number of APCs plated was the same in each well. In double APC combination wells the ratios were 50:50. In triple combination wells the ratio was 1/3 of each APC. LPS (1 μg/mL) and CpG (0.3 μg/mL) were added at the same time as the lysates. The following morning, $OVA_{323-339}$ peptide (2 μg/mL) was added 4 hours prior to the addition of 0.5 x 10^6 VPD450-labeled CD4+ T cells (10:1 ratio, T cell to APC). APCs and T cells were co-cultured for 72 hours, supernatants collected prior to harvest and stored at -80° C prior to analysis by anti-IFN-γ ELISA as described in Materials and Methods. Data was analysed in Exel and graphed in Prism. Summary data of 3 independent experiments. Statistically significant results were calculated by Kruskal-Wallis test followed by unadjusted Dunn's test. * $p < 0.05$; ** $p < 0.01$. Error bars = mean +/- s.e.m.

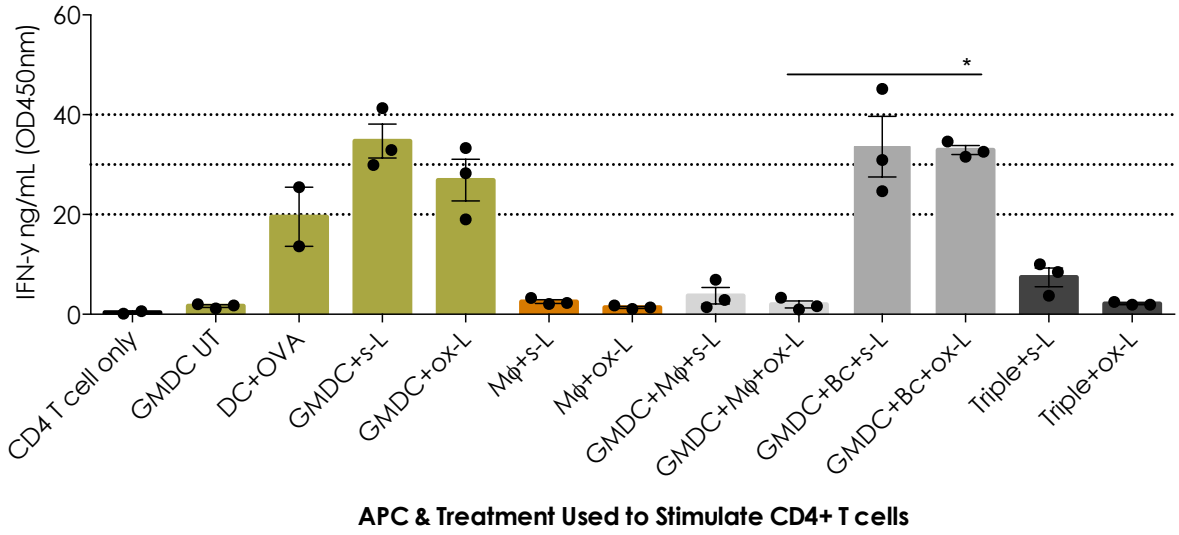

Figure 49 **CD4+ T cells primed with soluble or oxidised lysate-loaded GMDC or GMDC+B cell +LPS&CpG produce equivalent amounts of IFN-γ .** 5 x 10^4 Day 6 GMDC, Day10 M1 MΦs and freshly isolated splenic B cells, or combinations thereof, were pulsed overnight with whole OVA protein (50 μg/mL) and B16.OVA TL (1:1 ratio tumor cell to APC). The total number of APCs plated was the same in each well. In double APC combination wells the ratios were 50:50. In triple combination wells the ratio was 1/3 of each APC. LPS (1 μg/mL) and CpG (0.3 μg/mL) were added at the same time as the lysates. The following morning 0.5 x 10^6 VPD450-labeled CD4+ T cells (10:1 ratio, T cell to APC) were added. APCs and T cells were co-cultured for 72 hours, supernatants collected prior to harvest and stored at -80° C prior to analysis by anti-IFN-γ ELISA as described in Materials and Methods. Data was analysed in Exel and graphed in Prism. Summary data of 3 independent experiments. Statistically significant results were calculated by Kruskal-Wallis test followed by unadjusted Dunn's test. * $p < 0.05$. Error bars = mean +/- s.e.m.

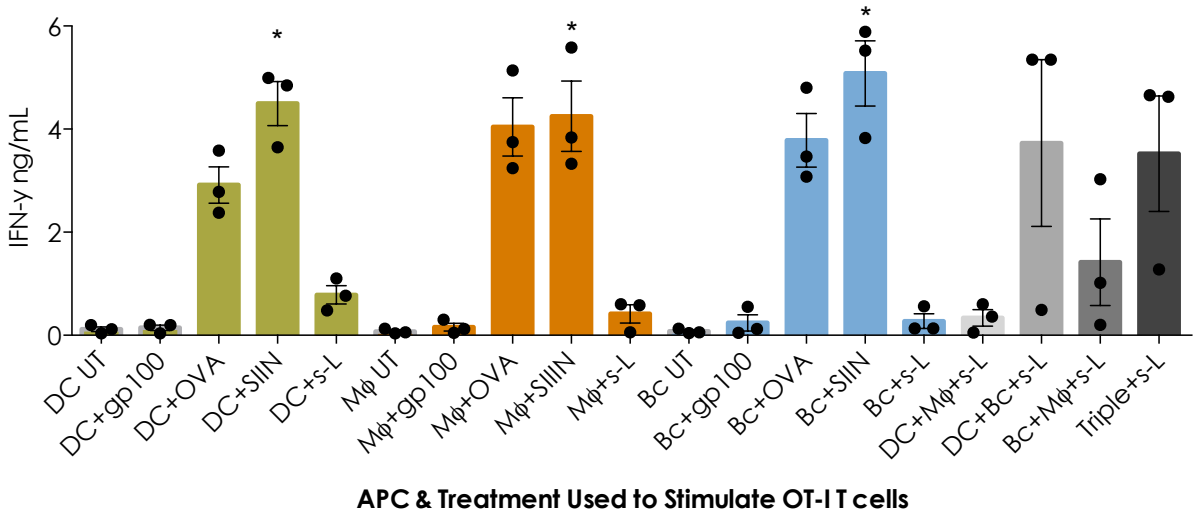

Figure 50 **No statistically significant increases in IFN-γ production are observed when CD8+ T cells are primed with unadjuvanted lysate-loaded GMDC, M1 MΦ , B cell or combinations thereof**. 5 x 10^4 Day 6 GMDC, Day10 M1 MΦs and freshly isolated splenic B cells, or combinations thereof, were pulsed overnight with whole OVA protein (50 μg/mL) and B16.OVA TL (1:1 ratio tumor cell to APC). The total number of APCs plated was the same in each well. In double APC combination wells the ratios were 50:50. In triple combination wells the ratio was 1/3 of each APC. LPS (1 μg/mL) and CpG (0.3 μg/mL) were added at the same time as the lysates. The following morning, SIINFEKL peptide (2 μg/mL) was added 4 hours prior to the addition of 0.5 x 10^6 VPD450-labeled CD8+ T cells (10:1 ratio, T cell to APC). APCs and T cells were co-cultured for 72 hours, supernatants collected prior to harvest and stored at -80°C prior to analysis by anti-IFN-γ ELISA as described in Materials and Methods. Data was analysed in Exel and graphed in Prism. Summary data of 3 independent experiments. Statistically significant results were calculated by Kruskal-Wallis test followed by unadjusted Dunn's test. * $p < 0.05$. Error bars = mean +/- s.e.m.

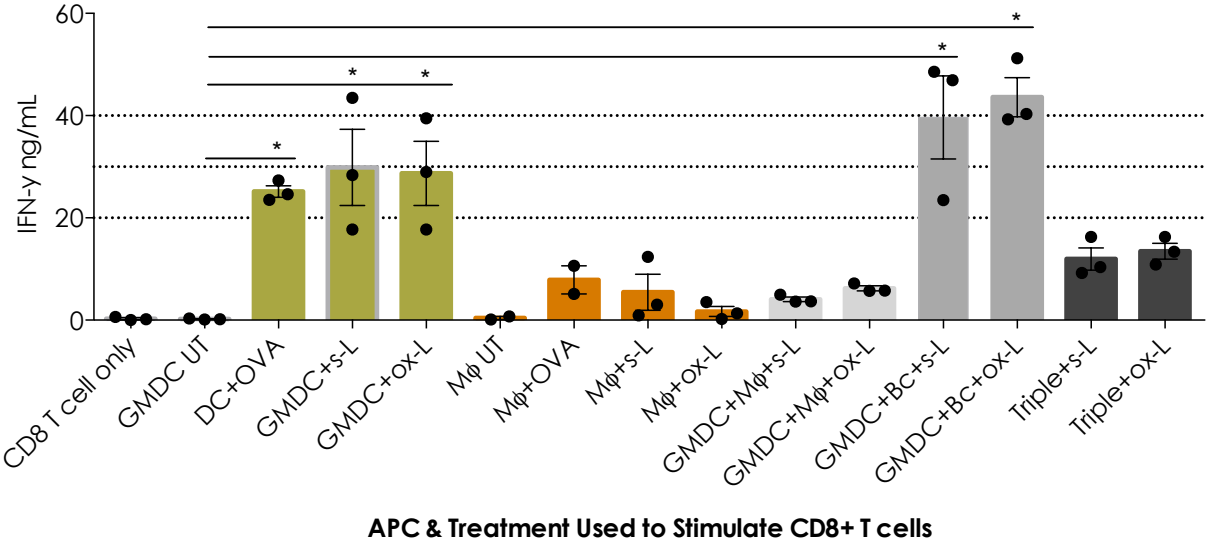

Figure 51 **When soluble or oxidised lysates are presented to CD8+ T cells by GMDC+B cell+LPS&CpG no increase in IFN-γ production is observed compared to presentation by GMDC alone**. 5 x 10^4 Day 6 GMDC, Day10 M1 MΦs and freshly isolated splenic B cells, or combinations thereof, were pulsed overnight with whole OVA protein (50 μg/mL) and B16.OVA TL (1:1 ratio tumor cell to APC). The total number of APCs plated was the same in each well. In double APC combination wells the ratios were 50:50. In triple combination wells the ratio was 1/3 of each APC. LPS (1 μg/mL) and CpG (0.3 μg/mL) were added at the same time as the lysates. The following morning 0.5 x 10^6 VPD450-labeled CD8+ T cells (10:1 ratio, T cell to APC) were added. APCs and T cells were co-cultured for 72 hours, supernatants collected prior to harvest and stored at -80° C prior to analysis by anti-IFN-γ ELISA as described in Materials and Methods. Data was analysed in Exel and graphed in Prism. Summary data of 3 independent experiments. In DC+OVA, MΦ UT, MΦ+OVA and Triple+s-L only two observations were recorded. Statistically significant results were calculated by Kruskal-Wallis test followed by unadjusted Dunn's test. * $p < 0.05$. Error bars = mean +/- s.e.m.

4.3.4.2 IL-12 Response to Soluble and Oxidised Lysates

IL-12 secreted by activated DCs after CD40 ligation is a crucial cytokine for driving T_H1 differentiation[66], CTL effector function[67] and the development of T cell memory[68]. We therefore examined the IL-12 in co-cultures of T cells primed by lysate-loaded APCs. After 48 hours exposure to soluble and oxidised lysate-loaded APCs cell conditioned media were collected for analysis by anti-IL-12 ELISA. All untreated samples produced less than 15 ng/mL IL-12, and all OVA-primed samples produced a minimum of 29 ng/mL (range 29 – 105 ng/mL) (Figure 52, Figure 53).

shows summary data for CD4+ T cell-APC co-cultures. GMDC loaded with soluble or oxidised lysate stimulated 38 ng/mL and 44 ng/mL IL-12 respectively, significantly more than the untreated samples (1.6 ng/mL; $p<0.05$). The combination of GMDC+B cell loaded with soluble lysate generated 28 ng/mL IL-12 while oxidised lysate yielded 30 ng/mL, The difference in IL-12 between GMDC and GMDC+B cell was not statistically significant for either soluble or oxidised lysate.

We did not see any proliferation in CD4+ T cells primed with GMDC+OVA, GMDC+MΦ+lysates or the triple combination+lysates. We did not expect to see IL-12 production in these groups, however one group was checked and there was no increased IL-12 in groups containing MΦs.

Figure 52 shows summary data for CD8+ T cell-APC co-cultures. These results showed a similar pattern to that of CD4+ T cells with no statistically differences in IL-12 production between groups.

However there appeared to be less IL-12 in GMDC+B cell groups so we compared IL-12 production in GMDC+B cell cultures prior to the addition of T cells (Figure 54). The combination of B cell+GMDC nearly tripled the average production of IL-12 in the soluble lysate-treated samples (64 ng/mL compared with 21 ng/mL in GMDC+soluble lysate; $p<0.05$). A similar response was seen in the oxidised lysate-treated samples with 26 ng/mL IL-12 in GMDC+oxidised lysate and 55 ng/mL in the GMDC+B cell+oxidised lysate groups, however this result did not reach statistical significance.

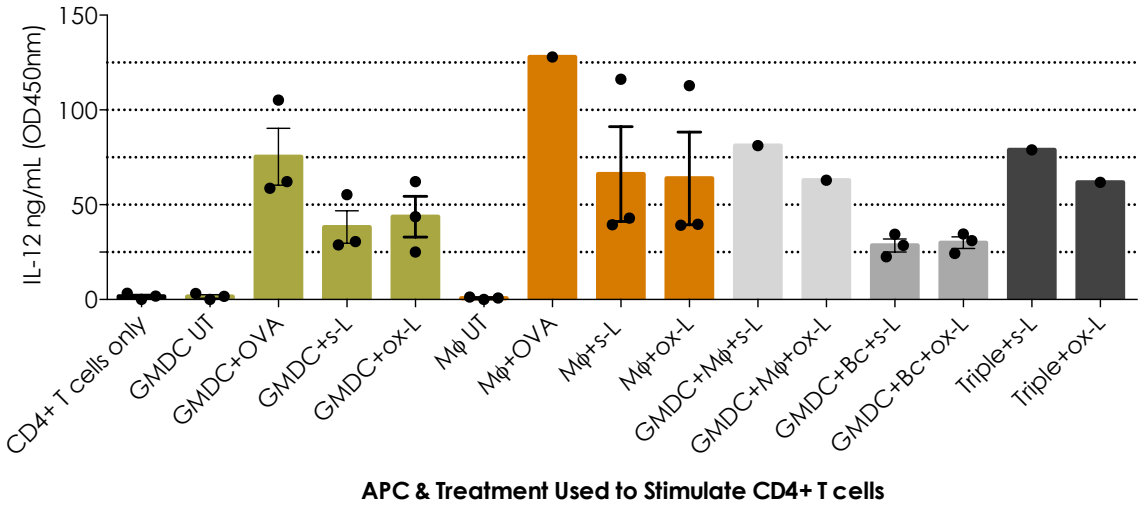

Figure 52 **The production of IL-12 is not increased when two or three APCs are used to prime CD4+ T cells with B16.OVA soluble or oxidized tumour lysate**. 5 x 10^4 Day 6 GMDC, Day10 M1 MΦs and freshly isolated splenic B cells, or combinations thereof, were pulsed overnight with whole OVA protein (50 µg/mL) and B16.OVA TL (1:1 ratio tumor cell to APC). The total number of APCs plated was the same in each well. In double APC combination wells the ratios were 50:50. In triple combination wells the ratio was 1/3 of each APC. LPS (1 µg/mL) and CpG (0.3 µg/mL) were added at the same time as the lysates. The following morning 0.5 x 10^6 VPD450-labeled CD4+ T cells (10:1 ratio, T cell to APC) were added. APCs and T cells were co-cultured for 72 hours, supernatants collected prior to harvest and stored at -80° C prior to analysis by anti-IFN-γ ELISA as described in Materials and Methods. Data was analysed in Exel and graphed in Prism. Summary data of 3 independent experiments. Statistically significant results were calculated by Kruskal-Wallis test followed by unadjusted Dunn's test. Error bars = mean +/- s.e.m.

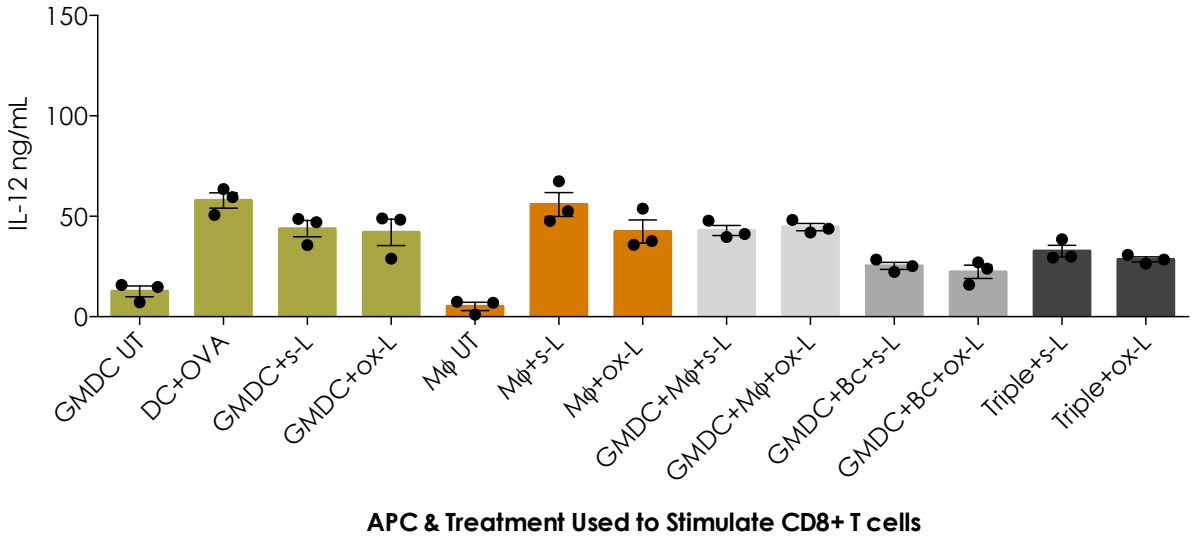

Figure 53 **The production of IL-12 is not increased when two or three APCs are used to prime CD8+ T cells with B16.OVA soluble or oxidized tumour lysate**. 5 x 10^4 Day 6 GMDC, Day10 M1 MΦs and freshly isolated splenic B cells, or combinations thereof, were pulsed overnight with whole OVA protein (50 µg/mL) and B16.OVA TL (1:1 ratio tumor cell to APC). The total number of APCs plated was the same in each well. In double APC combination wells the ratios were 50:50. In triple combination wells the ratio was 1/3 of each APC. LPS (1 µg/mL) and CpG (0.3 µg/mL) were added at the same time as the lysates. The following morning 0.5 x 10^6 VPD450-labeled CD8+ T cells (10:1 ratio, T cell to APC) were added. APCs and T cells were co-cultured for 72 hours, supernatants collected prior to harvest and stored at -80° C prior to analysis by anti-IL-12 ELISA as described in Materials and Methods. Data was analysed in Exel and graphed in Prism. Summary data of 3 independent experiments. In DC+OVA, MΦ UT, MΦ+OVA and Triple+s-L only two observations were recorded. Statistically significant results were calculated by Kruskal-Wallis test followed by unadjusted Dunn's test. Error bars = mean +/- s.e.m.

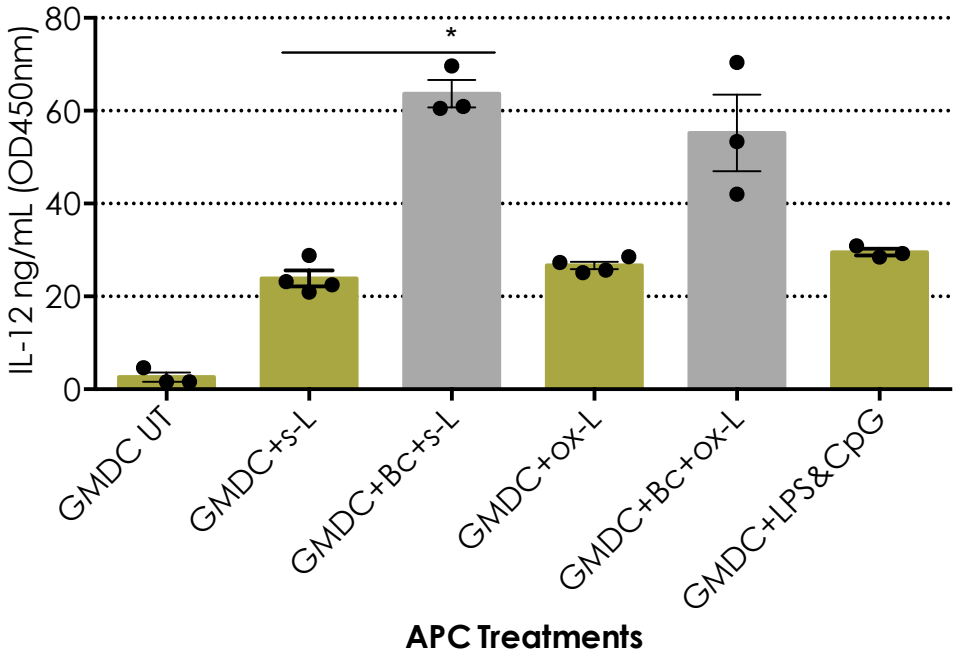

Figure 54 **The combination of LPS&CpG-activated, lysate-loaded GMDC+B cell induces a synergistic increase in IL-12 production over LPS&CpG alone**. 5 x 10^4 Day 6 GMDC, Day10 M1 MΦs and freshly isolated splenic B cells, or combinations thereof, were pulsed overnight with whole OVA protein (50 μg/mL) and B16.OVA TL (1:1 ratio tumor cell to APC). The total number of APCs plated was the same in each well. In double APC combination wells the ratios were 50:50. LPS (1 μg/mL) and CpG (0.3 μg/mL) were added at the same time as the lysates. Supernatants collected after 48 hours and stored at -80°C prior to analysis by anti-IL-12 ELISA as described in Materials and Methods. Data was analysed in Excel and graphed in Prism. Summary data of 3 independent experiments. Statistically significant results were calculated by Kruskal-Wallis test followed by unadjusted Dunn's test.* $p < 0.05$. Error bars = mean +/- s.e.m.

4.3.4.3 TNF-α Response to Soluble and Oxidised Lysates

TNF-α is produced by monocytic lineage cells, primarily MΦs (reviewed in [75] and [77]) and has been shown to cooperate with IFN-γ to mediate tumour rejection[79,80]. Interestingly, membrane-bound but not soluble TNF-α was shown to be responsible for anti-tumour response in one study[95]. Therefore we wished to evaluate the presence of soluble TNF-α in the cell-condition media of lysate-primed APC-T cell co-cultures.

Very low levels of TNF-α were detected in CD4+ or CD8+ T cell co-cultures after 72 hours of culture with the various protein, peptide or lysate-loaded APCs (Figure 55). No increase in TNF-α above untreated levels was observed in CD4+ or CD8+ T cells groups.

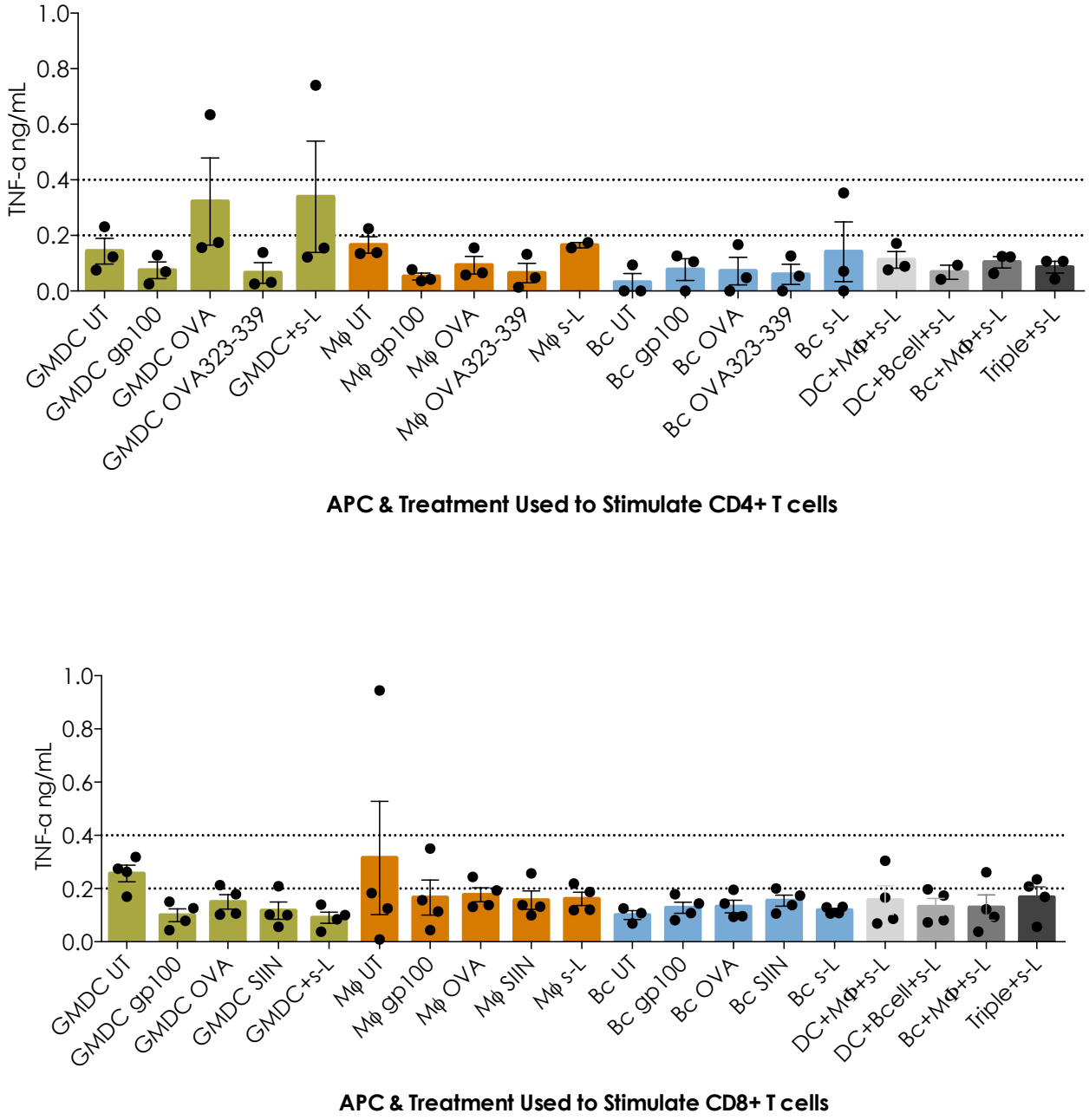

Figure 55 **Soluble TNF-α is not present in lysate-primed CD4+ or CD8+ T cell co-cultures**. 5 x 10^4 Day 6 GMDC, Day10 M1 MΦs and freshly isolated splenic B cells, or combinations thereof, were pulsed overnight with whole OVA protein (50 µg/mL) and B16.OVA TL (1:1 ratio tumor cell to APC). The total number of APCs plated was the same in each well. In double APC combination wells the ratios were 50:50. In triple combination wells the ratio was 1/3 of each APC. LPS (1 µg/mL) and CpG (0.3 µg/mL) were added at the same time as the lysates. The following morning $OVA^{323-339}$ or SIIN peptide (2 µg/mL) were added to the CD4+ or CD8+ wells respectively prior to the addition of 0.5 x 10^6 VPD450-labeled CD4+ or CD8+ T cells (10:1 ratio, T cell to APC). APCs and T cells were co-cultured for 72 hours, supernatants collected prior to harvest and stored at -80° C prior to analysis by anti-IFN-γ ELISA as described in Materials and Methods. Data was analysed in Exel and graphed in Prism. Summary data of 3 independent experiments. In DC+OVA, MΦ UT, MΦ+OVA and Triple+s-L only two observations were recorded. Statistically significant results were calculated by Kruskal-Wallis test followed by unadjusted Dunn's test. Error bars = mean +/- s.e.m.

4.3.4.4 IL-10 Response to Soluble and Oxidised Lysates

IL-10 is associated with T_{REG}s and immune suppression[96]. However IL-10 has also been shown to promote tumor regression (reviewed in [81,108]), therefore we wished to assess whether lysate-loaded APCs stimulated high or low levels of IL-10.

Little IL-10 was detected in CD4+ or CD8+ T cell-APC co-cultures (Figure 56). No differences were observed between APCs priming the T cells or between oxidised and soluble lysates.

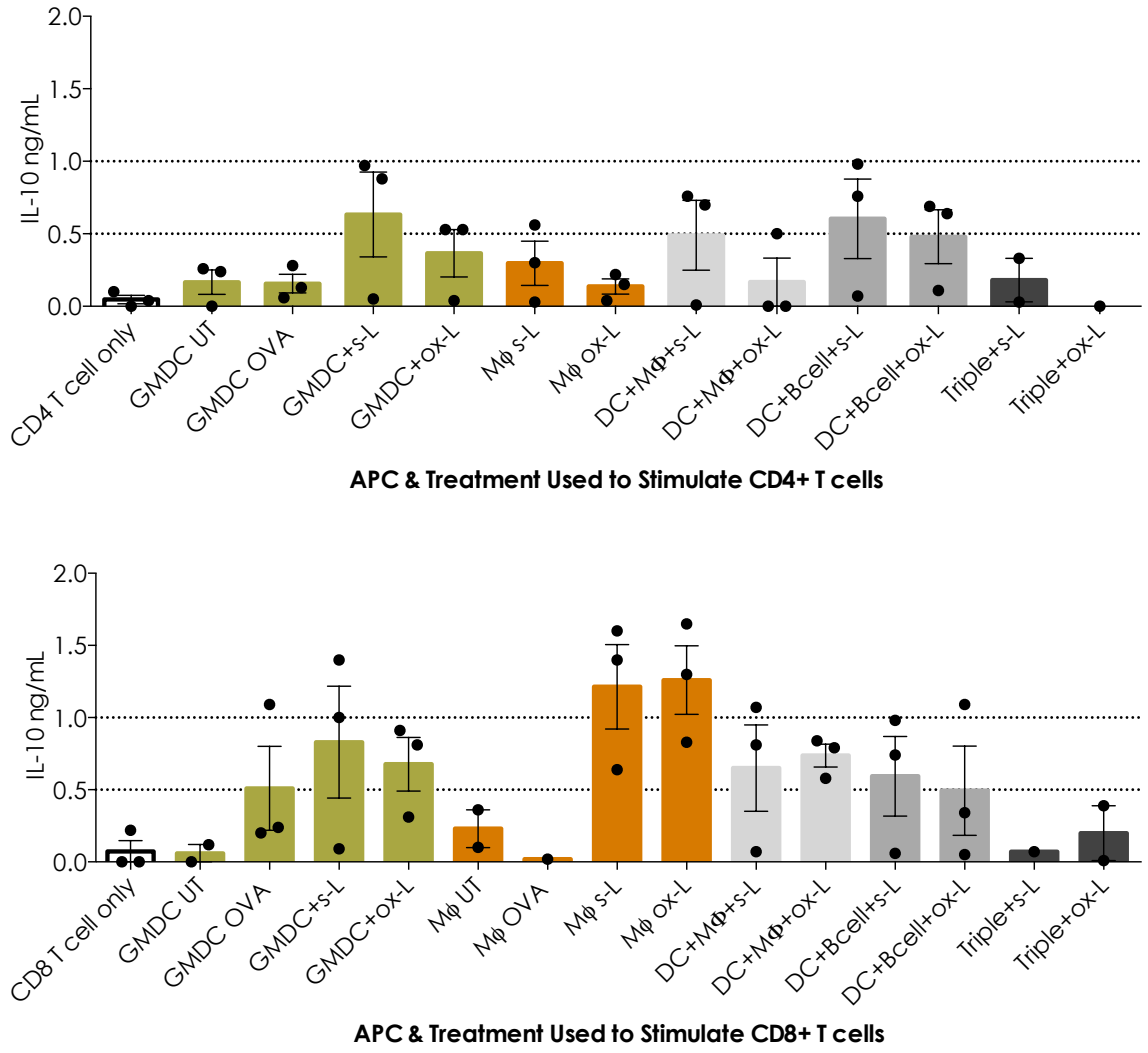

Figure 56 **No difference in IL-10 production is observed between oxidized lysate-primed T cell co-cultures and soluble lysate-primed co-cultures**. 5 x 10^4 Day 6 GMDC, Day10 M1 MΦs and freshly isolated splenic B cells, or combinations thereof, were pulsed overnight with whole OVA protein (50 µg/mL) and B16.OVA soluble or oxidised lysate (1:1 ratio tumor cell to APC). The total number of APCs plated was the same in each well. In double APC combination wells the ratios were 50:50. In triple combination wells the ratio was 1/3 of each APC. The following day CD4+ or CD8+ wells respectively prior to the addition of 0.5 x 10^6 VPD450-labeled CD4+ or CD8+ T cells (10:1 ratio, T cell to APC). APCs and T cells were co-cultured for 72 hours, supernatants collected prior to harvest and stored at -80° C prior to analysis by anti-IFN-γ ELISA as described in Materials and Methods. Data was analysed in Excel and graphed in Prism. Summary data of 3 independent experiments. For CD8+ DC UT, MΦ UT & triple+ox-L only two observations were recorded and in MΦ+OVA and Triple+s-L only one observation was recorded. Statistics were not applied due to the wide variation in data points. Error bars = mean +/- s.e.m.

4.3.5 Proliferation Response to Priming and Re-stimulation with Lysate-loaded APCs

The overarching aim of this thesis was the improvement of ACT for cancer. As such one of the key goals is the expansion of sufficient lysate-primed T cells for adoptive transfer into patients. We therefore investigated the fold expansion of CD4+ and CD8+ T cells when primed with the lysate-loaded APCs and APC combinations over a 10 day period. We also aimed to restimulate the primed T cells with the same APCs and APC combinations to ascertain what impact priming with one APC and boosting with the same or a different APC might have on T cell priming and restimulation.

We were unable to stimulate CD4+ T cells to expand in response to priming with soluble or oxidised lysate (Data not shown). Small numbers of live CD4+ T cells were observed in the oxidized lysate-primed samples and the GMDC+B cell and Triple combinations after 10 days. All CD8+ T cells primed with soluble lysate were dead by Day 10.

CD8+ T cells could be stimulated to expand approximately 2 fold when primed by GMDC presenting oxidized lysate antigen (Figure 57). When data from all the experiments are collated no combination of APC gives any advantage over the use of GMDC alone. However in two of the three experiments CD8+ T cells primed with oxidised lysate-loaded GMDC+B cell proliferated approximately two fold higher than T cells primed by GMDC alone (Figure 57).

Since soluble lysate-primed CD4+ and CD8+ T cells and oxidised lysate-primed CD4+ T cells were all dead by D10 we were unable to re-stimulate those lysate-primed T cells. In one experiment with oxidised lysate-primed CD8+ T cells, enough cells remained alive at D10 to attempt re-stimulation. The T cells were restimulated with the same APC, or APC combination, with which they had been primed. Alternatively the T cells were restimulated with a different APC or APC combination from that which was used for priming. By Day 20 the greatest fold expansion was observed in the “Primed with DC, Boosted with DC” and “Primed with MΦ, Boosted with MΦ” groups, however the fold expansion was only 1.13 and 1.01 respectively (data not shown). Attempts were also made to re-stimulate OVA-primed CD8+ T

cells with the same or different APC(s). Insufficient data was obtained for statistical analysis therefore this result is discussed in Appendix 1, Supplementary Figure 28.

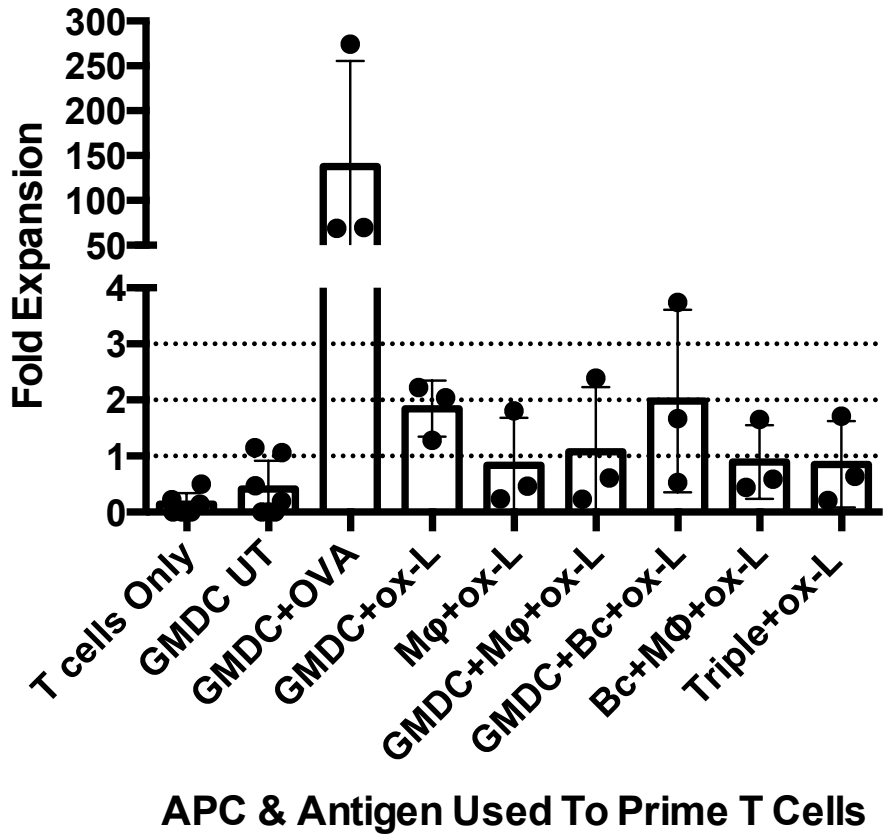

Figure 57 **No differences are observed between CD8+ T cells primed for 10 days with 1, 2 or 3 oxidised lysate-loaded APCs.** 1 x 10^5 Day 6 GMDC, Day10 M1 MΦs and freshly isolated splenic B cells, or combinations thereof, were pulsed overnight with whole OVA protein (50 μg/mL) and B16.OVA soluble or oxidised lysate (1:1 ratio tumor cell to APC). The total number of APCs plated was the same in each well. In double APC combination wells the ratios were 50:50. In triple combination wells the ratio was 1/3 of each APC. The following day CD4+ or CD8+ wells respectively prior to the addition of 1 x 10^6 CD8+ T cells (10:1 ratio, T cell to APC). APCs and T cells were co-cultured for 10 days with fresh media and cytokines being replaced every 2 days. Fold expansion was assessed by counting live cells under Trypan Blue dead cell exclusion staining. Summary data of 3 independent experiments. Error bars = mean +/- s.e.m. UT: untreated.

4.3.6 Naïve T cell Phenotype

We wished to observe the effect that priming with different lysate-loaded antigen presenting cells had on the resulting T cell phenotype. We therefore examined the phenotype of the naïve CD4+ and CD8+ T cells used in APC co-cultures and compared it to that of the primed T cells. Freshly isolated CD4+ and CD8+ T cells were analysed by Flow Cytometry for the presence of a range of molecules that collectively classify T cells as naïve. CD27, CD28, PD-1, CD44, CD122, CD127, CD62L and CCR7 were evaluated. Antibody panels used in T cell phenotyping are found in Materials and Methods Table 4.

Figure 58 shows the gating strategy used to phenotype both naïve and lysate-primed T cells. For the sake of simplicity only the GMDC+OVA, GMDC+oxidised lysate and GMDC+B cell+oxidised lysate plots are shown. For comparison a full set of plots are found in Appendix 1, Supplementary Figure 32. Figure 59 shows summary data for seven CD8+ and four CD4+ independent experiments assessing naïve T cell phenotypes.

The molecules CD27 and CD62L were both highly expressed on all freshly-isolated CD4 and CD8 T cells (Figure 59). CD8 and CD4 T cell expression of CD27 was indistinguishable at 86% and 89% respectively. CD62L expression on CD4+ T cells was slightly lower (74%) than that of CD8+ T cells (83%).

CD28 was consistently expressed at very low levels on both CD4+ and CD8+ T cells (5% and 1% respectively). Three different antibodies, two conjugated to FITC, one conjugated to PE, were compared and all returned the same result. We also observed very low expression of the early T cell activation marker CD69 on both CD4+ and CD8+ T cells as well as the T cell exhaustion marker PD-1.

There were slight differences in the expression of CD122 and CD127 on CD4+ and CD8+ T cells. CD122 expression was not expressed on either cell type. CD127 expression varied more between the two cell types with CD8+ cells having an average of 8% expression and CD4+ cells an average of 2%.

CD44 expression was identical with 15% of CD8+ T cells positive for this marker and CD4+ cells averaging 24%.

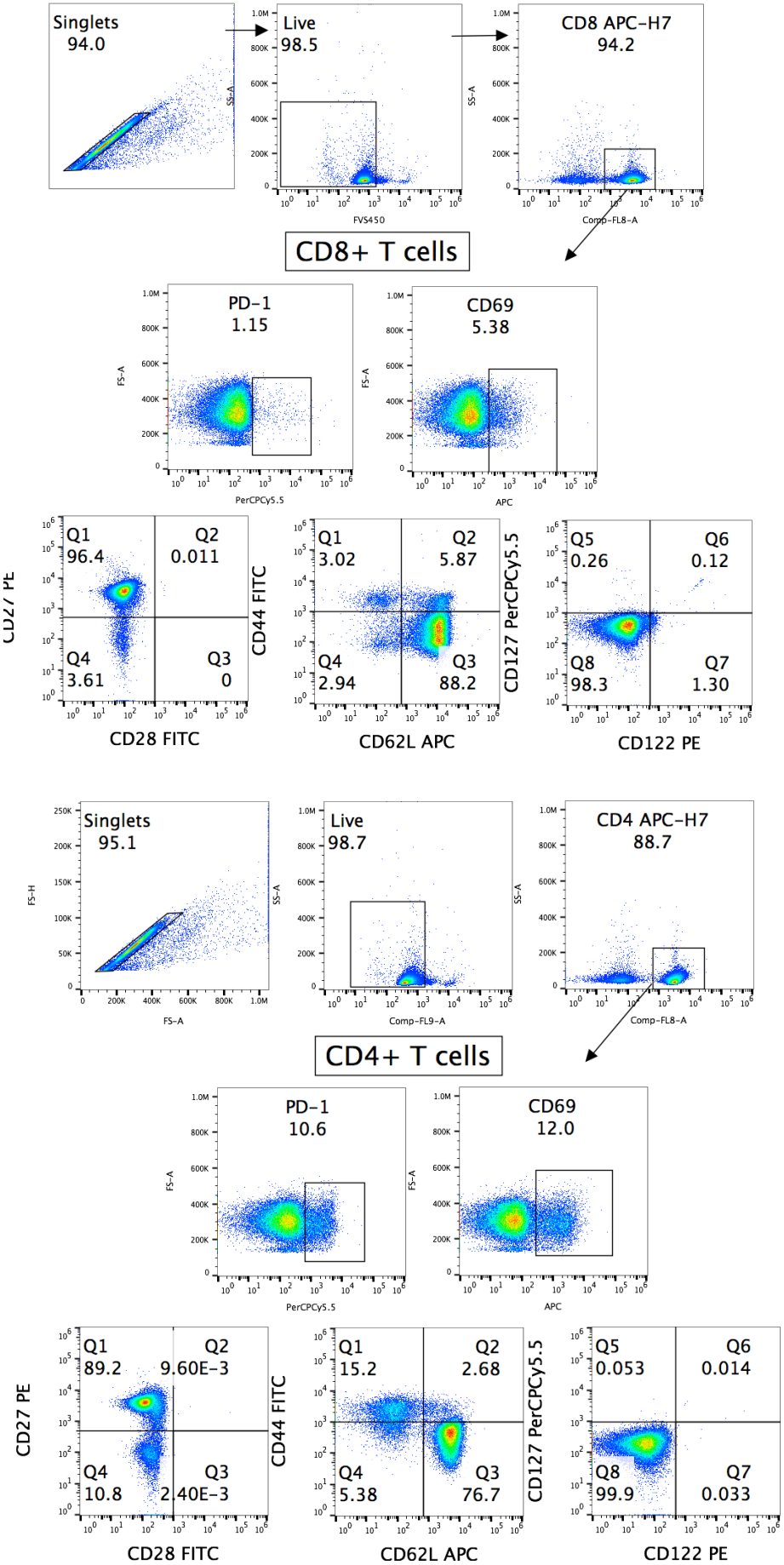

Figure 58 **Gating strategy for Day 0 T cells.** Freshly isolated OT-II and OT-I splenocytes were labelled with CD4 and CD 8 negative selection bead cocktails respectively and sorted by AutoMACS Pro as described in Materials & Methods. The resultant CD4+ and CD8+ T cells were stained with dead cell exclusion dye (FVS450) and labeled with mABs against the surface molecules CD3, CD8, CD27, CD28, CD44, CD122, CD127, PD-1, CD62L and CD69. Labeled cells were fixed in 4% PFA, stored overnight at 4°C and collected the following day on a Gallios Flow Cytometer. Data was analysed on FlowJo VX Software and graphed in Prism Representative data of 7 (CD8+) and 3 (CD4+) independent experiments.

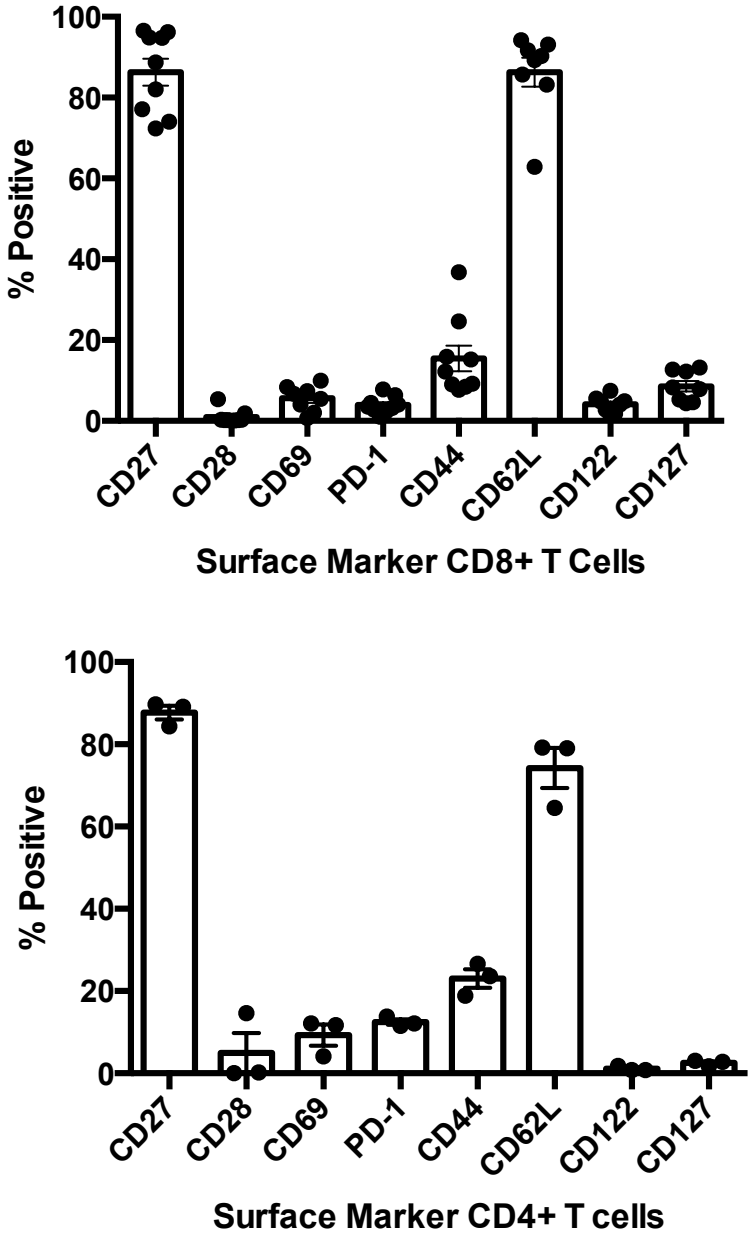

Figure 59 **Naïve CD4+ and CD8+ T cell phenotypes show similar profiles**. Freshly isolated OT-II and OT-I splenocytes were labeled with CD4 and CD8 negative selection bead cocktails respectively and sorted by AutoMACS Pro. The resultant CD4+ and CD8+ T cells were stained with dead cell exclusion dye and labeled with mABs against the surface molecules CD3, CD8, CD27, CD28, CD44, CD122, CD127, PD-1, CD62L and CD69. Labeled cells were fixed in 4% PFA, stored overnight at 4°C and collected the following day on a Gallios Flow Cytometer. Data was analysed on FlowJo VX Software. and graphed in Prism. Summary data of 7 (CD8+) and 3 (CD4+) independent experiments.

4.3.7 Phenotype of T Cells Primed with Lysate-loaded APCs

We wished to identify whether T cells primed with undefined tumour antigen would adopt the T_{CM} phenotype that has been shown to be more effective on a per cell basis than T_{EM} in ACT for cancer[109,141]. Therefore the 10-day lysate-experienced T cells were assayed using mABs against the same surface markers as were used to assess the naïve T cells and the the phenotypes compared. Figure 60 shows the gating strategy and representative naïve and Day 10 CD8+ T cell data from ten independent experiments. Figure 61 shows summary data of Day 10 CD8+ T cells primed by GMDC and GMDC+B cells. Only the results of GMDC and GMDC+B cell-primed T cells are reported in this chapter as they were the only groups for which the most complete data was obtained. Appendix 1, Supplementary Figure 32 shows a representative dot plot data set for comparison of bi-variate analysis between all groups. Appendix 1, Supplementary Figure 33 shows the full summary data available for all groups. Statistically significant differences were calcualated using the Kruskal-Wallis test, since a normal distribution can not be assumed with such low numbers. This test can be applied to groups containing different numbers of samples. The Kruskal-Wallis was followed by Dunn's post test Unadjusted p values were used to compensate for the loss of power in the Dunn's post test.

CD8+ T cells primed by GMDC+OVA, GMDC+Bc+OVA, GMDC+ox-L or GMDC+Bc+ox-L all had reduced CD27 percent positivity compared to naïve T cells (). (GMDC+OVA: 69%; GMDC+ox-L: 39%; GMDC+Bc+OVA: 57%; GMDC+Bc+ox-L: 47% ($p<0.05$); naïve: 82%). One outlier had a marked impact on the mean of the GMDC+ox-L group, therefore while this result is technically statistically significant a further repeat is required to clarify the situation. No differences were observed between the GMDC and GMDC+B cell-primed groups.

The CD44 percent positivity response was the opposite to that of CD27 with GMDC+OVA and GMDC+B cell+OVA groups having higher percent positivity compared with naïve T cells (74% ($p<0.05$), 46% (ns) and 15% respectively). GMDC+ox-L and GMDC+Bc+ox-L-primed T cells also showed increased CD44 over naïve (73% (ns) and 66% ($p<0.05$), respectively. However once again outliers in these groups mean that a further repeat is required.

CD62L decreased significantly across all groups compared to naïve T cells. GMDC+OVA (14%), GMDC+B cell+OVA (23%), GMDC+ox-L (15%) and GMDC+Bc+ox-L (20%).

At Day 10 CD28 and CD127 was detected at less than 1% across all treatment groups and fewer than 3% CD8+ T cells were positive for PD-1 and CD122 across all groups. CD69 percent positivity was low (<3%) across all groups (Appendix 1, Supplementary Figure 33).

Appendix 1, Supplementary Figure 32 shows representative Flow Cytometric data highlighting the difference in percentages of CD8+ T_{EM}, T_{CM} and T_N/T_{SCM} cells between groups primed with the various APCs. Figure 28 shows the same data for GMDC and GMDC+B cell groups alone. The GMDC and GMDC+B cell groups cluster together with similar percentages of CD44+CD62L- T_{EM} and CD44+CD62L T_{CM} cells. CD8+ T cells primed with oxidized lysate-loaded GMDC and GMDC+B cell exhibited 73% and 76% T_{EM}, and 21% and 19% T_{CM} respectively. Approximately 5% and 4% of these cells displayed a CD44-CD62L+ T_N/T_{SCM} phenotype. By contrast CD8+ T cells primed with oxidized lysate-loaded M1 MΦs, DC+MΦ, Bc+MΦ or the Triple combination displayed lower percentages of T_{EM} (36%, 44%, 40% and 43% respectively) and higher proportions of T_{CM} (53%, 42%, 44% and 44% respectively). Thus, while these data remain preliminary, the phenotypes generated in response to priming suggest that CD8+ T cells have the capacity be activated into T_{EM} and T_{CM} by oxidised lysate antigens.

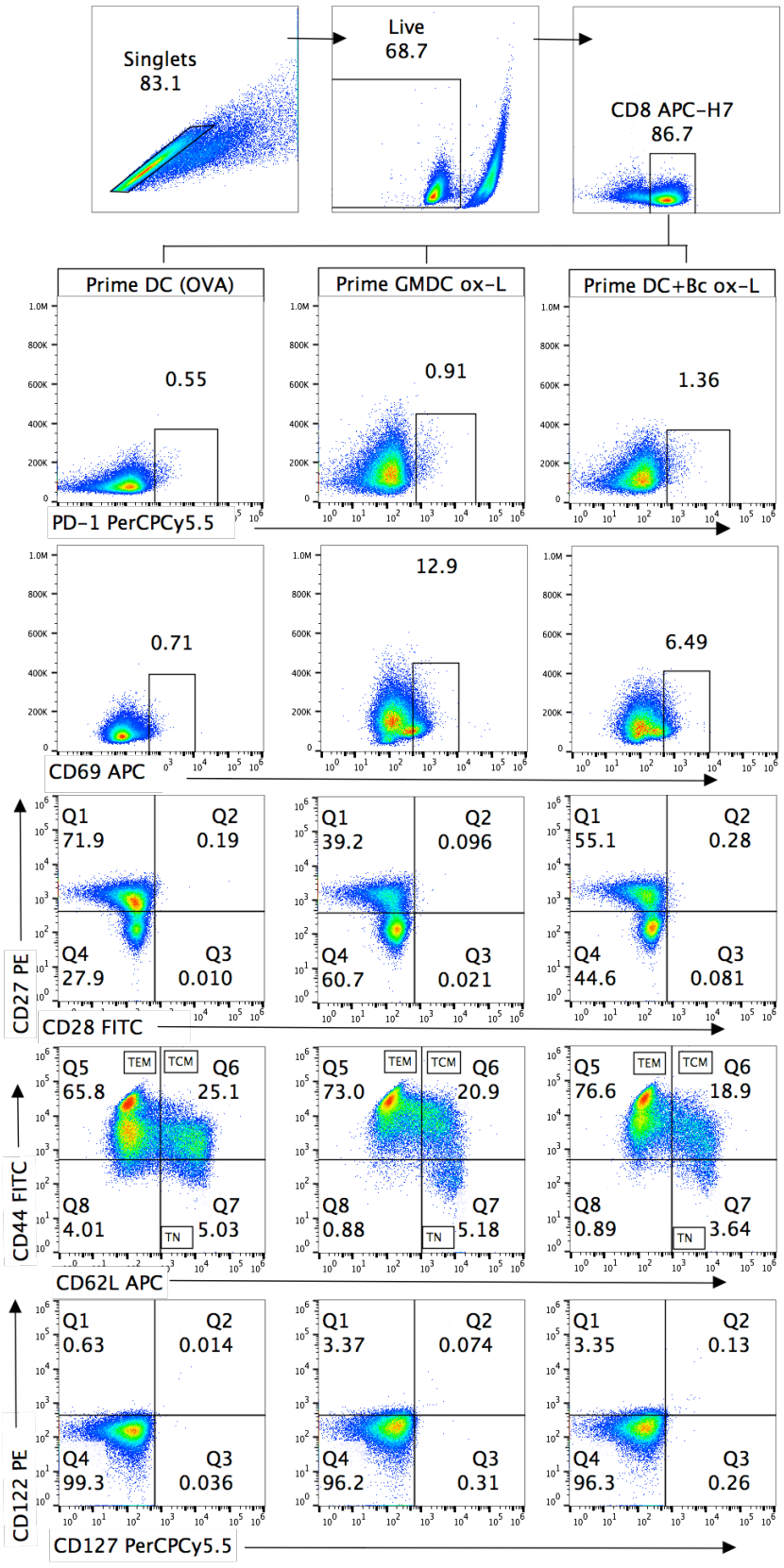

Figure 60 **Gating strategy and raw data for Day 10 CD8+ T cells primed with GMDC or GMDC+B cell loaded with oxidised lysate.** Day 6 GMDC and freshly isolated B cells were prepared as previously described. 1 x 10^5 GMDC or 0.5 x 10^5 GMDC and 0.5 x 10^5 B cells were pulsed overnight with whole OVA protein (50 μ g/mL) and B16.OVA oxidised lysate (1:1 ratio tumor cell to APC) plus LPS&CpG. The following day freshly isolated OT-II and OT-I splenocytes were labeled with CD4 and CD 8 negative selection bead cocktails respectively and sorted by AutoMACS Pro and plated with lysate-loaded APCs (10:1 ratio, T cell to APC). APCs and T cells were co-cultured for 10 days with fresh media and cytokines being replaced every 2 days. The resultant CD8+ T cells were stained with dead cell exclusion dye and labeled with mABs against the surface molecules CD3, CD8, CD27, CD28, CD44, CD122, CD127, PD-1, CD62L and CD69. Labeled cells were fixed in 4% PFA, stored overnight at 4°C and collected the following day on a Gallios Flow Cytometer. Data was analysed on FlowJo VX Software and graphed in Prism. Representative data of 9 independent naïve T cell experiments and 3 independent 10-day lysate-primed T cell experiments.

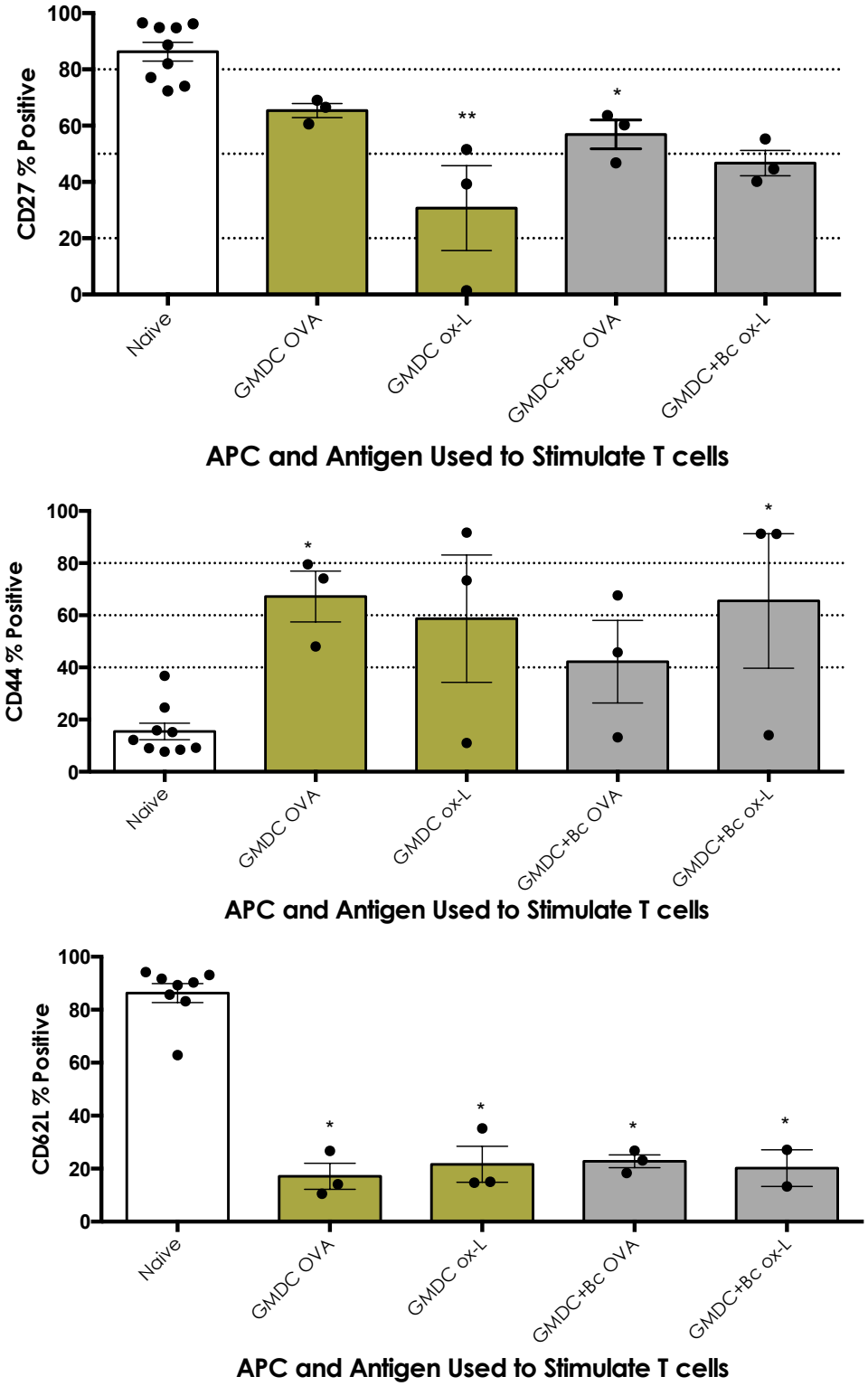

Figure 61 **Surface phenotype of Day 10 CD8+ T cells primed by oxidized lysate-loaded GMDC or GMDC+B cells.** Day 6 GMDC and freshly isolated B cells were prepared as previously described. 1 x 10^5 GMDC or 0.5 x 10^5 GMDC and 0.5 x 10^5 B cells were pulsed overnight with whole OVA protein (50 µg/mL) and B16.OVA oxidised lysate (1:1 ratio tumor cell to APC) plus LPS&CpG. The following day freshly isolated OT-II and OT-I splenocytes were labeled with CD4 and CD 8 negative selection bead cocktails respectively and sorted by AutoMACS Pro and plated with lysate-loaded APCs (10:1 ratio, T cell to APC). APCs and T cells were co-cultured for 10 days with fresh media and cytokines being replaced every 2 days. The resultant CD8+ T cells were stained with dead cell exclusion dye and labeled with mABs against the surface molecules CD3, CD8, CD27, CD28, CD44, CD122, CD127, PD-1, CD62L and CD69. Labeled cells were fixed in 4% PFA, stored overnight at 4°C and collected the following day on a Gallios Flow Cytometer. Data was analysed on FlowJo VX Software and graphed in Prism. Summary data of 3 - 9 independent experiments. Statistically significant results were calculated by Kruskal-Wallis test followed by unadjusted Dunn's test. * $p < 0.05$; ** $p<0.01$. Error bars = mean +/- s.e.m.

4.4 Discussion

We are aware of one other study directly comparing DCs, MΦs and B cells and their role in the immune response to the model antigen OVA using four different adjuvants[142]. The setting for that study was infectious disease rather than cancer. In addition the Mamula laboratory has looked at the interplay of DC, B cells and MΦs antigen presentation in the setting of autoimmune disease and cancer[143,144]. To our knowledge, this is the first study to compare the T cell response to being primed with combinations of lysate-loaded APCs.

4.4.1 Tumour Lysate Dose Titrations

It has been shown that the number of tumour cells plays a major role in whether or not CTLs can effectively eliminate tumours, with tumour cell numbers over a certain threshold impairing CTL function[145]. The amount[146–148], and the affinity[149,150], of antigen being presented to T cells, as well as the number of responding T cells[146], all play critical roles in determining the magnitude of the T cell response. Tumour lysate has been variously used by different groups at ratios of between 1:1 up to 100:1[6,11,151,152] (tumour cell:DC). We carried out concentration titrations to compare the T cell response to priming by APCs loaded with different volumes of lysate equating to ratios of 3:1, 6:1, 12:1 and 30:1 tumour cells to DCs. The greatest proliferation response by CD8+ T cells was observed at the 6:1 ratio and initial experiments were carried out with this ratio. Higher concentrations appeared to induce an inhibitory effect, which was not unexpected given that lysates are known to be immunosuppressive. However it has been suggested that this suppression may have been due to the protease inhibitors in which the lysed tumour cells were resuspended. While the use of protease inhibitors was specifically recommended for this project we did note that Chiang *et al* did not resuspend their lysed tumour cells in protease inhibitors[3]. It would be interesting to compare the T cell response to lysate created without these inhibitors.

4.4.2 The T cell Proliferation Response is Reduced When Lysate-Loaded APCs are Activated with LPS&CpG

Antigen presented by immature APC can induce immunological tolerance[153–155] therefore immune-activating stimuli must be a part of any anti-cancer therapy. The combination of the immunostimulatory adjuvants IFN-γ and monophosphoryl lipid A

(MPL) (a clinical grade, low-toxicity form of bacterial lipopolysaccharide)[4] has been used clinically with tumour lysate. Other groups have experimented with whole tumour lysate and TLR agonists such as LPS, CpG and alpha-galactylceramide[156,157]. However all of these experiments were carried out using DCs as the only APC. Thus it was necessary to consider which TL preparation in conjunction with which immune activating stimuli would lead to improved T cell activation in the OVA-transgenic mice model when DC or MΦ or B cells or a combination thereof were used as the APC(s).

A common understanding at the time of beginning these experiments was that phagocytic capacity is reduced in activated DCs compared to immature DCs[158]. We therefore reasoned that we might see a greater T cell response if APC were loaded with lysate prior to adjuvant stimulation. Two experiments suggested this might be the case, however, incorrect data analysis led to the decision to continue loading the APCs with lysate plus activation stimuli overnight. More comprehensive reading of the literature revealed that while macropinocytic uptake activities of activated DCs may be reduced[146], FC-γ-R-mediated, DEC205-mediated, clathrin-mediated and phagocytic acquisition mechanisms remain intact in activated DCs[146,159–161]. Therefore the decision to load with lysates plus activation stimuli overnight serendipitously proved to be the better decision since it fostered enhanced B cell activation, which allowed the cooperative effects of the GMDC+B cell combination to be observed. However an improved approach might be to load the GMDC and B cells differentially. It would be interesting to compare B cells loaded with LPS&CpG overnight followed by lysate loading for 6 hours the following day, and GMDC loaded with lysates overnight followed by LPS&CpG activation for 4-6 hours the following day. By optimally activating both cell types individually prior to combining them, rather than treating them identically as was done in this project, the T cell response may be enhanced.

In vitro T cells may be activated via antigen-specific APC-mediated means or non-specific mechanisms such as cyokines. It is common to study T cell activation responses via the use of non-specific T cell activators. Such activators include mitogenic lectins such as phytohemagglutinin (PHA) or Concanavalin A, or the

protein kinase C (PKC) signal transduction activator phorbol 12-myristate 13-acetate (PMA) together with IL-2-inducing ionomycin[240]. The use of beads coated with anti-CD3 and anti-CD28 mABs replicates stimulation by APCs to a degree, yielding a slightly more physiologically relevant result[162].

None of these artificial stimulants reproduce *in vivo* priming by an APC presenting peptide on MHC, but can provide a useful functional control to verify that a given cell can produce cytokines. Assessing the T cell response *in vitro* to APC+peptide stimulation provides a more relevant model of what may happen *in vivo*. Thus, rather than inducing an artificially high result by stimulating our lysate-primed T cells with mitogens or mABs we evaluated the T cell response to the defined antigen OVA, along with undefined TAA, presented by APCs. In this case presentation of whole OVA provides a more meaningful functional control than anti-CD3/anti-CD28.

We hypothesized that treating lysate-loaded APCs with LPS&CpG would increase the T cell proliferation response allowing us to observe more clearly the effect of the different APC combinations on the T cell response. Unexpectedly, instead of increasing CD8+ T cell proliferation the proliferation response fell from an average of approximately 20% when primed by unactivated GMDCs to an average of around 10% when primed by GMDCs that had been pulsed overnight with lysate plus LPS&CpG. In two experiments APCs were pulsed overnight with lysate and activation stimuli added the following morning. In that scenario CD8+ T cell proliferation was not greater than in T cells primed with APCs loaded overnight with lysate plus LPS&CpG, and for CD4+ T cells the proliferation was less (data not shown), so we continued pulsing overnight with lysates plus activation stimuli. As mentioned previously, this ultimately proved to be a better decision.

4.4.3 The APC Presenting Lysate Antigens, Not the Lysate Type, Determines T Cell Proliferation Response

In vitro T cell fluorescence diluting assays are widely used to study naïve and antigen-specific T cell proliferation. We assessed the 72 hour T cell proliferation response to soluble and oxidized lysate and we expected that the oxidised lysate would yield greater T cell proliferation. However, interestingly, these results demonstrated that the greatest T cell proliferation occurred only when GMDC+B cells presented oxidized

lysate antigens to CD8+ T cells. Thus the APC presenting the lysate was important, not simply the oxidised lysate antigens.

Previous work in our laboratory demonstrated that small numbers of proliferating antigen-specific murine T cells were generated in response to priming with soluble lysate[139]. This has also been demonstrated in human studies of parathyroid carcinoma lysate-loaded DC-primed T cells, in which *in vitro* T cell proliferation assays returned stimulation indices of 1.8-5.7[163]. However other studies using tumour lysate-loaded DCs have demonstrated the ability to generate large numbers of T cells[23]. Because the soluble lysate generated a low T cell response when presented by GMDCs we experimented with increasing the immunogenicity of the tumour cells. Recent work by Chiang *et al* had identified sodium hypochlorite oxidation of ovarian cancer cells (SK-OV-3) as a more effective method of generating highly immunostimulatory tumour lysate (TL) than heat-treated or irradiated cells. This was assessed by the ability of DC to take up TL, produce T_H1-priming cytokines, stimulate an MLR and produce T cells that could control tumour growth *in vivo*[3,4,36,164]. Others have also reported on the immune enhancing properties of hypochlorous acid[165]. This approach appeared promising therefore we compared the T cell response to soluble and oxidised lysates.

Contrary to Chiang's results we saw no increase in T cell proliferation in the oxidized lysate samples when presented by GMDC. OVA protein was detected in the oxidized lysate, therefore the absence of OVA was not the reason for the lack of improved proliferation. We posited that the antibody used in the Western Blot was able to recognise oxidized OVA epitope whereas the OT-I T cells could not. However this theory was disproved in later experiments in which GMDC+B cell were combined (Figure 46) and in *in vivo* experiments.

Rather than differing between the two lysates *per se*, in our hands the CD8+ T cell proliferation response differed according to which APC was presenting the lysate antigens. For CD4+ T cells, no improvement over GMDC-stimulated proliferation was observed in any soluble or oxidised lysate-primed group (Figure 46). No difference in proliferation was observed between soluble and oxidized lysate-primed

CD8+ T cells when the lysates were presented by any APC or APC combination except GMDC+B cell. The exception was when M1 MΦs presented oxidised antigens the CD8+ T cell proliferation was less than that induced by presentation of soluble lysate.

HOCl is a physiologically relevant adjuvant found in sites of inflammation where it is produced by activated granulocytes, which flood into these areas along with antigen-presenting DCs[166]. Rather than just bactericidal activity inside the phagolysosome these volatile, strong oxidizing agents also act in the intracellular milieu to cause oxidative changes to lipids[167], enzymes[168] and extracellular matrix proteins[169]. The adjuvant effects of HOCl are independent of TLR signaling[170] with HOCl-oxidised OVA taken up more efficiently by APCs and degraded more efficiently by proteinases. The enhanced uptake is mediated via a specific receptor, which binds preferentially to chlorinated OVA. HOCl may improve the targeting of these glycoprotein antigens to scavenger receptors on the surface of APCs, suggesting a novel mechanism that links innate and adaptive immunity. Thus plausible evidence exists for the concept of enhanced tumour antigen presentation and T cell activation by HOCl-oxidised material *in vivo*.

In tumour lysate non-immunostimulatory elements, such as tolerising self-antigens, may vastly outnumber the immunostimulatory components, which in this setting is primarily the model antigen OVA. We reasoned that high amounts of self antigen in the lysate would have an inhibitory effect on T cell activation via normal tolerance mechanisms, however, the object of this study was to activate T cells against undefined antigens therefore determining which factors were inhibitory to T cells was outside the remit of this thesis. Nevertheless, an interesting report has identified the intracellular accumulation of lipids in DCs as inhibitory to T cell stimulation in tumour-associated DCs (TADC)[171]. Tumour-produced reactive oxygen species (ROS) caused endoplasmic reticulum (ER) stress in TADC. ER stress led to the IRE1α-mediated activation of the transcription factor XBP1, which switches on lipid-synthesis genes. This increase in intracellular lipids interfered with DC antigen presentation and hindered their anti-tumour role. DC-initiated T cell immunity was restored when XBP1 was silenced. It is possible that this mechanism played a role in

down regulating the T cell response to oxidized lysate presented by GMDC and that this was overcome or ameliorated by the combination of GMDC+B cell, but examination of this theory was beyond the scope of this project.

We noticed the low response to SIINFEKL and so compared our SIINFEKL to that of three other laboratories and found identical responses across the batches. The ability of CD8+ and CD4+ T cells to respond to whole OVA was more in line with expected proliferation rates and was a more relevant control since the whole protein, like the lysates, must be processed for presentation on MHC molecules whereas peptides are able to enter the MHC-peptide groove without processing via MHC-I recycling through early endosomes[172]. Thus while the SIINFEKL response was lower than we would have liked, we retained a relevant control that demonstrated the functionality of the APCs and the T cells.

4.4.3.1 Lysate-loaded GMDC Stimulate Low Levels of CD8+ T Cell Proliferation

The stimulation of T cell proliferation by freeze-thaw lysate-loaded DCs has been attempted unsuccessfully by other groups[11,14,24,173]. However it must be noted that significant differences exist between the preparation methods of the freeze-thaw lysates and methods of DC loading used in this current study and the four studies cited (soluble fraction versus whole portion lysate; sonication prior to freeze-thaw lysis; and timing of lysate loading on to DCs to name just three). Hatfield, who used the soluble fraction of freeze-thaw lysate[11], suggested that the unsuccessful proliferation response to B16.OVA freeze-thaw lysate-pulsed GMDCs may be due to soluble OVA stimulating less cross-presentation than cell-bound OVA, which was reported by Li *et al*[163].

In our early lysate-loaded GMDC-T co-cultures lysate was loaded on to the GMDCs at a ratio of 6:1 and resulted in an average of 20% CD8+ proliferation (figure 40, left). In the interests of attempting to reproduce the oxidised lysate experiments of Chiang *et al* later experiments used the 1:1 ratio employed by Chiang *et al* in their oxidation assays. We continued to observe a T cell proliferation response however it was lower than that observed in the 6:1 ratio assays (Figure 46). This reduced proliferation response may reflect the reduced amount of OVA available to the APCs for presentation to the T cells.

The increased expression of CD40 on GMDC loaded with oxidized lysate does not appear to be a key factor in why GMDC+ox-L stimulated greater T cell proliferation than s-L. CD40 expression increased to 53% on ox-L-pulsed GMDC ($p<0.05$), which was greater than s-L (19%). However CD40 expression was also upregulated on M1 MΦs in response to s-L and ox-L, though this increase was not statistically significant, and neither s-L nor ox-L-loaded M1 MΦs were able to stimulate T cell proliferation (Figure 46). CD40 ligation is required to link the innate and cognate immune responses (reviewed in[165,174]), but in this case does not explain the enhanced T cell activation by ox-L-loaded GMDC-primed T cells.

Finally, we noted that while GMDC were able to present OVA peptides to both CD4+ and CD8+ T cells, demonstrating cross-priming ability, the IL4DC could only present OVA to CD4+ T cells. Proliferation clusters were observed in GMDC+OVA-primed CD4+ and CD8+ wells whereas proliferation clusters were only observed in IL4DC+OVA-primed CD4+ wells and not in the CD8+ wells. Thus in addition to the lower viability of lysate-loaded IL4DC compared with lysate-loaded GMDC this gave us further justification for continuing with GMDC instead of IL4DC.

While both CD8- and CD8+ DC are able to efficiently capture antigen and present it on MHC-Class II, CD8+ DCs are superior to CD8- DC at cross-presenting captured antigen[175,176]. This helps to explain why these CD8-CD11b^{HI} DCs were unable to stimulate strong CD8+ T cell proliferation, though it fails to explain why they did not stimulate CD4+ T cell proliferation. Merad suggests that, in addition to cross-presentation, the increased CD8+ T cell priming potential of CD8+ DCs may also lie outside their cross-presentation abilities as they express more MHC-I-related genes than CD11b+ cDCs[172,177]. They also produce more of the T_H1, CTL-promoting cytokines IL-12[178–180] and IL-15[181], as well as expressing the chemokine receptor XCR1. The chemoattractant XCL1 is expressed by CD8+ T cells after antigen presentation and promotes CD8+ DC expansion and IFN-γ production. The interaction of binding to its ligand XCR1 on CD8+ DC further promotes CD8+ CTL differentation[182]. Thus these CD8- GMDCs may represent a less than ideal population of cells for presentation of undefined tumour antigens to T cells. It has been reported

that generation of CD8α+ DCs can be achieved using Flt3L[183,184] and it would be interesting to compare the ability of these DCs to elicit a CD8+ T cell reponse to undefined tumour antigens. Though it has been reported that GM-CSF-differentiated DCs are superior to Flt3L DCs in the *in vivo* anti-tumour response[185,186].

4.4.3.2 Lysate-loaded MΦs Do Not Stimulate T Cell Proliferation

We hypothesised that lysate-loaded MΦs may be superior to GMDCs at stimulating T cell proliferation as they excel at clearing apoptotic and necrotic cells and debris[187,188]. Despite showing increased CD40, lysate-loaded M1 MΦs were unable to stimulate a CD4+ or CD8+ T cell response (Figure 45, Figure 47). The reason appears to lie in a loss of presentation ability as MHC-II percent positivity was markedly reduced in MΦs loaded with s-L+LPS&CpG and ox-L+LPS&CpG.

IFN-γ is a strong stimulator of MΦ cytotoxic capacity and MHC-II expression and is secreted by activated T cells, along with GM-CSF (reviewed in[189]). IFN-γ alone blocks the actions of M-CSF in bone marrow progenitor cells but the combination of GM-CSF, M-CSF and IFN-γ fosters mononuclear phagocyte proliferation[190]. MΦs may have performed better had we included IFN-γ in the adjuvants. However there were high levels of IFN-γ in the supernatants, 5% GM-CSF was added to the media, and activated T cells in the co-cultures should be secreting GM-CSF also, so there should have been some MΦ stimulation. However this was obviously insufficient to overcome the lack of T cell activation by lysate-loaded MΦs.

4.4.3.3 Lysate-Loaded B cell Do Not Stimulate T Cell Proliferation

Researchers have begun to explore the possible use of B cells as alternative APCs in anti-cancer cell-based therapies [46,191–195]. Two early studies utilized murine and human B cells loaded with melanoma lysates to activate CD4+ T cells[45,46]. We furthered this by assessing CD8+ responses such as have been observed by others[46,196,197].

Whole OVA protein and OVA peptides both generated strong T cell responses, demonstrating that the splenic B cells were able to present antigen and stimulate T cells, but that they weren't able to do so when presenting soluble or oxidised lysate antigens.

4.4.3.4 Lysate-Loaded GMDC+B Cells Stimulate Greater CD8+ T Cell Proliferation Than GMDC Alone

Steinman and Dhodapkar stated in 2001 that the immature DC is best for loading antigens for immunotherapy, whereas the mature DC represents the optimal cell for presentation of MHC-peptide complexes to T cells[154]. We began our experiments loading lysate on to APCs overnight and adding adjuvants the following morning, and in hindsight, should have continued with this method. However, despite the reduced overall proliferation of CD8+ T cells primed by GMDC loaded overnight with lysate plus adjuvant, a serendipitous outcome to continuing with loading the APCs overnight with lysate plus adjuvants was the appearance of increased proliferation in the GMDC+B cell group. Whereas we had previously consistently observed increased CD8+ T cell proliferation in each of the double and triple APC combination groups, compared to GMDC alone, we had been unable to discern differences between these combination groups. The addition of LPS and CpG "broke the tie" and allowed the GMDC+B cell combination to emerge as a stronger contender for inducing superior CD8+ T cell activation. The GMDC+B cell combination was superior to GMDC alone at stimulating C8+ T cell proliferation when presenting both both soluble and oxidised lysates (Figure 46). However in the presence of oxidized lysate the increase in CD8+ T cell proliferation was more than double compared with soluble lysate.

Shirota and colleagues demonstrated that CpG-conjugated antigen stimulated B cells to cross-present antigen to CD8+ T cells and drive a T_H1 response via strong IL-12 production[8365]. This was the first time B cells had been reported to share the DC characteristics of non-specific antigen capture and T_H1 differentiation from unprimed T cells. A later study also described how CD8- Flt3L-generated BMDCs, as well as splenic CD8- DCs, could be induced to cross-present dead cell-associated antigens, only after treatment with CpG[198].

Unconjugated CpG was loaded onto the APCs at the same time as the lysates, and was thus available to be picked up by the GMDC and B cells at the same time as the lysate. While IL-12 production by lysate-loaded B cells alone was not measured, a synergistic increase in IL-12 production over that produced by lysate-loaded GMDC was observed in overnight co-cultures of lysate-loaded GMDC+B cells (Figure 54).

These data, combined with the improvement in CD8+ T cell proliferation, suggest that the unconjugated CpG has had a similar effect to those described by Shirota and de Brito. This observation may be useful in the clinic the use of unconjugated CpG regularly takes place.

4.4.4 Lysate-Loaded GMDC+B Cells Stimulate Enhanced IFN-γ But Not TNF-α or IL-10 Production

The requirement in anti-tumour immunity for not just IFN-γ alone but polyfunctional T cells secreting multiple effector cytokines, is being increasingly recognised[80,199] . IFN-γ, IL-12, TNF-α and IL-10 were selected as a minimum indication of T cell polyfunctionality in this study. IL-10 was included to observe whether lysate induces a regulatory T cell response. Finally, IL-2 production in response to oxidized tumour lysate has been used as a measure of improved T cell response[200].

IFN-γ secretion by CD8+ and CD4+ T cells follows recognition of p-MHC-I or p-MHC-II respectively and IL-12 signaling. Loss of tumour control by both CD4+ and CD8+ T cells is observed in mice deficient in IFN-γ[201,202] or the IFN-γ-R[203–205]. Tumour mediated CTL-destruction is mediated by not only direct killing mechanisms such as the perforin-granzyme system[206], but also by the anti-angiogenic effects of IFN-γ[203–205,207]. Qin *et al* demonstrated in a variety of tumours (fibrosarcoma, ras-transformed fibroblasts, colon carcinoma, and plasmacytoma) that IFN-γ-mediated inhibition of tumour-induced angiogenesis always preceded rejection of tumours by CD8+ T cells.

CD8+ T effector cells can eliminate tumours in the absence of perforin but not IFN-γ, although CD8+ memory cells cannot eliminate tumours in the absence of perforin[202]. Further anti-tumour effects of IFN-γ include the upregulation of MHC expression on tumours so that peptide presentation is enhanced[154,208]. It has also been shown that tumour-specific CD4+ T cells can eliminate MHC Class II negative cancers in mice, in the absence of CD8+ T cells, via the indirect effects of IFN-γ on host cells[209]. Mice that were immunocompetent, apart from their capacity to produce IFN-γ, were shown to be more susceptible to spontaneous tumour development[210], and to carcinogen-induced tumours[211].

DCs loaded with freeze-thaw lysate of medullary thyroid carcinoma have been shown to induce high intracellular IFN-γ in patient CD8+ T cells[2]. Thus the importance of this cytokine in the anti-tumour immune response is well established, as well as precedence for lysate-loaded DC induced IFN-γ productions.

In line with other studies that found no IFN-γresponse to freeze-thaw lysate-loaded DCs[11] our unactivated lysate-loaded GMDCs stimulated very limited amounts of IFN-γ release by CD8+ T cells When oxidised lysate was used as the antigen source the combination of GMDC+B cell caused an approximately 50% increase in IFN-γ levels compared to GMDC alone in CD8+ T cell cultures, however this difference was not statistically significant.

For CD4+ T cells only the GMDC+LPS&CpG and GMDC+B cell+LPS&CpG groups showed strikingly higher levels of IFN-γ. However the increased IFN-γ in the GMDC+B cell+ox-L-primed CD4+ T cells was only significant when compared to the GMDC+MΦ group.

Thus these IFN-γ data supported the CD8+ T cell proliferation data, which showed increased proliferation in response to priming by oxidised lysate-loaded GMDC+B cell, indicative of enhanced T cell function.

In contrast to Wölfl and Greenberg who found that IL-12 production was the only factor that correlated with fully functional T_H1-primed T cell populations[212] we observed reduced IL-12 levels in soluble and oxidised lysate-primed CD4+ and CD8+ T cell co-cultures compared with OVA-stimulated cells. This was in contrast to elevated IL-12 recorded in lysate-loaded GMDC+B cell cultures. IL-12 levels in GMDC+B cell-primed T cell cultures were lower than in GMDC-primed CD4+ and CD8+ T cells cultures, irrespective of whether they were presenting soluble or oxidised lysate antigens. Given that IL-12 drives the differentiation of Type 1 cells during specific antigenic activation, and given the increased proliferation of CD8+ T cells in response to priming by oxidised lysate-loaded GMDC+B cell it is possible that the reduced level of IL-12 is indicative of this cytokine being used up by the proliferating T cells.

No TNF-α was observed in lysate-primed CD4+ and CD8+ T cell cultures (Figure 55). One explanation for the absence of TNF-α observed may lie in the fact that TNF-α is transiently upregulated within 1-3 hours after CpG exposure[213]. This may also help explain why unactivated M1 MΦs were more effective than CpG+LPS-activated MΦs - they may have been exposed to CpG for an excessive amount of time[213]. Crabtree's data argues that 1-3 hours of CpG exposure results in enhanced TNF-α production, whereas longer time periods (6-9 hours) results in TNF-α inhibition and increased IL-10, possibly as a result of autocrine negative feedback regulation. However we found no TNF-α or IL-10 in the cell culture media of T cells primed with any of our APCs, whether they had been activated with CpG&LPS or not. Thus we have no evidence for this mechanism of TNF-α, or IL-10, inhibition.

A more recent study reported that membrane-bound TNF-α may have a protective role in cancer while the soluble form may be responsible for TNF-α's confusing pro-tumoural effects[95]. On the basis of those results the low levels of soluble TNF-α in this study may represent a positive rather than a negative finding. However membrane-bound TNF-α was not adequately assessed in this study so that observation that remains to be further explored.

The presence of high levels of IL-10 is indicative of a skew toward immunoregulatory functions, via induction of tolerogenic DC function[98] and T_{REG} formation[96], or response resolution. Therefore we were concerned to ascertain that our lysate-loaded APCs did not stimulate a regulatory T cell response.

Hatfield *et al* reported that while overall levels of IL-10 were low, small but statistically significant increases were observed in lysate-treated DCs[11]. In a similar manner, for both CD4+ and CD8+ T cells, we also consistently observed low levels of IL-10 that were slightly increased in the presence of soluble- and oxidised lysate-loaded APCs. A consistent pattern of less IL-10 in oxidized lysate-primed cultures was observed across all APCs for both CD4+ and CD8+ T cells, though no differences were statistically significant. In GMDC+B cell-primed CD8+ T cells IL-10 was slightly lower in the soluble lysate group compared to GMDC-primed cells, though again, not to a statistically significant degree.

In light of the consistently low levels of IL-10 we were not concerned about the generation of T_{REG}s and so the expression of CD25 or FoxP3 was not analysed on our lysate-primed cells. Further, with the induction of costimulatory molecules and IL-12 production in response to the lysates we reasoned that a tolerogenic state was likely not being induced.

4.4.5 Long Term *In Vitro* Culture of Lysate-Primed T Cells Was Not Achieved

Since significant reductions in viability were observed in lysate-primed T cells after 72 hours the commencement of ten-day priming experiments highlighted the need for cell culture conditions that would support T cells survival throughout this longer time period. The inclusion of IL-2, IL-7, IL-15, IL-21 and GM-CSF in the cell culture medium yielded no improvements in fold expansion. Limited improvement in viability was observed at the higher cytokine doses in both GMDC- and IL4DC-primed T cells (Appendix 1, Supplementary Figures 24 and 25). Efforts at supporting the long-term culture of lysate-primed T cells were unsuccessful and require further optimization.

4.4.6 Lysate-loaded APCs Are Unable to Stimulate T Cell Expansion

We attempted to examine the differences in fold expansion of T cells primed with one APC or APC combination and re-stimulated with the same APC(s) or another APC(s). These experiments established that lysate-loaded APCs have a negative effect on T cell viability. By Day 10 all CD4+ T cells were dead in both the soluble and oxidised lysate-primed groups. CD8+ T cells died in the soluble lysate-primed groups also therefore we were only able to analyse the fold expansion of oxidised lysate-primed CD8+ T cells (Figure 57). The most obvious advantage of using oxidised lysate over soluble lysate was that some CD8+ T cells remained alive at Day 10 whereas in the soluble lysate-treated wells all T cells were dead by Day 10. Although our aim was to measure Fold Expansion, in reality by the end of 10 days we were simply measuring viability.

The two conditions that stood out in these cells were the GMDC-primed and GMDC+B cell-primed (1.84 and 1.98 FE respectively). In two experiments an

approximate doubling of fold expansion was observed in the GMDC+B cell group compared with GMDC alone. However, while the GDMC-primed cells cluster around an approximate 2-fold expansion, the range of results in the GMDC+B cell condition makes this data on its own less convincing. Nevertheless the GMDC+B cell group was the only group in which T cells were split during 10-day priming. In no other groups were sufficient cells generated to require splitting into more than one well. This observation required confirmation that T cells rather than B cells were proliferating, since CpG is a strong mitogen of B cells. This was confirmed by a CD19 and CD11c Dump Channel, which verified that even in these wells the percentage of CD19+ or CD11c+ cells was 10% at the most and usually 5% or less (Appendix 1, Supplementary Figure 17). For this reason we continued to be particularly interested in the comparison between the GMDC and GMDC+B cell groups as no enhanced fold expansion was observed in any other 10-day-primed group.

4.4.7 Lysate-loaded APCs Induce T_{CM} and T_{EM} T Cell Phenotypes

By Day 10 sufficient numbers of live lysate-primed CD8+ T cells were often unavailable for phenotyping, and even fewer remained by Day 20. No CD4+ T cells survived 10-day priming. However we were able to harness the power of Flow Cytometry which allows analysis of rare cell populations to assess what changes in phenotype could be observed between naïve and Day-10 lysate-primed CD8+ T cells. Three observations were measured wherever possible despite the difficulties in obtaining three robust repeats,

The naïve CD4+ and CD8+ T cells were unexpectedly found to have very low expression of CD28 a key co-stimulatory molecule in the CD28/CTLA4/B7 (CD80/CD86) pathway. CD28 is known to be critical in the priming of antigen-specific CD4 T cells, however, in line with findings from other studies that showed CD28-independent CD8+ T cell priming[214–216], our lysate-primed CD8+CD28- T cells were able to proliferate in response to OVA antigen. In accordance with these other studies, antigen-specific lysate-primed T cells were observed, as evidenced by T cell proliferation and IFN-γ responses, and the lack of CD28 did not affect the generation of memory T cells, albeit possibly at lower frequencies.

Loss of CD27 in fully differentiated effector T cells may be responsible for, or contribute to, CD8+ T cell impaired expansion and survival after ACT[69]. In three consecutive clinical trials that yielded 49 – 72% response rates, the number of CD8+CD27+ cells was highly correlated with the persistence of adoptively transferred TILs[112]. Therefore it was encouraging to observe 40% and 50% CD27 expression respectively in CD8+ T cells primed by oxidized lysate-loaded GMDC and GMDC+B cell. Interestingly, while these CD27 expression levels were lower than those recorded in the MΦ, DC+MΦ, Bc+MΦ or Triple combination-primed groups, in our hands this did not correlate with reduced *in vivo* function, as will be discussed in Chapter 5.

Our naïve T cells were CD28- and, unsurprisingly, did not show upregulation of CD28 in response to priming with lysate. Romero and colleagues have shown heterogenicity of CD27 and CD28 expression in human CD8+ T cells and suggested that stepwise loss of both molecules characterised T cell differentiation[217]. They grouped T_{EM} cells into four subsets on the basis of CD27CD28 expression: EM_1 ($CD27^+CD28^+$), EM_2 ($CD27^+CD28^-$), EM_3 ($CD27^-CD28^-$) and EM_4($CD27^-CD28^+$) On this basis our cells would fall into the EM_2 subset which they describe as producing characteristic effector mediators. The late-stage effector CD27-CD28- group are further differentiated and have undergone further rounds of division than the CD27+CD28- group.

CD44 is a molecule that is expressed at low levels on naïve T cells and upregulated in response to antigen exposure. Compared with naïve levels we observed an approximate tripling in the expression of CD44 in CD8+ T cells in response to priming with OVA-loaded GMDC. The response to oxidised lysate-loaded GMDC generated similar levels of CD44 to the OVA-primed cells in two out of three experiments. No differences were observed between the GMDC and GMDC+B cell-primed groups.

When assessing the functionality of the CD62L molecule, Gattinoni and colleagues reported that $CD62L^{HI}$ subset had superior anti-tumour properties *in vivo* compared to

the $CD62L^{LO}$ subset[69]. Interestingly, CD62L positivity returned an opposite trend to that of CD44, with OVA-, ox-L+GMDC- and ox-L+GMDC+B cell-primed T cells displaying lower levels than CD8+ T cells primed by other APCs. And in bi-variate CD44+CD62L analysis the GMDC+OVA-, GMDC+ox-L- and GMDC+Bc+ox-L-primed CD8+ T cells had lower percentages of double positive cells compared to cells primed with M1 MΦs or the other APCs or APC combinations. Thus we were able to successfully generate CD8+ T_{EM} cells, but fewer T_{CM}. However, in the GMDC and GMDC+B cell-primed groups the percentage of naïve or T_{SCM}(CD62L+CD44-) cells was also lower (Figure 60, Figure 61), suggesting that the GMDC and GMDC+B cell combination were able to activate more T cells in response to lysate antigens. In addition CD62L+ cells are classified as early effector cells, as opposed to the effector cells which lose CD62L expression[69], therefore it may be that these cells retained an early effector phenotype such as have been described as being more effective in ACT[109,218,219]. The lower percentages of T_{CM} generated by the GMDC and GMDC+B cell combination, which primed superior CD8+ T cell responses, may reflect higher levels of IL-2 production in these co-cultures, however IL-2 was not measured.

Upregulation of PD-1 would not have been an unexpected response to the 'self-antigens' found in the lysate since PD-1 upregulation is a means of regulating autoimmune responses and promoting self-tolerance. However no increases in PD-1 were observed on CD8+ T cells in response to ox-L (Appendix 1, Supplementary Figure 33) and overall levels of PD-1 remained <3%, indicating that priming with oxidized lysate was not inducing significant levels of T cell exhaustion.

No upregulation of CD122 was observed in any lysate-primed CD8+ T cells. The lack of CD122 was of some concern given that CD122 upregulation should be observed on T_{EFF} and T_{CM} cells, thus its absence implies lack of differentiation. Although CD122 upregulation is transient on T_{EFF} therefore its lack may be due to the timing of the observation. CD8+CD122+PD-1+ cells have been reported to be T_{REG}s, whereas CD8+CD122+PD-1- cells were true memory cells[220,221]. Thus despite the lack of CD122, the absence of PD-1 on these lysate-primed cells points to a lack of regulatory function, which would be a desireable feature in cells destined for ACT.

Likewise, no upregulation of the IL-7 receptor CD127 was observed, suggesting a lack of memory phenotype. Sensitivity to IL-7 and IL-2 instills in T_{CM} a greater capacity than T_{EM} to persist *in vivo* and a greater proliferative potential, thus rendering them more efficient mediators in anti-tumour immunity. CD8+CD127$^{-/LO}$ cells have been assigned to the short-lived effector category[222]. Thus the lack of CD127 was concerning due to its implications of IL-7 insensitivity in these cells, which implies reduced persistence and memory differentiation *in vivo*.

Overall these data demonstrated that priming CD8+ T cells with undefined tumour antigens in the form of oxidized tumour lysate leads to the formation of T cells with an appropriate phenotype for use in ACT for cancer.

With regards to the aims of this chapter, these results established that when oxidized lysate is used to prime CD8+ T cells, the GMDC+B cell combination resulted in significantly greater T cell proliferation than GMDC alone. No statistically significant increases in IFN-γ, or TNF-α or IL-10, were observed in CD8+ T cells primed with soluble or oxidised lysate-loaded GMDC+B cell compared to GMDC alone. Less IL-12 was measured in the GMDC and GMDC+B cell-primed groups compared to other APC-primed T cells and the GMDC and GMDC+B cell-primed CD8+ T cells also clustered together in terms of their T_{CM} and T_{EM} phenotypes at Day 10.

Taken together these data supported our hypothesis in that the combination of a GMDC and a B cell might represent an enhanced method of priming CD8+ T cells with oxidized lysate antigens, We therefore examined whether these enhancements would translate into an improved effector function by consequently examining the *in vivo* cytotoxic capacity of these cells.

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5 Cytotoxic Function of Lysate-Primed T cells

5.1 Introduction

Adoptive cell therapy with activated T cells has proven to be the most promising approach for the treatment of advanced melanoma[1–8]. In the clinic, the capacity of tumour-specific cells to release IFN-γ and to kill the patients' tumor cells *in vitro* have been the primary criteria used to select cells for adoptive transfer to cancer patients[9–11]. While some clinical success has been reported with the use of these cells[12,13], an association between immune assay response and patient antitumour response has not been clearly established for most current *in vitro* measurements of cellular immune responses[14,15]. This could be due to the use of peripheral blood T cells, which may not be the optimal source of TAA-reactive T cells, since cytolytic CTLs in peripheral blood are rare and require multiple rounds of expansion prior to lysis testing which may introduce artefacts[14,16,17]. In one study regression was observed in two patients in the absence of *in vitro* CTL lysis[14,18], thus there remains a disconnect between the *in vitro* capacities of tumour-specific T cells and their *in vivo* efficacy.

An inverse relationship between *in vitro* effector function and *in vivo* antitumor efficacy has been noted in several studies[9,11,19–21] and retrospective analyses of ACT in melanoma patients demonstrate that *in vivo* effectiveness is not predicted by these *in vitro* criteria. Objective clinical responses are not always observed after adoptive transfer of IFN-γ producing, cytolytic tumor-specific CD8+ lymphocytes[11,19,20]. Gattinoni notes that "*Specifically IFN-γ release and cytotoxicity were negatively correlated with in vivo antitumor efficacy*"[9]. We had observed IFN-γ production in our lysate-primed T cells and therefore wished to know if these *in vitro* capabilities would transfer to a tumour antigen-specific response *in vivo*.

Activated CD4+ and CD8+ T cells are essential to the regression of established cancers, whether individually, or in concert. Both CD4+ and CD8+ CTLs display direct and indirect cytotoxic functions. Clinical work using APC-activated T cells has

focused largely on the use of DCs as the APC and tumour associated peptides as the source of antigen. A number of clinical trials have been conducted, in various cancer types, using undefined antigens in the form of tumour lysate, all utilising a DC as the APC[22–32]. While B cells are entering the clinic as a viable alternative APC to DCs[33], we are not aware of any clinical trials that have utilized B cells or MΦs to present lysate antigens to T cells.

We had identified that our lysate-primed CD8+ T cells were able to proliferate in response to the presentation of oxidised lysate by GMDC+B cells. We had also demonstrated that lysate antigen-primed CD8+ T cells produced IFN-γ, particularly in response to priming by GMDC or GMDC+B cell. Oxidised lysate-primed CD8+ T cells also displayed T_{EM} and T_{CM} phenotypes in varying proportions, according to the APC(s) that had presented the lysate antigens. Thus these lysate antigen-primed T cells displayed the potential for some clonal expansion, and cytotoxic functions based on their phenotype and ability to produce IFN-γ. It has been observed in a murine adoptive cell therapy model that more-differentiated cells are less effective *in vivo*[9] so we were interested to assess the *in vivo* effectiveness of our lysate-primed cells.

Since *in vitro* results do not necessarily reflect what occurs *in vivo* we examined whether our IFN-γ-producing T_{EM} and T_{CM} cells would deliver cytolytic functions *in vivo* by measuring the ability of lysate-primed T cells to lyse peptide-pulsed target cells *in vivo*.

Work in our laboratory[34] and others has demonstrated the importance of the T_{CM} phenotype, particularly CD62L positivity, in the anti-cancer response[35–37]. A study to identify the characteristics of transferred CD8+ T lymphocytes that are effective *in vivo* versus cells with impaired *in vivo* function identified that less differentiated, early effector T cells proliferated more vigorously both *in vitro* and *in vivo*[9]. However, while both $CD62L^{LO}$ and $CD62L^{HI}$ cells both proliferated well *in vitro*, only the $CD62L^{HI}$ group produced superior *in vivo* antitumor results. These cells preferentially homed to the lymph nodes, where it was demonstrated that they interacted with tumour-antigen-bearing APC to bring about eradication of established tumours.

It had been proposed that T_{CM} and T_{EM} are not necessarily discrete subsets, but rather form part of a linear differentiation continuum that proceeds from naïve to effector to T_{EM} to T_{CM}[37]**.** However an interesting study published in 2015 that analysed the transcriptional profiles of differentiating T cells revealed an unexpected step-wise progression that followed a T_N ➔ T_{CM} ➔ T_{EM} ➔ T_{EFF} pattern[38]. Rather than more differentiated, this puts T_{CM} cells as less-differentiated cells than T_{EFF} and may help to explain their superior abilities in ACT[35], particularly in light of Gattinoni's observation of an inverse correlation between *in vitro* full effector differentiation and *in vivo* effectiveness[9]. Having identified that the lysate-primed CD8+ T cells displayed T_{CM} and T_{EM} subpopulations we proceeded to assess their effector functions.

We investigated the ability of anti-OVA CD4+ or CD8+ T cells that had been primed with lysate-loaded GMDC or GMDC+B cell to kill OVA peptide-loaded target cells *in vivo* after adoptive transfer into wild type mice. At the time of conducting the *in vivo* cytotoxicity assays M1 MΦs, along with the combination of DC+MΦ, Bc+MΦ and the triple combination, had been eliminated as being less effective than a GMDC alone or the GMDC+B cell combination and therefore these APCs and APC combinations were not tested.

The optimal characteristics of the ideal T cell for ACT for cancer remain to be defined and we were interested to examine whether or not the CD62L status, or T_{EM} to T_{CM} ratio, of our lysate-primed T cells prior to injection correlated with *in vivo* killing, even if their *in vivo* proliferation and tumour eradication was not assessed in this study.

5.2 Objectives

The aim of the studies in this chapter was to assess the *in vivo* functional capacity of CD8+ T cells primed with lysate-derived tumour antigens. The *ex vivo* T cell response to re-stimulation with a lysate-loaded GMDC or GMDC+B cell was also examined. We hypothesized that priming T cells with GMDC+B cell would result in enhanced *in vivo* cytotoxicity of target cells compared with a GMDC alone. We also hypothesized that re-stimulating *ex vivo* tumour-draining lymph node-derived cells with the combination of GMDC+B cell would result in enhanced expansion of these cells compared with a GMDC alone.

To address these hypotheses the following objectives were undertaken:

1. Determine the *in vivo* cytotoxic response of adoptively transferred T cells primed by lysate-loaded GMDC versus GMDC+B cell

2. Determine the fold expansion and T cell phenotype that results from restimulating *ex vivo* tumour-draining lymph node-derived cells with GMDC versus GMDC+B cell

5.3 Results

5.3.1 Adoptively Transferred T Cells Primed With Lysate-Loaded Dendritic Cells+B cells Display Enhanced *In Vivo* Cytotoxicity

Our *in vitro* results indicated that the GMDC+B cell combination might yield a superior anti-tumor response based on slight increases in proliferation and IFN-γ production, but not *in vitro* cytotoxicity (Appendix 1, Supplementary Figures 31, 32). Therefore the GMDC+B cell combination was compared against GMDC alone in the *in vivo* cytotoxicity response. The effector T cells were generated as usual by co-culture with lysate-loaded GMDC or GMDC+B cell. Target cells were pulsed with SIINFEKL or $OVA_{323-339}$ peptide, or left unpulsed as a control against non-specific killing. Peptide-loaded target cells were stained with fluorescent dyes and i.v. injected into wild type mice. The following day a 50/50 mix of CD4+ and CD8+ effector T cells primed by either soluble lysate- or oxidised lysate-loaded GMDCs or lysate-loaded GMDC+B cells was adoptively transferred by i.v. injection. Twenty-four hours after transfer the spleens were harvested and killing of the peptide-loaded targets assessed by Flow Cytometry.

Figure 63 shows the gating strategy and raw phenotype data of GMDC+B cell soluble lysate, GMDC oxidised lysate and GMDC+B cell oxidised lysate-primed CD8+ T cells. Raw data for CD4+ T cells is found in Appendix 1, Supplementary Figure 33. In the first experiment sufficient cells were generated for injection only and not for phenotyping. In the second experiment sufficient cells were generated by GMDC+soluble lysate-priming for injection however insufficient cells remained for assessment of T cell phenotype therefore T cell phenotype data is only available for the GMDC+Bc+s-L, GMDC+ox-L and GMDC+Bc+ox-L groups (Figure 63). Summary data is shown in Appendix 1, Supplementary Figure 34. T cell viability in the GMDC oxidised lysate groups was consistently greater than 40% whereas viability in the GMDC+B cell groups was considerably lower at 20-30%. The phenotype of these cells was only measured once and so these results are discussed in Appendix 1 Section 7.33.

shows the results of CD8+ T cell-mediated lysis from two independent *in vivo* cytotoxicity experiments. In both experiments, 100% lysis was achieved in the OVA-

primed T cells. Unexpectedly, in both experiments, 100% lysis also occurred in the mice that were injected with PBS instead of effector cells as a negative control. In the APC-primed T cells identical killing trends were observed in both experiments.

Twenty-four hours after transfer of targets 49% of SIINFEKL-pulsed cells were eliminated by GMDC+soluble lysate-primed CD8+ T cells. The percentage of cells lysed increased to 61% for GMDC+Bc+soluble lysate-primed CD8+ T cells. Lysis was higher again in the GMDC+oxidised lysate-primed CD8+ T cells (84%), and the greatest cell lysis, 95%, was achieved in the GMDC+Bc+oxidised lysate-primed group.

Transferred CD4+ effector cells appeared to elicit no killing in either experiment (Appendix 1, Supplementary Figure 35). Unexpectedly the number of unpulsed cells was less than the number of $OVA_{323-339}$ peptide-pulsed cells in both experiments, which resulted in negative lysis values, a biological impossibility. The trend of fewer target cells in the GMDC+B cell primed CD4+ groups compared to the GMDC groups was the same as CD8+ T cells but does not alter the fact that these results do not demonstrate killing by CD4+ T cells due to the negative values.

Negative binomial regression, which accounts for an over-dispersed Poisson distribution was used to compare the number of live SIIN and $OVA_{323-339}$-pulsed cells that remained after 24 hours *in vivo* in mice that had received adoptively transferred lysate-primed effector T cells. The model included terms for the groups and adjusted for the number of cells collected. Although no adjustment was made for multiple comparisons it should be noted that all the p values were <0.001 therefore these results are highly unlikely to be due to chance.

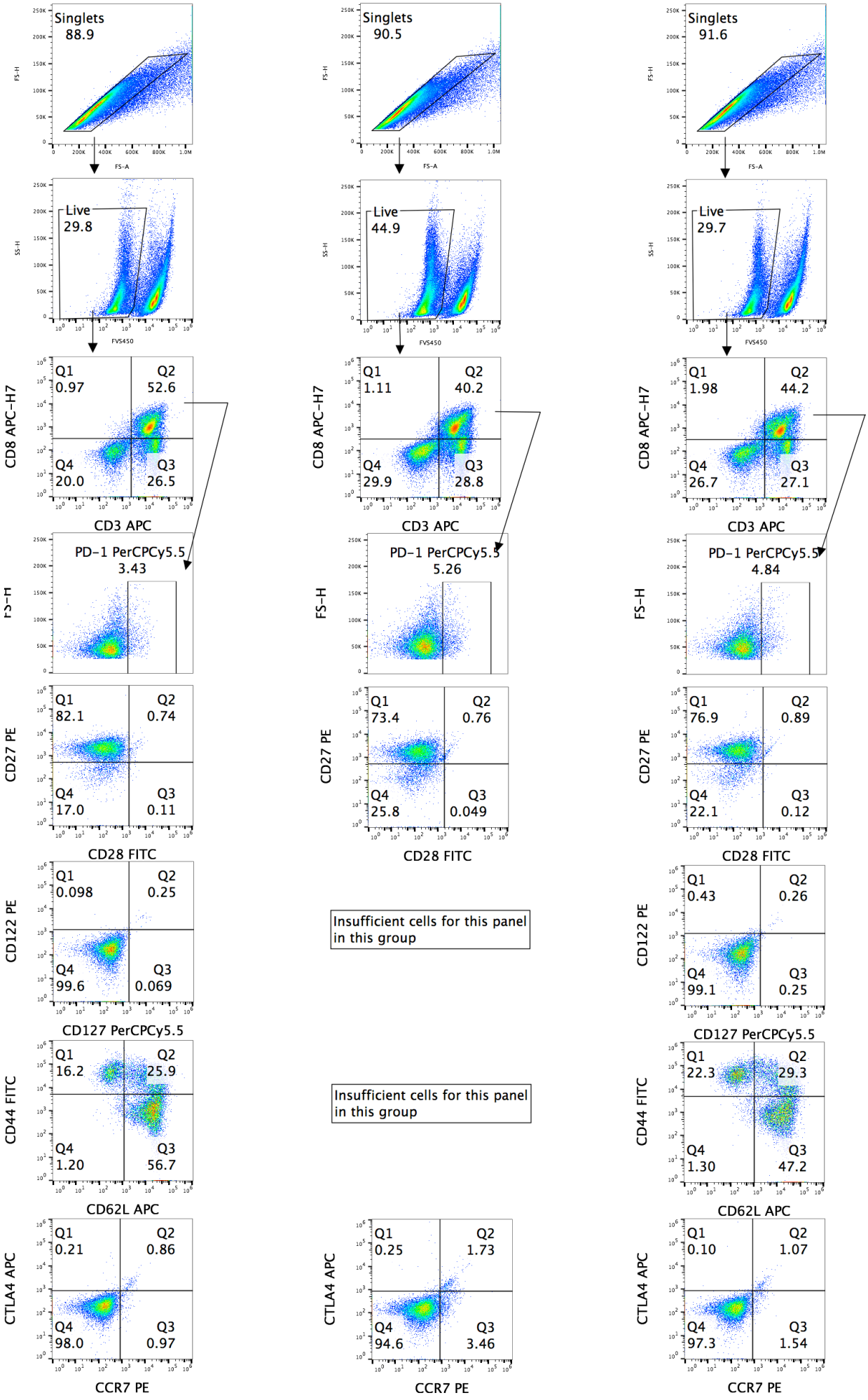
Singlets
88.9
Live
29.8
Q1
0.97
Q2
52.6
Q4
20.0
Q3
26.5
CD8 APC-H7
CD3 APC
PD-1 PerCPCy5.5
3.43
Q1
82.1
Q2
0.74
Q4
17.0
Q3
0.11
CD27 PE
CD28 FITC
Q1
0.098
Q2
0.25
Q4
99.6
Q3
0.069
CD122 PE
CD127 PerCPCy5.5
Q1
16.2
Q2
25.9
Q4
1.20
Q3
56.7
CD44 FITC
CD62L APC
Q1
0.21
Q2
0.86
Q4
98.0
Q3
0.97
CTLA4 APC
CCR7 PE
Singlets
90.5
Live
44.9
Q1
1.11
Q2
40.2
Q4
29.9
Q3
28.8
CD8 APC-H7
CD3 APC
PD-1 PerCPCy5.5
5.26
FS-H
Q1
73.4
Q2
0.76
Q4
25.8
Q3
0.049
CD27 PE
CD28 FITC
Insufficient cells for this panel in this group
Insufficient cells for this panel in this group
Q1
0.25
Q2
1.73
Q4
94.6
Q3
3.46
CTLA4 APC
CCR7 PE
Singlets
91.6
Live
29.7
Q1
1.98
Q2
44.2
Q4
26.7
Q3
27.1
CD8 APC-H7
CD3 APC
PD-1 PerCPCy5.5
4.84
FS-H
Q1
76.9
Q2
0.89
Q4
22.1
Q3
0.12
CD27 PE
CD28 FITC
Q1
0.43
Q2
0.26
Q4
99.1
Q3
0.25
CD122 PE
CD127 PerCPCy5.5
Q1
22.3
Q2
29.3
Q4
1.30
Q3
47.2
CD44 FITC
CD62L APC
Q1
0.10
Q2
1.07
Q4
97.3
Q3
1.54
CTLA4 APC
CCR7 PE

Figure 62 **Gating strategy & raw data showing phenotype of adoptively transferred T cells.** Immature Day 6 C57/BL6 BMDC, +/- spleen-derived B cells, were pulsed overnight with whole OVA protein (50 µg/mL) and soluble lysate or Oxidised lysate (1:1 ratio tumor cell to APC). The following day CD4+ and CD8+ T cells were added to the APCs (10:1) and co-cultured for 3 or 4 days. Cells were harvested for injection into CD57BL/6 mice and surplus cells were analysed for phenotype by Flow Cytometry. Cells were labeled with FVS450 (BD Biosciences) dead cell exclusion dye and mABs against CD3, CD4, CD8, CCR7, CD154 (CTLA4), CD27, CD28, CD44, CD62L, CD122, CD127 and PD-1. Cells were fixed in 4% paraformaldehyde and stored at 4°C overnight prior to collection on a Gallios. Data shown is from 1 of 2 experiments. A) T cells primed with GMDC+B cell+soluble lysate B) T cells primed with GMDC+oxidised lysate C) T cells primed with GMDC+B cell +oxidised lysate.

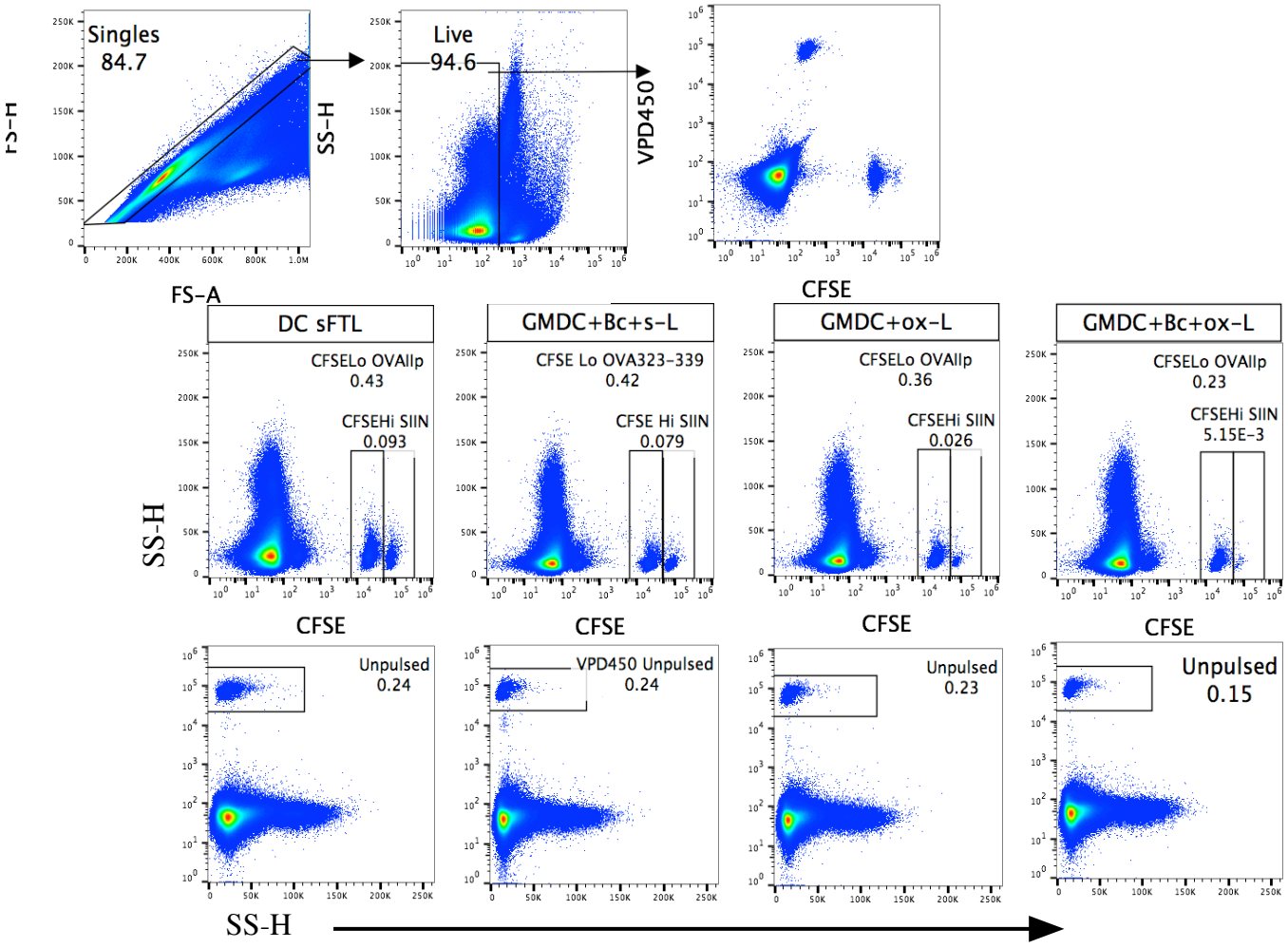

Figure 63 **Gating strategy and raw data showing loss of SIIN-pulsed T cells in in vivo cytotoxicity assays**. Day 6 C57BL/6 GMDC and spleen-derived B cells were prepared as previously described. APCs were pulsed overnight with whole OVA protein (50 µg/mL) and soluble or oxidised lysate (1:1 ratio tumor cell to APC). The following day CD4+ and CD8+ T cells were added to the APCs (10:1) and co-cultured for 3 days. Effector cells were resuspended at 1 x 107 c/mL in sterile DPBS and 200 µL injected per mouse. The following day C57BL/6 splenocytes were pulsed for 3 hours with SIINFEKL or $OVA_{323-339}$ peptide or left unpulsed. The 3 groups of target cells were dyed with $CFSE^{HI}$ (25 µM), $CFSE^{LO}$ (2.5 µM) and VPD450 (10 µM) respectively. Dye-labelled cells were resuspended at 50 e 6 c/mL in DPBS and equal volumes of the target populations mixed together. 200 µL per mouse (10 e 6 target cells; 3.33 e 6 per target per mouse) were i.v. injected. 24-hours after adoptive transfer mice were sacrificed and splenocytes prepared for acquisition by Flow Cytometry. Cells were labeled with live/dead NIR dead cell exclusion dye, fixed in 4% paraformaldehyde and stored at 4°C overnight prior to collection on a Gallios. Representative flow dot plots are shown from 1 of 2 experiments.

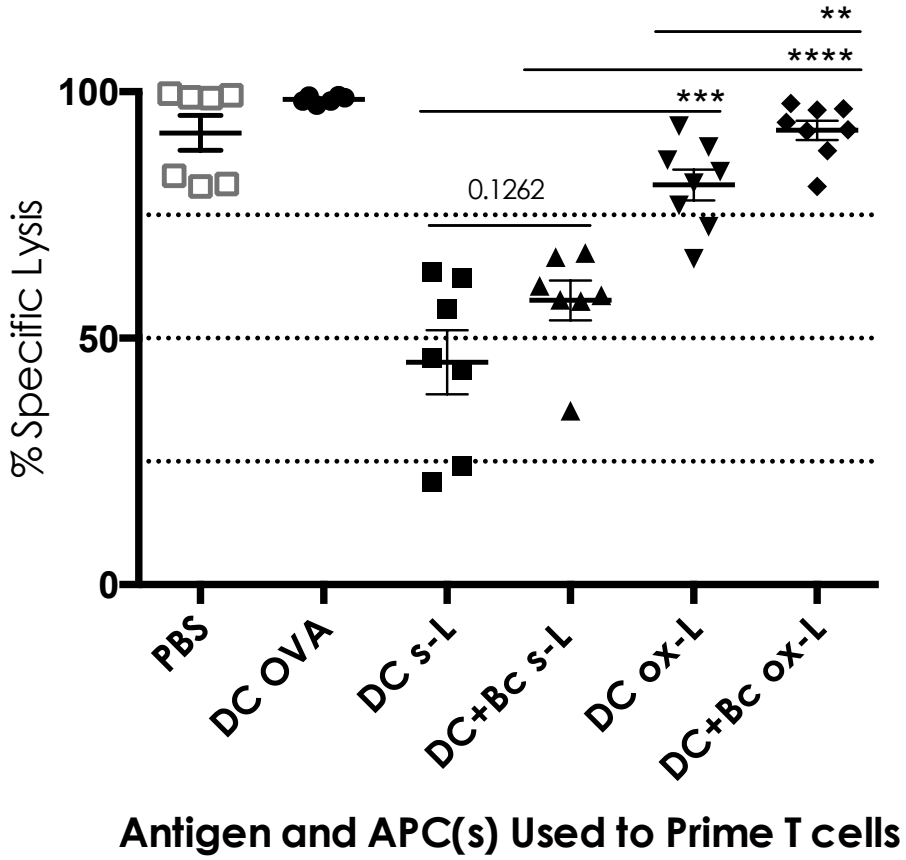

Figure 64 **Priming T cells with Oxidised lysate results in greater in vivo cytotoxicity by CD8+ effector cells than soluble lysate and the combination of lysate-loaded GMDC+B cell is superior to GMDC alone**. Immature Day 6 C57/BL6 BMDC, +/- spleen-derived B cells, were pulsed overnight with whole OVA protein (50 μg/mL) and soluble lysate or oxidised lysate (1:1 ratio tumor cell to APC). The following day CD4+ and CD8+ T cells were added to the APCs (10:1) and co-cultured for 3 or 4 days. Lysate-primed effector T cells were i.v. injected into CD57BL/6 mice. The following day C57BL/6 target splenocytes were incubated with SIINFEKL peptide (1 μg/mL), $OVA_{323-339}$ peptide (5 μg/mL), or left unpulsed. Target cells were labeled 25 μM CFSE ($CFSE^{HI}$), 2.5 μM CFSE ($CFSE^{LO}$) or 10 μM VPD450 and mixed together for injection into recipient mice (1/3 each SIIN-pulsed, $OVA_{323-339}$-pulsed and unpulsed). Legend: each symbol represents a single mouse; (n = 3-7 per group). $^{**}p$ <0.01, $^{***}p$<0.001 and **** P <0.0001 (Negative binomial regression). Data are from 2 independent experiments. Error bars = s.e.m.

5.3.2 *Ex Vivo* Re-stimulation of Tumour Draining Lymph Node Cells

The final data of this thesis pertains to the attempt to restimulate cells isolated from the tumour draining lymph nodes (tu dLN) of B16.OVA tumour-bearing mice. Single cell suspensions were created from the dLNs and the cells frozen for later use. Cryopreserved cells were recovered from liquid nitrogen storage and allowed to recover overnight prior to co-culture with lysate-loaded GMDC or GMDC+B cells.
In addition to IL-2, IL-7, IL-15 and IL-21, anti-CD28 was included in the medium in an attempt to augment the tumour-specific T cells' fold expansion. Including α-CD28 with the T cells provides extra costimulation for the antigen-specific T cells but will not drive non-specific proliferation, as α-CD3 will do.

We also experimented with the use of mABs against CTLA-4 and PD-1 as an indicator of whether these checkpoint inhibitors might be useful in augmenting the response of *ex vivo*-derived cells restimulated with lysate tumour antigens. Those observations are discussed in Appendix 1, Section 7.33.

Cell conditioned media was analysed by ELISA for the presence of IFN-γ and IL-12. Consistent with the *in vitro* results in Chapter 4 (Chapter 4, Figures 16 and 17), these preliminary observations showed a pattern of increased IFN-γ (Figure 65A) and decreased IL-12 (Figure 8B) in the GMDC+B cell groups compared to the GMDC-primed cells.

In cells treated with checkpoint blockade mABs, cytokine production was strongly decreased in mAB-treated groups compared with cells not exposed to mABs (Figure 8C).

Due to the low numbers of cells that were recovered we were unable to test more than one parameter at a time and therefore these results remain as preliminary observations.

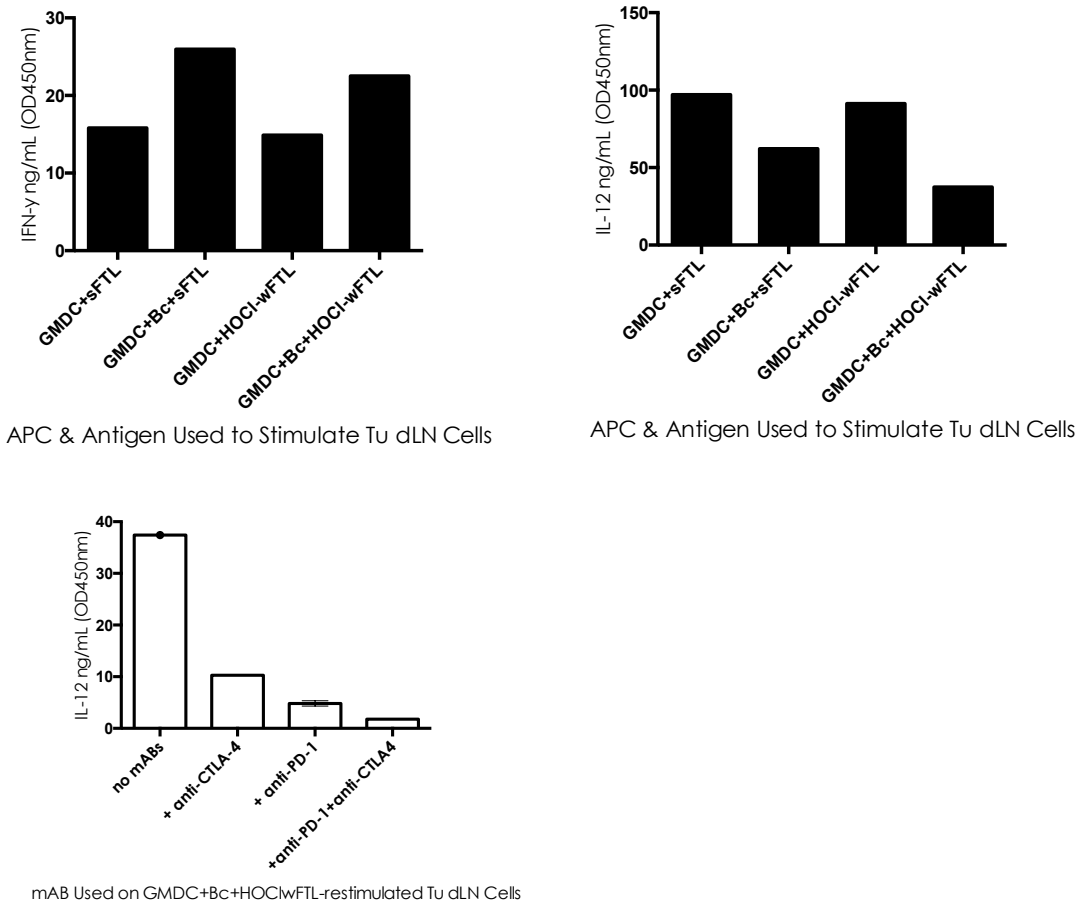

Figure 65 **IFN-γ production is increased, while IL-12 production is decreased, in one set of tumour draining lymph node cultures re-stimulated with oxidised lysate-loaded GMDC+B cell**. Wild type C57BL/6 mice were injected with 50,000 B16.OVA cells and sacrificed when tumours reached 150 mm^2. Tumour draining lymph nodes were excised, single cell suspensions prepared and the cells cryopreserved for future use. Cryopreserved cells were retrieved from liquid nitrogen, thawed and allowed to rest overnight prior to adding to lysate-loaded APCs at a ratio of 10:1, +/- α−PD-1 and α-CTLA-4 as indicated. Cells were cultured for 10 days and fed no less than every other day. Cell conditioned media were harvested, stored at -20° and analysed by anti-IFN-γ and anti-IL-12 ELISA. Insufficient cells were obtained to test more than 2 conditions.in any given experiment. GMDC+s-L and GMDC+Bc+s-L were compared in one experiment. GMDC+ox-L and GMDC+Bc+ox-L were compared in one experiment. Each column in the lower graph shows the result from one experiment.

5.4 Discussion

5.4.1 *In vivo* Cytotoxicity

The *in vitro* data discussed in Chapter 4 demonstrated that the combination of GMDC+B cell resulted in enhanced T cell proliferation and IFN-γ production when compared with GMDC alone – particularly in oxidised lysate-primed CD8+ T cell responses. Our *in vivo* cytotoxicity data corroborated those results with highly significant improvements in target cell lysis by CD8+ T cells primed with GMDC+B cell as opposed to a GMDC alone. In line with Chiang's findings[39–41], oxidized lysate also proved more effective as an antigen source than soluble fraction lysate *in vivo*. However, our data demonstrated that the T cell response to oxidized lysate was enhanced to an even greater degree when presented by the combination of GMDC+B cell.

In order for self-antigens to be used as anti-tumour targets tolerance must be overcome. Oxidation provides an effective means of increasing the immunogenicity of poorly immunogenic molecules[40,42–45]. It is interesting to note that the production of reactive oxygen species, including hypochlorous acid and hydrogen peroxide, by neutrophils is one cooperative strategy used between the innate and adaptive immune systems[44].

IL-12 production by DCs has been used as a measure of improved immunogenicity of oxidized tumour lysate[46]. In this study IL-12 production in lysate-loaded APC cell cultures was noted to increase in response to oxidised lysate. However in APC-T cell cultures the IL-12 levels decreased in response to oxidized lysate compared to the soluble fraction of freeze-thaw lysate. Nonetheless, *in vivo* the oxidized lysate-primed CD8+ T cells outperformed CD8+ T cells primed with soluble fraction lysate (Figure 64Figure 2). This result is in line with another study which also found a negative correlation between the IL-12 response and the *in vivo* anti-tumor response when apoptotic-necrotic lysate-loaded DC were used as the APC[47]. Thus increased IL-12 does not always correspond to an improved anti-tumor response and more must be done to understand this mechanism of action. The authors of the aforementioned study concluded that the anti-tumour response was unconnected to the IL-12-dependent mechanisms and that therefore other non-DC immune cells in the starting

bone marrow-derived, GM-CSF-stimulated APC population were involved. In this present study I hypothesise that the reduced levels of IL-12 may be due to the IL-12 being consumed by the more activated T cells in the oxidised lysate groups.

Work by Constant *et al* provides one possible explanation as to why the GMDCs in this study could competently prime CD4+ and CD8+ T cells with peptides, but were less efficient with lysate, which comprises numerous different proteins. They demonstrated that the type of antigen, peptide versus protein, was important. DCs can efficiently present peptide to naïve CD4+ T cells but are poor presenters of the same antigen in its protein form[48,49]. Using μMT KO mice, which lack B cells, they showed impaired priming against protein, but not peptide antigens. The presence of B cells was required for DCs to prime with protein antigen. *In vivo* uptake experiments demonstrated preferential uptake of peptide antigens by DC, while B cells took up protein antigens. Both DC and activated B cells express the costimulatory molecules essential for effective T cell priming and *in vivo* protein-primed B cells showed upregulation of CD80 and CD86[48]. We did not observe upregulation of these molecules in response to *in vitro* loading with lysates. However our CD80, CD86 data are *in vitro* results and may not reflect the *in vivo* situation as studied by Constant *et al*.

Kleindienst and Brocker[50] used a transgenic mouse model in which B cells or DCs, or both, expressed MHC Class II. They showed that DCs alone are sufficient to prime naïve T cells *in vivo* after immunization with a peptide antigen. Interestingly, on their own neither naïve nor activated B cells could activate naïve CD4+ T cells, however if DCs were present B cells supported and enhanced CD4+ T cell priming. In our hands there was a significant increase in the MFI of MHC-II on both B cells and GMDC in response to soluble lysate but only B cells upregulated MHC-II in response to oxidised lysate. However the absolute MFI values were greater on B cells than GMDC, which reflects an increased capacity for presenting lysate proteins to naïve CD4+ T cells.

B cells express clonal, antigen-specific surface immunoglobulins (Ig) that stimulate antigen uptake[51–54]. Antigen taken up by B cell surface Ig is processed and presented

on MHC Class II[55]. While early studies indicated that B cells induced T_H2 responses and did not activate T_H1 cells[56–60] Shirota *et al* argued showed that B cells could efficiently take up and present peptides to T_H cells, as long as the antigen was conjugated to CpG. In addition CpG-activated B cells produced IL-12 in quantities sufficient to drive a T_H1 response[61]. Importantly for this thesis, Shirota and colleagues demonstrated that CpG-activated B cells achieved this irrespective of the antigen specificity of the surface Ig. In other words, the antigen uptake occurred in an antigen non-specific manner, a feature that was previously believed to be a characteristic of DCs and MΦs.

It has been demonstrated that antigen-specific B cells can cross-present antigen[62,63]. In this study we used B cells that were not specific for the model antigen OVA, which begs the question, how can these non-OVA-specific B cells enhance the CD8+ T cell response? The non-classical MHC-I, which is conserved in mice[64], may provide the answer. In 2013 Goodridge and colleagues described a novel extracellular cross-presentation mechanism, which may explain the ability of non-professional APCs to cross-present MHC-I-restricted peptides to CTLs, despite their reduced intracellular cross-presentation machinery compared to DCs[65]. During presentation of endogenous antigen and the cross presentation of exogenous antigen acquired proteins are degraded into 8-10 amino acid sequences in the proteasome. In the ER these epitopes are loaded onto MHC-I by the chaperones TAP and tapasin for delivery to the plasma membrane, where they can be recognised by CTL TCRs. It was previously believed that MHC-I molecules required peptide binding to form stable complexes for delivery to the surface. However it is now known that during activation lymphoid and myeloid cells also upregulate empty MHC-I in an open conformation (MHC-I OC)[64,66–68]. In addition HLA-F, one of the non-classical MHC-molecules in humans, is upregulated in a peptide-free format, and this open conformation HLA-F binds MHC-I OC on activated lymphocytes and monocytes[66,67]. They discovered that HLF-F assists loading of peptides into MHC-I in B cells. HLA-F and MHC-I-expressing B cells took up native proteins and polypeptides. HLA-F and MHC-I dissociated in the lysosome where further protein processing and binding of peptides to MHC-I occurred. Finally, on presentation of viral or tumour antigen by these B cells, CTLs lysed the antigen-specific B cells. Importantly TAP and tapasin were not required in this pathway,

which also exists in activated HLA-F-expressing CTLs, and presents the intriguing notion of self-presentation in inflammatory settings[68]. Interestingly the external binding to HLA-F and MHC-I required denatured protein, such as may be found in oxidized lysate. HLA-F has a murine ortholog meaning that this scenario is not impossible in mice also.

Wykes *et al* and others have also demonstrated that DC can transfer native antigen to naïve B cells to stimulate antibody production in T-dependent and T-independent manners[69–71]. Kushnir *et al* showed initiation of DC-B cell clusters within 1 hour which persisted for up to 48 hours indicative of significant inter-cellular interactions[72]. Wykes points out that since DCs can be a source of antigen for B cells, which can then upregulate CD40L, and given that DCs and B cells can form stable clusters, the B cells are likely to receive CD40 cross-talk from the DCs. In addition, since DCs can induce B cell proliferation independent of CD40, Wykes proposed that this would result in B cell clonal expansion prior to T cell interaction and increase the likelihood that antigen-specific B cells would encounter an antigen-specific T cell[73,74]. In the context of this study this mechanism seems less likely since B cells and DCs formed less than 10% of the 72-hour proliferation population, thus little B cell expansion had occurred.

Work by Gattinoni *et al*[9] addressed the issue that is currently faced in the clinic, namely, that whereas less-differentiated T cells are more effective *in vivo* the *ex vivo* expansion of small precursor frequencies into high numbers of tumour-specific T cells for adoptive transfer necessarily forces T cell differentiation. By comparing T cells expanded in the presence of IL-2 with T cells expanded in the presence of IL-15 they observed less differentiation and superior *in vivo* anti-tumour efficacy in the IL-15-expanded group. The observation that less-differentiated effector cells are more effective in the anti-tumour response has been corroborated by work in our laboratory[34], therefore we used Day 4 primed T cells for the first *in vivo* cytotoxicity assay and Day 3 cells for the second assay. We expanded our lysate-loaded APC-primed T cells in the presence of IL-2 for the first 24 hours only, but retained IL-15 in the cell culture medium until the cells were harvested for transfer. The phenotype of

these cells resembled a mix of early effector and effector cells, with relatively low percentages of T_{CM}.

We used Day 3 lysate-primed T cells for the second assay due to the significant reduction in T cell numbers after longer periods in culture, which resulted in insufficient cell numbers for injection. Previous work has demonstrated that memory-like T cells develop 1 day after antigen encounter, but optimally after 3 days[75]. Therefore in order to have sufficient quantities of cells to inject we reasoned that Day 3 cells would suffice.

Work in the Busch lab has demonstrated that transfer of just one antigen-specific naïve CD8+ T cell resulted in retrieval of clonally expanded cells following immunization[76]. Taken together these data from the Busch[76] and Restifo[9] labs argue that the extremely high numbers of T cells currently utilized in ACT may not necessarily be required, and that fewer, less-differentiated, tumour-specific T cells may be sufficient to achieve tumour regression. Though whether fewer lysate-primed T cells would be sufficient to overcome immune suppression in cancer-bearing mice or cancer patients remains to be tested in animal models, or in the clinic.

Insufficient cells remained for phenotyping in the first *in vivo* assay therefore phenotype data only exists for the cells used in the second *in vivo* assay. While this single observation can be commented on, no conclusions can be drawn and therefore these results are discussed in Appendix 1, Section 8.35. However it is interesting to compare these 3 day-primed cells with the 10-day primed cells. Only slight differences were observed between the phenotypes of T cells generated by priming with GMDC+Bc+soluble lysate, GMDC+oxidised lysate and GMDC+Bc+oxidised lysate (Figure 62). Our cells did not fall neatly into the definitions outlined by Restifo *et al*[9]. Instead of an early effector, effector or late effector phenotype our cell populations displayed a phenotype that demonstrated a mix of $CD62L^{HI}$ (indicative of naïve or T_{CM}) $CCR7^{LO}$ (indicative of early effector or effector cells) $CD27^{HI}$ (T_N to early effector). No correlations were observed between CD62L positivity and *in vivo* effectiveness as the difference in the percentage of cells positive for CD62L between GMDC+Bc+soluble lysate-primed T cells and GMDC+Bc+oxidised lysate-primed T

cells was slight (83 and 77% respectively). No CD62L data exists for T cells primed with soluble lysate-loaded GMDC alone as, due to lower numbers in this group these cells could only be stained with two of three panels.

CD44 is an indicative marker for effector memory T cells and is expressed at a lower level on naïve T cells compared to memory T cells[77,78]. Cells that are double positive for CD62L and CD44 are not addressed in Restifo's schematic, however this combination is also used to identify T_{CM} cells. The proportion of T_{CM} between GMDC+Bc+soluble lysate and GMDC+Bc+oxidised lysate was very similar (26 and 29% respectively) and these small differences are unlikely to be statistically significant. Tumour trials would also be required to ascertain if these differences have a significant impact on T cell ability to control tumour growth. However the three populations resulting from bi-variate analysis of CD44 and CD62L point toward a mix of T_{EM}, T_{CM}, the recently named memory stem cells (T_{SCM})[85,86] and early effectors. The left to right spread of CD62L positivity in particular points to a mix of T_{SCM} and early effectors.

CD127, also known as IL-7 receptor α (IL-7Rα), is expressed by most resting T cells and downregulated following T cell activation. Long-living T cells are characterized by constitutive CD127 expression[79,80]. The $CD127^{HIGH}$ population has been shown in adoptive cell transfer experiments to represent long living T central memory (T_{CM}) cells, especially when combined with $CD62L^{HIGH}$ status[88]. The combination of CD122 and CD127 are used to assess survival potential and responsiveness of cells to homeostatic cytokine signaling *in vivo*. Cells that are negative for CD122 display unresponsiveness to IL-2, which is indicative of reduced long-term persistence *in vivo*. In the same manner cells that are negative for CD127 display unresponsiveness to IL-7, which is also indicative of reduced long-term persistence *in vivo*. Our lysate-primed cells were CD127-CD122-, which suggests that they might not be ideal candidates for ACT. Their usefulness would need to be further analysed by assessment of *in vivo* persistence after ACT.

Finally, overexpression of inhibitory receptors such as PD-1 is one indicator of T cell exhaustion (reviewed in [81]) and high PD-1 expression correlates with impaired T cell

function[82]. PD-1 negativity indicates that these cells do not appear to be exhausted by priming with lysates. Although one study has shown that very low PD-1 expression can be sufficient to inhibit T cell expansion, TNF-α and IL-2 production[81].

As a final comment, in both *in vivo* experiments, while 100% lysis was achieved in the OVA-primed T cells, 100% lysis also occurred in the mice that were treated with PBS. Therefore these results remain preliminary until minimal lysis can be achieved in PBS-treated negative controls. However the fact that a treatment-dependent result, as opposed to 100% lysis, was observed in the four APC-primed T cell groups, points to a technical error rather than a failure of the assay. We are yet to ascertain the source of this technical error.

5.4.3 *Ex Vivo* Re-stimulation of Tumour Draining Lymph Node Cells

While the analysis of *in vitro*-priming of T cells by various APCs can shed light on whether or not multiple APCs may play a role in *in vivo* priming, ultimately clinicians have no control over this aspect of their patients' treatment. Patients arrive at the clinic with cancer-primed T cells that are ineffective. Thus the clinician's role is to re-activate these T cells against the patients' tumour, or to re-educate them to recognise the patient's tumour. While our studies are still far from the clinic setting, re-stimulation of *ex vivo*-derived tumour draining lymph node cells is a more clinically relevant scenario.

The conditions under which the *ex vivo*-derived cells were cultured are clearly still far from optimal as evidenced by the low fold expansion and poor viability of these cells after 10 days in culture. An improved approach may be to attempt to coordinate the retrieval of tumour draining lymph node cells with the culture of the APCs, such that freshly isolated lymph node cells may be added directly to lysate-loaded APCs without the cryopreservation step. This will result in higher starting lymph node cell numbers, which will allow analysis of multiple parameters, instead of one parameter at a time. Alternatively, a study discovered after these experiments were completed, utilised anti-CD3 and anti-CD40 mABs to stimulate the T cells, B cells and DCs in the *ex vivo* tumour dLN cells[92]. Removal of either B cells or DCs significantly diminished the anti-tumour responses of the tumour dLN cells highlighting the importance of antigen presentation and CD40 stimulation. We used anti-CD28 only in

our tumour dLN cells and an improvement would be to compare the response of both anti-CD40 and anti-CD28 engagement.

While acknowledging the highly preliminary nature of these data, an interesting pattern of increased IFN-γ and reduced IL-12 production was observed in these cells when re-stimulated with GMDC+B cell (Figure 65). This result correlates with the pattern of increased IFN-γ and reduced IL-12 observed in lysate-loaded GMDC+B cell T cell co-cultures. These data contrast with that of Li *et al* who observed increased IL-12, and inhibited IL-4, in their anti-CD3+anti-CD40-stimulated tumour dLN cells[92]. As stated above we would like to repeat these experiments using anti-CD28 and anti-CD40 to compare the response to anti-CD28 alone and to Li's anti-CD3+anti-CD40 combination.

The upregulation of CTLA4 is important in T cell anergy. If the high level of T cell death is due to GMDCs killing off self-reactive T cells then blocking T cell inhibition with monoclonal antibodies against CTLA4 may result in a reduction of T cell death, as well as anergy. Our preliminary attempts to test this theory demonstrated a small increase in fold expansion over cells treated with α-PD-1 (**Figure 3**), and an apparent synergy when α-CTLA-4 and α-PD-1 were both used. However, this small synergistic increase did not result in greater fold expansion than when cells were primed with GMDC+Bc+oxidised lysate+ α-CD28. Therefore these preliminary results suggest that the inclusion of α-PD-1 and α-CTLA-4 yield no additional benefit.

Taken together these *in vitro*, *in vivo* and *ex vivo* results continue to lend weight to the argument for the use of oxidised lysate antigens in stimulating an anti-tumour response. They also demonstrate that an improvement in CTL-mediated killing can be achieved when CD8+ T cells are primed with lysate-loaded GMDC+B cell as opposed to a GMDC alone, particularly oxidized lysate.

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6 Discussion of Results & Future Directions

"only a fraction of patients treated with currently available immunotherapies respond to them. The challenge now is to figure out how to unleash and strengthen the power of the immune response to turn more non-responders into responders"

AACR 2015 Report, CRI website

http://cancerresearch.org/news-publications/our-blog/april-2015/aacr-2015-report-from-day-5

6.1 Results

The overarching aim of this thesis was to enhance adoptive cell therapy for cancer. DCs are currently in clinical use for ACT for cancer, however despite some notable successes, robust and consistent tumour regressions remain elusive. Thus some investigators are turning to alternative APCs as potential adjuncts in cancer immunotherapy. Several populations of APC can individually activate an anti-tumour T cell response, but they may also act in concert to achieve this. Therefore we examined the three professional APCs, DCs, MΦs and B cells for their ability to present lysate tumour antigens to T cells, individually and in combination.

Previous work with soluble freeze-thaw lysate had yielded low T cell proliferation. We therefore manipulated the potentially immunosuppressive lysate to make the antigens more immunogenic and compared the APC response to loading with soluble and oxidised lysate. GMDCs upregulated CD40 in response to oxidised lysate, while B cells upregulated CD86. MHC-II was only upregulated on B cells in response to LPS&CpG rather than lysate.

We hypothesised that the use of two or more APC would yield an augmented T cell response to lysate tumour antigens. We used a model system in which B16 tumour cells have been engineered to express ovalbumin. The high frequency of antigen-specific T cells in this model allows assessment of both MHC Class II-mediated CD4+ and MHC Class I-mediated CD8+ T cell responses following priming by GMDC,. M1 MΦ and B cells. Following lysate exposure DC upregulated CD40 and B cells upregulated CD86. MHC-II was upregulated on B cells in response to

LPS&CpG. Combining GMDC+B cells resulted in enhanced IL-12 production after oxidised lysate loading.

We next examined whether combining APCs would augment the T cell response to presentation of lysate antigen and discovered that the combination of GMDC+B cell resulted in increased proliferation and IFN-γ production in CD8+, but not CD4+, T cells compared with a GMDC alone. Finally, *in vivo* cytotoxicity assays demonstrated the superior cytotoxicity of CD8+ T cells primed by GMDC+B cells over a GMDC alone.

We compared the ability of two kinds of lysate to generate a T cell response and found that oxidized lysate resulted in better T cell viability, enhanced CD8+ T cell proliferation and *in vivo* killing compared with soluble lysate, particularly when GMDC+B cell presented the oxidised lysate antigens. Oxidised lysate did not increase IFN-γ production in CD8+ T cell cultures.

Our results support the hypothesis that different APCs possess different roles in the priming of $CD8^+$ T cell responses against lysate tumour antigens and that has implications for the design and administration of APC-mediated ACT for cancer.

6.2 The Problem: DCs Presenting Defined Peptides Do Not Consistently Yield Durable T Cell Responses

The numbers of DCs recovered from cancer patients are lower than from healthy volunteers, and monocyte-derived DC generation *ex vivo* can also be diminished (reviewed in[1]). Removal of primary tumours can result in significant recovery of DC numbers[2–4], however DCs still only represent less than 1% of circulating leukocytes[5]. Moreover, since *ex vivo* tumour experienced DC function may be impaired their use is avoided by the use of GM-CSF+IL-4 to generate DCs from monocyte precursors. However the limited response to monocyte-derived DC-mediated ACT has forced the immunotherapy community to re-visit the use of naturally occurring blood DCs[6].

Much work has been done showing that the tumour microenvironment can be highly immunosuppressive. Cytokines secreted by pancreatic carcinoma cells have resulted

in a tolerogenic DC phenotype[7] and in melanoma constitutive activation of the B-RAF signal path promotes excessive production of the DC suppressing factors IL-10, IL-6 and VEG-F[8]. Tumour glycoproteins such as MUC-1 and CEA have been observed to interact with DC-SIGN, leading to antigenic retention in early endosomal compartments, which inhibited effective processing and presentation[9]. Additionally, MUC-1, a potent chemoattractant for iDC, also suppresses IL-12 production by DCs[10].

DCs from mice in late stage cancer expressed higher levels of PD-L1 along with high arginase activity. These data correlate with DCs observed in human ovarian cancer. Tumour-derived PGE2 and mature TGF-β1 induced the up-regulation of PD-L1 in immunocompetent DCs causing their T cell suppressive activity. The final sentence of Scarlett's abstract summarises by saying: "… *we … show that aggressive cancer progression after a comparatively long latency period is primarily driven by the mobilization of immunosuppressive microenvironmental leukocytes, rather than loss of tumour immunogenicity*"[11].

Thus, despite the DCs impressive antigen presenting credentials, in the cancer patient the growing tumour often negatively impacts this professional APC, which is critical to mounting an anti-tumour response, rendering the DC unable to effectively perform the task for which it is so exquisitely designed.

6.3 A Potential Solution: Combine GMDC +B cell During Antigen Presentation

B cells possess many of the same biological attributes as DC, including high MHC expression and antigen-specific T cell-differentiating cytokine profiles. These reasons, along with their relative ease of use makes them an alternative to DCs in immunotherapy[12,13]. Autologous B cells vastly outnumber DCs and can be readily isolated from patient blood[14], and further expanded by CD40L activation. Monocyte-differentiated DCs are significantly lower in number than B cells to begin with, and are not so easily expanded as required for multiple transfers in therapeutic schedules[15–17]. Reinfused B cells migrate to lymph tissue, the principal site of T cell

activation and, depending on their antigenic load, can avoid CTL-mediated destruction, which can plague antigen delivery by infused DCs. In the LNs B cells can deliver antigen to follicular DCs which in turn present antigen to CD8+ CTLs and T_{CM} cells[17]. Indeed novel ways to enhance antigen-non-specific uptake by B cells are already being explored[18].

The key finding of this thesis is that the combination of GMDC+B cell yields an improved T cell response, *in vitro* and *in vivo*, over GMDC alone presenting oxidised lysate-derived antigens. DC-B cell cooperation has been observed in other investigations. Dubois *et al* published *in vitro* evidence in 1997[19] and 1999 of direct DC-B cell CD40L-CD40 interaction[20]. In 2000 Wyckes and Macpherson showed that CD40 on DC was important for B cell survival but not proliferation[21]. Other studies have shown DC-B cell clustering *in vivo* and that DC can increase B cell proliferation. For this reason it was important to show that in the DC+B cell +T cell co-cultures it was T cell proliferation that was occurring, not just B cell proliferation. A flow cytometric dump channel showed that in most cases less than 10% of the cells in the culture were CD11c+ or CD19+ demonstrating that it was T cells and not B cells or DCs that were proliferating.

Clinical studies by Nielsen and colleagues have identified that CD20+ TILs with an atypical CD27 negative phenotype, in conjunction with CD8+ TILS, correlate with favourable prognoses in ovarian cancer[22]. Interestingly Nielsen's findings did not show a link with increased levels of serum antibodies against tumour antigens, indicating this effect was not antibody mediated. This was despite the B cells being of the IgG type and therefore having undergone somatic hypermutation and class switching. These activated B cells were acting in a manner independent of the antibodies they were secreting. While in the Nielsen study patient serum antibodies did not correlate with protection, this is not always the case.

Other studies have reported correlations between serum antibody levels and tumour responses[23]. Valmori *et al* did not directly observe whether or not the B cells and T cells interacted, but they did use CpG as an adjuvant to a peptide vaccine therapy and noted that this was a potent activator of B cells. They proposed that these B cells may

support long-term persistence of CD8+ CTLs, and that their lymphotoxin and cytokines may positively affect both local (tertiary) lymphoid structures and T cell polarization[24]. Others have also reported on the short lifespan of antigen-presenting DCs as being a limiting factor in their T cell stimulating capacity and that this is an aspect that needs further investigation[25]. Moseman *et al* have also reported on the lymphotoxin-mediated role of B cells in protection against VSV infection[26]. Lymphotoxin activation of MΦs, not the adaptive antibody response, was required. Our results argue for a role in the CTL adaptive response as improvements were observed in the CD8+ but not CD4+ groups, and it is CD4+ T cells, not CD8+ T cells that are involved in antibody production by B cells[27]. Analysis of the AB response was not conducted in this study, though samples have been stored for analysis of anti-OVA antibodies in the cell supernatants.

The current dogma of DC, CD4+, B cell activation is that DCs become activated by the uptake of antigen, activate CD4+ T cells, which then activate B cells. While this thesis doesn't argue against this dogma, it argues that the order of the steps can be manipulated, and the immune response enhanced, if the B cells are activated at the same time as the DCs, rather than relying on B cells to be activated by DC-activated T cells.

Wang also argues that there are multiple paths for activation of naive CD8+ T cells and other sources of CD4-independent help[28,29]. "*CD8+ T cells can provide self-help if they are present at a sufficiently high precursor frequency. The important variable is the total number of T cells responding*." Whether Wang's studies present a physiologically relevant scenario, or an artifact of *in vitro* studies with artificially high numbers of T cells remains to be verified.

6.3.1 The CpG Factor

In 2002 Shirota *et al* challenged the notion that B cells lack antigen non-specific capture, priming of naïve T cells and T_H1 induction capacity[30]. Theirs was the first study to demonstrate that when OVA antigen was conjugated to CpG, B cells assumed the same functional capacity as DCs. Unconjugated CpG was utilised as an immune stimulant in our study, yet we also observed significant increases in MHC-II

on B cells in response to LPS&CpG, demonstrating increased antigen presentation capacity in the lysate+LPS&CpG-loaded B cells.

Another intriguing example of CpG-mediated anti-tumour effects is a study that found that intratumoural injection of the TLR9 agonist CpG along with anti-OX40L and/or anti-CTLA4 antibodies, to eliminate T_{REG}s in the tumour microenvironment, initiated a systemic anti-tumour response and long-lasting protection in mice[31]. Conversely, systemic delivery of antibodies and CpG ODNs had an immediate anti-tumour effect but mice later relapsed. The study authors attributed the results to the depletion of T_{REG}s by mABs but in light of the results of this thesis, it seems plausible that the anti-tumour effect observed in the intratumoural injection groups arose from the effects of damaged tumour cells being exposed to activated DCs and B cells in the tumour which were able to more effectively prime the CTLs.

In a similar vein, Brok and colleagues reported that *in situ* cryoablation, another scenario that induces a form of *in situ* tumour lysate, accompanied by CpG, resulted in an enhanced anti-tumour response[32]. They also only assessed the DCs, which they found were more effective in this scenario, however it is likely that CpG enhanced the B cell function also.

6.3.2 CD40-CD40L Connections

Immunological dogma says that priming of CD8+ CTLs usually requires "help" from activated CD4+ T cells. Yet it has been known since 1998 that ligation of CD40L with CD40 can replace CD4+ T helper cell priming of helper-dependent CTLs[33]. CD40-CD40L interactions can take place between APC and CD8+ T cells and signaling through CD40 on APCs can replace CD4+ 'help'[34].

Mamula and Janeway pointed out that early studies in mice whose B cell development was suppressed from birth, had defective T cell proliferation that could be rescued with antigen-specific B cells, suggesting an important role for B cells in the T cell response to antigen[35]. The MHC II-restricted CD4+ T cell levels of these B cell-deficient mice were normal, showing that B cells are required for particular aspects of CD4+ T cell priming. These authors proposed a model in which DC present primarily high affinity peptides to CD4+ T helper cells via MHC II. These CD4 T_H cells,

primed against immunodominant peptides, then activate antigen-specific B cells inducing costimulatory capacity in B cells, probably principally via the CD40L-CD40 interaction. The activated B cells are then able to act as APCs that can initiate naïve T cell responses. More recent studies have also demonstrated that DCs primarily prime against tight-binding, high affinity peptides while MΦs broaden the T cell response by presenting more loosely binding epitopes[36]. Thus both B cells and MΦs have the potential to assist DCs in widening the adaptive response against the full range of tumour antigens found in tumour lysate.

Wykes *et al* demonstrated a direct DC-B cell interaction, independent of T cells, that was able to initiate antibody production[37]. They showed that DC could retain native antigen, as opposed to processing it and presenting it as peptides on MHC, and that DC could transfer this native antigen to B cells *in vivo*. In 2008 and 2014 Harvey *et al* reported on their findings that human B cells transfer BCR-captured antigen to MΦs and DCs via Scavenger Receptor A on MΦs, resulting in MΦs and DCs acquiring more antigen in the presence of B cells than in their absence[38,39]. Scavenger Receptor A had previously been assigned a role in DC 'nibbling' – a process whereby they capture antigen from live cells[40]. Harvey has now shown that human DC can also 'nibble' membrane-bound BCR-antigen complexes from B cells. While DCs tend to present and cross-present high affinity peptides on MHC II and MHC I respectively, is it possible that nibbling antigen complexes from B cells is another way for increasing the amount of peptide presented? Albeit possibly only the high affinity peptides? This hypothesis could be tested by comparing the uptake of antigen and the level of surface MHC-bound specific peptides on DC cultured with B cells in the presence and absence of specific inhibitors of DC 'nibbling'

As an alternative mechanism to B cells cross-presenting the lysate peptides on MHC-I Shultz suggests that B cells could act as accessory cells for CD8+ T cell activation by way of their IL-1 or IL-6 production[48]. Klarnet *et al* discovered that a subset of CD8+ CTLs produced IL-2 and that this production was IL-1α-mediated[41]. These T cells may be critical in the anti-tumour response as they don't require CD4+ T helper cells to become activated but rather respond to IL-1α via their IL-1 receptors. The interaction of IL-1α with IL-1 receptors on these tumour antigen-specific CTLs is

necessary for IL-2 production, which then acts in an autocrine and paracrine manner to stimulate proliferation of both the IL-2 producing and non-IL-2 producing CD8+ T cells. In this scenario, IL-1α-producing B cells would play an important role in CD8+CTL activation in environments where tumour antigen-specific CD4+ T cells are lacking. IL-1 was not measured in this study thus we currently have no evidence to support this theory.

6.3.3 A proposed model

Based on the data from this study and the observations of other investigators the following scenario is proposed to explain how the combination of oxidised lysate-loaded GMDC+B cell enhances the CD8+ T cell response to tumour antigens.

- DCs and B cells acquire and process lysate antigens
- Mutual antigen transfer occurs: DCs present and/or transfer undegraded antigen to B cells[42,43] and B cells transfer antigen to DCs[39]
- Costimulatory CD40 is induced on DC by oxidised lysate; CD86 is upregulated on B cells by oxidised lysate; and CpG&LPS upregulates MHC-II on B cells
- B cells become competent APCs capable of initiating naïve T cell responses

- As yet unidentified cell-cell or cytokine mediated interactions between DCs and B cells induces IL-12 production by DCs, and possibly by B cells
- Elevated levels of IL-12 provide the critical third signal (over and above Signals 1 and 2) for optimal CD8+ IFN-γ production and effector function[44]

6.4 Challenges

6.4.1 OVA-Specific Effects?

OVA is a metal-binding transport protein that can bind to many other proteins. Zinkernagel has questioned whether this is a special property of OVA which may have given rise to some of the OVA-mediated immune responses observed and suggests this needs to be further explored[45]. With regards to this study OVA is utilised as an imitation tumour antigen to allow the CD4+ and CD8+ T cell response to be explored. However it is recognised that OVA-specific effects may be at play in this model system, which may not readily translate to a truly undefined lysate setting.

OVA is an immunodominant foreign antigen while tumour antigens are altered self antigens. While B16.OVA provides a useful tool for proof of principle studies, these studies need to be taken into an 'altered self' setting. A more clinically relevant scenario would be to generate tumour-specific T cells in tumour-bearing WT mice. The tumours could be surgically excised from the mice, to mimic a patient biopsy. Undefined lysate could be created from the 'biopsies' and loaded onto APCs for re-stimulation of T cells from tumour-bearing mice. Reinfusion of these T cells into mice who have had tumours excised would mimic ACT in a patient. These mice could be monitored to evaluate whether these T cells can prevent the return of tumours.

6.4.2 A Dendritic Cell By Any Other Name

There is controversy surrounding the relationship of DCs with the mononuclear phagocyte system (MPS). Significant overlaps of function and marker expression in bone marrow- and monocyte-derived cells makes the demarcation of DC from MΦ difficult, especially in non-lymphoid tissue.

On the basis of a large meta-analysis of multiple public domain gene expression array datasets Mabbot *et a*l found that BMDC, cultured in GM-CSF, identified clearly as phagocytes and were distinct from isolated lymphoid tissue DC that showed low expression of phagocytic genes. They suggested that a better delineation between these two cells would be to call them phagocytic APCs (antigen-presenting MΦs) and non-phagocytic APCs (classical DCs)[46]. Hume has also questioned the identification of DCs and MΦs on the basis of surface markers[47,48] and makes the point (personal email correspondance, May 2015) that antigen presentation is a function, not a cell type.

When this study commenced the prevailing consensus was that GM-CSF-differentiated bone marrow APCs were considered a model for dendritic cells. By the end of this study the tide of opinion had turned toward calling these cells GM-CSF-stimulated antigen-presenting cells and the consensus is that they represent a type of antigen-presenting MΦ, not a DC. Under this logic the APCs studied in this project could all be considered MΦs and thus it remains to be seen how a Flt-3 ligand-differentiated cell (i.e. a "true" DC) stacks up against these cells. Unfortunately due to the timing of these developments, this question remains to be answered.

6.4.3 Further Optimisation of T Cell Numbers

Not unlike the situation in the clinic, we experienced difficulties obtaining T cell numbers high enough for phenotyping the cells used in *in vivo* studies. This problem has to be addressed, probably through further optimisation of the growth conditions to encourage the T cells to keep expanding. This may be achieved by the inclusion of anti-CD28 +/- anti-CD3 mABs to stimulate explansion of all the clones that are generated with the priming. Alternatively, the use of protease inhibitors in the lysate may be responsible for the low T cell numbers and so comparison of the T cell response to lysate made with and without these inhibitors should be made.

Nonetheless Stemberger *et al* showed that diverse effector and memory subsets can develop from a single naïve CD8+ precursor using *in vivo* ACT of a single, antigen-specific naïve CD8+ T cell[49]. *In vivo* T_{EM} proliferate poorly, despite having rapid effector function, while T_{CM} expand rapidly but have delayed effector function. T_{EM} are small in number out in the periphery but if they encounter their cognate antigen they're very powerful. Thus the quality of these cells may be more important than the quantity.

On the other hand T_{CM} in the lymphoid organs will rapidly expand to generate overwhelming T cell numbers as well as exerting effector functions once they reach the peripheral site of infection or tumour. In other words though small in number the T_{EM} attempt to hold the problem at bay while the T_{CM} get activated and come to the rescue with superior numbers. Applying this to the clinic, it is possible that in future we may see effective ACT achieved with a combination of smaller numbers of highly specific and highly effective T_{EM} and larger numbers of T_{CM}. The intriguing work of Busch and Schumacher with minimal T cell numbers in the bacterial infection and allo-HST settings[49–53] highlights an opportunity for testing low dose T cell numbers in ACT for cancer.

6.4.4 How to Overcome T cell 'Stunning' in the Face of Tumour Lysate

A 2008 study reported that very few peptide-MHC complexes were necessary for the induction of full-fledged T cell activation and effector differentiation[54]. They observed a "Goldilocks effect" in which the amount of antigen had to be not too much, nor too

little, but "just right" for optimal T cell activation. They observed that: "*T cells rapidly formed tight stable contacts with DCs presenting large numbers of cognate pMHC complexes. However, T cells did not engage in immediate tight contacts with DCs with low antigen densities; instead, they accumulated signals through hours of signal integration during serial encounters with many DCs until sufficient antigenic stimulation enabled the transition from phase one to phase two*" (stable T cell-DC interactions).

So, if a group of DCs arrive in the lymph nodes bearing a significant amount of antigen, the phase 1 'sampling' of antigen timing to reach a critical threshold, will be shorter, and continuation to phase 2 and activation faster, than if DCs bearing smaller amounts of antigen arrive in the LN. This is due to the serial sampling taking longer to reach critical activation threshold, not unlike bacterial quorum sensing.

This is relevant to cancer because APCs are less likely to arrive with large amounts of tumour antigen, but rather more likely to arrive with small amounts of cancerous antigen over a longer period of time. This could help explain why T cells are not interested in the tumour antigens – because there is insufficient antigen to reach activation threshold. The T cell sees its cognate antigen but due to the lack of sufficient signal integration to form a 'quorum' the T cell passes to anergy instead of activation. Therefore in ACT its critical to get the right amount of antigen.

In this study our APC:T cell ratio was 1:10 so the density of APCs was likely adequate. However the number of peptide-MHC complexes per APC (ie MHC-SIIN) in the lysates may be too low and may be the limiting factor in determining overall antigen dose. Experiments to assess the number of peptide-MHC complexes on the surface of the lysate-loaded APCs may yield further insights for the presentation of lysate TAAs.

6.5 Limitations

For many of these assays using tumour lysates, a similarly prepared lysate from the non-OVA expressing B16F10 cells would have been an excellent negative control. We used the irrelevant melanoma peptide gp100 as the negative control, since the OVA transgenic mice should only bind OVA peptides, not gp100 or any other peptide in the lysate. In hindsight, including the B16 parental

line as a further control would have controlled for unexpected non-specific binding, and thus this is a limitation of these assays.

Considering that our overall aim was the improvement of adoptive cell therapy for cancer, the value of this thesis would have had more impact if we had demonstrated evidence of enhanced tumor regression or clearance by DC+B cell-primed T cells in a preclinical tumour model. The *in vitro* work was necessary to provide a rationale for which combination(s), if any, were worth trying in expensive, time-consuming *in vivo* trials. Unfortunately infections in the mice colony for a significant period of the thesis tenure resulted in significant delays due to the need for many repeats. Many experiments had proliferation in untreated wells rendering the entire experiment invalid. This proliferation was not due to contamination or experimental technique as other students and staff had the same issue. The unfortunate outcome was that planned tumor trials were unable to be conducted within the time frame. To build on the results of this thesis, another lab member is currently conducting trials of oxidized lysate in a pre-clinical model of colorectal cancer. Planning is also well underway to conduct trials comparing oxidized lysate presented by DC+B cells in a xenograft model of human brain tumors at Children's National Medical Centre in Washington DC. It is hoped that the groundwork laid by this thesis will prove fruitful in future trials.

6.6 Future Directions

6.6.1 Tumour Trials

Rolf Zinkernagel has written: "*The difference between "what is possible under any experimental condition" versus "what happens physiologically" is a crucial distinction*"[45]**.** While the results of our *in vivo* cytotoxicity assays are promising, further *in vivo* data in the form of tumour trials is required to assess the ability of T cells primed with GMDC+B cell presenting lysate antigens to eliminate established tumours and to resist tumour challenge. In particular, as mentioned above, the use of truly undefined lysate antigens from tumour-bearing mice would represent a significant step toward clinical applicability should the same observations of increased tumour control by T cells primed by oxidised lysate—loaded GMDC+B cells hold true.

T cell degeneracy and promiscuity[55] make it reasonable to assume that when APCs are pulsed with lysate from tumour cells, in addition to the highly peptide-specific TCR-p-MHC interactions, there will also be lower affinity interactions with other p-MHC complexes, generating a broader repertoire of T cell responses to other unidentified TAAs in the lysate. Tumour antigen expression is not only unique to each patient, it is also highly heterogeneous within the tumour tissue down to the single cell level[56,57],

resulting in immune activity against multiple TAAs within the patient[56,58,59]. Thus T cell degeneracy and promiscuity combined with tumour heterogeneity further argues for the choice of lysate as opposed to a limited range of defined antigens as the TAA source in ACT for cancer.

6.6.2 Which is the Best DC?

Most clinical studies use *in vitro*-generated monocyte-derived DCs as opposed to the less populous peripheral blood DCs[60]. The most recent edition of Clinical and Translational Immunology reported on the Tenth Human Leukocyte Differentiation Antigen (HLDA) Workshop, in which the authors state: "*To date, the use of monocyte-derived DCs has failed to provide strong clinical data to argue that these should be the cells of choice*"[6,61]. Optimal parameters, including which is the superior DC for activating anti-tumour T cells, is ongoing so the full potential of DC-mediated immunomodulation may be realized. Repeating these experiments using tumour biopsy-derived material and peripheral blood DCs may be enlightening. As would repeating the experiments using Flt3L-generated DCs, which more closely resemble lymphoid CD8+ DCs.

Interestingly, Gérard and colleagues found that T cell-T cell interactions had a stronger impact on T cell expansion and effector memory balance than DC-T cell interactions[62]. Scarlett *et al* showed a DC-mediated immune response that transitioned from elimination to escape during tumour development[11]. These two reports have a strong bearing on this study since they demonstrate opposite ends of the DC-T cell interaction spectrum and highlight both the DC-T cell relationship as well as the importance of T cell-T cell and other cellular contacts during T cell maturation.

The APC-T cell contact is obviously critical in initiating the T cell response, but then its importance may be lessened during T cell maturation. However, as tumourigenesis proceeds the DC-T cell interaction comes back into the limelight as the DCs lose anti-tumour reactivity in response to tumour-produced PGE2 and TGF-β1, reducing CD8+ T cell proliferation and production of IFN-γ and granzyme-B, thus eliminating their tumour-eradicating faculties. Since, in the Scarlett model, the tumour retained immunogenicity[11], is it possible that during the immunoediting process a second APC

could step in at this point to reignite the T cells? Why are DC so responsive to PGE2 and TGF-β1? Are other APCs more resistant to the immunosuppressive effects of PGE2 and TGF-β1?

Perhaps TAA-specific B cells in the splenic germinal centres, whose proximity to the T cell zone facilitates their priming of naïve CD4+ T cells, could step in to take up where the DCs have left off? Confirmation of the existence of cytotoxic CD4+ T cells[63] has shed light on the importance of CD4+ T cells in the anti-tumour response, and as such their relationship with B cells may also prove to be important in tumour elimination since B cells are required for protection against certain tumours[64,65].

6.6.3 Enhanced Definition of Optimal GMDC and B Cell Activation and Lysate Loading

The decision to load GMDC+B cell with lysates plus activation stimuli overnight serendipitously enabled enhanced B cell activation, which allowed the cooperative effects of the GMDC+B cell combination to be observed. However, further optimization of GMDC and B cell activation is required. A superior scenario may involve pulsing GMDC overnight with lysate, followed by 4 hours of activation stimuli. For the B cells pulsing overnight with activation stimuli prior to adding lysates would be a better approach. The two optimally activated cells would then be combined immediately prior to the addition of the T cells. In this manner the T cell enhancing properties of this combinatorial approach may be further augmented.

However this optimization must be changed for translation into human cells since human DCs do not express TLR9 and human B cells do not express TLR4 thus in all likelihood this combination of immune stimulants will not work in the human setting. The combination of CD40L and CpG induces IL-12 production from human B cells[66] while the combination of CD40L and monophosphoryl lipid A (MPL-A) triggers synergistic increases in IL-12 in human DCs compared with either agonist alone[67]. Further examination of CD40L, CpG and MPL-A in the activation of lysate-loaded GMDC+B cell is warranted.

6.6.4 Broaden Analysis of APC-Mediated Tumour Responses to Include B cells & MΦs

In 2011 Beatty and colleagues reported on a clinical trial in which anti-CD40 mABs plus gemcitabine chemotherapy were tested in a small cohort of pancreatic ductal adenocarcinoma (PDA) patients[68]. Gemcitabine therapy was used prior to delivery of the mAB to enhance tumour antigen presentation by APCs. The CD40 mABs were used for their T cell-stimulating properties and tumour regressions were observed in some patients. In testing the mechanism of action in a mouse model they unexpectedly discovered that tumour regression required MΦs, but not T cells or gemcitabine. CD40-activated MΦs rapidly infiltrated tumours, lysed tumour cells and mediated tumour stroma destruction, demonstrating MΦ-mediated, T cell-independent elimination of these tumours. The role of B cells was not examined in this study but it would be interesting to explore given the work with CD40-activated B cells which showed MΦ transfer of antigen to B cells[38,69], B cell transfer of antigen to DCs[39] and tumour regression[15,70].

Examples abound of the utility of CpG in the anti-tumour setting[71–76]. However in the majority of cases the reactions of only two immune cells are observed: the DCs and the T cells. I pose the question, have the B cell responses been investigated in these approaches? Were the tumour regressions really CD4+-mediated? Did they look at CD8+ T cells? B cells as professional APCs is a paradigm shift in thinking for immunotherapists but perhaps one we need to consider.

Or alternatively, could the combination of CD169+ MΦs[77–79] and *in situ*-generated "tumour lysate" be harnessed to stimulate TAA-specific T cells in the face of DC immunosuppression? CD169+CD11c+ MΦs, carrying out their general scavenging function, bind phosphatidyl serine (PS) on the surface of dead cells entering the LN via lymph flow. These MΦs phagocytose the apoptotic cells and cross-present antigen to CD8+ T cells[80]. Therefore killing tumour cells *in vivo* i.e. *in vivo*-generation of tumour lysate, may be useful due to dead cells flowing into the lymph nodes and spleen via lymph and blood flow, where CD169+CD11c+ MΦs can engulf them. This rapid process sees cells arriving in the LNs within hours. DCs in the area of the lysed tumour cells will also collect antigen and migrate back to the LNs to present antigen to T cells. These DCs start arriving within days, a much slower process. However, if

these DCs are insufficiently activated due to immune-suppressive factors in the tumour environment, or due to the fact that tumour cells are self cells, then cross-presentation of these antigens will not lead to a robust anti-tumour T cell result, but rather T cell anergy or immune tolerance to these antigens. Thus the combination of *in situ* tumour lysate may activate the clinical potential for a response by CD169+ MΦs.

6.6.5 Re-think MΦs In Cancer Immunotherapy: MΦs in the Driving Seat

Cancer has been described as a smouldering, unhealing wound[81]. Thus it is unsurprising that tumour-promoting MΦs secrete large quantities of growth-promoting ornithine in an attempt to "heal the wound". Tumour-protective MΦs, on the other hand, produce large quantities of nitric oxide (NO)[82], just as they do when fulfilling their anti-pathogen role[83,84]. Thus the key to harnessing the MΦs tumour-fighting capacity may lie in defining the conditions that switch MΦs from tumour-promoting arginine production[85] to tumour protective NO production[86]. It is already known that TGF-β is one of the key cytokines regulating this balance between NO and ornithine, with TGF-β inhibiting NO production[87–94]. MΦs do not require T or B adaptive immune cells to become activated[68,95,96], thus directing the MΦ response may result in efficient anti-tumour T cell outcomes.

This simple proposition will nonetheless require fine regulation since NO suppresses tumour growth but also suppresses CTLs[97–100]. Thus overproduction of NO by MΦs inhibits the beneficial T cell response against tumours and refined clinical control will be required to focus their nitric oxide synthase activities toward producing just enough NO to be toxic to tumours but not so much that it suppresses activity of TILs. Even so, manipulating MΦ activities toward the required disease-suppressing role, rather than eliminating them altogether, may represent the more useful approach.

A mechanistic explanation for the loss of response to lysate in LPS+CpG activated MΦs may lie in the downregulation of IRAK-4. This phenomenon has been implicated in LPS- and CpG DNA-induced tolerance in macrophages[101]. Modulation of the MΦ inflammatory response to lysate via IRAK phosphorylation and degradation has not been conducted on our cells, but represents an intriguing option for future analysis.

6.6.6 Manipulating the Combined CD4+ CD8+ T Cell Response To Lysate Antigens

The results of this study suggest that CD4+ T cells on their own may not be useful in the lysate setting. However certain experiments hinted at possible synergies with the CD4+CD8+ combinations. Studies in our lab and others[102] point to the requirement for both CD4+ and CD8+ T cells in the effective eradication of cancer – particularly in melanoma. Therefore future studies could include examining how the CD4+ T cell response could be more successfully included. For example, previous work has shown that defined antigen can be used to activate both CD4+ and CD8+ T cells. Therefore would priming CD4+ T cells with defined peptide(s), and CD8+ T cells with lysate lysate peptides, augment the CD8+ activity and increase T cell memory compared with using CD8+ T cells alone?

6.6.7 Bringing Other Players Into The Game

The role of immune cells other than DC, B cells and MΦs play is currently being more appreciated. The work of Dranoff and colleagues in 1993 hinted at this when they observed massive infiltration of "*immature dividing monocytes, granulocytes (predominantly eosinophils) and activated lymphocytes*" at the vaccination site in mice vaccinated with B16.OVA cells engineered to express GM-CSF in which a strong anti-tumour response was seen[103].

A furtherance of this current study would be to include neutrophils (polymorphonuclear cells; PMNs) in the analysis, for example, DC+PMN. Recent work is focusing on the anti-tumour activity of PMNs, natural killer (NK) and NK T cells. Darcy and colleagues found in a mouse model of breast cancer that depletion of PMNs or NK cells or CD8+ T cells was detrimental to the anti-tumour response showing that both the adaptive and innate arms of the immune system are involved[104]. Another study showed that murine PMNs can act as professional APCs capable of directing a T_H1 and T_H17 response[105]. We are aware that these cells could also be important in the anti-tumour immune response however time constraints did not allow this project to look at the impact of PMNs, NK or NK T cells, however future study could include these cells. It is recognised of course that while these innate cells may be useful in the induction of tumour killing they will never be utilised as cell-based therapies on their own since they do not induce immune memory. The advantage of

including innate immune cells will only lie in their augmentation of the adaptive, memory-inducing response.

6.6.8 Mechanisms of Action

We began preliminary investigations of the mechanisms of action with two inconclusive experiments assessing the MHC-I response on lysate-loaded APCs (data not shown). Further investigations into how the combination of GMDC+B cell improves the T cell response could include analysis of the CD40, CD80 and CD86 responses on the APCs. For example calcium flux, which has been shown to be important for the efficiency of costimulation[106]. Analysis of the calcium flux in lysate-loaded GMDCs versus B cells versus when the two APCs are co-cultured during lysate loading may reveal differences between the cells and differences when these two cells are interacting during lysate antigen loading. Analysis of signaling pathways with inhibitors may also give insight into which pathways are being activated, or not activated, while gene expression profiles will do the same for gene activation. The DeMAND method of genome-wide analysis of network perturbation is utilised to identify targets, effectors and activity modulators of small-molecule compounds[107]. This method clarifies mechanism of action proteins based on global dysregulation of their molecular interactions and could equally be applied to the analysis of alterations in lysate-loaded APC networks compared to un-loaded controls, or lysate-primed T cells compared to un-primed controls.

6.6.9 Does the GMDC+B Cell Augmentation of the CD8+ T Cell Response Hold True For Lysate Colorectal Cancer Antigens?

"There has been limited study of DC vaccines in the adjuvant setting after resection of metastatic tumours in general, and ***colorectal cancer*** *in particular"*[108]

In addition to our position as world leaders on the melanoma "scoreboard", Australia and New Zealand also have the highest rates of colorectal cancer (CRC) in the world. Laparoscopic resection of CRC combined with polychemotherapeutic regimens such as fluorouracil, leucovorin, and oxaliplatin (FOLFOX6) or fluorouracil, leucovorin, and irinotecan (FOLFIRI) are the current standard of care for CRC. Chemotherapy in metastatic CRC seeks primarily to prolong survival but is not curative[109]. Targeted monoclonal antibodies (mAB) may also be included, examples of which include bevacizumab, which slows angiogenesis by inhibiting vascular endothelial growth factor A [VEGF-A].

Currently around 50% of all CRC cases will progress to advanced metastatic disease leading to death in most patients[109]. While many ACT studies have been carried out in melanoma, little work on ACT for CRC has been undertaken to date. Since our aim was ultimately to translate the work of this thesis into human colon carcinoma studies are already underway in our laboratory to transfect the MC38 murine colon carcinoma cell line to express membrane bound OVA and utilise this cell line as a source of tumour lysate.

6.7 Concluding Remarks

The most important finding from this study was that combining GMDCs and B cells, could augment the CD8+ T cell response to oxidised tumour lysate antigens. In the same way that combination immunotherapies are currently favoured for cancer treatment, the results of this thesis suggest that combinations of APC should be considered in ACT for cancer in the future.

I propose that the following road map (**M.A.P.**) for APC-mediated ACT may represent a better approach:

- **MANIPULATE** MΦs: direct their arginine substrate to the NOS path to promote an M1 phenotype
- **ACTIVATE** B cells: use CD40L+CpG to enhance IL-12 production, which drives T_H1 differentiation and results in strong IFN-γ production from these cells
- **POTENTIATE** DCs: combining DCs with these appropriately activated B cells and/or MΦs may result in more effective antigen presentation by DCs

In recognition of the current dogma which states that B cells can't activate CD8+ T cells I leave the reader with the words of Nobel laureate Ralph Zinkernagel, Professor of Experimental Immunology at the University of Zurich:

"*In biology, particularly in immunology, nothing is impossible*"

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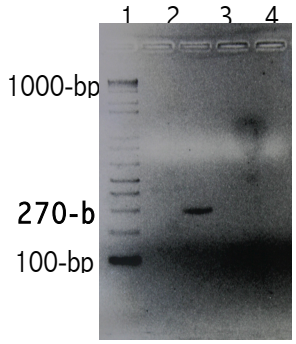
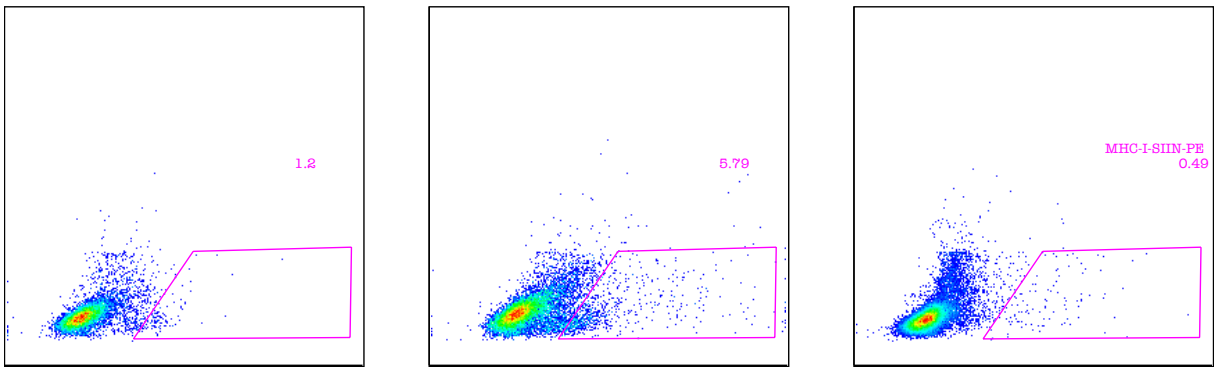
7 Appendix 1, Supplementary Data

8.1 B16.OVA Melanoma is not contaminated with *Mycoplasma* DNA



Supplementary Figure 1 B16.OVA melanoma cell line is not contaminated with *Mycoplasma*. B16OVA cells that had been grown for at least three subcultures without antibiotics were harvested, resuspended in PBS and stored at -20°C until DNA extraction. DNA was extracted using Purelink DNA Mini-kit from Invitrogen (Cat:K1820-01) according to the manufacturer's instructions. The DNA concentration was measured via NanoDrop, and diluted to 10 ng/µL in miliQ water. The primers used amplified DNA from highly conserved regions of the *16SrRNA* gene, which do not detect eukaryotic DNA or bacteria closely related to *Mycoplasmas*. Lane 1, 100-bp DNA ladder; lane 2, negative control (mQ); lane 3, positive control; lane 4, half dilution of DNA sample (13 ng/µL); lane 5, one third dilution of DNA sample (8.7 ng/µl).

7.2 MHC-I-SIIN is Not Expressed By B16F10 Cells and Is Expressed at Low Levels on B16.OVA cells



Supplementary Figure 2 No MHC-I-SIIN is expressed on the surface of B16F10 cells. B16.OVA and B16F10 cells were cultured as described under B16OVA cells in Materials & Methods. Geneticin was not added to B16F10 cultures. Following 5 hours incubation with Brefeldin A cells were stained with antibodies to H-2Kb bound to SIINFEKL conjugated to PE. Cells were fixed with 2% paraformaldehyde, stored overnight at 4°C and acquired the following day on a Gallios Flow Cytometer. Data was analysed on FlowJo version 9. Representative dot plots of B16.OVA and B16F10 cells after Brefeldin A treatment. Representative data from three independent experiments in which results were very similar.

7.3 Protein Concentrations in Tumour Lysates

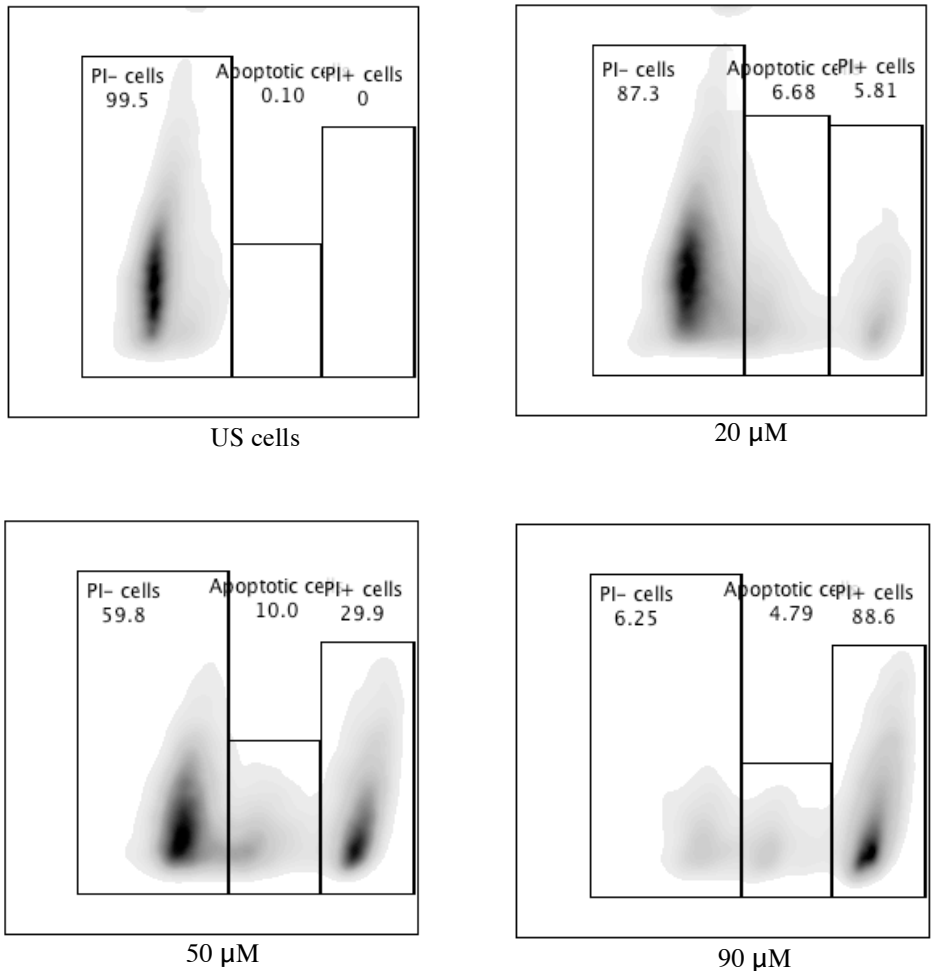
Supplementary Table 1 Protein concentrations in tumor lysates

BATCH	AVERAGE PROTEIN CONCENTRATION	METHOD USED TO ASSAY PROTEIN CONCENTRATION
TL1	Unreliable results	ND
TL2	9.38 mg/mL	ND
TL3	9.49 mg/mL	ND
TL4	Not measured	
TL5	13.22 mg/mL	ND
TL6	13.29 mg/mL	ND
TL7	11.6 mg/mL	ND
TL8	8.92 mg/mL	ND
TL9	16.97 mg/mL	BCA
TL10	10.48 mg/mL	BCA

The protein content of each batch of tumor lysate (TL) was assessed by NanoDrop™ (ND; ThermoFisher Scientific Inc) where 1 A280 = 1 mg/mL, or by BCA Assay as specified in the Table.

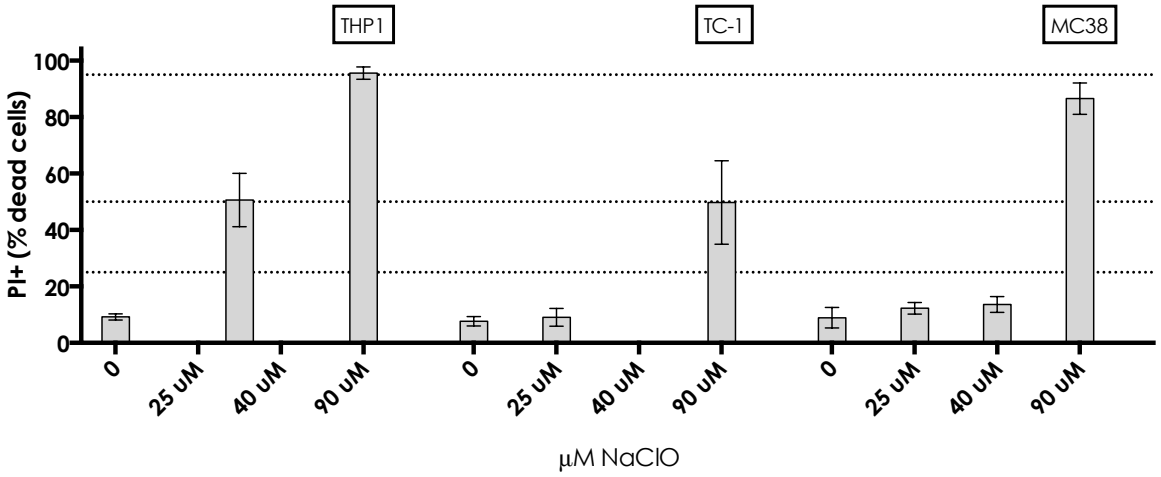
7.4 Sample B16.OVA Oxidation Raw Data

Supplementary Figure 3 shows sample raw data of the dose-dependent death of HOCl-oxidised B16.OVA cells.



Supplementary Figure 3 B16.OVA cells display concentration-dependent levels of live, apoptotic and dead cells after HOCl-mediated cell death. B16.OVA cells were harvested and resuspended in varying doses of HOCl as indicated in the figure. After 1 hour at 37°C cells were labeled with PI and acquired on a Gallios Flow Cytometer within 1 hour. Data was analysed and graphed with FlowJo software. Representative data from multiple independent experiments. US: Unstained; PI: propidium iodide.

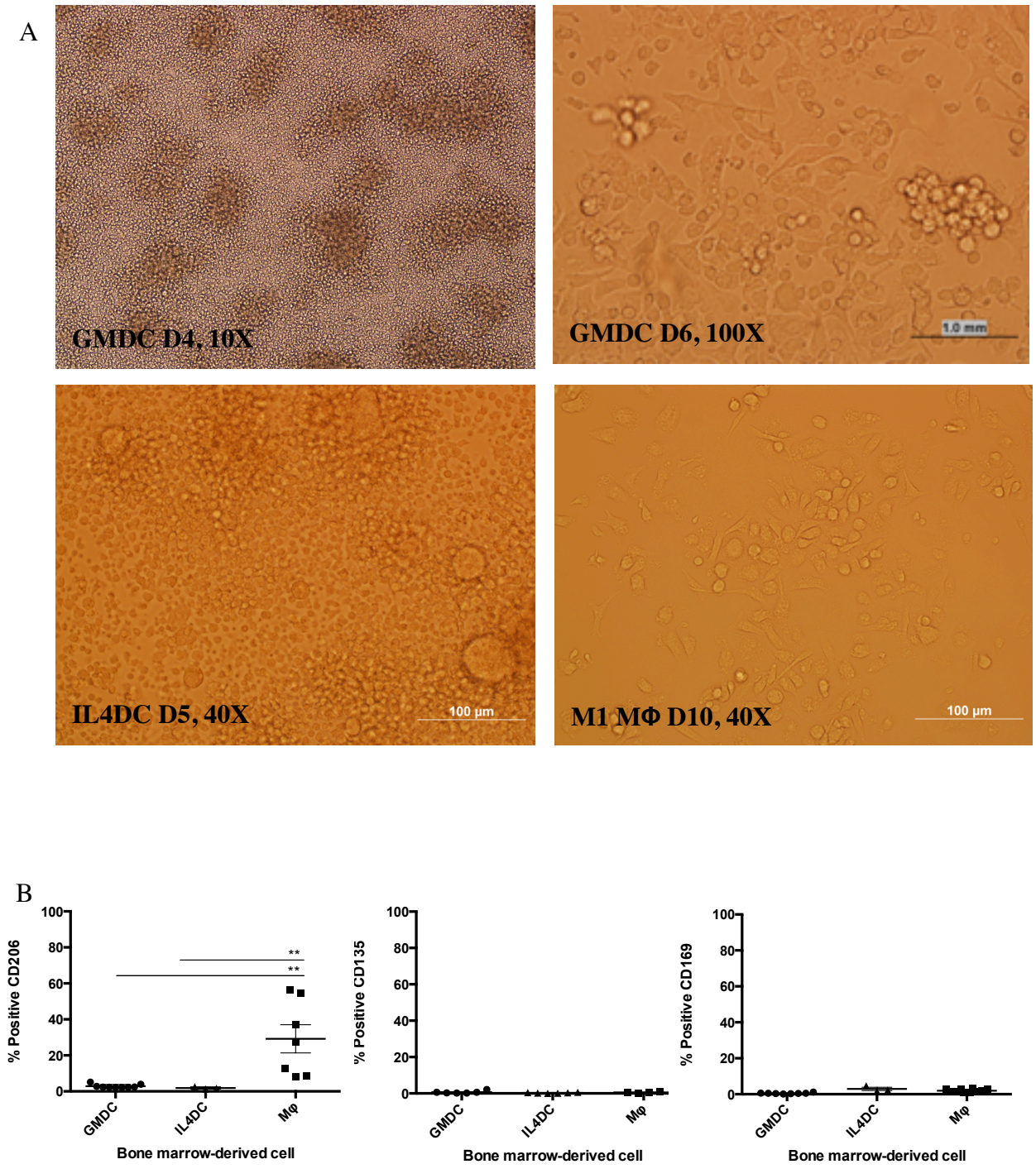
7.5 Three Non-Melanoma Cell Lines Require Different HOCl Concentrations to Achieve 95% Cell Death by Oxidation



Supplementary Figure 4 Three cell lines require different HOCl concentrations to achieve 95% cell death by oxidation. The colon carcinoma cell line MC38, the human monocytic leukaemia cell line THP-1 and the murine lung epithelial tumour cell line TC1 were incubated for 1 hour at 37°C in the HOCl concentrations indicated. Cells were washed, labelled with PI and acquired by Flow Cytometry within the hour. Cell death aws measured by the percentage of PI positive cells. Data was analysed and graphed with FlowJo software. Results of 3-5 experiments. Error bars = mean + s.e.m.

7.6 Unactivated GMDC, IL4DC and MΦ Morphology & Phenotypes

The different magnifications below best illustrate the differences between the cell cultures. IL4DCs and MΦs at 40X show the difference in cell size and cell numbers, which were always much lower in MΦ cultures compared to DCs. DCs at 10X shows the global view of the DC clusters which were much smaller in MΦ cultures and not obvious at 10X magnification, therefore that image was not shown. Finally, GMDC at 100X gives a close-up of one of the clusters seeding off into the medium. These clusters are present to a greatly reduced degree in MΦ cultures. This difference can be observed between the DC 100X and MΦ 40X magnifications.



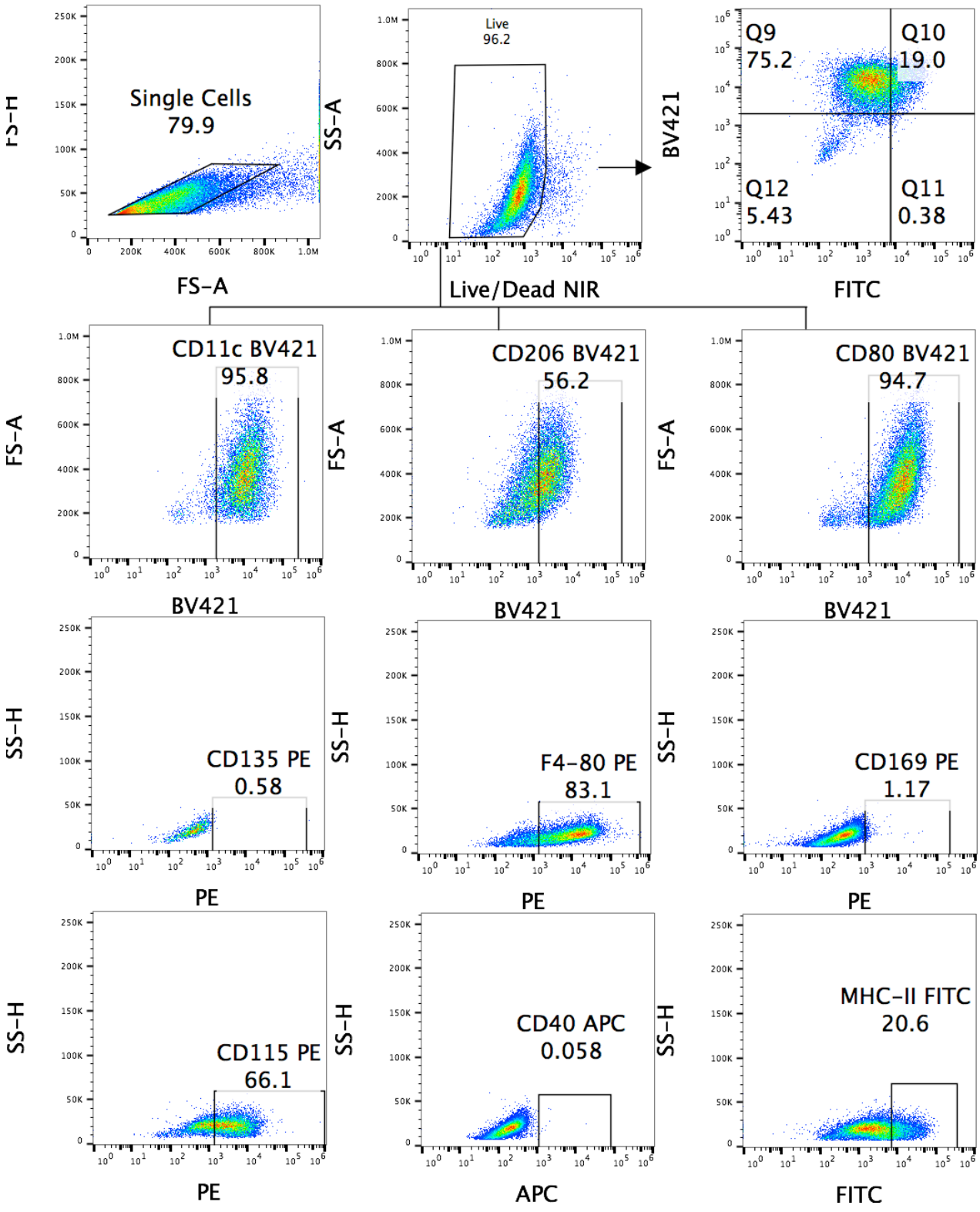
Supplementary Figure 5 GMDC, IL4DC and MΦ populations differ in their gross morphological characteristics,and CD206 expression but are all negative for CD135 and CD169. Bone marrow-derived precursor cells were incubated for 10 days in GM-CSF (5 ng/mL)+IL-3 (5 ng/mL)+FCS10% (MΦ); 6 days in GM-CSF (20 ng/mL)+FCS (5%) (GMDC); or 6 days in GM-CSF (20 ng/mL)+IL-4 (10 ng/mL)+FCS (5%) (IL4DC). Cell cultures were imaged prior to harvest. A) Top left: D6 unactivated GMDC 10X magnification showing typical pattern of background adherent and semi-adherent cells along with non-adherent cells seeding off into culture medium. **A**) Top right: D6 GMDC 100X magnification showing typical immature GMDC population with background adherent cells and smaller, round, non-adherent clusters seeding off. A) Bottom left: D5 unactivated IL4DC 40X magnification showing 60 μM diameter large clear cells among the smaller immature dendritic cells. A) Bottom right: D10 unactivated M1 MΦ 40X magnification, showing firmly adherent, rounded and elongated cells. Example images from representative experiments. **B**) Cells were harvested, stained with dead cell exclusion dye and labeled with fluorophore-conjugated antibodies against a range of common DC and MΦ identifying markers: CD11c, MHC-II, F4/80, CD115, CD135, CD8α; as well as the costimulatory markers CD40, CD80 and CD86. Cells were fixed with 4% paraformaldehyde, stored overnight at 4°C and collected the following day on a Gallios Flow Cytometer (Beckman Coulter). Data were analysed on FlowJo Version X and graphed in Prism. Summary data showing the mean ± s.e.m of six (GMDC, IL4DC) and four (MΦ) independent experiments. Statistically significant differences were calculated by Kruskal-Wallis one-way ANOVA by ranks followed by Dunn's post test. * $p<0.05$; ** $p<0.01$.

7.7 Antibody Panels Used To Assess GMDC, IL4DC and MΦ Response to Lysates

Supplementary Supplementary Table 2 Antibody panels used in experiments to assess GMDC, IL4DC and MΦ activation response to lysates

	Panel 1	**Panel 2**
FL1	MHC-II-FITC **1/800 (0.125 µL)**	MHC-II-FITC **1/800 (0.125 µL)**
FL2	CD11c-PE **(1/400) 0.25 µL**	MHC-I-SIIN-PE **(1/400) 0.25 µL**
FL3		
FL4		MHC-I-PerCP-Cy5.5 **(1/400) 0.25 µL**
FL5	CD86-PE-Cy7 **(1/400) 0.25 µL**	
FL6	CD40-APC **(1/400) 0.25 µL**	CD11c-APC **(1/400) 0.25 µL**
FL7		
FL8	L/D NIR 0.005 µL/test	L/D NIR 0.005 µL/test
FL9	CD80-BV421 **(1/400) 0.25 µL**	
FL10		

7.8 Sample Raw Data and Gating Strategy for MΦ Phenotype

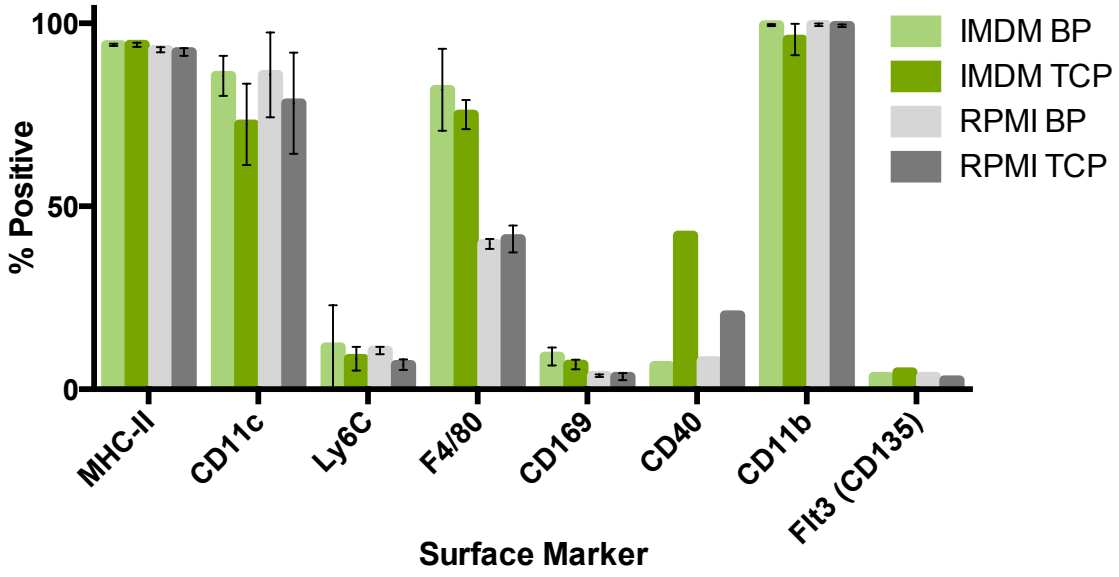


Supplementary Figure 6 Gating Strategy Used in MΦ Phenotype Analysis. D10 M1 MΦ were prepared as previously described. Cells were stained with Live/Dead exclusion dye and Fc receptor block followed by surface molecule staining with the following monoclonal antibodies: MHC-II-FITC, CD11c-BV421, CD135-PE, CD169-PE, CD115-PE, F4/80-PE, CD40-APC, CD206-BV421, CD80-BV421. Antibody-stained cells were washed twice and fixed in 4% paraformaldehyde. Cells were stored overnight at 4°C and acquired the following day on a Gallios Flow Cytometer. 5 e 4 - 1 e 5 cells were collected per sample wherever possible. The gating strategy consisted of doublet exclusion followed by dead cell exclusion and then gating on the cells of interest using unstained cells and single stained cells as negative gating controls. Data was analysed and graphed in FlowJo software Version X. Representative data from numerous independent experiments.

7.9 Media Alters M1 MΦ Surface Phenotype but Bacteriological Petri Dishes Have No Impact

We compared M1 MΦs grown in RPMI and IMDM on tissue culture plates (TCP) and bacteriological petri dishes (BP) (Supplementary Figure). There was no difference in the expression of F4/80, or any other marker measured, on cells grown on TCPs versus BPs. However the RPMI medium had a strong negative impact on the levels of this marker. Even though the downregulation of F4/80 was not statistically significant when analysed by either Mann Whitney U test or one-way ANOVA, we elected to use IMDM to avoid downregulation of F4/80.

CD40 was higher on TCP than on BP in both IMDM and RPMI. However CD40 was only measured in one experiment, therefore we cannot be sure that this was not due to chance.



Supplementary Figure 7 Bone marrow macrophages downregulate F4/80 when cultured in RPMI. Day 10 M1 MΦs cultured in RPMI or IMDM on tissue culture plates (TCP) or sterile bacteriological (non-tissue culture-treated) plates (BP) were harvested, stained with dead cell exclusion dye and labeled with mABs against MHC-II, CD11c, Ly6C, F4/80, CD169, CD40, CD11b and Flt3 (CD135). Antibody-stained cells were washed twice, fixed in 4% paraformaldehyde, and stored overnight at 4°C. Cells were collected the following day on a Gallios Flow Cytometer. 3 e 4 cells were collected in each sample. The gating strategy consisted of doublet exclusion followed by dead cell exclusion and then gating on the cells of interest using unstained cells and single stained cells as negative gating controls. Data was analysed on FlowJo software Version X and graphed in Prism. Summary data of two independent experiments. CD40 and Flt3 were only measured in the second experiment. Error bars = mean + s.e.m.

7.10 GMDC, M1 MΦ and B Cell Response to Activation Stimuli

Various APC activation stimuli are in current use in bench studies and clinical trials around the world[1–9]. To determine the best parameters for activating these APCs their responses to different combinations and concentrations of the cytokine interferon gamma (IFN-γ) and two TLR agonists, CpG or LPS were tested (Supplementary Figure 6). Supplementary Table 3 shows the concentrations and combinations of these three immune stimuli that were compared for their ability to upregulate MHC-II, CD40, and CD80 on GMDCs, M1 MΦs and B cells. It has been noted that these LPS concentrations are high, however they were chosen based on previous studies in the literature.

Supplementary Table 3 Immune stimuli combinations and Doses Tested on GMDCs, M1 MΦs and B Cells

IFN-γ	LPS	CpG
10 U/mL	1 μg/mL	_
50 U/mL	5 μg/mL	_
100 U/mL	10 μg/mL	_
50 U/mL	100 μg/mL	_
_	1 μg/mL	0.3 μg/mL
_	5 μg/mL	0.03 μg/mL
_	10 μg/mL	0.5 μg/mL
_	50 μg/mL	3 μg/mL
_	100 μg/mL	6 μg/mL
10 U/mL	_	0.5 μg/mL
50 U/mL	_	3 μg/mL
100 U/mL	_	5 μg/mL

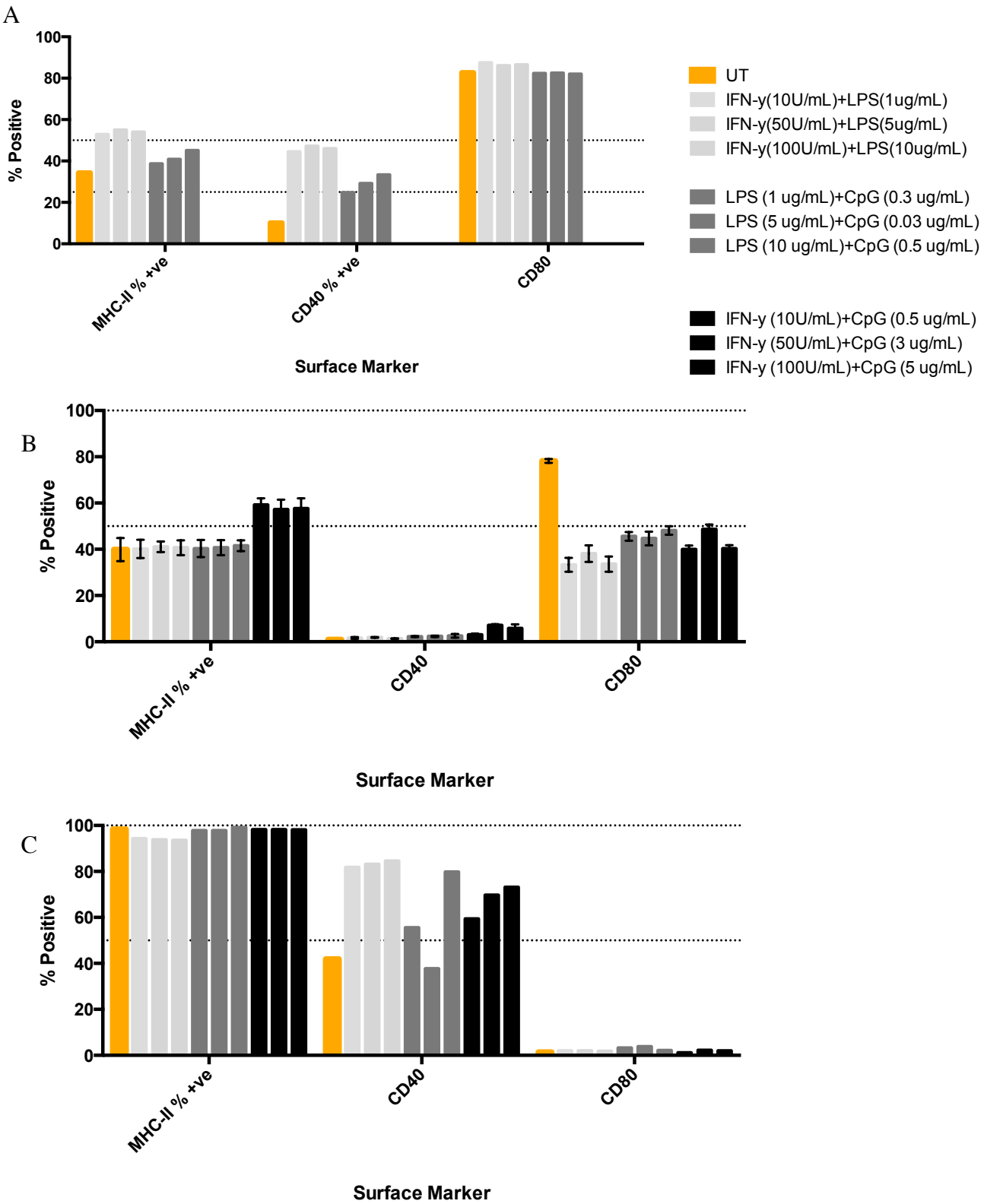
A stimulant concentration titration experiment was carried out on GMDCs. The combinations of LPS+CpG and LPS+IFN-γ yielded no statistically significant increases in the percentage of GMDC positive for CD40, CD80 or MHC-II (Supplementary Figure 6a). This contrasted with the IFN-γ+CpG combination, which reduced MHC-II, CD40 and CD80 expression. For M1 MΦs none of the activators tested stimulated any increase in CD40 or MHC-II and downregulation of CD80 occurred in the presence of all stimulant doses when compared with UT (Supplementary Figure 6b). Finally these combinations were also assessed on B cells in one pilot experiment, which yielded small, non-statistically significant increases in CD40 but no response by CD80. Thus no one combination was ideal for any of these APCs. The triple combination of IFN-γ+CpG+LPS was tested on GMDCs only and results were identical to those of the other combinations.

It was not noted at the time of analysing these experiments that the MHC-II on UT GMDCs was lower than normal while the MHC-II and CD40 on UT B cells was higher than normal (Supplementary Figure 6c).

At the time the results of these experiments were incorrectly analysed leading to the erroneous conclusion that LPS was the common stimulating factor for GMDC and MΦs, whereas the combinations of LPS+CpG and IFN- γ +CpG enhanced the B cell response. Later reanalysis after the experiments were concluded is shown in Supplementary Figure 6. These results show that none of these cytokine TLR combinations resulted in upregulation of MHC-II, CD40 or CD80.

However, the T_H1/T_H2 cytokine response elicited by these stimuli was also evaluated in one LegendPlex (BioLegend, USA) assay (Supplementary Figure 10). The combinations of LPS+CpG and IFN- γ+CpG elicited the highest IFN-γ and TNF-α responses from GMDC, B cell and M2 MΦs. M2 MΦs were inadvertently analysed by bead array instead of M1 MΦs and we did not have access to a further bead array at that time, thus this data set does not exist for M1 MΦs.

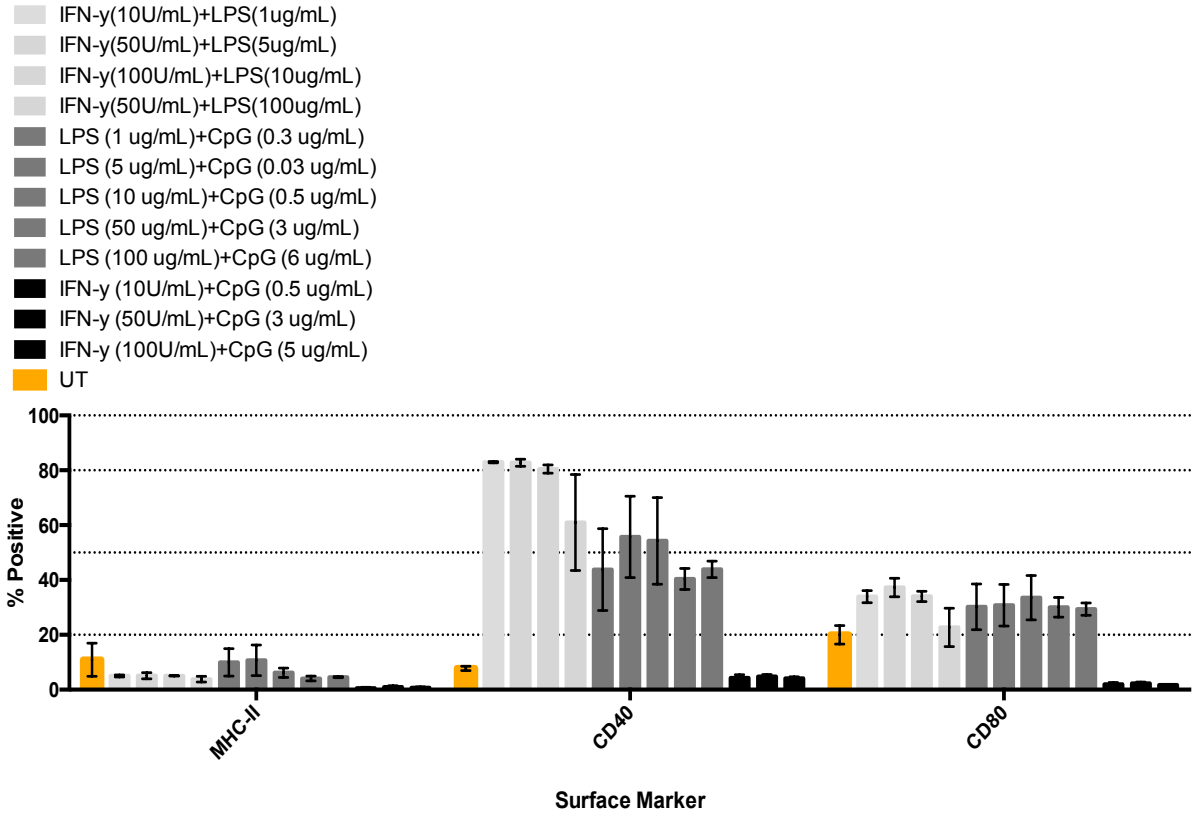
We observed no difference between these stimuli in the activation response of the M1 MΦs. The inclusion of IFN-γ appeared important for GMDC activation while CpG appeared to be required for B cell activation but the IFN-γ+CpG combination yielded a very poor costimulatory marker response for GMDCs. Therefore based on the surface molecule response, as analysed at the time, and LegendPlex cytokine data, the combination of LPS and CpG was chosen as the APC activator for future experiments. However it is acknowledged that these optimisation experiments should have been conducted more robustly.



Supplementary Figure 8 GMDC, M1 Macrophage and B cell responses to immune stimuli concentrations. **A)**Day 6 bone marrow-derived GMDC, **B)** Day 10 bone marrow-derived macrophages and **C)** B cells were prepared as previously described and pulsed overnight with the immune stimuli concentrations described in the legend. Cells were harvested, stained with dead cell exclusion dye and mABs against the surface markers MHC-II, CD40 and CD80. Cells were fixed with 4% paraformaldehyde, stored overnight at 4° C and acquired the following day by Gallios Flow Cytometer. Data were analysed on Flow Jo Version X and graphed in Prism. Summary data of 1 (GMDC & B cell) and 3 (M1 MΦ) experiments are shown. Error bars = mean +/- s.e.m.

7.11 M2 MΦ Response to Activation Stimuli

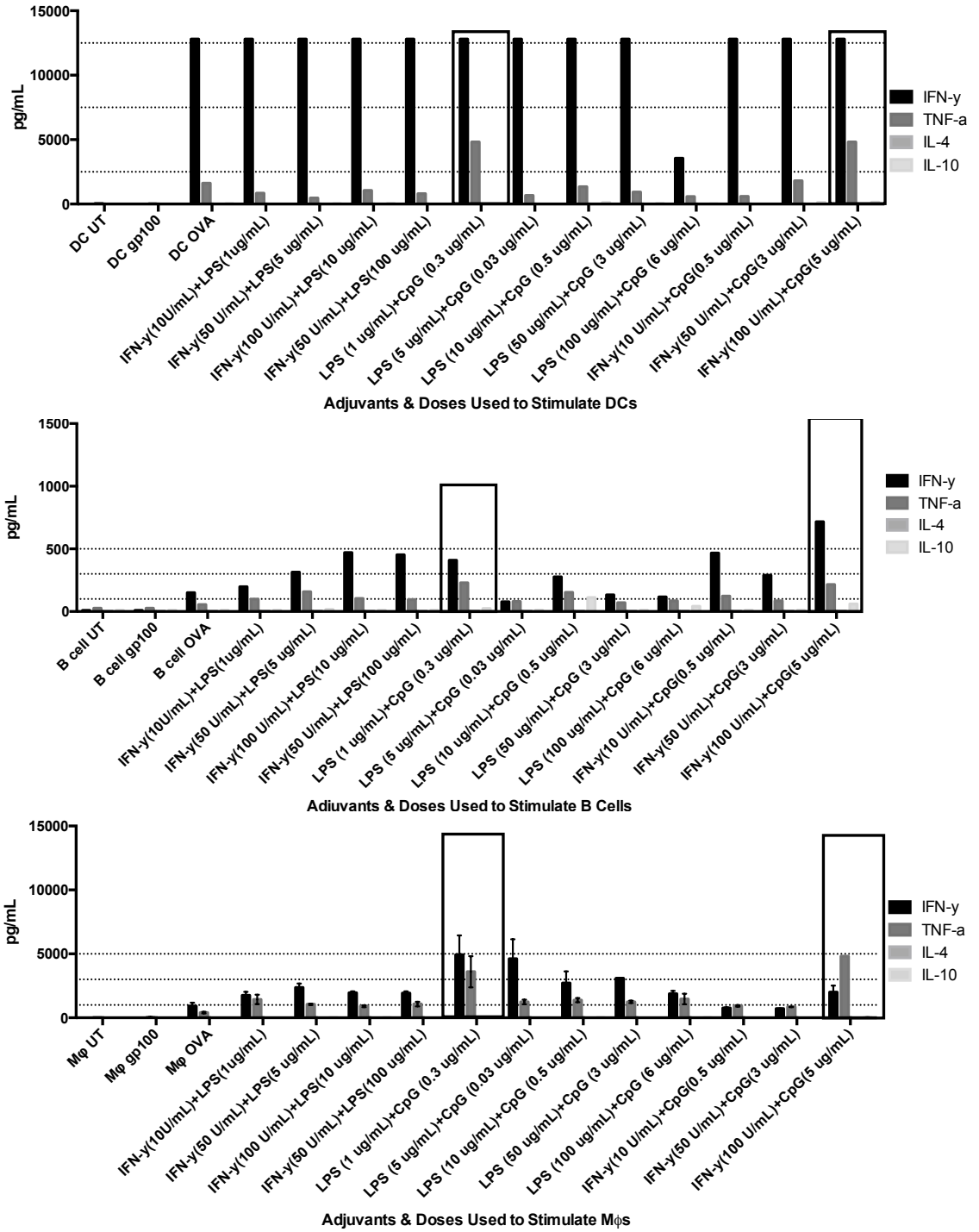
On M2 MΦs all doses of IFN-γ+LPS yielded statistically significant upregulation of CD40 and CD80 compared with UT (Supplementary Figure 9). All doses of LPS+CpG also significantly upregulated CD40 and CD80 molecules on M2 MΦ though to a lesser degree than IFN-γ+LPS. The combination of IFN-γ+CpG stimulated no increase in CD40 or CD80 on MΦs as compared to the UT cells. No differences were seen in the percentage of M2 MΦs positive for MHC-II or CD169, nor was there any change in the MHC-II MFI in response to any combination of adjuvants.



Supplementary Figure 9 M2 macrophage responses to activation stimuli. Day 10 bone marrow-derived precursor cells were prepared as previously described and pulsed overnight with the immune stimuli concentrations described in the legend. Cells were harvested, stained with dead cell exclusion dye and mABs against the surface markers MHC-II, CD40 and CD80. Cells were fixed with 4% paraformaldehyde, stored overnight at 4° C and acquired the following day by Gallios Flow Cytometer. Data were analysed on Flow Jo Version X and graphed in Prism. Summary data of 3 experiments are shown. Error bars = mean +/- s.e.m.

7.12 Legendplex Analysis of Th1/Th2 Cytokine Response to Activation Stimuli

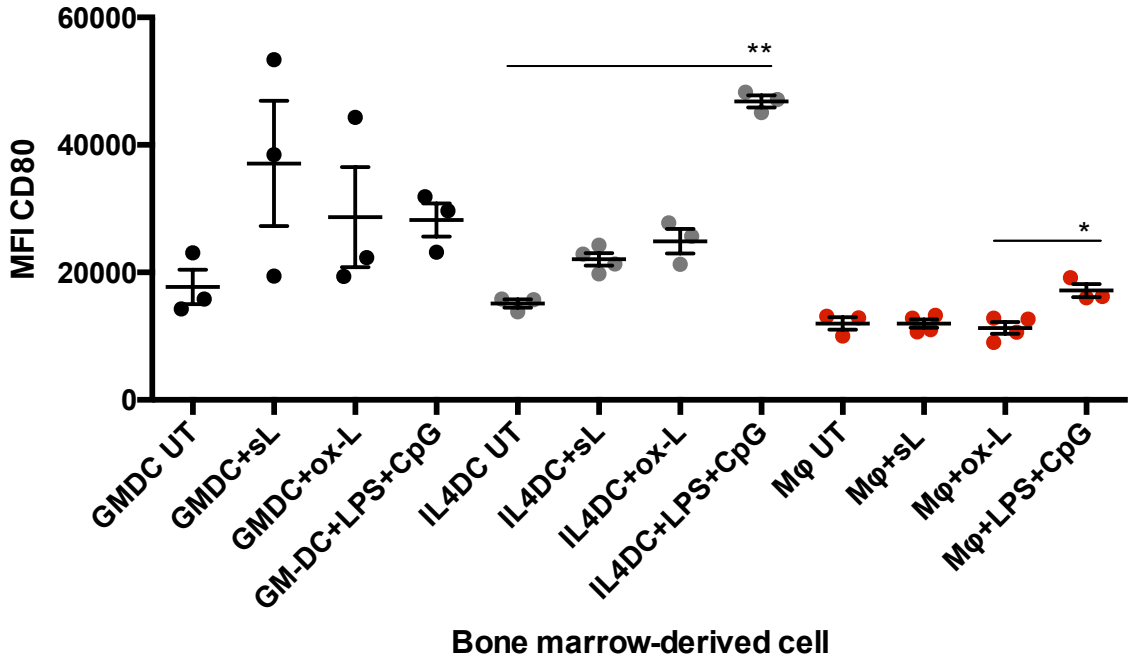
We analysed the cell culture conditioned media (supernatants) of GMDC, B cells and M2 MΦs cultured overnight with the adjuvants described on the x axes in Supplementary Figure 10. Supernatants were analysed by LegendPlex bead array for the presence of the two T_H1 cytokines, IFN-γ and TNF-α and the two T_H2 cytokines, IL-4 and IL-10. Based on these data, the surface molecule responses and the T cell proliferation responses LPS+CpG were chosen as the adjuvants for continued experiments.



Supplementary Figure 10 GMDC, B cells and M2 Macrophage Cytokine Responses Differ Between Adjuvant Types. GMDC, M2 MΦs and B cells were prepared as previously described. APCs were pulsed overnight with the adjuvant concentrations described on the x axes. Cell culture supernatants were stored at -20°C prior to analysis by LegendPlex bead array on a Gallios Flow Cytometer. The data was analysed on Legendplex softwared and graphed in Prism. Summary data of 1 experiment are shown. Error bars = mean with s.e.m. of triplicate readings.

7.13 GMDC, IL4DC and MΦ Activation Response To Soluble & Oxidised lysates

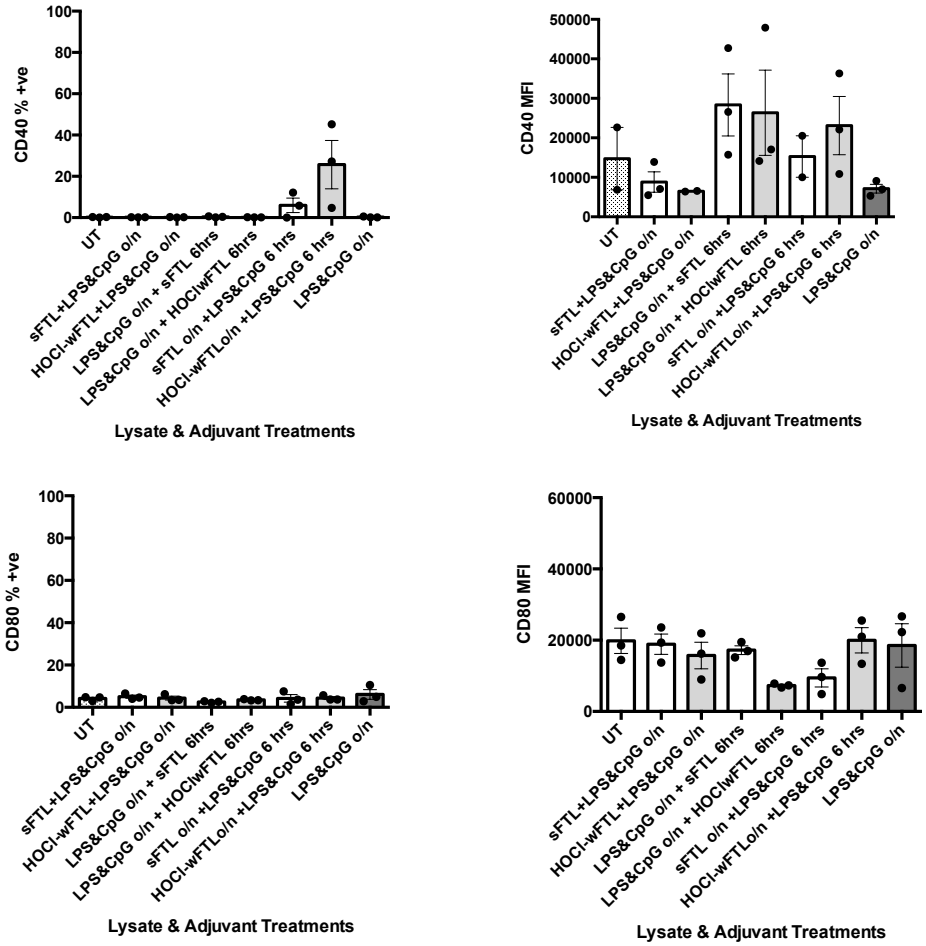
DCs and MΦs vary in size and granularity and therefore auto fluorescence. For this reason these cells were acquired using different voltages. Thus while direct comparisons of MFI cannot be drawn between DCs and MΦs in the manner shown in these graphs the aim of these experiments was to assess the response of the individual APCs to the lysates. Given that no change in the MFI of CD40 was recorded for MΦs (Supplementary Figure 11) it was deemed unnecessary to directly compare the results between MΦs and the two DCs. A significant change in CD80 MFI was observed for both IL4DCs and MΦs therefore we could have directly compared the change of this molecule between GMDC, IL4DC and MΦs. However in both cases these significant fold increases only related to the difference between untreated and positive control-treated cells thus calculation of this fold change was also deemed unnecessary since the change merely acted as a biological control rather than a result *per se*. In no instance did the median fluorescence intensity of MHC-II, CD40, CD80 or CD86 increase in response to either lysate.



Supplementary Figure 11 GMDC, IL4DC and MΦ Vary In Their CD80 Response to s-L and ox-L. GMDC, M1 MΦs and B cells were prepared as previously described. APCs were pulsed overnight with soluble or oxidised lysate (1:1 ratio tumour cell:APC), LPS (1 μgmL) and CpG (0.3 μg/mL). After 24 hours cells were prepared for Flow Cytometric analysis of CD80 expression on the three APCs in response to s-L and,ox-L +/- LPS & CpG. Summary data of 4 independent experiments,. Statistically significant differences were calculated using the Kruskal-Wallis test followed by Dunn's test, without Bonferroni correction. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. Error bars = mean ± s.e.m.

7.14 CD40 and CD80 Are Not Upregulated on B Cells in Response to Soluble or Oxidised Lysate Plus Activation Stimuli

In contrast to MHC-II and CD86 (Chapter 3, Figure 13) neither CD40 nor CD80 percent positivity, nor MFI, increased significantly in response to any lysate plus activation stimuli scenario (Supplementary Figure 12).

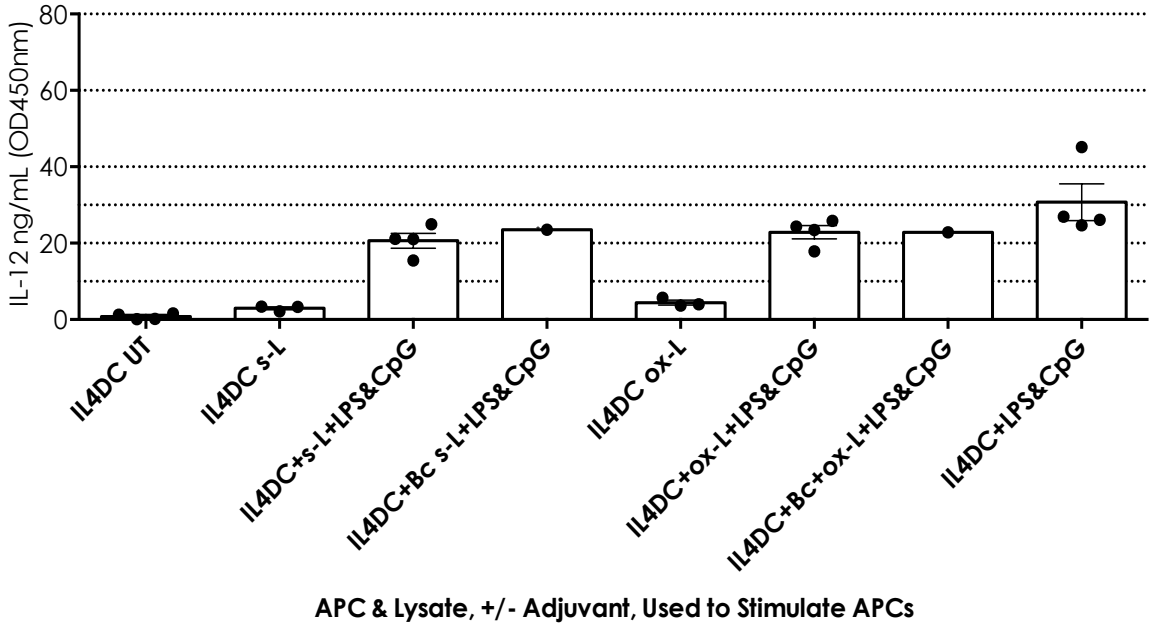


Supplementary Figure 12 The percentage of B Cells positive for CD40 or CD80 does not increase in response to s-L or ox-L plus activation stimuli. Splenic B cells were isolated by magnetic bead separation. B cells were pulsed overnight with s-L and ox-L (1:1 ratio tumour cell:APC) + LPS (1 μg/mL) & CpG (0.3 μg/mL) as indicated on the x axes. The day after lysate loading cells were prepared for Flow Cytometric analysis of antigen presentation and costimulatory molecule expression in response to s-L and ox-L. Summary data of 3 independent experiments. Statistical analysis was not required. Error bars = mean ± s.e.m. UT: untreated; o/n: overnight.

7.15 No Difference Is Observed In the Amount of IL-12 Produced by IL4DCs Compared to GMDCs

The APC populations were compared for their ability to produce IL-12 in response to the two tumour lysates (Chapter 3, Figure 37). After 24 and 48 hours exposure to soluble lysate and oxidised lysate in the presence or absence of LPS & CpG cell conditioned media were collected and stored at -20°C for analysis by anti-IL-12 ELISA.

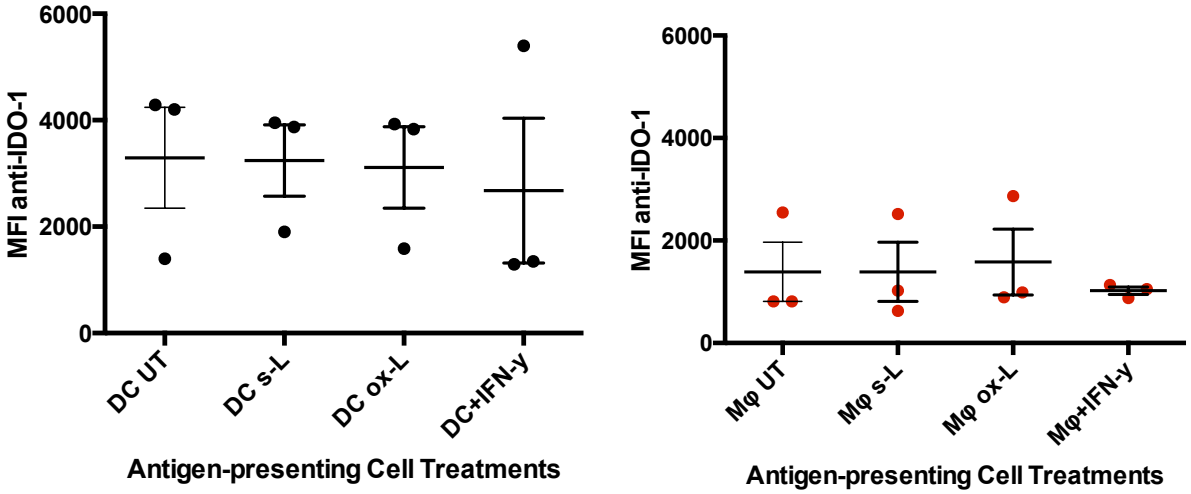
GMDC, M1 MΦs and IL4DC all produced small amounts of IL-12 in response to soluble lysate (5.7 ng/mL, 9.7 ng/mL and 2 ng/mL respectively) (Supplementary Figure 13). Differences between the APCs were not statistically significant. All three APCs also produced IL-12 in response to oxidised lysate, however GMDC and M1 MΦs produced more IL-12 (11 ng/mL and 8 ng/mL respectively) than IL4DC (4.4 ng/mL; $p<0.05$). There was no difference in IL-12 production between soluble lysate- and oxidised lysate-loaded APCs.



Supplementary Figure 13 IL4DCs do not produce more IL-12 than GMDCs in response to lysate loading. GMDC, M1 MΦs and B cells were prepared as previously described. APCs were pulsed overnight with s-L and ox-L (1:1 ratio tumour cell:APC) +/- LPS+CpG. After 24 and 48 hours cell conditioned media (supernatants) were collected and stored at -20°C. Supernatants were analysed by anti-IL-12 ELISA. Observations from 1 (IL4DC+B cell), 3 (IL4DC+s-L; IL4DC+ox-L) and 4 (remaining bars) independent experiments showing IL-12 production after 48 hours exposure to s-L and ox-L +/- LPS+CpG. Statistically significant differences were calculated by Kruskal-Wallis one-way ANOVA followed by Dunn's post test. Error bars = mean ± s.e.m.

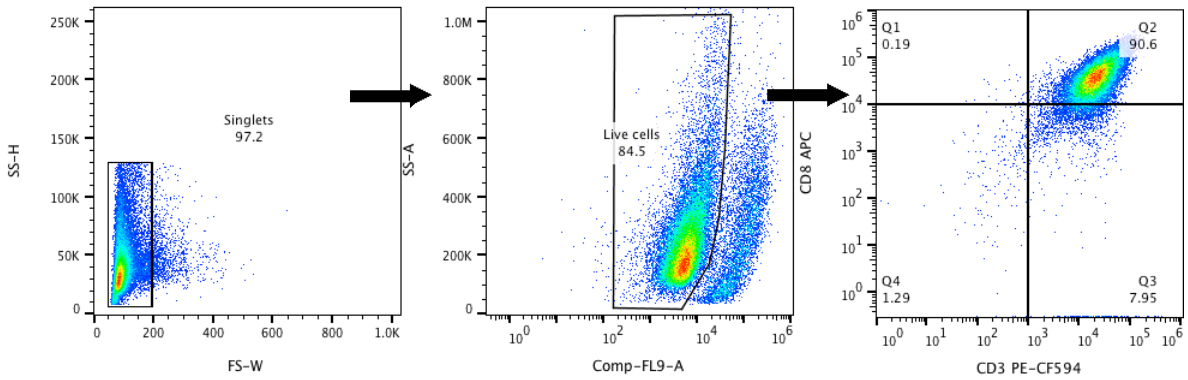
7.16 Lysate-loaded APC Do Not Express IDO

It has been shown that DCs expressing indoleamine 2,3-dioxygenase (IDO) can suppress antigen-specific T-cell responses by killing T cells or inhibiting T-cell proliferation[10–12]. We tested IDO expression by DCs and found no difference in the median fluorescence intensity of IDO-1 between UT and lysate-treated cells (Supplementary Figure 14). These data combined with the extremely low numbers of B220+ cells in the co-cultures (data not shown) mean that IDO production was unlikely to play a major role in any lysate-mediated suppression.



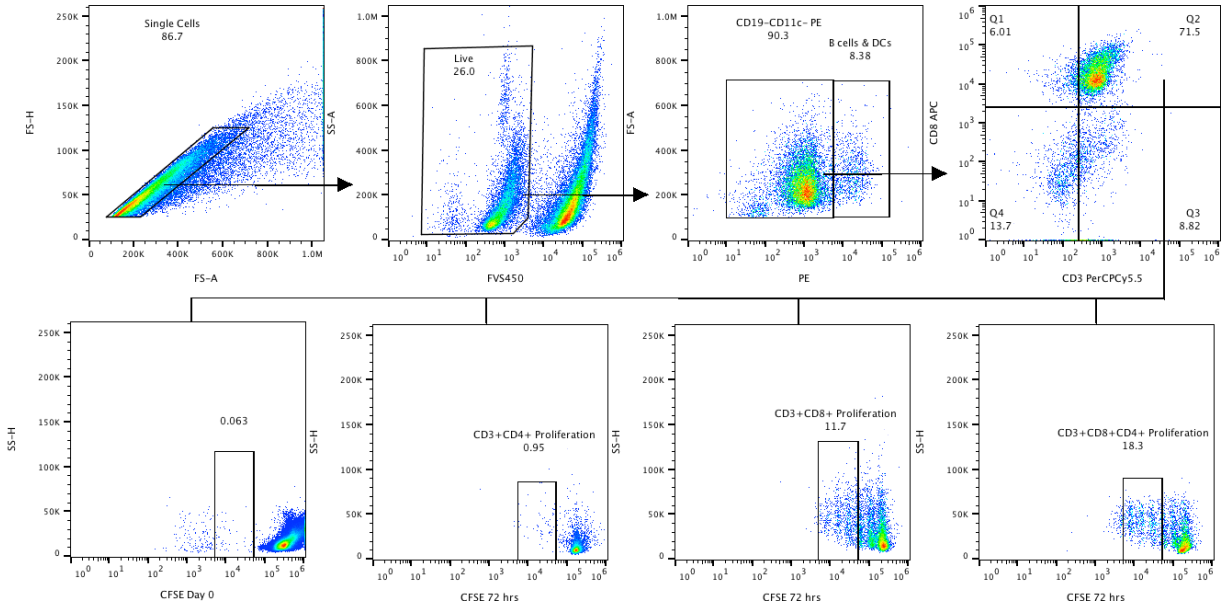
Supplementary Figure 14 DCs and macrophages do not upregulate IDO-1 in response to soluble or oxidised lysate. Bone marrow-derived GMDC and MΦ were generated as previously described. 50,000 APCs were pulsed overnight with s-L and ox-L (1:1 ratio tumour cell:APC) and LPS+CpG. After 24 hours cells were harvested, stained with live/dead exclusion dye, labeled with mABs against surface CD11c, fixed, permeabilised and labeled with mABs against intracellular IDO-1. Cells were stored overnight at 4°C and acquired by Gallios Flow Cytometer the next day. Data were analysed on FlowJo Version 10 and graphed in Prism . Summary data showing median fluorescence intensity of anti-IDO-1 after 24 hour exposure to s-L and ox-L or IFN-γ. Error bars = mean ± s.e.m of 3 independent experiments. UT: untreated.

7.17 Gating Strategy for Assessment of T Cell Purity after AutoMACS Sorting



Supplementary Figure 15 Flow cytometry gating strategy for assessment of T cell purity. CD8+ and CD4+ T cells were isolated by positive or negative selection magnetic beads (AutoMACS Pro, Miltenyi Biotech), labeled with live cell exclusion dye (VPD450, BD Biosciences) and incubated with mABs against the surface markers CD8, CD3, CD27, CD28, CD44, CD62L, CD69, CD122 and CD127. Cells were fixed in 4% paraformaldehyde, stored overnight at 4°C and analysed the next day on a Gallios (Beckman Coulter). Fluorescence Minus One and unstained autofluorescence controls were used to guide the setting of positive and negative gates. Representative data from 15 independent experiments.

7.18 GMDC+B Cell-T Cell Proliferation Gating Strategy



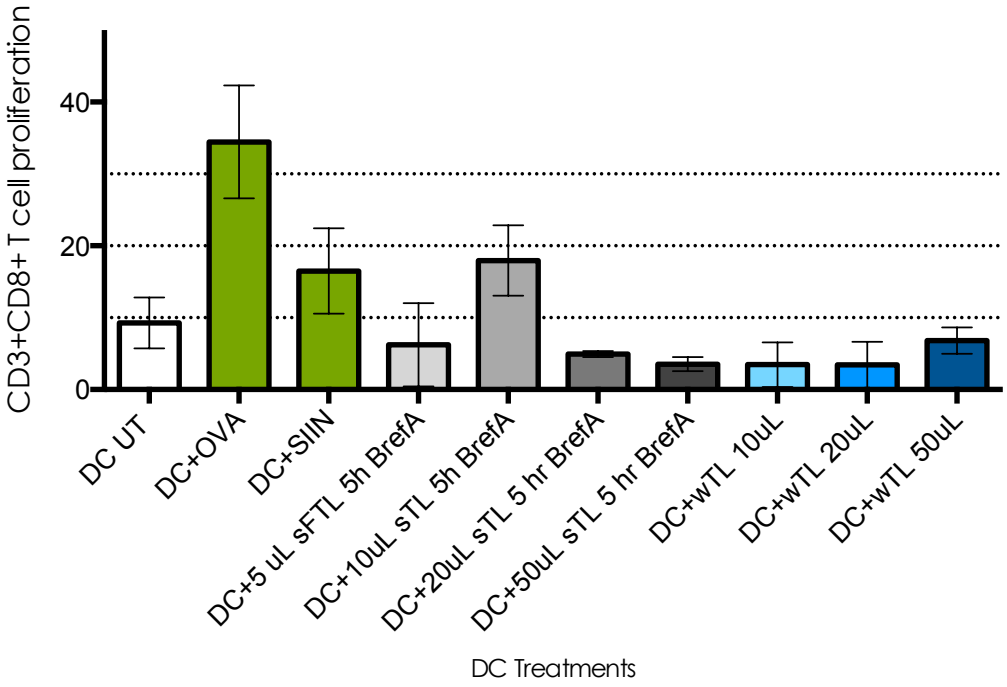
Supplementary Figure 16 Gating strategy used in GMDC+B cell-T cell proliferation co-cultures. GMDCs and B cells were prepared as previously described. APCs were pulsed with whole OVA protein [50 µg/mL], or tumor lysate [6:1]) for 24 hours and co-cultured with CFSE-labeled CD4+ or CD8+ OT T cells, or both. Cells were harvested at 72 hours, stained with dead cell exclusion dye, and labeled with mABs against anti-CD19-PE anti-CD11c-PE (dump), anti-CD8α-APC, anti-CD3-PerCPCy5.5. Cells were fixed with 4% paraformaldehyde, stored overnight at 4°C and collected the following day on a Gallios Flow Cytometer. Data was analysed and graphed in FlowJo Version X. Dead cells (Live/Dead+) were gated out, followed by gating out of CD19+ and CD11c+ cells. Gating on CD3+CD8+ (or CD3+CD4+) double positive cells was used for assessment of T cell proliferation at 72 hours. Proliferation was expressed as the percentage of cells that had reduced fluorescence compared with untreated cells. Day 0 CFSE-stained cells, unstained cells and single stain control cells were used in every experiment to set gates. Representative data from numerous experiments.

7.19 Tumour Lysate Dose Titrations

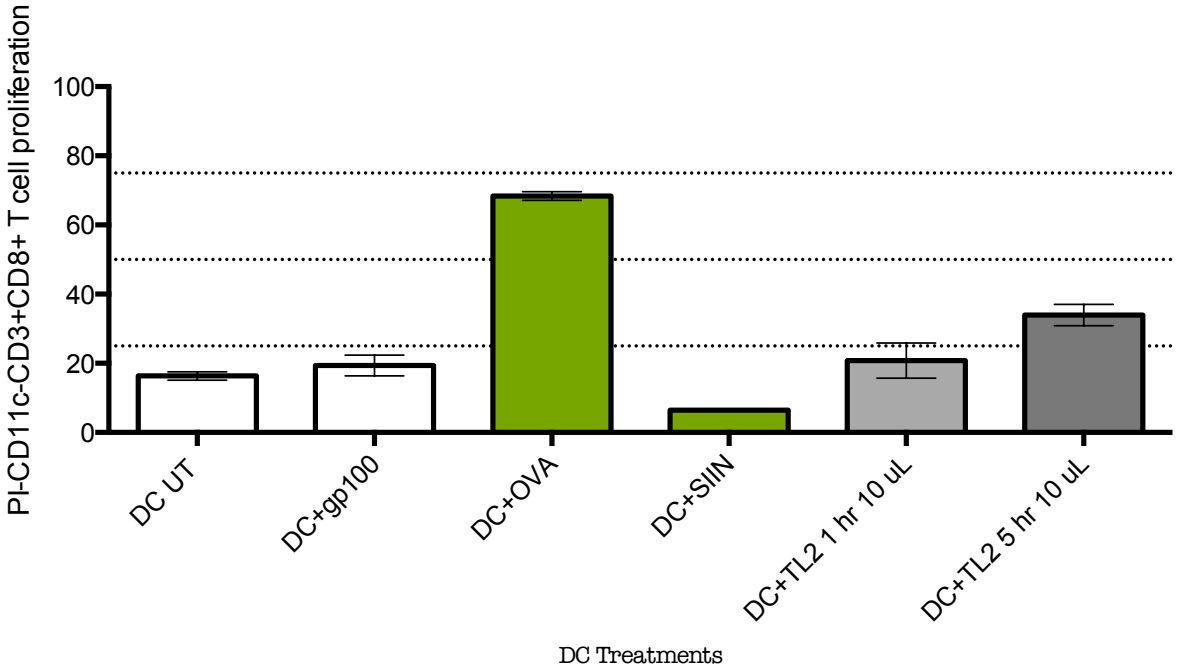
B16.OVA tumour lysate contains OVA protein and therefore the peptide sequences recognised by the T cell receptors of OVA transgenic mice should be presented on MHC molecules after processing by APC. To identify which concentration of tumour lysate would generate the best T cell proliferation we pulsed GMDCs overnight with different volumes of tumour lysate. The following day VPD-450-labeled T cells were added to the lysate-pulsed APCs and after 72 hours the cells were analysed by Flow Cytometry for their proliferative response. The B16.OVA cells were resuspended at 2.4 x 10^7 c/mL prior to freeze-thaw lysis. Therefore 5 μL of lysate equated to the contents of approximately 1.2 x 10^5 lysed tumour cells. Unsorted splenocytes were cultured with the GMDCs at a ratio of 5 T cells to 1 GMDC. With whole freeze-thaw lysate no differences were seen between lysate doses and the proliferation stimulated with whole FTL was much lower overall - 18% for 10 μL of soluble lysate compared with 3%, 3% and 7% for 10, 20 and 50 μL of whole freeze-thaw lysate lysate respectively (Supplementary Figure 17).

In one experiment 5 μL soluble lysate was insufficient to stimulate T cell proliferation, 10 μL stimulated T cell proliferation and 20 μL inhibited proliferation. In a repeat of this experiment 10 μL of sFTL again yielded the highest T cell proliferation, with 20 μL and 50 μL reducing proliferation in a dose-dependent manner.

We also compared lysate made with tumour cells that had been incubated in the protein transport inhibitor Brefeldin A for 1 hour or 5 hours (Supplementary Figure 18). The first experiment returned no difference between these two lysates and the second yielded 34% proliferation for cells that had been Brefeldin A-treated for 5 hours, compared with 21% proliferation for cells that had been incubated in Brefeldin A for 1 hour. We reasoned that this second experiment made more sense and used 10 μL of 5-hour Brefeldin A-treated soluble lysate in future experiments. No dose response was seen between lysate doses in OT-II CD4+ T cells (data not shown).



Supplementary Figure 17 Ten 10 µL of 5-hour Brefeldin-A-treated soluble freeze thaw lysate stimulates optimal CD8+ T cell proliferation. Day 6 C57/BL6 GMDC, were pulsed overnight with whole OVA protein (50 - 100 µg/mL) and the B16.OVA s-L doses indicated. The following day the APC were pulsed with SIINFEKL peptide (2 µg/mL) for at least 4 hours prior to addition of unsorted, VPD-labeled OT-I splenocytes at a ratio of 10 T cells:1 DC. 72 hours later proliferation was assessed by Flow Cytometry (Fortessa LSR). No statistically significant differences were recorded. Error bars = s.e.m of 2-3 experiments carried out in duplicate or triplicate.



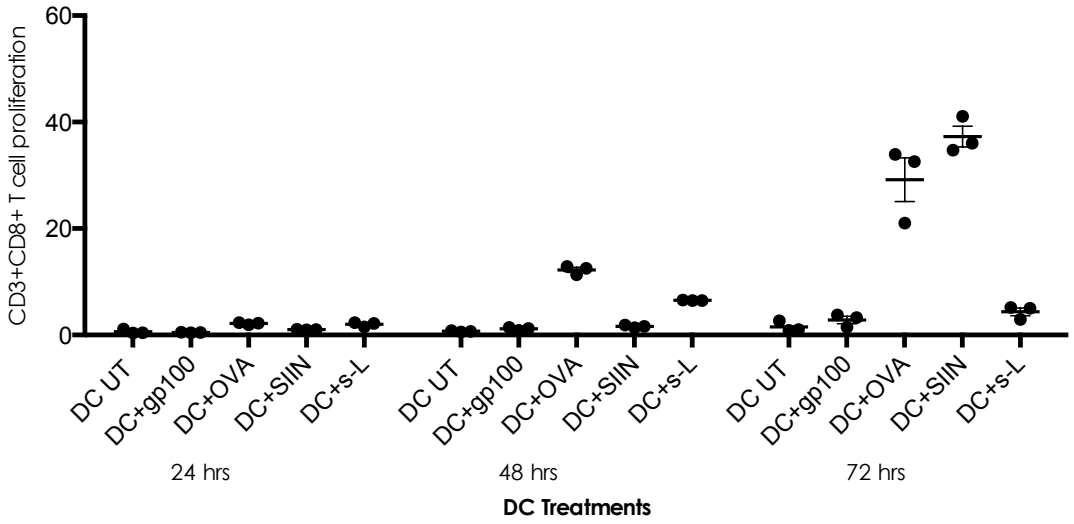
Supplementary Figure 18 Tumour cells treated with Brefeldin A for 5 hours stimulate stronger CD8+ T cell proliferation than tumour cells treated with Brefeldin A for 1 hour. Day 6 C57/BL6 GMDC, were pulsed overnight with whole OVA protein (50 µg/mL) and the lysates of B16.OVA s-L from cells treated with Brefeldin A for 1 hr or 5 hrs prior to freeze-thaw lysis, at the doses indicated. The following day the APC were pulsed with SIINFEKL peptide (2 µg/mL) for at least 4 hours prior to addition of unsorted, VPD-labeled OT-I splenocytes at a ratio of 10 T cells:1 DC. 72 hours later proliferation was assessed by Flow Cytometry (Fortessa LSR). No statistically significant differences were recorded. Error bars = s.e.m of 1 experiment plated in triplicate and carried out in tandem (2 identical plates).

7.20 CD8+ T Cell Proliferation Is Observed After 72 Hour Incubation with Lysate-Loaded GMDCs But Not with MΦs or B cells

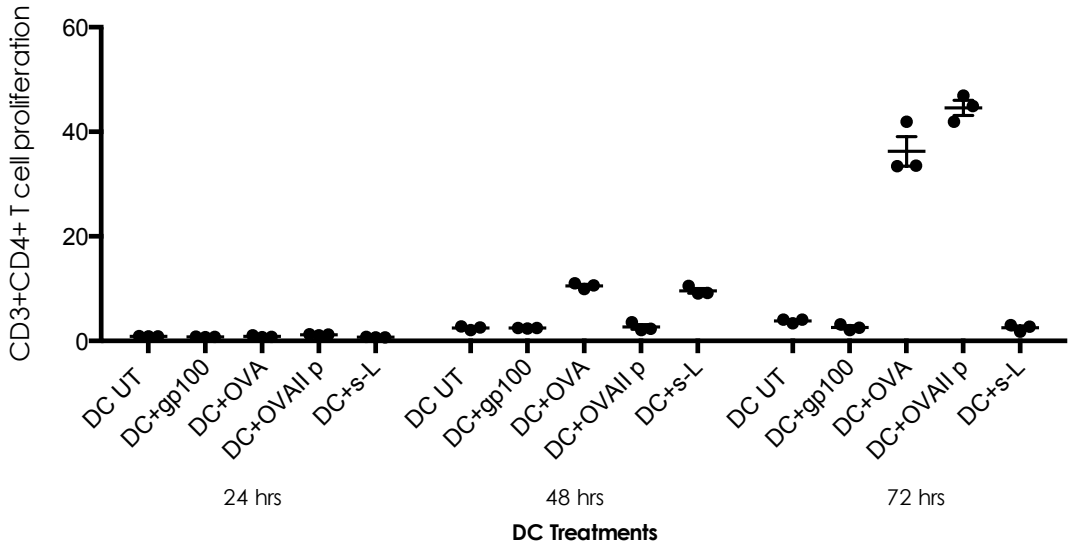
To verify the optimal time point for harvesting undefined antigen-primed T cells we assessed proliferation at 24, 48 and 72-hours (Supplementary Figure 19). Initial experiments indicated that significant CD8+ T cell proliferation in response to soluble lysate could sometimes be achieved when priming with GMDCs (Supplementary Figure 19A). However the level of response was usually more moderate and overall CD8+ proliferation averaged approximately 20%. Initial experiments also indicated that CD4+ T cells could proliferate in response to undefined lysate antigens (Supplementary Figure 19B). However these early data could not be reproduced and Supplementary Figure 19C shows the CD4+ T cell response that was consistently observed throughout remaining experiments.

Supplementary Figure 19D and E show the lack of CD4+ and CD8+ T cell proliferation in response to soluble lysate-loaded M1 MΦs. Supplementary Figure 19F and G show the lack of CD4+ and CD8+ T cell proliferation in response to soluble lysate-loaded B cells.

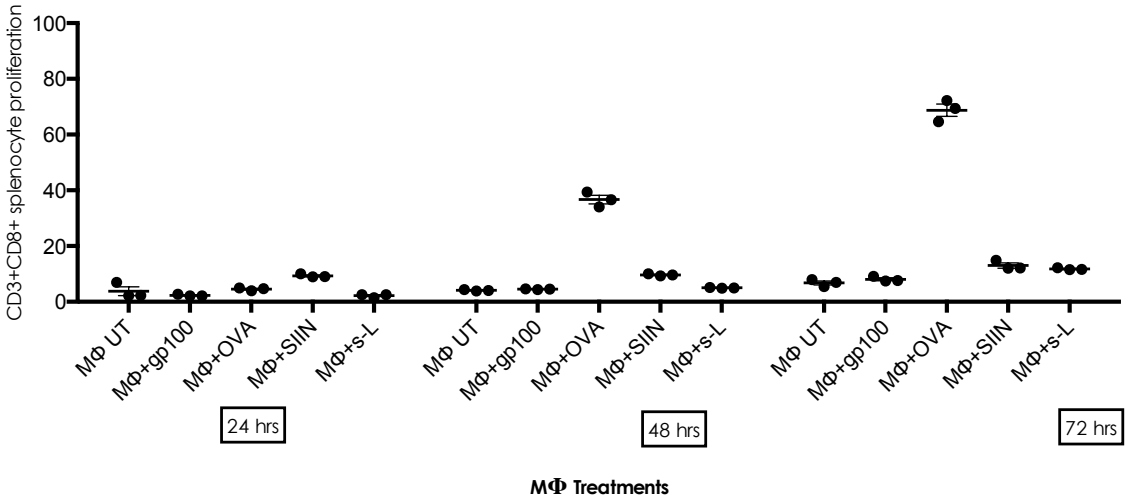
19A



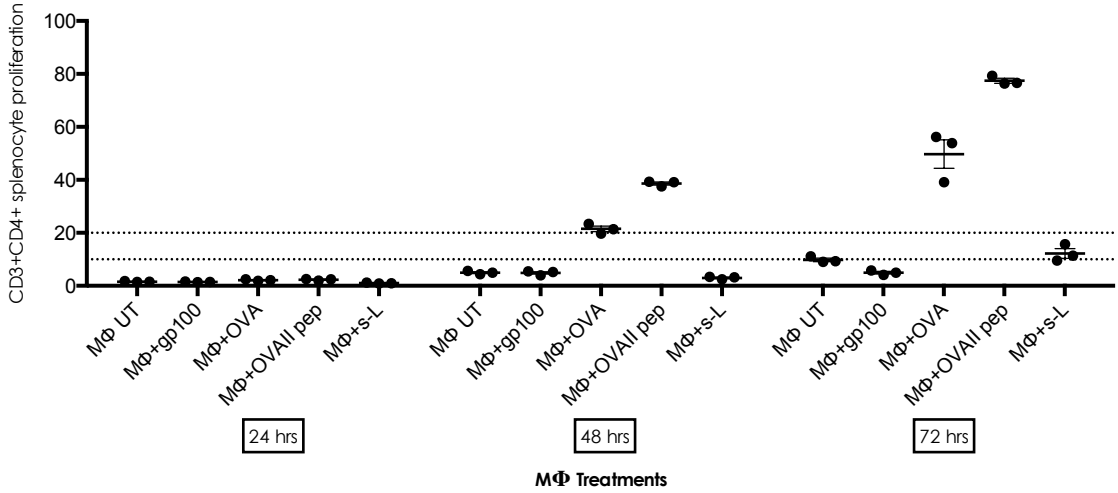
19B



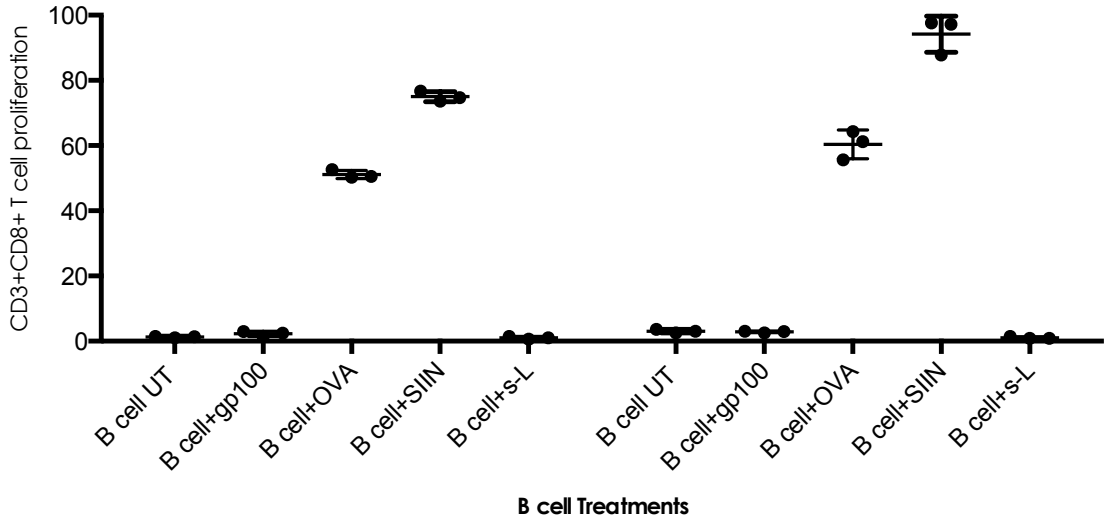
19C



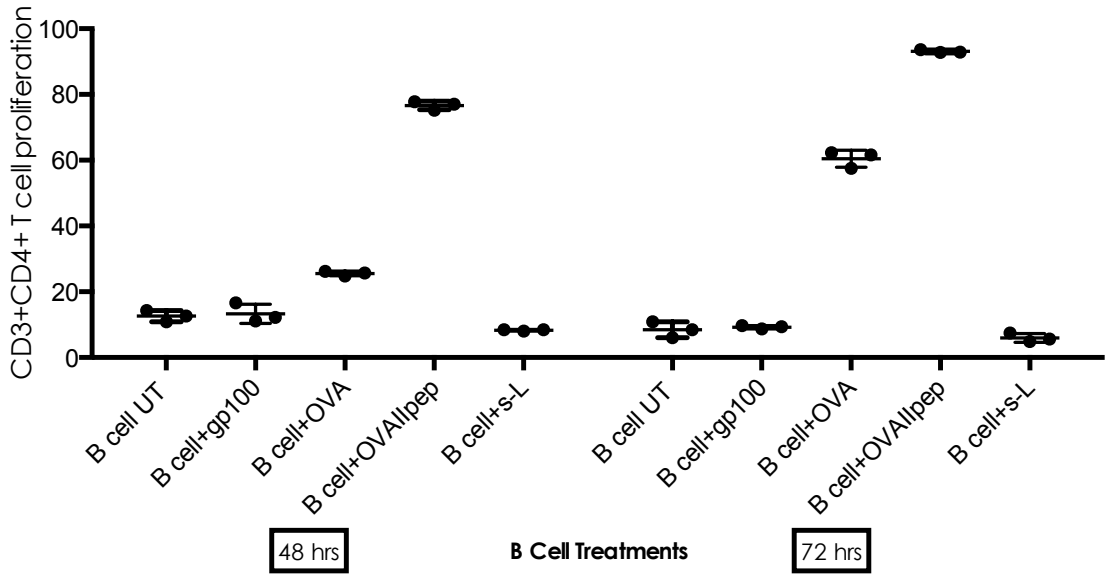
19D



19E



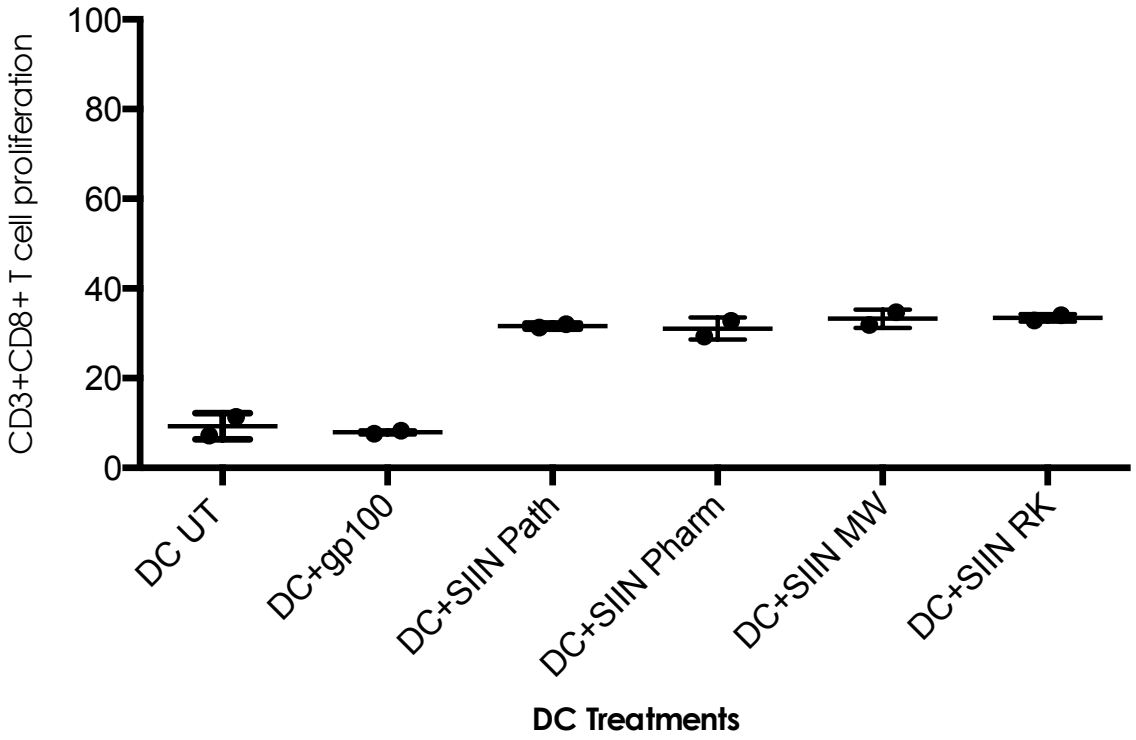
19F



Supplementary Figure 19 Optimal CD8+ and CD4+ T cell proliferation response to cognate antigen is observed at 72 hours. Bone marrow-derived precursor cells were incubated for 6 days in GM-CSF (20 ng/mL)+FCS (5%) (**GMDC**) and 10 days in GM-CSF (5 ng/mL+10% FCS (**M1 MΦ**). **B cells** were freshly isolated from C57BL/6 spleens by magnetic bead isolation (AutoMACS Pro). 4 x 10^4 APCs were pulsed overnight with the soluble fraction of freeze-thaw lysate (sTL) (6:1 ratio tumour cell:APC) or whole OVA protein (50 μg/mL). The following morning 2 μg/mL SIIN peptide was added for at least 4 hours prior to the addition of VPD450-stained OT-I splenocytes (10 T cell:1 DC). After 72 hours cells were stained with dead cell exclusion dye, labeled with mABs to surface markers and fixed in 2% paraformaldehyde. Cells were stored overnight at 4°C and collected the following day on an LSR Fortessa Flow Cytometer. Data was analysed on FlowJo Software version 9 and graphed in Prism...Summary data of T cell proliferation after 72 hours exposure to sFTL-loaded GMDC, M1 MΦ or B cells. The results of 1 representative experiment, plated in triplicate are shown for each APC. Statistically significant differences were assessed by Kruskal-Wallis one-way ANOVA followed by Dunn's post test with Bonferroni correction. $p<0.05$;. Error bars = mean ± s.e.m.

7.21 SIINFEKL Response Is Consistently Low Across Different SIINFEKL Sources

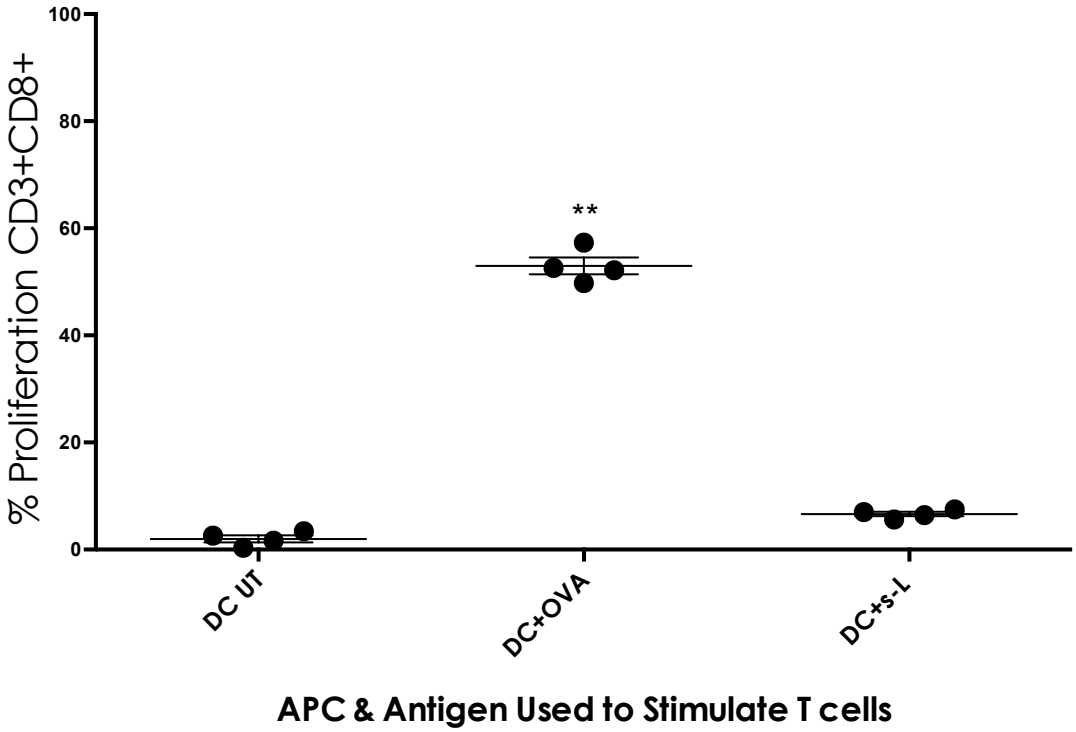
The CD8+ T cell response to SIINFEKL peptide was lower than expected and so we compared the GMDC-primed T cell response to our SIINFEKL and to the SIINFEKL of three other independent labs (Supplementary Figure 20). There was no concentration-response to different doses of SIINFEKL (2, 4, 8, 10 μg/mL; data not shown) and no differences in the response to SIINFEKL from the four different sources indicating that the source of the low response did not lie in our batch of SIINFEKL



Supplementary Figure 20 The source of low CD8+ T cell response to SIINFEKL peptide did not lie with our batch of SIINFEKL. Day 6 GMDC were loaded with SIINFEKL peptide (2,μg/mL) was added for 4 hours prior to the addition of unsorted, Violet Proliferation Dye-labeled OT-I splenocytes (10:1 ratio T cells to DC). 72 hours later the cultures were dyed with dead cell exclusion dye and labeled with mABs against CD11c, CD3 and CD8. Cells were fixed in 2% PFA, stored overnight and proliferation assessed by Flow Cytometry the following day on an LSR Fortessa Flow Cytometer. Flow data was analysed on FlowJo software V9 and graphed in Prism Summary data of 1 experiment, 2 plates plated in triplicate. Path: our laboratory's SIINFEKL; Pharm, MW and RK correspond to independent laboratories sources of SIINFEKL.

7.22 Activated Soluble Lysate-Loaded GMDCs Induce Less CD8+ Proliferation Than Unactivated GMDCs

When compared to Figure 43 in Chapter 4 we observed that GMDC loaded overnight with sFTL and LPS&CpG stimulate less CD4+ and CD8+ proliferation than GMDCs loaded with sFTL alone (Supplementary Figure 21). The GMDC continued to induce an approximately 3 fold proliferation over UT cells (p<0.05 when calculated using Mann Whitney U test; ns when differences between the 3 groups are assessed by Kruskal-Wallis followed by Bonferroni-adjusted Dunn's post test). That the reduction in proliferation could be attributable to the use of LPS&CpG was not recognized at the time. Nonetheless, the addition of LPS&CpG ultimately allowed the superiority of the GMDC+B cell combination to be identified.

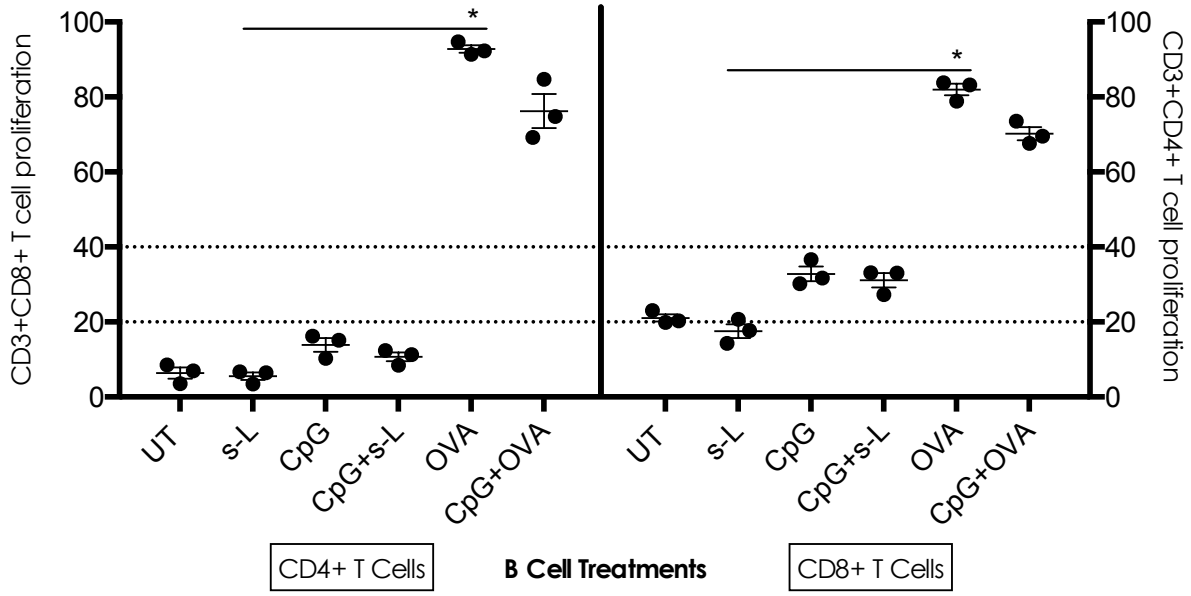


Supplementary Figure 21 GMDCs pulsed overnight with sFTL and activation stimuli induce less CD8+ T cell proliferation than lysate-loaded GMDCs alone. Day 6 GMDC, prepared as described previously. 5 x 10^4 GMDCs were pulsed overnight with lysate (6:1 tumour cell:APC) or OVA protein (50 μg/mL). The following morning the irrelevant melanoma peptide gp100 and $OVA_{257-264}$ peptide (SIINFEKL) or $OVA_{329-337}$ peptide were added 4 hours prior to the addition of 0.5 x 10^6 Violet Proliferation Dye 450-labelled CD4+ or CD8+ T cells. 72 hours later cells were stained with dead cell discrimination dye and labeled with mABs against CD11c, CD3 and CD8. Cells were fixed in 2% paraformaldehyde and stored overnight at 4°C and analysed the following day on an LSR Fortessa Flow Cytometer. Flow data was analysed on FlowJo software V9 and graphed in Prism. Summary data of the mean of 4 independent experiments. Statistical differences were assessed by Kruskal-Wallis test followed by Dunn's post test with Bonferroni correction. ** p<0.01.. Error bars = mean ± s.e.m.

7.23 Activated B cells Stimulate More T Cell Proliferation Than Unactivated B Cells

We compared the T cell stimulatory capacity of immature B cells loaded with lysate and that of B cells activated for 24 hours with CpG prior to the addition of lysate. Unactivated lysate-loaded B cells were unable to stimulate CD4+ or CD8+ T cell proliferation (Supplementary Figure 22). No improvement in CD4+ or CD8+ proliferation was observed in CpG-loaded B cell groups.

The addition of CpG to the lysate yielded no significant increases in T cell proliferation over UT, lysate-loaded or OVA-primed groups, thus these results were interpreted to mean there was no advantage to using activated B cells for presenting lysate antigen and experiments were continued for some time using unactivated B cells. This may explain the delay in seeing the advantage of using a DC+Bc combination.



Supplementary Figure 22 Activating B cells with CpG alone does not stimulate greater CD4+ or CD8+ T cell proliferation than unactivated B cells. Freshly isolated C57/BL6 splenocytes were labeled with anti-CD43 magnetic beads and B cells isolated by AutoMACS Pro. The negative fraction was loaded with soluble freeze-thaw lysate (5.0 x 10^5 cells/well) and/or CpG (2 μg/mL) overnight and the following day unsorted, Violet Proliferation Dye-labeled OT-I or OT-II splenocytes were added to the B cells at a ratio of 10 T cells to 1 B cell. 72 hours later the cultures were stained with dead cell exclusion dye and labelled with mABs against CD19, CD3, CD8 or CD4. Cells were fixed in 2% PFA, stored overnight and proliferation assessed by Flow Cytometry the following day on a Gallios Flow Cytometer.. Flow data was analysed on FlowJo software V9 and graphed in Prism. Statistically significant differences were calculated using Kruskal-Wallis followed by Dunn's test with Bonferroni adjustment. *p<0.05. Summary data of 1 experiment showing the mean +/- the s.e.m of triplicates.

7.24 T Cells Vary in Their Viability Response To Soluble and Oxidised lysates

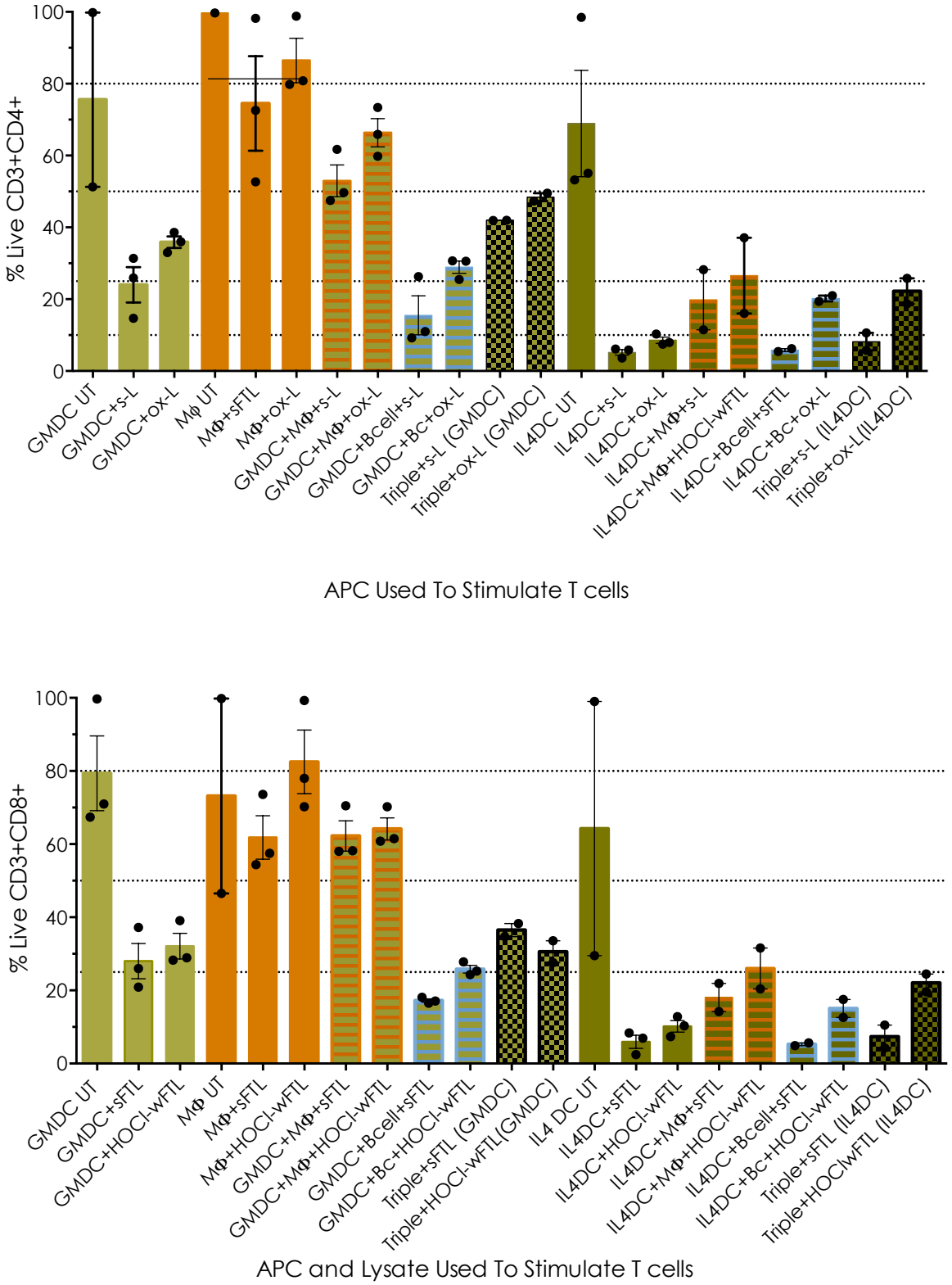
During the 72-hour proliferation assays we observed large reductions in the viability of both CD4+ and CD8+ T cells primed with APCs loaded with either lysate (Supplementary Figure 23). The one exception to this observation was the viability of CD8+ T cells primed by oxidised lysate-loaded MΦs. Consistent with the excellent viability of MΦs loaded with both lysates (Figure 36), T cells primed by oxidised lysate-loaded MΦs showed the best overall viability (Supplementary Figure 23).

The viability of CD4+ and CD8+ T cells primed by soluble lysate-loaded M1 MΦs was higher than the viability of T cells primed by GMDC+s-L (CD4+: 73% versus 24% respectively; CD8+: 62% versus 28% respectively).

The combination of an M1 MΦ with a GMDC showed a trend toward rescued viability in both CD4+ and CD8+ T cell cultures compared with GMDC alone. When CD8+ T cells were primed with GMDC loaded with s-L, live T cells constituted only 22% of the population compared with 53% in GMDC+MΦ+s-L-primed samples. The same trend was seen oxidized lysate groups with 32% live T cells in the GMDC-primed samples versus 64% in the GMDC+MΦ-primed wells. The same pattern of viability rescue in the GMDC+MΦ groups was observed in the CD4+ T cells. However none of these differences were statistically significant. There was no difference in viability observed between GMDC-primed and GMDC+Bc-primed samples.

CD4+ and CD8+ T cells primed by lysate-loaded IL4DC showed the worst viability overall. Experiments using IL4DCs combined with other APCs were only conducted twice due to the overall low viability. Therefore, while there was a general trend of the lowest live cells in soluble lysate groups, nothing statistically significant can be calculated. Given that the viability of IL4DC-primed T cells was so low we discontinued the evaluation of these APCs.

Together these data demonstrate a negative effect of these lysates on T cell viability, particularly when presented by GMDC or GMDC+B cells. It has been suggested that the use of protease inhibitors during lysate generation may be responsible for the negative impact on the T cells. However it was assumed that the small volumes of lysate would not contain sufficient protease inhibitors to impact the T cells significantly and so comparison of lysate +/- protease inhibitors was not conducted during this project. Given that Chiang *et al* do not use protease inhibitors during lysate creation the results of such a comparison would be very interesting.



Supplementary Figure 23 CD4+ and CD8+ OT-I T Cells Viability Response to Presentation of soluble and oxidised lysate by Different APCs. Bone marrow-derived precursor cells were prepared as previously described. APCs were pulsed overnight with s-L and ox-L (1:1 ratio tumour cell:APC) and LPS+CpG. The following day CD4+ and CD8+ cells were isolated from spleens of OT-I mice by AutoMACS Pro (Miltenyi Biotech) negative selection bead separation and stained with CFSE prior to being added to the lysate-pulsed APCs at a ratio of 1 T cell:10 APCs. After 72 hours cells were stained with dead cell exclusion dye (Fixable Viability Stain 450, BD Biosciences), labeled with mABs to surface markers and fixed in 4% paraformaldehyde. Cells were stored overnight at 4°C and collected the next day on a Gallios Flow Cytometer. Data was analysed on FlowJo version X and graphed in Prism..Summary data of T cell viability after 72 hours exposure to s-L and ox-L. Results of two (IL4DC) and three (GMDC, MΦ B cell) independent experiments. Statistically significant differences were assessed by Kruskal-Wallis test followed by unadjusted Dunn's test and by Mann Whitney U. Error bars = mean ± s.e.m.

7.24.1 Lysate-Loaded APCs Reduce T Cell Viability

CD4+ and CD8+ T cell viability was higher in oxidised lysate-primed T cells than soluble lysate-primed T cells (Supplementary Figure 23). This result contrasted with GMDC viability, which dropped strikingly when GMDC were pulsed with oxidised lysate+LPS&CpG. IL4DC-primed T cell viability was significantly lower than that of GMDC-primed T cells. These results paralleled the reduced IL4DC viability compared to GMDC when loaded with lysate. Thus, while the oxidised lysate had a negative impact on GMDC and IL4DC viability T cell viability was consistently higher when T cells were primed with oxidised lysate-loaded APC. We therefore continued to evaluate its usefulness.

With the exception of MΦs there were only small differences in viability between CD8+ T cells primed with soluble or oxidised lysate irrespective of which APC(s) presented the lysate-derived antigens. There were, however, differences in the number of live T cells depending on which APC(s) presented the lysate-derived antigens. GMDC alone and GMDC+Bc yielded the lowest numbers of live T cells and these numbers were similar between these two lysate groups. A small improvement in T cell viability was observed in the triple combination group but the best viability occurred in the MΦ and GMDC+MΦ groups.

We observed that by Day 3 lysate-primed T cells were in clumps of dead or dying cells and large numbers of cells had disappeared (Appendix 1, Supplementary Figure 23), presumably phagocytosed by the APCs as part of homeostatic clonal deletion of autoreactive T cells[13] or as a result of activation induced cell death (AICD)[14]. Lysate contains a mixture of TAAs and self antigens. In the periphery self-antigen is presented on DCs and other non-professional APCs such as Lymph Node Stromal Cells (LNSCs)[15]. For activation of lymphocytes to occur, both antigen and costimulatory 'second' signals must be present. Absence of either will result in failure of lymphocyte activation and self-antigens presented to auto-reactive T cells in the periphery will result in T cell anergy or deletion via AICD mechanisms. T cell activation causes upregulation of the death receptor Fas (CD95) and its ligand Fas-ligand (Fas-L) resulting in cell suicide and sororicide[16]. AICD forms part of normal homeostatic peripheral tolerance mechanisms against self reactive mature T cells and is induced by repeated antigenic stimulation, such as may be found in our lysate-loaded APC-T cell co-cultures[14]. Only activated T cells are sensitive to this form of apoptotic death, which is enhanced by IL-2 and is Fas-dependent.

One reason for reduced T cell viability in our samples may be the induction of AICD by APCs via Fas-FasL-mediated pathways. Freshly isolated CD8+FasL+ murine lymphoid DC have been shown to induce apoptosis in activated CD4+ T cells[17]. The DCs in our study were CD8 negative however another study demonstrated FasL+CD8- DC also induced AICD showing that CD8 expression may be unrelated to this function[18]. The expression of Fas or FasL on the T cells or APCs was not assessed, therefore we cannot ascribe the cause of death to Fas-FasL-mediated signaling. But it remains a biologically plausible explanation.

Tumour-infiltrating MΦs have been shown to strongly induce apoptosis in activated CD8+ T cells via MΦ-associated TNF and Nitric oxide (NO). However we saw the lowest T cell death in our MΦ-stimulated samples. In addition the conditioned cell media from all our samples were negative for TNF-α. Thus it is unlikely that TNF-α played a role in the T cell death. Nitric oxide (NO) plays a dual role in immuno-pathologies[19] and immune-mediated defense against micro-organisms[20]. It is known that high dose NO inhibits IL-12 production in MΦs whereas low dose NO enhances differentiation of murine T_H1 cells[21]. Given that we saw excellent levels of IL-12 expression across GMDC-, MΦ- and IL4DC-lysate-primed T cells (Figure 17, Figure 16) it is also unlikely that NO was responsible for the T cell death.

The use of chemotherapy to reduce tumour burden prior to ACT restores the CTL's ability to overcome the tumour. These effects have been ascribed to the combination of de-bulking by direct chemotherapeutic tumour killing, as well as CTL activation by the dead and dying tumour cells[22] and the reduction in circulating T_{REGS}[23]. In this study the deletion and inactivation of CTLs was clearly seen in *in vitro* assays in which the APCs had been pulsed with soluble lysate. The APCs were clearly able to process and present TAA as evidenced by their ability to stimulate T cell proliferation. However after 72 hours the T cell proliferation ceased and the T cells appeared 'stunned' and began to die off. Proliferation did not cease and die-off did not occur in T cells activated by APCs pulsed with OVA protein. This points to a failure, not at the level of antigen presentation but at the level of T cell function post-antigen presentation. Something in the lysate switches off the CTLs, or, the immune environment induced by presentation of lysate antigens induces a T cell-destructive milieu. In a study by Prato and colleagues they state that "*it is still unclear whether CTL inactivation occurs as a result of recognition of cognate Ag or is mediated by soluble factors acting in a non-antigen–specific fashion*"[24]. This current study does not clarify that issue and we recognise the need for dissection of the pathways involved in T cell activation to identify at what point, and by what

mechanisms, the CTLs are deactivated. We also recognise the need for comparison of lysates generated with and without protease inhibitors to ascertain their impact on T cell 'stunning'.

The hypothesis of APC phagocytosis of lysate-primed T cells could be tested by an uptake assay in which T cells are stained with CFSE prior to culturing with APCs, harvested on Day 2 of co-culture and gated on CFSE+CD11c+MHC-II+CD3- cells. If the theory is correct two populations should be observed: a larger population of CFSE+CD11c+MHC-II+CD3- cells and a much smaller fraction of proliferating, TAA-reactive CFSE+CD3+ T cell. This assay would provide a proxy method for quantifying the percentage of potentially tumour-reactive T cells in the cultures. These T cells could potentially also be tested with soluble peptide MHC tetramers to confirm true tumour reactivity.

To summarise, striking differences in both APC viability and T cell viability were noted between the two types of lysate. Despite the higher cell death in oxidised lysate-loaded APCs we continued to compare the response to the two lysates because of the improved viability in the T cells primed with oxidised lysate-loaded APCs.

As a final comment on the low T cell numbers, interesting work in the Busch and Schumacher labs has demonstrated that as few as one T cell can yield the diverse range of cells that are required for an effective immune response[25–28]. Furthermore Busch *et al* have evaluated a lower limit of antigen-specific T cells required for protective immunity in a mouse model of bacterial infection as well as preliminary human data in the stem cell transplantation setting[29]. They reported that as few as 1-100 $CD62L^{HI}$ CD8+ T cells proliferated, differentiated into different subpopulations and conferred immunity. Other groups have argued the case for observing physiologically relevant doses of T cells and demonstrated that seeding as few as ~10–50 TCR-transgenic T cells, which constitute a fraction of the endogenous repertoire, allowed vigorous proliferation and analysis of TCR-transgenic cells after infection in a scenario representing normal physiology for any individual TCR[30]. These observations bear important relevance to ACT for cancer with its current requirement of high numbers of TAA-specific T cells. While the low numbers of viable T cells generated in this study were disappointing, the high numbers that have been used in clinical trial to date may not necessarily be required – though this hypothesis remains to be tested. Nevertheless, despite the less than ideal T cell proliferation we were able to generate an *in vivo* response with these lysate antigen-primed T cells. Thus the quality of the cells generated may be more important than the quality.

7.25 Supporting Long Term *In Vitro* Culture of T Cells

The choice of exogenous cytokines in growth media is an important factor for directing the outcome of anti-tumour immune responses. Highly regulated cytokines are important for for driving differentiation of different T cell subsets from their uncommitted precursors as well as for supporting naïve and antigen-experienced T cell survival.

Lysate had a negative effect on both APC and T cell viability. We therefore evaluated IL-2, IL-7, IL-15 and IL-21 by carrying out concentration titrations to verify if they would have any improvement on viability or fold expansion of lysate-primed CD4+ or CD8+ T cells. During a personal discussion Professor Susanne Heinzel (Walter and Eliza Hall Institute, 10.7.15) mentioned that high cytokine doses are needed to see a good T cell response. For this reason we chose the 10-100 ng/mL range for our concentration titrations. Supplementary Table 4 shows the cytokine concentration titrations examined.

The combination of IL-7, IL-15 and IL-21 has been shown to preferentially expand CD8+CD44+CD62L+ central memory T cells (T_{CM}) in ACT for murine melanoma[31]. Therefore we compared the proliferation of lysate-primed T cells cultured in the doses of IL-2, IL-7, IL-15 and IL-21specified in Supplementary Table 4.

Supplementary Table 4 Cytokine Dose Titrations

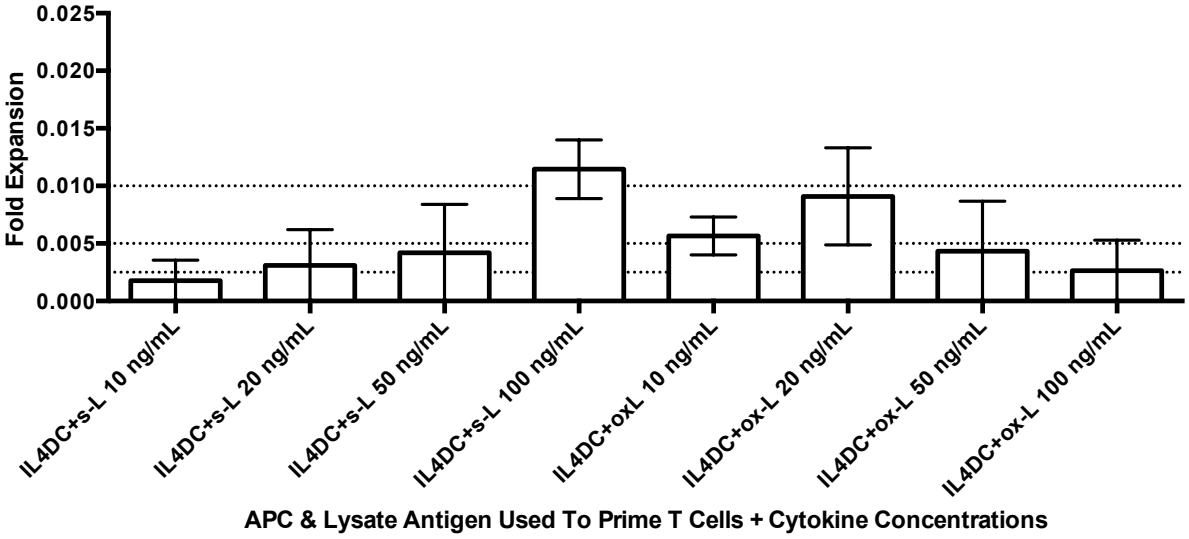
IL-2	2 ng/mL	2 ng/mL	2 ng/mL	2 ng/mL
IL-7	10 ng/mL	20 ng/mL	50 ng/mL	100 ng/mL
IL-15	10 ng/mL	20 ng/mL	50 ng/mL	100 ng/mL
IL-21	10 ng/mL	20 ng/mL	50 ng/mL	100 ng/mL
GM-CSF	5 ng/mL	5 ng/mL	5 ng/mL	5 ng/mL

IL-2 was added for the first 24 to 48 hours only as previous work from our laboratory showed that prolonged IL-2 led to activation induced cell death (AICD)[32]. GM-CSF was also included in the T cell medium as GM-CSF-differentiated DCs can die when GM-CSF is removed from the culture medium[33], therefore we wished to eliminate GM-CSF-starvation-induced DC death as a confounding variable. Also GM-CSF has been used successfully as an adjunct in human trials of immunotherapy for cancer due to its effects on DCs which skew T cells toward Th1 responses[34–36].

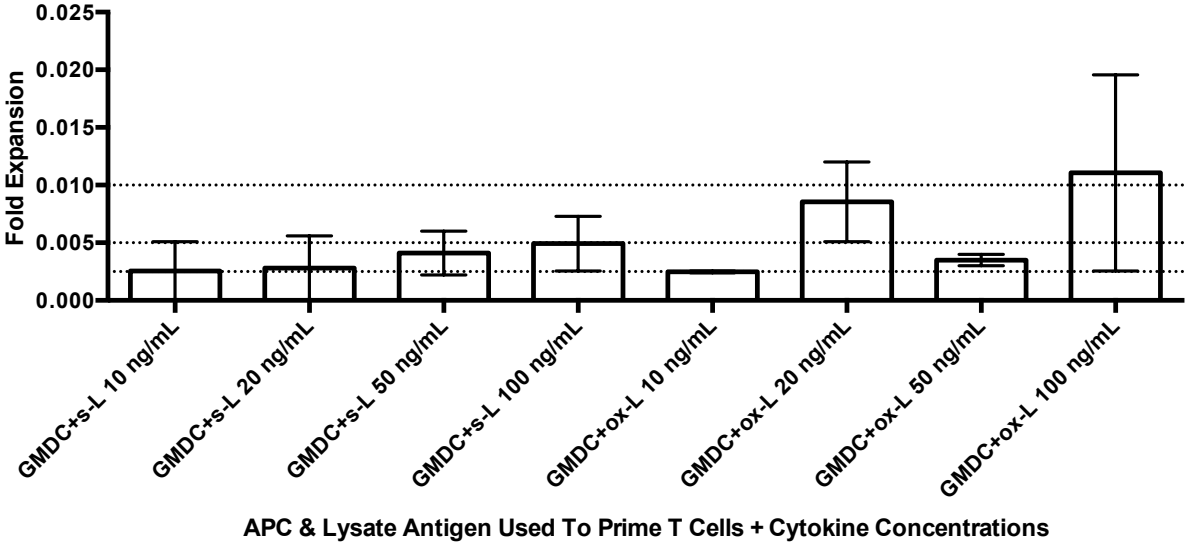
No improvement in fold expansion was observed with any of these cytokines (Supplementary Figures 24, 25). The "fold expansion" was far too low to be considered fold expansion *per se*, but slight, dose-dependent improvements in viability were observed.

The 100 ng/mL of IL-7, IL-15 and IL-21 was the only dose that trended toward an improvement in FE/viability for CD8+ T cells primed by IL4DC pulsed with s-L compared with 10 ng/mL (Supplementary Figure 24). There was no concentration-dependent improvement in FE/viability for T cells primed by IL4DC loaded with ox-L.

The "fold expansion" doubled from 0.002 at the 10 ng/mL dose to 0.005 for the 100 ng/mL dose in sFTL-loaded GMDC-primed CD8+ T cells. For T cells primed by GMDC loaded with HOCl-wFTL 100 ng/mL of these cytokines also generated the highest FE/viability (0.011 FE compared with 0.0025 FE for 10 ng/mL) (Supplementary Figure 25). However this data had a very wide response range (0.003 to 0.02). We continued with the 100 ng/mL dose in order to maximize cell viability.



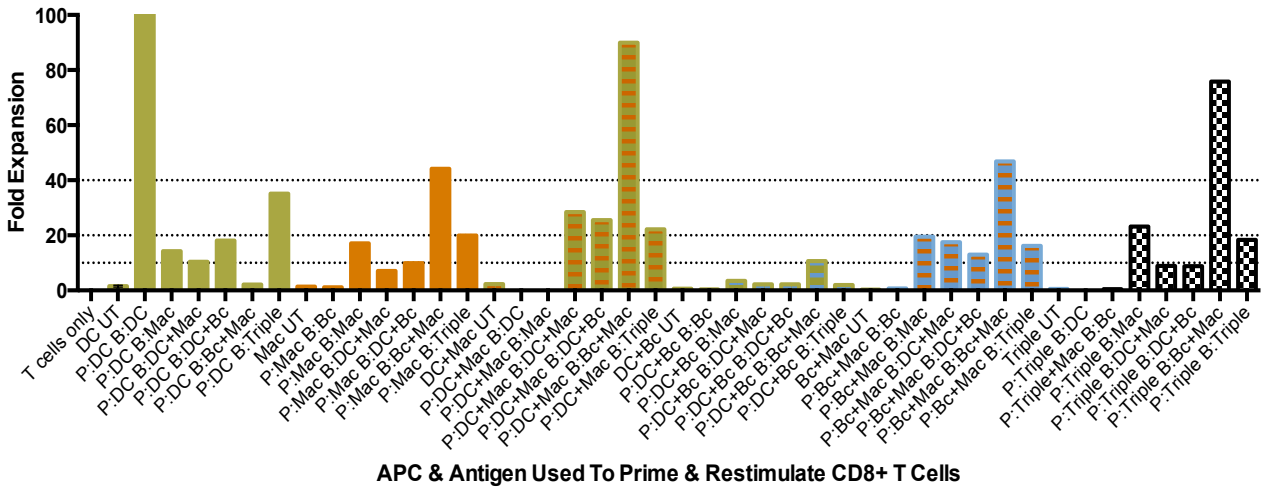
Supplementary Figure 24 CD8+ T cells primed by IL4DCs loaded with soluble lysate show no concentration-dependent improvements in viability when cultured for 10 days with IL7, IL-15 and IL-21. IL4DCs were prepared as previously described and pulsed overnight with s-L and ox-L (1:1 ratio tumour cell:APC) and LPS+CpG. The following day CD8+ cells were isolated from spleens of OT-I mice by AutoMACS Pro negative selection bead separation and added to the lysate-pulsed APCs at a ratio of 1 T cell:10 APCs. After 10 days live cells were counted by Trypan Blue exclusion. Results were graphed in Prism. Summary data of T cell fold expansion after days co-culture with s-L and ox-L-loaded APCs. Results of three independent experiments. No statistically significant differences were calculated. Error bars = mean ± s.e.m.



Supplementary Figure 25 CD8+ T cells primed by GMDCs loaded with s-L and ox-L show no concentration-dependent improvements in viability when cultured for 10 days with IL7, IL-15 and IL-21. GMDCs were prepared as previously described and pulsed overnight with s-L and ox-L (1:1 ratio tumour cell:APC) and LPS+CpG. The following day CD8+ cells were isolated from spleens of OT-I mice by AutoMACS Pro negative selection bead separation and added to the lysate-pulsed APCs at a ratio of 1 T cell:10 APCs. After 10 days live cells were counted by Trypan Blue exclusion. Results were graphed in Prism. Summary data of T cell fold expansion after days co-culture with s-L and ox-L-loaded APCs. Results of three independent experiments. No statistically significant differences were calculated. Error bars = mean ± s.e.m.

7.26 The Combination of B Cell+MΦ Does Not Induce Greater Fold Expansion of CD8+ T Cells Than GMDC Alone When Restimulating OVA-Primed Cells

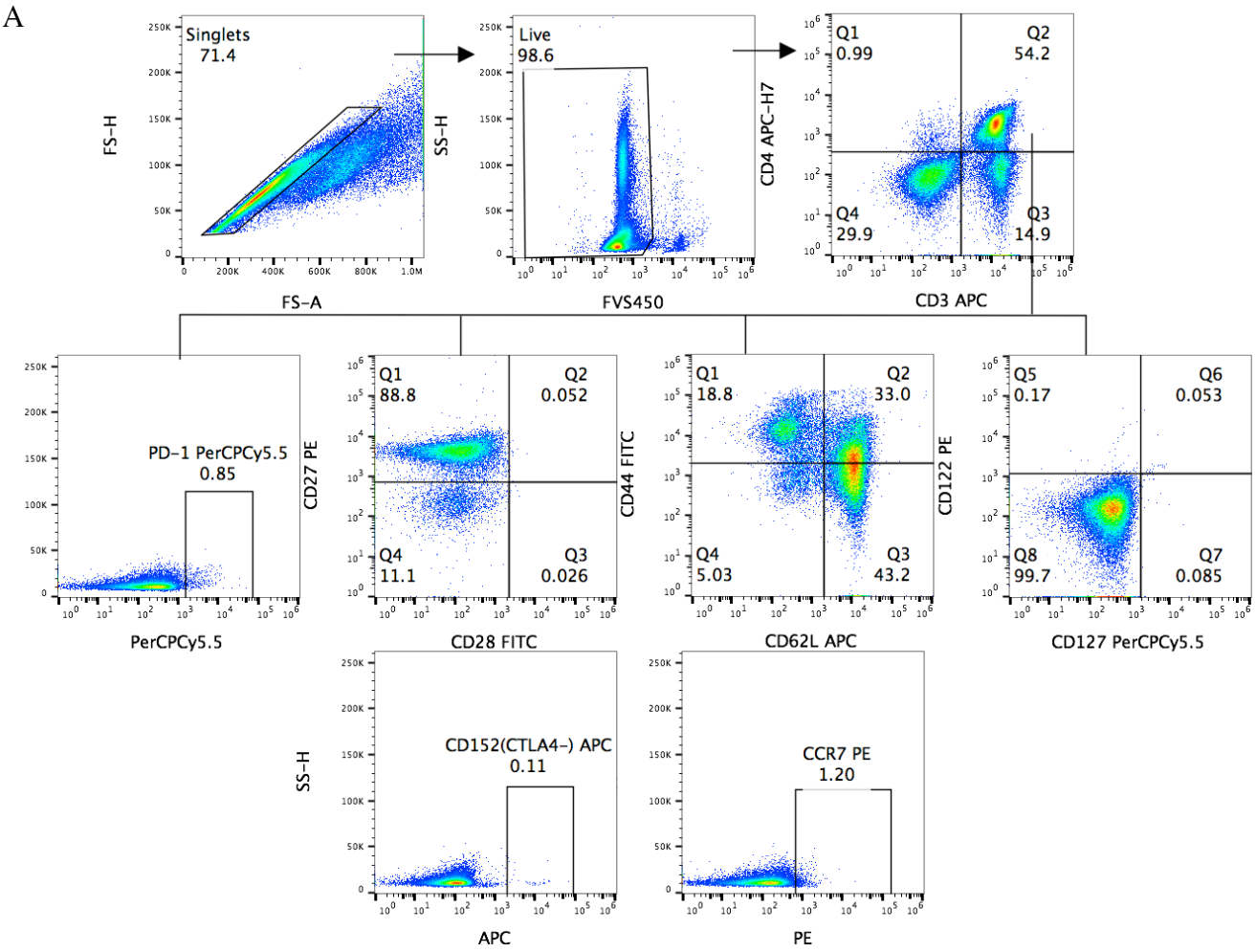
We attempted to re-stimulate OVA-primed CD8+ T cells with the same or different APC(s). In our hands OVA-primed CD8+ T cell proliferation was variable, and usually less than that observed by others in our lab and in other groups. Therefore limited data also exists for OVA-primed and re-stimulated CD8+ T cells (Supplementary Figure 26). In contrast to the oxidised lysate-primed and re-stimulated CD8+ T cells, the combination of B cell+MΦ during re-stimulation resulted in an enhanced fold expansion of OVA-primed and restimulated CD8+ T cells, irrespective of which APC(s) had primed the T cells. This was the case in every group except the GMDC-primed T cells, which saw the greatest fold expansion in cells primed and restimulated with a GMDC. Conversely, CD8+ T cells primed by a GMDC presenting whole OVA protein and restimulated by the Bc+MΦ combination presenting OVA protein resulted in the lowest fold expansion. In the OVA-primed CD8+ T cells priming with a GMDC and restimulating with a GMDC yielded the greatest fold expansion. In no group was restimulation with the Bc+MΦ combination able to induce greater fold expansion than restimulation with a GMDC alone. However this experiment was only successfully conducted once and thus this data remains preliminary.



Supplementary Figure 26 Irrespective of which APC(s) are used to prime CD8+ T cells, the combination of B cell+MΦ has a positive effect on the expansion of restimulated CD8+ T cells, except where T cells have been primed by an OVA-pulsed GMDC. GMDC, M1 MΦs and B cells were prepared as previously described. 5.0 x 10^5 individual APCs, or combinations thereof, were pulsed overnight with whole OVA protein (50 µg/mL), and LPS+CpG. The following day CD8+ T cells were isolated by magnetic bead negative selection and added to APCs (10:1 Tcell:APC). APCs and T cells were co-cultured for 10 days. T cells were counted and re-stimulated with OVA-pulsed APCs as indicated on the x-axis. Cells were cultured for a further 10 days and fold expansion assessed by counting live cells under Trypan Blue dead cell exclusion staining. Results were graphed in Prism. Summary data of 1 experiment..

7.27 Gating Strategy for Assessment of T Cell Phenotype

A



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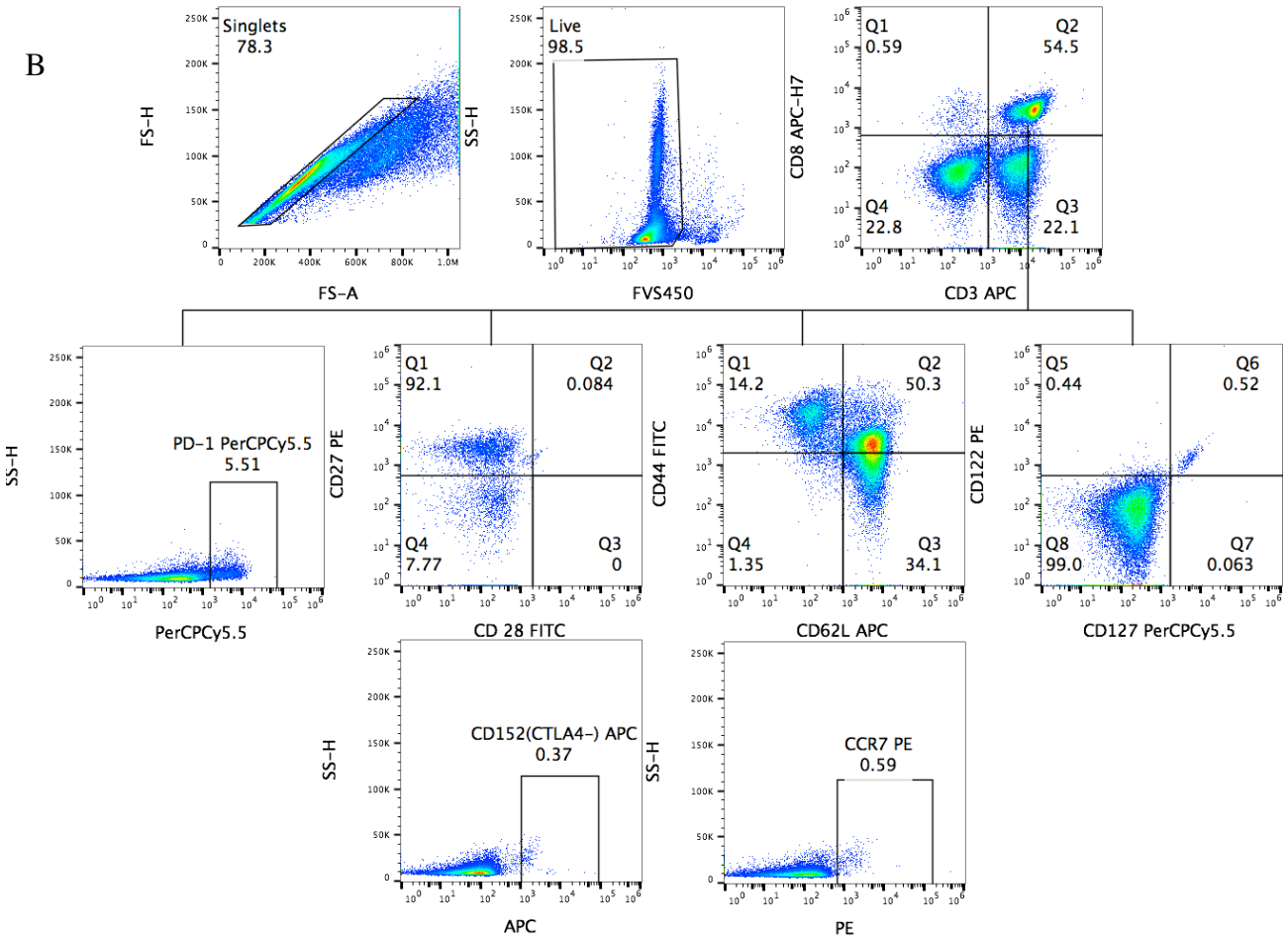
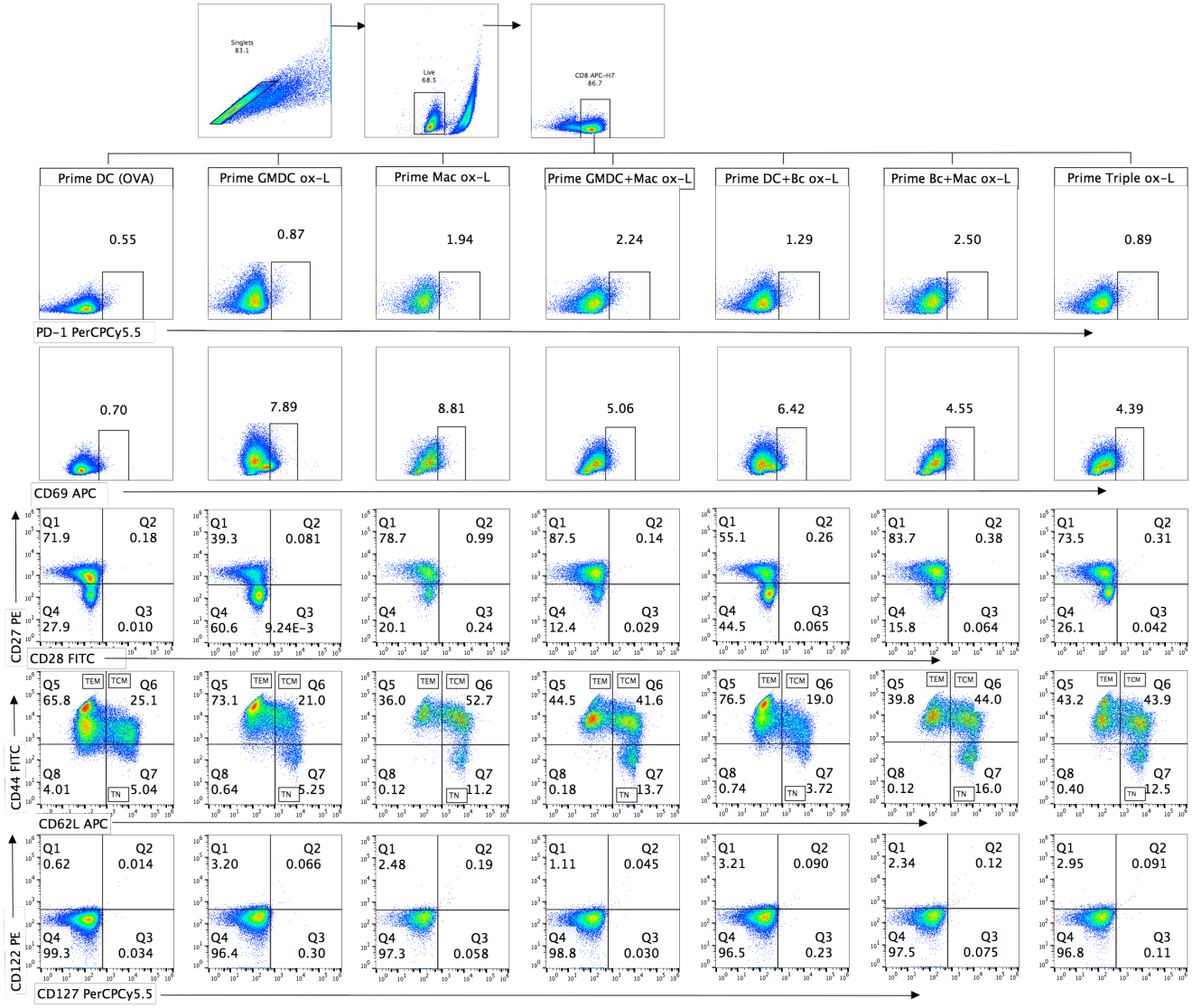


Supplementary Figure 27 Flow cytometry gating strategy for assessment of phenotype in naïve CD4+ and CD8+ T cells. A) CD4+ and **B**) CD8+ T cells were isolated by negative selection magnetic beads (AutoMACS Pro), labeled with live cell exclusion dye and incubated with mABs against the surface markers CD8, CD27, CD28, CD44, CD62L, CD69, CD122 and CD127. Cells were fixed in 4% paraformaldehyde, stored overnight at 4°C and acquired the next day on a Gallios Flow Cytometer. Data was analysed and graphed in FlowJo Version X. The quadrant markers for bivariate dot plots were set based on unstained autofluorescence controls and verified with single stain control cells and Fluorescence Minus One controls. Representative data from 15 independent experiments.

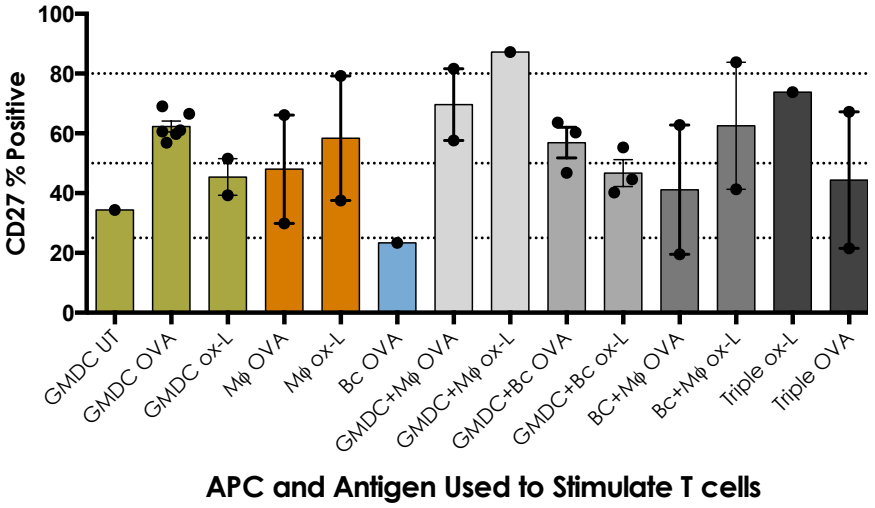
7.28 Gating Strategy and Raw Data of Day 10 Primed CD8+ T Cell Phenotype

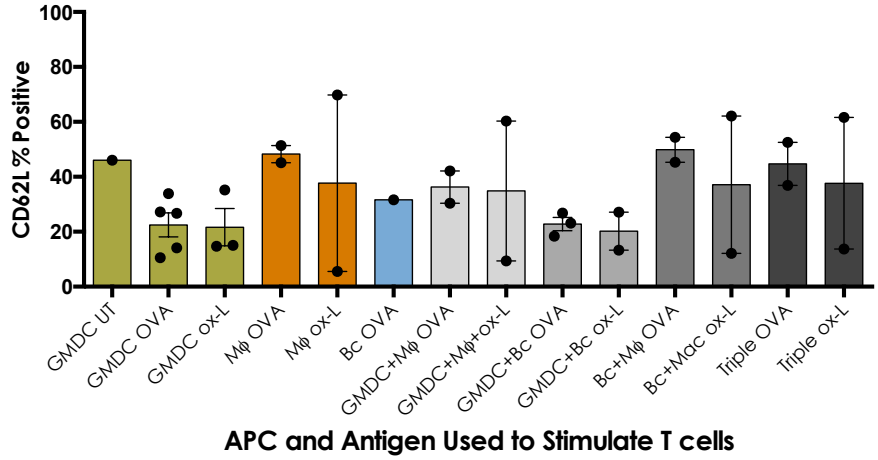


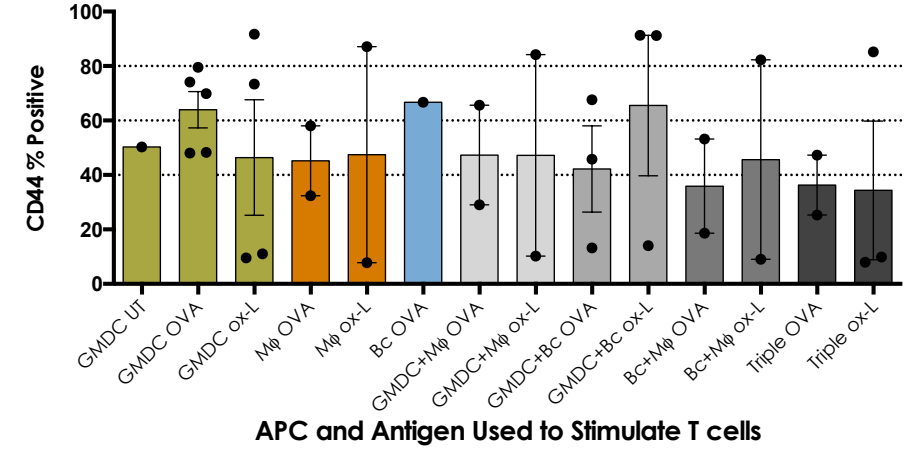
Supplementary Figure 28 Sample raw data of Day 10 oxidised lysate-primed CD8+ T cells. Day 6 GMDC and freshly isolated B cells were prepared as previously described. 1 x 10^5 GMDC or 0.5 x 10^5 GMDC and 0.5 x 10^5 B cells were pulsed overnight with whole OVA protein (50 µg/mL) and B16.OVA oxidised lysate (1:1 ratio tumor cell to APC) plus LPS&CpG. The following day freshly isolated OT-II and OT-I splenocytes were labeled with CD4 and CD 8 negative selection bead cocktails respectively and sorted by AutoMACS Pro and plated with lysate-loaded APCs (10:1 ratio, T cell to APC). APCs and T cells were co-cultured for 10 days with fresh media and cytokines being replaced every 2 days. The resultant CD8+ T cells were stained with dead cell exclusion dye (FVS450) and labeled with mABs against the surface molecules CD3, CD8, CD27, CD28, CD44, CD122, CD127, PD-1, CD62L and CD69. Labeled cells were fixed in 4% PFA, stored overnight at 4°C and collected the following day on a Gallios Flow Cytometer. Data was analysed and graphed in FlowJo VX Software. Representative data of 7 independent experiments.

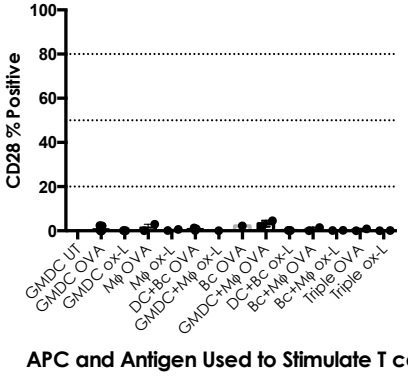
7.29 Phenotype of T Cells Primed with Lysate-loaded APCs

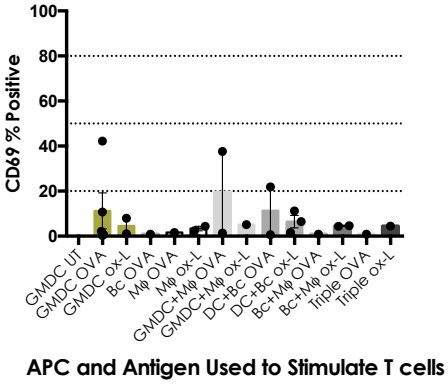
Wherever possible within the liminations of this technically challenging assay we assessed the phenotype of T cells primed with lysate tumour antigens. Supplementary Figure 29 shows the summary data that could be obtained. While results for OVA-primed T cells could be obtained, despite methodology modifications, sufficient live lysate-primed T cells for phenotyping were not always available at Day 10. Lysate-primed CD8+ T cells were consistently negative for CD28, PD-1, CD122 and CD127 We observed up to 13% expression of CD127 on naïve CD8+ T cells and we compared two different anti-CD127 antibodies on different fluorophores. Therefore it does not appear that the low expression of CD127 on lysate-primed T cells was due to issues with the antibody. We also compared three different antibodies conjugated to two different fluorophores for CD28 to eliminate antibody binding as the issue for the non-existent CD28. Other students in our lab have experienced the same issues with CD122 expression therefore, once again we were satisfied that the problem was not with the antibody. Due to the lack of data and variability in results insufficient data is available draw robust conclusions.

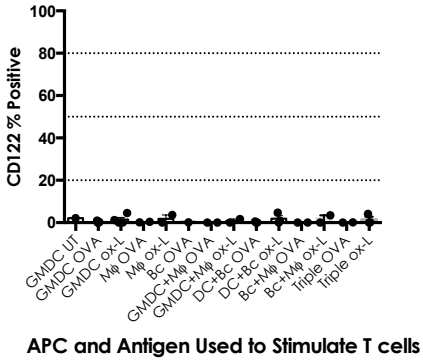


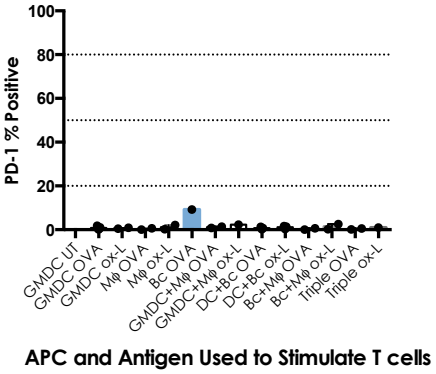


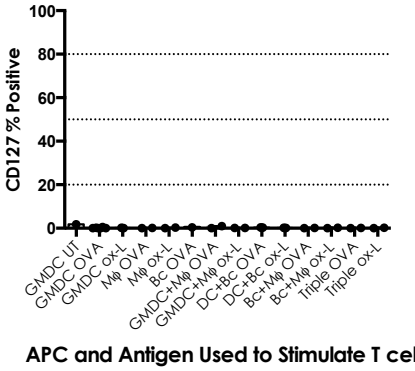








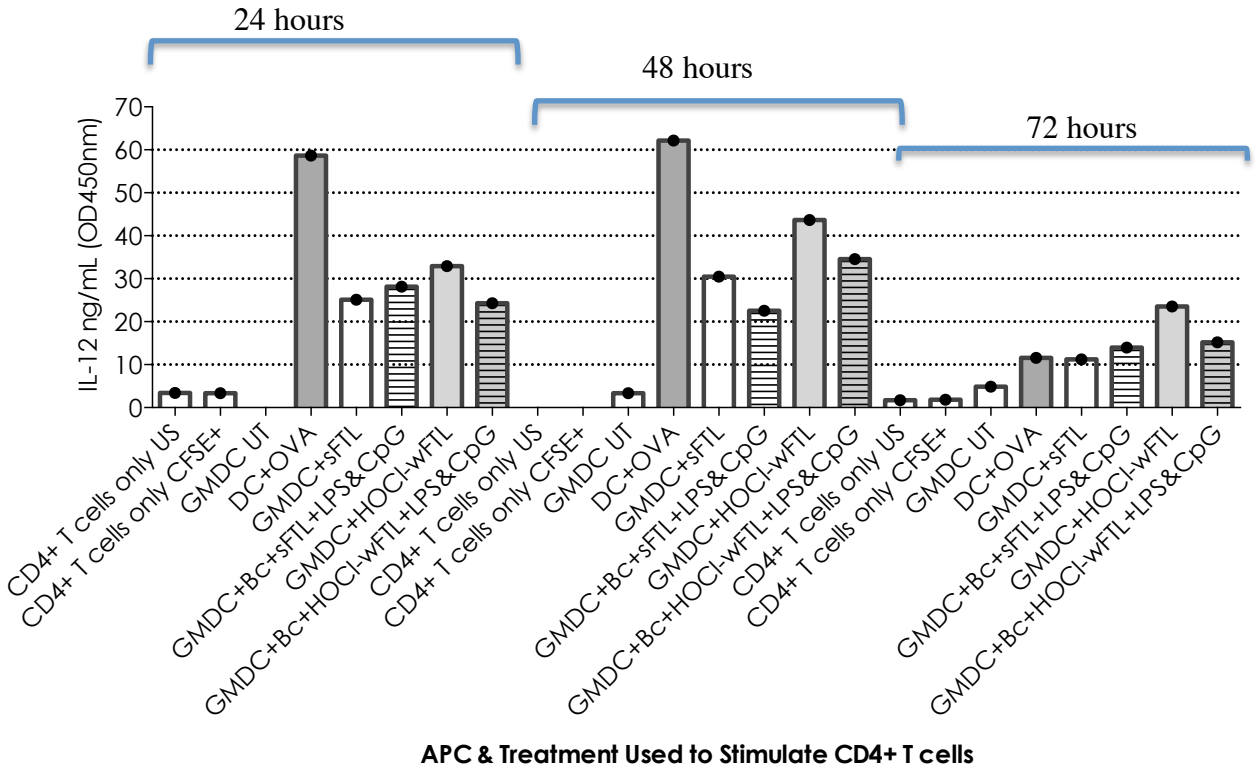




Supplementary Figure 29 Day 10 CD8+ T cells primed by oxidized lysate-loaded APCs do not express CD28, PD-1, CD122 or CD127. GMDC, M1 MΦs and spleen-derived B cells, or combinations thereof, were prepared as previously described and pulsed overnight with whole OVA protein (50 µg/mL), B16.OVA ox-L (1:1 tumour cell:APC) and LPS+CpG. The following day CD8+ T cells were isolated by magnetic bead negative selection (AutoMACS Pro), co-cultured with APCs for 10 days and then harvested for phenotype analysis. Cells were stained with dead cell exclusion dye and labeled with mABs against the surface molecules CD8, CD27, CD28, CD44, CD69, CD122, CD127, CD62L and PD-1. Cells were fixed with 4% paraformaldehyde, stored overnight at 4°C and acquired on a Gallios Flow Cytometer the following day. Data was analysed in FlowJo Version X and results graphed in Prism . Summary data of 2 - 8 independent experiments.

7.30 Optimal IL-12 Levels Are Observed at 24-48 hours

Lysate-loaded APCs and CD4+ or CD8+ T cells were co-cultured for 72 hours and conditioned cell media removed for anti-IL-12 ELISA assays at 24, 48 and 72 hours. Supplementary Figure 30 shows summary data of one set of data from one representative experiment taken at 24, 48 and 72 hour time points, demonstrating the elevated IL-12 levels at 24, 48 and 72 hours.



Supplementary Figure 30 Optimal IL-12 levels are observed at 24-48 hours of APC-T cell co-cultures Day 6 GMDC, Day10 M1 MΦs and freshly isolated splenic B cells, or combinations thereof, were pulsed overnight with whole OVA protein (50 μg/mL) and sFTL or Ox-L (1:1 ratio tumor cell to APC). LPS (1 μg/mL) and CpG (0.3 μg/mL) were added at the same time as the lysates. The following morning, CFSE-labeled CD4+ or CD8+ T cells were added (10:1 ratio, T cell to APC). APCs and T cells were co-cultured for 72 hours and conditioned cell media removed at 24, 48 and 72 hours. Supernatants were stored at -20°C for assessment by anti-IL-12 ELISA. Results were analysed in Excel and graphed in Prism. .Summary data of 1 experiment showing representative results from multiple experiments.

7.31 *In Vitro* Cytotoxicity

DCs loaded with freeze-thaw lysate of medullary thyroid carcinoma have demonstrated tumour cell cytotoxicity after *in vitro* restimulation[37]. Having demonstrated that combinations of GMDC+B cell can stimulate improved CD8+ T cell proliferation and IFN-γ production we evaluated whether or not combined APCs were superior to GMDCs in their capacity to generate *in vitro* cytotoxicity using B16.OVA tumour lysate as the source of tumour antigen.

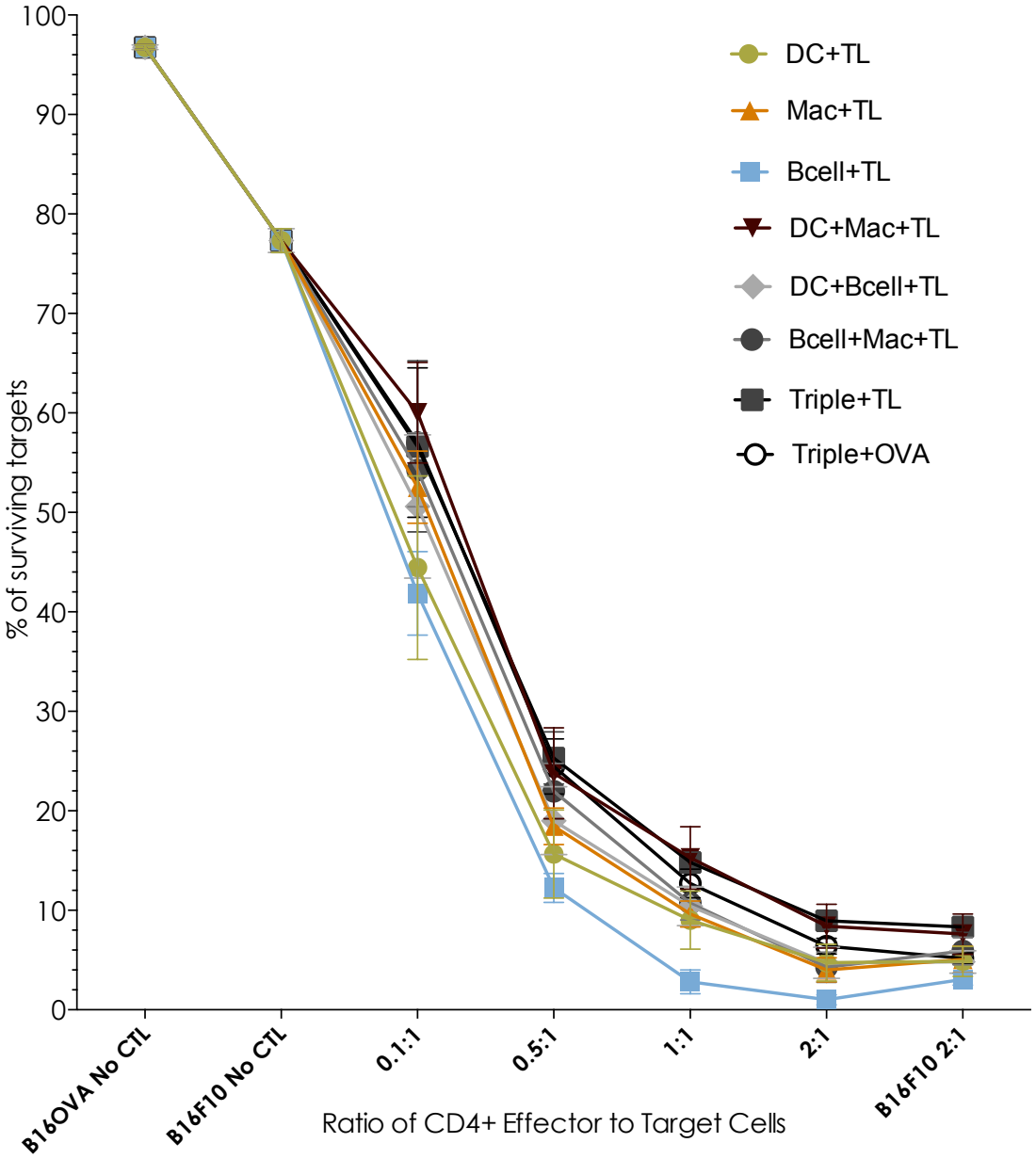
Effector T cells were generated by co-culture with soluble lysate-loaded APCs. These experiments were conducted prior to the work with oxidised lysate and were not repeated with oxidised lysate. Target B16.OVA cells were stained with VPD450 and allowed to form a monolayer prior to the addition of the effector T cells.

In B16.OVA tumor samples with no added cytotoxic T lymphocytes (CTLs) the survival ranged from 96 – 97% across all samples, as expected (Supplementary Figures 31, 32). Each group of lysate-primed CD4+ and CD8+ effector T cells demonstrated ratio-dependent increases in cytotoxicity as the ratio of effector cells to tumor cells was increased from 0.1:1 to 2:1.

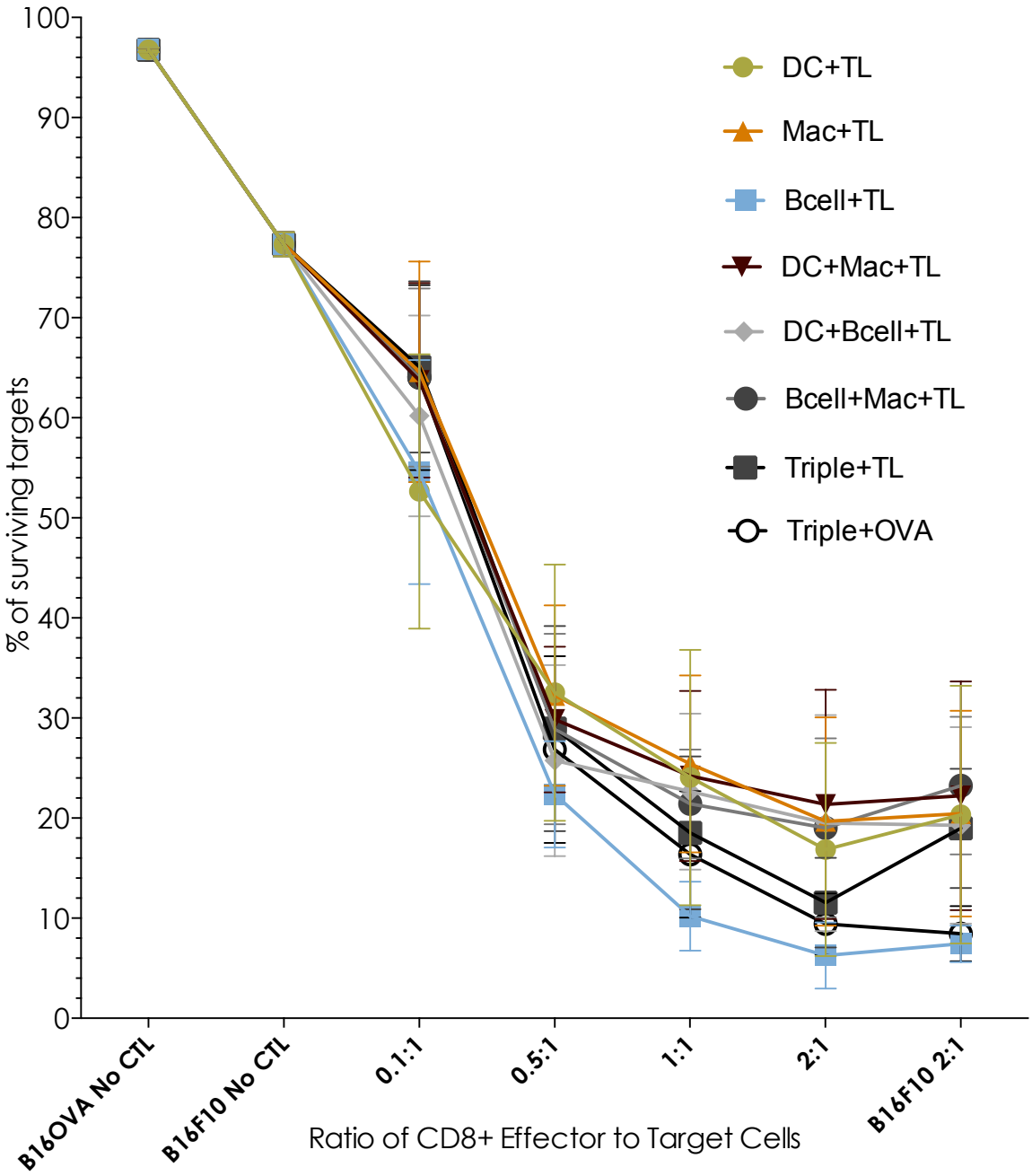
In the CD4+ T cell tests maximal killing (1% survival; range 0.5%-2%) was observed in the B cell-primed samples while 5% survival was observed in GMDC cell-primed samples (range 1%-7%). No differences were observed between the MΦ, DC+MΦ, DC+B cell, Bc+MΦ or groups (4%, 5%, 5% and 4% respectively). The Triple Combination survival was the highest on 9%. The differences between B cell-primed T cell killing and the DC+MΦ and Triple combination groups were statistically significant (p=0.013 and 0.015 respectively). Differences were assessed by negative bionomial regression that accounted for Poisson over-dispersion.

B cell-primed cells also generated maximal cytotoxicity in CD8+ T cells (6% survival). This was significantly lower than GMDC alone (17% survival; p=0.018). Once again no difference was observed between MΦ-primed T cells (20% survival) or the double combination groups of DC+MΦ, DC+Bc and B cell+MΦ (21%, 20% and 19% respectively). And as with the CD4+ T cells survival in the Triple combination group was lower than in the double combinations on 12%. In the MΦ and combination-primed groups killing was significantly lower than that achieved by B cell-primed T cells (p=0.003 or less).

In these assays lysis of B16.F10 cells was compared as a control against non-specific lysis. However the level of B16.F10 killing was virtually identical to that of B16.OVA cells suggesting that either the B16.F10 cells were incorrectly labeled, or the lysis observed was non-specific.



Supplementary Figure 31 B cells presenting lysate antigens demonstrate a trend toward inducing increased in vitro cytotoxicity in CD4+ T cells compared with GMDCs. Day 6 GMDCs, Day 10 M1 MΦs and splenic B cells (or combinations thereof) were pulsed overnight with freeze-thaw lysate and co-cultured for 72 hours with OT-II T cells. B16.OVA cells were labelled with VPD450 proliferation dye, and allowed to form a monolayer before the effector T cells were added to the tumor cells for 6 hours. The cells were harvested, stained with propidium iodide and analysed by Flow Cytometry. Alternatively cells were stained with dead cell exclusion dye**,** fixed in 2% paraformaldehyde and stored overnight at 4°C prior to collection on a Gallios Flow Cytometer. Data was analysed on FlowJo Version 9 and graphed in Prism**.** Statistically significant differences were calculated using negative binomal regression. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 as compared to B cell+TL. Error bars = mean with s.e.m. of 3 independent experiments.



Supplementary Figure 32 B cells presenting lysate antigens demonstrate a trend toward inducing increased in vitro cytotoxicity in CD8+ T cells. Day 6 GMDCs, Day 10 M1 MΦs and splenic B cells (or combinations thereof) were pulsed overnight with freeze-thaw lysate and co-cultured for 72 hours with OT-I T cells. B16.OVA cells were labelled with VPD450 proliferation dye, and allowed to form a monolayer before the effector T cells were added to the tumor cells for 6 hours. The cells were harvested, stained with propidium iodide and analysed by Flow Cytometry. Alternatively cells were stained with dead cell exclusion dye, fixed in 2% paraformaldehyde and stored overnight at 4°C prior to collection on a Gallios Flow Cytometer. Data was analysed on FlowJo Version 9 and graphed in Prism. Statistically significant differences were calculated using negative binomal regression. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 as compared to B cell+TL. Error bars = mean with s.e.m. of 3 independent experiments.

7.31.1 Discussion of *In Vitro* Cytotoxicity

We attempted to assess the *in vitro* cytotoxic capacity of unsorted splenocytes primed with DCs, MΦs B cells and combinations of these three APCs using the VITAL assay. These experiments were conducted in the first year of this thesis, graphed and and analysed at that time. Re-analysis of these experiments immediately prior to submission of this thesis revealed that these experiments had not been conducted according to the methods described by Hermans *et al*[38]. Hermans *et al* plated peptide-loaded and control cells in the same well. We plated B16.OVA targets separately to B16F10 controls. Thus the analysis employed by Hermans had to be modified accordingly. The results graphed in this thesis are the percentages of surviving cells, as opposed to the adjusted specific lysis calculated in the Hermans method. Thus these data ideally needs to be repeated according to the Hermans method so that specific lysis can be calculated. Nonetheless distinct ratio-specific responses were observed and significant levels of cytotoxicity were achieved with the lysate-primed cells. Interestingly, in both the CD4+ and CD8+ T cell groups the greatest cytotoxicity was achieved by soluble lysate-loaded B cell-primed T cells.

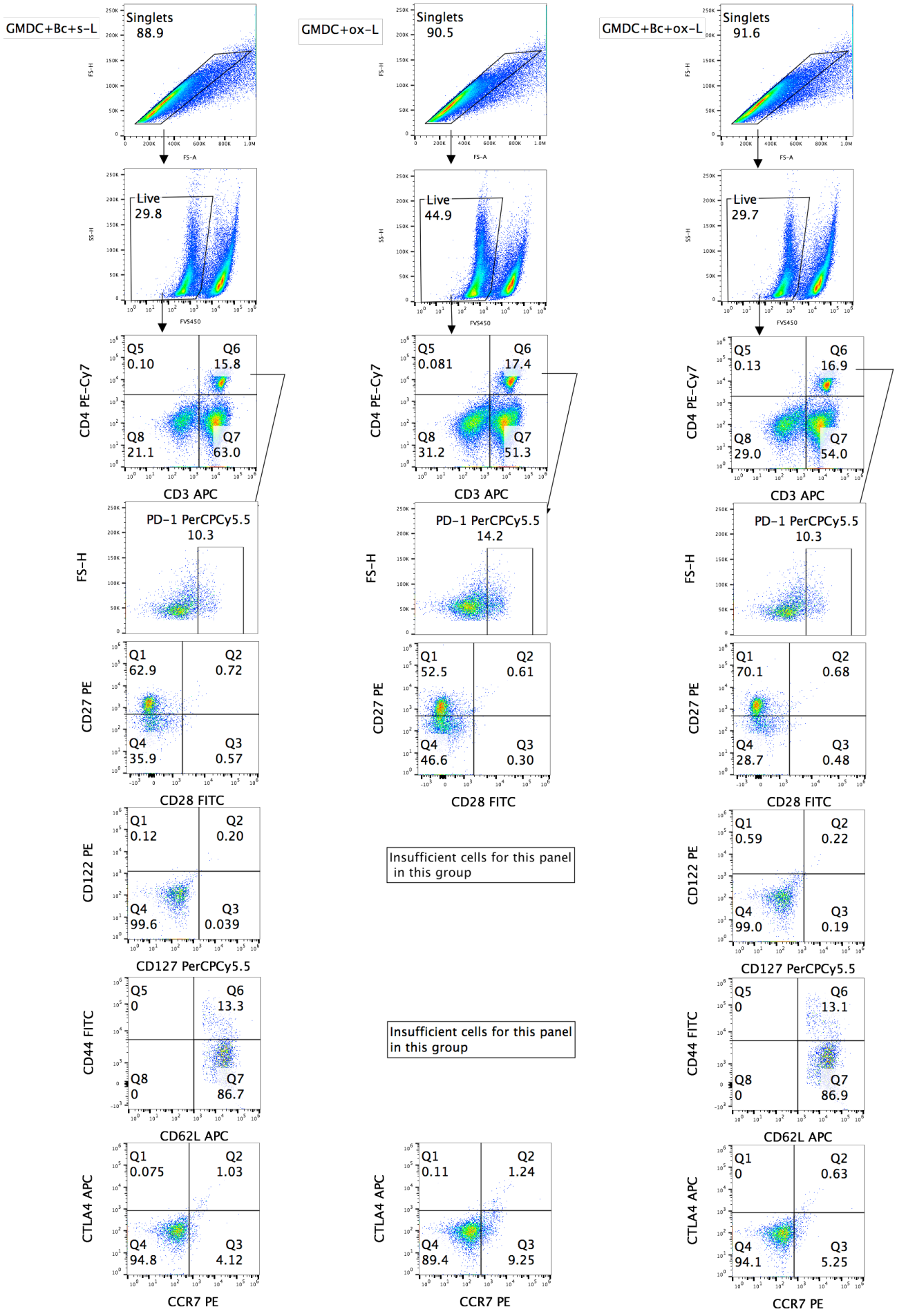
These assays were carried out using unactivated APCs, prior to our experiments with activation stimuli or oxidized lysate. They have not been conducted with activated or oxidised lysate-loaded APCs. While interesting, it is redundant in light of the *in vivo* cytotoxicity results to assess whether greater killing is achieved by activated, oxidised lysate-loaded GMDC+B cells.

We initially followed the method developed by Hermans *et al* that employs propidium iodide (PI) to assess the level of tumor cell killing by cytotoxic T cells[38]. While conducting these assays we experimented with using a dead cell exclusion dye and fixing the cells prior to analysis on a Flow Cytometer. This proved to be a more useful method than analysing cell death with PI, which requires flow cytometric analysis within the hour and is not practical on large sample sizes. The new method makes the assay more flexible as fixed cells can be stored for analysis at a later time point. This improved method reduces artefacts that may be introduced by the delay in harvesting tumour targets in the final wells compared to the initial wells when analysing high numbers of samples. It also reduces artefacts introduced by the delay in collecting cells which have PI left sitting on them for longer periods than the first tubes. The cells can be stained almost simultaneously with dead cell exclusion dye, as opposed to the PI method where PI must be added only shortly prior to collection to avoid false positives.

In CD4+ and CD8+ T cells we observed the same degree of killing of the B16F10 negative control cells as was observed in the 2:1 CTL:tumour cell ratio groups. This is indicative of non-specific cell killing, which may be cytokine mediated.

A limitation of the VITAL Assay includes the fact that, in contrast to the method described by Hermans in which only T cells were added to the tumour targets, we were not able to isolate T cells from T cell+APC co-culture wells. We merely harvested all non-adherent cells and added them to the tumour monolayers. For this reason, and the fact that the assays were conducted using unsorted splenocytes rather than isolated T cells, our T cell numbers will be less accurate. This may explain the wide error bars in these data, as well as the minimal differences in killing between groups. An improved method, in addition to using isolated T cells instead of unsorted splenocytes, may be to use magnetic beads that attach to DCs, B cells and MΦs but not T cells. If the plate were rested on a magnetic block during T cell harvest, this would reduce the number of non-T cells collected and added to the tumour targets. While these results show the same trend as that of Hermans T cell only method, namely increased cytotoxicity with increasing CTL:tumor ratio these data do not support antigen-specific killing by the lysate-loaded T cells.

7.32 Raw Data for Lysate-Primed CD4+ T Cells Used in *In Vivo* Cytotoxicity



Supplementary Figure 33 Gating strategy and raw data showing phenotypes of lLysate-primed CD4+ T cells used in *In Vivo* cytotoxicity assays. Day 6 C57BL/6 GMDC, +/- spleen-derived B cells, were pulsed overnight with whole OVA protein (50 μg/mL) and soluble lysate or oxidised lysate (1:1 ratio tumor cell to APC)+LPS (1 μg/mL) & CpG (0.3 μg/mL).. The following day CD4+ and CD8+ T cells were added to the APCs (10:1) and co-cultured for 3 or 4 days. Cells were harvested for injection into CD57BL/6 mice and surplus cells were analysed for phenotype by Flow Cytometry. Cells were labeled with FVS450 (BD Biosciences) dead cell exclusion dye and mABs against CD3, CD4, CD8, CCR7, CD154 (CTLA4), CD27, CD28, CD44, CD62L, CD122, CD127 and PD-1. Cells were fixed in 4% paraformaldehyde and stored at 4°C overnight prior to collection on a Gallios. Data shown is from 1 of 2 experiments. A) T cells primed with GMDC+B cell+soluble lysate B) T cells primed with GMDC+oxidised lysate C) T cells primed with GMDC+B cell +oxidised lysate.

7.33 Summary Data of Effector T Cell Phenotypes Used In Vivo Cytotoxicity Assays

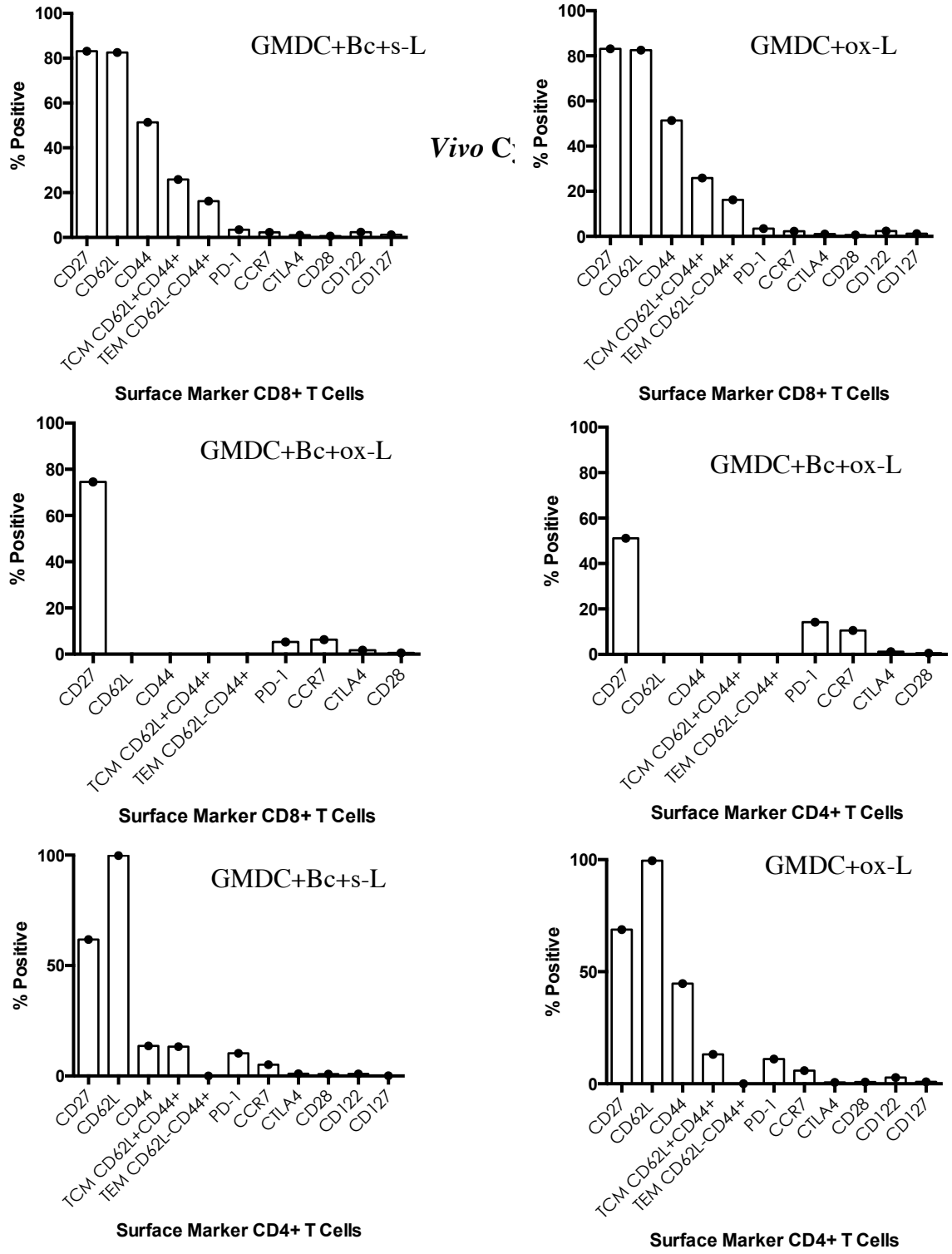
No difference was observed in the T_{CM} (CD62L+CD44+) subset between T cells primed with GMDC+Bc+soluble lysate and GMDC+Bc+oxidised lysate (26 and 29% respectively) (Supplementary Figure 34). Minor differences were observed in the percentage of cells displaying a T_{EM} phenotype (GMDC+Bc+soluble lysate: 15%; GMDC+Bc+oxidised lysate: 22%) suggestive of different responses to the two lysates. However no conclusions can be drawn from one experiment. Likewise the small differences in percentages of naïve or T stem cell memory phenotypes (GMDC+Bc+soluble lysate: 57%; GMDC+Bc+oxidised lysate: 48%) remain as one off observations.

Small increases were observed in the percentages of both CD8+ and CD4+ T cells positive for CCR7 when primed with GMDC+oxidised lysate. CD8+ T cells primed with GMDC+B cell+soluble lysate were 2.3% positive for CCR7, 3.3% positive when primed with GMDC+B cell+oxidised lysate and 6.3% positive when primed with GMDC+oxidised lysate. CD4+ T cells demonstrated the same pattern with an approximate doubling of CCR7 positivity in the GMDC+oxidised lysate group (GMDC+B cell+soluble lysate: 5.35%; GMDC+B cell+oxidised lysate: 5.85%; GMDC+oxidised lysate: 10.5%).

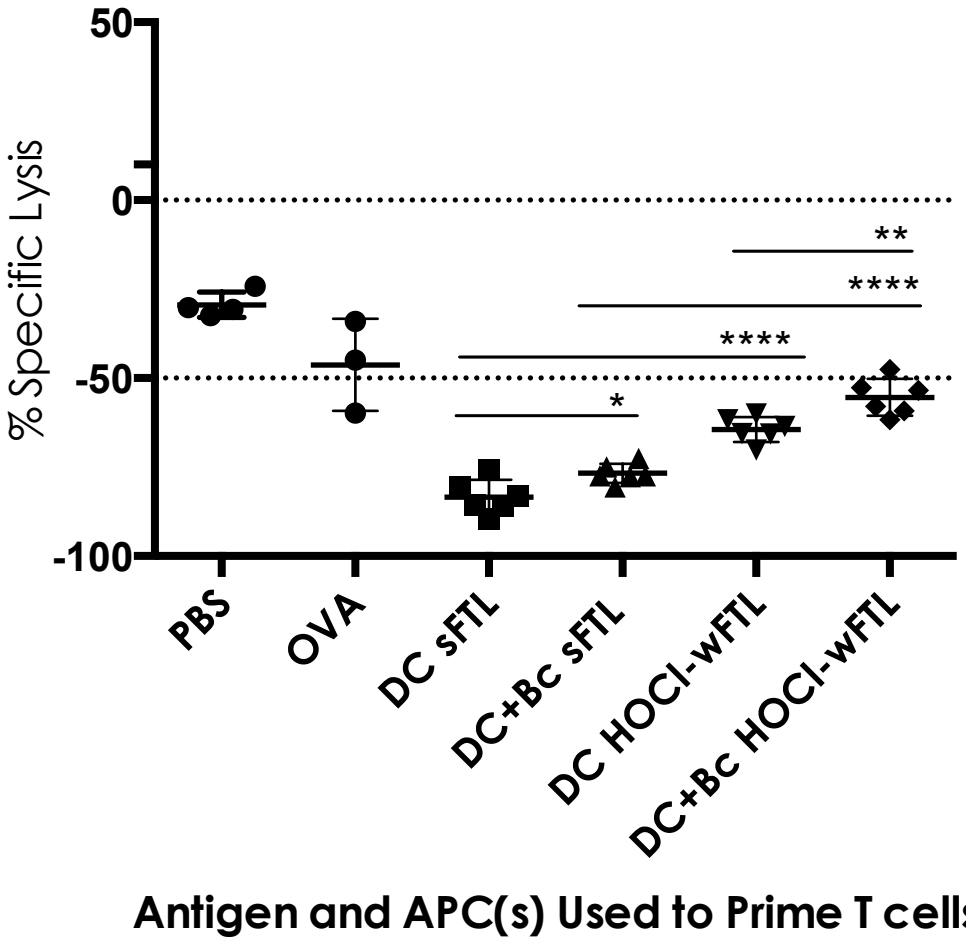
CD27 and CD44 expression was lower on CD4+ T cells than CD8+ T cells and consistent with previous experiments all cells exhibited high expression of CD62L.

CTLA4 percentages were less than 2% across all samples, however, once again the GMDC+oxidised lysate group showed an approximate doubling compared to the other two groups. PD-1 was consistently low (5% or less) and CD28, CD122 and CD127 were absent from these cells, as they had been in previous assays.

In the only group that we can directly compare with 10-day lysate-primed cells (GMDC+ox-L) CD44 and CD27 was lower CD62L higher on these 3-day-primed cells. These differences suggest that the cells used in the *in vivo* assays were less differentiated than 10-day-primed cells.



Supplementary Figure 34 Summary phenotype data of CD4+ and CD8+ effector T cells used in in vivo cytotoxicity assays. Day 6 C57BL/6 GMDC, +/- spleen-derived B cells, were pulsed overnight with whole OVA protein (50 μg/mL) and soluble lysate or oxidised lysate (1:1 ratio tumor cell to APC)+LPS (1 μg/mL) & CpG (0.3 μg/mL). The following day CD4+ and CD8+ T cells were added to the APCs (10:1) and co-cultured for 3 or 4 days. Cells were harvested for injection into CD57BL/6 mice and surplus cells were analysed for phenotype by Flow Cytometry. Cells were labeled with FVS450 (BD Biosciences) dead cell exclusion dye and mABs against CD3, CD4, CD8, CCR7, CD154 (CTLA4), CD27, CD28, CD44, CD62L, CD122, CD127 and PD-1. Cells were fixed in 4% paraformaldehyde and stored at 4°C overnight prior to collection on a Gallios. Data shown is from 1 of 2 experiments. A) T cells primed with GMDC+B cell+soluble lysate B) T cells primed with GMDC+oxidised lysate C) T cells primed with GMDC+B cell +oxidised lysate.



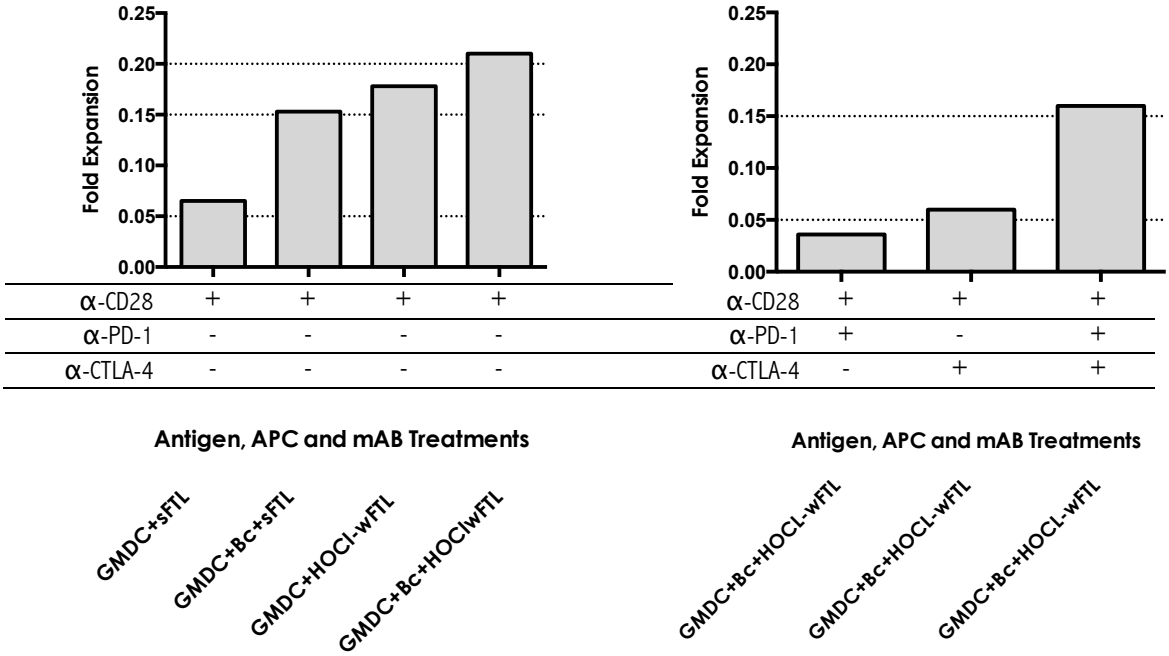
Supplementary Figure 35 No *in vivo* cytotoxicity is observed by CD4+ T cells. Day 6 C57BL/6 GMDC, +/- spleen-derived B cells, were pulsed overnight with whole OVA protein (50 μg/mL) and s-L or ox-L (1:1 ratio tumor cell to APC))+LPS (1 μ g/mL) & CpG (0.3 μ g/mL). The following day CD4+ and CD8+ T cells were added to the APCs (10:1) and co-cultured for 3 or 4 days. Lysate-primed effector T cells were i.v. injected into CD57BL/6 mice. The following day C57BL/6 target splenocytes were incubated with SIINFEKL peptide (1 μg/mL), $OVA_{323-339}$ peptide (5 μg/mL), or left unpulsed. Target cells were labeled 25 μM CFSE ($CFSE^{HI}$), 2.5 μM CFSE ($CFSE^{LO}$) or 10 μM VPD450 and mixed together for injection into recipient mice (1/3 each SIIN-pulsed, $OVA_{323-339}$-pulsed and unpulsed). Legend: each symbol represents a single mouse; (n = 3-7 per group). ** p <0.01, *** p<0.001 and **** P <0.0001 (Student's unpaired t-test). Data are from 2 independent experiments. Error bars = s.e.m.

7.35 *Ex Vivo* Expansion of Tumour Draining Lymph Node Cells

Once a cancer is established T cell control mechanisms have become impaired. The T cells may retain the capacity to recognise the tumour, as demonstrated by *ex vivo* expansion of tumour-specific T cells from patients, however tumor escape mechanisms may gain control over the immune response and in the tumour microenvironment the T cells are rendered ineffective. In the clinic the DC is the APC of choice for restimulating *ex vivo* derived TAA-specific patient T cells. Activation of naïve T cells *in vitro* is achieved more easily than activation of T cells from tumour bearing hosts whose T cells often display markers of exhaustion and are susceptible to AICD. Moreover generation of peptide-specific CTLs from patients is complex, time-consuming and laborious[39]. The isolation of tumour-reactive T cells is not easily achieved in all melanoma patients, nor is their *ex vivo* expansion to therapeutically useful numbers always attainable. TIL therapy can be even more difficult in non-melamona patients[40]. This is one of the reasons for the uses of alternative tumour-reactive T cells such as TCR-modified and CAR T cells. We used a murine model to mimic a clinically relevant scenario in which we obtained cells from the draining LNs of a tumour-bearing host and undertook a preliminary comparison of the ability of GMDC and GMDC+ B cell to restimulate these *ex vivo*-derived cells.

Fold expansion in these experiments was minimal and due to the low numbers of cells obtained from each tumour-bearing moue each condition was only tested once. Therefore no conclusions can be drawn from these results until they are repeated – preferably with improved methods that results in significantly greater fold expansion.

The use of α-PD-1 or α-CTLA4 reduced FE even further compared to cells restimulated without checkpoint inhibitors (Supplementary Figure 36). However once again no conclusions can be drawn from these preliminary observations.



Supplementary Figure 36 Single observations of fold expansion in tumour-draining lymph node cells +/- mABs against PD-1 and CTLA4. Wild type C57BL/6 mice were injected with 50,000 B16.OVA cells and sacrificed when tumours reached 150 mm^2. Tumour draining lymph nodes were excised, single cell suspensions prepared and the cells cryopreserved for future use. Cryopreserved cells were retrieved from liquid nitrogen, thawed and allowed to rest overnight prior to adding to lysate-loaded APCs at a ratio of 10:1. Cells were cultured for 10 days with feeding taking place no less than every other day. Cells were harvested and counted for fold expansion by Trypan Blue exclusion. Sufficient cells were harvested to test one condition per 3-4 tumour-bearing mice therefore each column is the result from 1 of 7 independent experiments.

7.36 Statistical Analysis of In Vivo Cytotoxicity Data

Negative binomial regression, which accounts for an over-dispersed Poisson distribution, was used to compare the number of live SIIN and OVA-$_{323-339}$-pulsed cells that remained after 24 hours *in vivo* in mice that had received adoptively transferred lysate-primed effector T cells. The model included terms for the groups and adjusted for the number of cells collected. Although no adjustment was made for multiple comparisons it should be noted that all the *p* values were <0.000 therefore these results are highly unlikely to be due to chance.

7.36.1 Negative Binomial Regression Analysis of SIINFEKL-Pulsed Target Cell Lysis

```
sum cfsehisiincells

    Variable |       Obs       Mean    Std. Dev.       Min        Max
-------------+--------------------------------------------------------
cfsehisiin~s |        31    812.3871    766.1442        18       2215

. nbreg cfsehisiincells b5.group, exp(unpulsed) irr

Fitting Poisson model:

Iteration 0:   log likelihood = -1173.2933
Iteration 1:   log likelihood = -992.03972
Iteration 2:   log likelihood = -987.97669
Iteration 3:   log likelihood = -987.95935
Iteration 4:   log likelihood = -987.95935

Fitting constant-only model:

Iteration 0:   log likelihood = -238.40137
Iteration 1:   log likelihood = -237.20897
Iteration 2:   log likelihood = -237.20876
Iteration 3:   log likelihood = -237.20876

Fitting full model:

Iteration 0:   log likelihood = -225.17312  (not concave)
Iteration 1:   log likelihood =  -208.9186
Iteration 2:   log likelihood = -197.82067
Iteration 3:   log likelihood = -193.45411
Iteration 4:   log likelihood = -192.85388
Iteration 5:   log likelihood = -192.85283
Iteration 6:   log likelihood = -192.85283


Negative binomial regression      Number of obs      =        31
                                  LR chi2(5)         =     88.71
Dispersion     = mean             Prob > chi2        =    0.0000
Log likelihood = -192.85283       Pseudo R2          =    0.1870

------------------------------------------------------------------------------
cfsehisiin~s |        IRR   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
       group |
           1 |   54.15918   13.54218    15.96   0.000     33.17678    88.41173
           2 |   40.67912   10.17332    14.82   0.000     24.91708    66.41192
           3 |   17.33085   4.339188    11.39   0.000     10.60966     28.3099
           4 |   5.515192   1.387944     6.79   0.000     3.367818    9.031763
           6 |   .9243781   .2616743    -0.28   0.781     .5307503    1.609937
             |
       _cons |   .0094875   .0020018   -22.08   0.000     .0062741    .0143465
ln(unpulsed) |          1  (exposure)
-------------+----------------------------------------------------------------
      /lnalpha |  -2.230603   .2675437                -2.754979   -1.706227
-------------+----------------------------------------------------------------
```

```
      alpha |   .1074636   .0287512                 .0636104    .1815496
------------------------------------------------------------------------------
LR test of alpha=0: chibar2(01) = 1590.21           Prob >= chibar2 = 0.000
```

```
. lincom 1.group-2.group, eform
 ( 1)  [cfsehisiincells]1.group - [cfsehisiincells]2.group = 0(GMDC+sFTL vs GMDC+Bc+sFTL)
------------------------------------------------------------------------------
cfsehisiin~s |      exp(b)   Std. Err.       z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         (1) |   1.331375   .2527206     1.51   0.132     .9177535    1.931412
```

```
. lincom 1.group-3.group, eform
 ( 1)  [cfsehisiincells]1.group - [cfsehisiincells]3.group = 0 (GMDC+sFTL vs GMDC+HOClwFTL)
------------------------------------------------------------------------------
cfsehisiin~s |      exp(b)   Std. Err.       z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         (1) |   3.125016   .5943684     5.99   0.000     2.152566    4.536785
```

```
. lincom 1.group-4.group, eform
 ( 1)  [cfsehisiincells]1.group - [cfsehisiincells]4.group = 0 (GMDC+sFTL vs GMDC+Bc+HOClwFTL)
------------------------------------------------------------------------------
cfsehisiin~s |      exp(b)   Std. Err.       z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         (1) |   9.819999   1.884309    11.91   0.000     6.741845    14.30356
```

```
. lincom 1.group-6.group, eform
 ( 1)  [cfsehisiincells]1.group - [cfsehisiincells]6.group = 0 (GMDC+sFTL vs PBS)
------------------------------------------------------------------------------
cfsehisiin~s |      exp(b)   Std. Err.       z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         (1) |   58.58986    13.5674    17.58   0.000     37.2146    92.24259
```

```
. lincom 2.group-3.group, eform
 ( 1)  [cfsehisiincells]2.group - [cfsehisiincells]3.group = 0 (GMDC+Bc+sFTL vs GMDC+HOClwFTL)
------------------------------------------------------------------------------
cfsehisiin~s |      exp(b)   Std. Err.       z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         (1) |   2.347209   .4465646     4.48   0.000     1.61662    3.407971
```

```
. lincom 2.group-4.group, eform
 ( 1)  [cfsehisiincells]2.group - [cfsehisiincells]4.group = 0 (GMDC+Bc+sFTL vs GMDC+Bc+HOClwFTL)
------------------------------------------------------------------------------
cfsehisiin~s |      exp(b)   Std. Err.       z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         (1) |   7.375831   1.415723    10.41   0.000     5.063265    10.74463
```

```
. lincom 2.group-6.group, eform
 ( 1)  [cfsehisiincells]2.group - [cfsehisiincells]6.group = 0 (GMDC+Bc+sFTL vs PBS)
------------------------------------------------------------------------------
cfsehisiin~s |      exp(b)   Std. Err.       z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         (1) |   44.00702   10.19259    16.34   0.000     27.94942     69.2901
```

```
. lincom 3.group-4.group, eform
 ( 1)  [cfsehisiincells]3.group - [cfsehisiincells]4.group = 0 (GMDC+HOClwFTL vs GMDC+Bc+HOClwFTL)
------------------------------------------------------------------------------
cfsehisiin~s |      exp(b)   Std. Err.       z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         (1) |   3.142383   .6043249     5.95   0.000     2.155564    4.580968
```

```
. lincom 3.group-6.group, eform
 ( 1)  [cfsehisiincells]3.group - [cfsehisiincells]6.group = 0 (GMDC+HOClwFTL vs PBS)
------------------------------------------------------------------------------
cfsehisiin~s |      exp(b)   Std. Err.       z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         (1) |   18.74866   4.348241    12.64   0.000     11.90028    29.53814
```

```
. lincom 3.group -5.group, eform
 ( 1)  [cfsehisiincells]3.group - [cfsehisiincells]5b.group = 0 (GMDC+HOClwFTL vs GMDC+OVA)
------------------------------------------------------------------------------
cfsehisiin~s |      exp(b)   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         (1) |   17.43849   1.650077    30.21   0.000     14.48658    20.99191
```

7.36.2 Negative Binomial Regression Analysis of $OVA_{323-339}$-Pulsed Target Cell Lysis

```
sum vpd450ovaiipcells

    Variable |       Obs        Mean    Std. Dev.       Min        Max
-------------+--------------------------------------------------------
vpd450ovai~s |        31     6033.29    1280.342      3118       9005

. nbreg vpd450ovaiipcells b5.group, exp(unpulsed) irr

Fitting Poisson model:
Iteration 0:   log likelihood = -252.27203
Iteration 1:   log likelihood = -252.26399
Iteration 2:   log likelihood = -252.26399

Fitting constant-only model:
Iteration 0:   log likelihood = -300.36388
Iteration 1:   log likelihood = -247.66715
Iteration 2:   log likelihood =  -246.1204
Iteration 3:   log likelihood =  -245.8812
Iteration 4:   log likelihood = -245.88095
Iteration 5:   log likelihood = -245.88095

Fitting full model:
Iteration 0:   log likelihood = -231.55316
Iteration 1:   log likelihood = -208.07264
Iteration 2:   log likelihood = -206.26215
Iteration 3:   log likelihood = -205.79948
Iteration 4:   log likelihood = -205.79796
Iteration 5:   log likelihood = -205.79796

Negative binomial regression            Number of obs      =        31
                                        LR chi2(5)         =     80.17
Dispersion     = mean                   Prob > chi2        =    0.0000
Log likelihood = -205.79796             Pseudo R2          =    0.1630

------------------------------------------------------------------------------
vpd450ovai~s |        IRR   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
       group |
           1 |   1.253654   .0276089    10.26   0.000     1.200693    1.308951
           2 |    1.20825   .0266041     8.59   0.000     1.157216    1.261535
           3 |     1.1241   .0247726     5.31   0.000      1.07658    1.173717
           4 |   1.061771   .0235287     2.70   0.007     1.016643    1.108903
           6 |   .8852598   .0214371    -5.03   0.000     .8442253    .9282889
             |
       _cons |   1.46274   .0263761    21.09   0.000     1.411947    1.515361
ln(unpulsed) |          1  (exposure)
-------------+----------------------------------------------------------------
    /lnalpha |  -7.123038   .3088993                     -7.728469   -6.517606
-------------+----------------------------------------------------------------
       alpha |   .0008063   .0002491                      .0004401    .0014772
------------------------------------------------------------------------------
LR test of alpha=0: chibar2(01) = 92.93                Prob >= chibar2 = 0.000
```

```
. lincom 1.group-2.group, eform
( 1)  [vpd450ovaiipcells]1.group - [vpd450ovaiipcells]2.group = 0 (GMDC+sFTL vs GMDC+Bc+sFTL)
------------------------------------------------------------------------------
vpd450ovai~s |      exp(b)   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         (1) |   1.037578   .0185465     2.06   0.039     1.001857    1.074573
```

```
. lincom 1.group-3.group, eform
 ( 1)  [vpd450ovaiipcells]1.group - [vpd450ovaiipcells]3.group = 0 (GMDC+sFTL vs GMDC+HOClwFTL)
------------------------------------------------------------------------------
vpd450ovai~s |     exp(b)   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         (1) |   1.115252   .0199611     6.09   0.000     1.076807    1.155069
```

```
. lincom 1.group-4.group, eform
 ( 1)  [vpd450ovaiipcells]1.group - [vpd450ovaiipcells]4.group = 0 (GMDC+sFTL vs GMDC+Bc+HOClwFTL)
------------------------------------------------------------------------------
vpd450ovai~s |     exp(b)   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         (1) |    1.18072   .0213089     9.20   0.000     1.139685    1.223232
```

```
. lincom 1.group-6.group, eform
 ( 1)  [vpd450ovaiipcells]1.group - [vpd450ovaiipcells]6.group = 0 (GMDC+sFTL vs PBS)
------------------------------------------------------------------------------
vpd450ovai~s |     exp(b)   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         (1) |   1.416142   .0290621    16.95   0.000     1.360312    1.474264
```

```
. lincom 2.group-3.group, eform

 ( 1)  [vpd450ovaiipcells]2.group - [vpd450ovaiipcells]3.group = 0 (GMDC+Bc+sFTL vs GMDC+HOClwFTL)
------------------------------------------------------------------------------
vpd450ovai~s |     exp(b)   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         (1) |    1.07486    .019233     4.03   0.000     1.037818    1.113225
```

```
. lincom 2.group-4.group, eform
 ( 1)  [vpd450ovaiipcells]2.group - [vpd450ovaiipcells]4.group = 0 (GMDC+Bc+sFTL vs GMDC+Bc+HOClwFTL)
------------------------------------------------------------------------------
vpd450ovai~s |     exp(b)   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         (1) |   1.137957   .0205327     7.16   0.000     1.098417    1.178921
```

```
. lincom 2.group-6.group, eform
 ( 1)  [vpd450ovaiipcells]2.group - [vpd450ovaiipcells]6.group = 0 (GMDC+Bc+sFTL vs PBS)
------------------------------------------------------------------------------
vpd450ovai~s |     exp(b)   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------

         (1) |   1.364854    .028002    15.16   0.000      1.31106    1.420855
```

```
. lincom 3.group-4.group, eform
 ( 1)  [vpd450ovaiipcells]3.group - [vpd450ovaiipcells]4.group = 0 (GMDC+HOClwFTL vs GMDC+Bc+HOClwFTL)
------------------------------------------------------------------------------
vpd450ovai~s |     exp(b)   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         (1) |   1.058702   .0191273     3.16   0.002      1.02187    1.096863
```

```
. lincom 3.group-6.group, eform
 ( 1)  [vpd450ovaiipcells]3.group - [vpd450ovaiipcells]6.group = 0 (GMDC+HOClwFTL vs PBS)
------------------------------------------------------------------------------
vpd450ovai~s |     exp(b)   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         (1) |   1.269796   .0260778    11.63   0.000      1.2197    1.32195
```

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