Defining the mechanisms by which lenalidomide can modulate the human T cell alloresponse to improve the outcome of allogeneic haematopoietic stem cell transplantation

Caroline Besley

September 2016

Submitted in partial fulfilment of the requirements of the Degree of Doctor of Philosophy.

Queen Mary University of London

Centre for Haemato-Oncology

Barts Cancer Institute

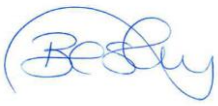
Statement of originality

I, Caroline Besley, confirm that the research included within this thesis is my own work or that where it has been carried out in collaboration with, or supported by others, that this is duly acknowledged below and my contribution indicated. Previously published material is also acknowledged below.

I attest that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge break any UK law, infringe any third party's copyright or other Intellectual Property Right, or contain any confidential material.

I accept that the College has the right to use plagiarism detection software to check the electronic version of the thesis.

I confirm that this thesis has not been previously submitted for the award of a degree, by this or any other university.

Signature: 

Date: 28th September 2016

Details of publications relating to this thesis

1. Stem cell transplantation for indolent lymphomas.

   Besley C, LeDieu R.

   Current Medical Literature – Leukaemia and Lymphoma 2015. 22(2): 33-44.

2. TNFRSF14 aberrations in follicular lymphoma increase clinically significant allogeneic T cell responses.

   Kotsiou E, Okosun J, **Besley C**, Iqbal S, Matthews J, Fitzgibbon J, Gribben JG, Davies JK.

   Blood. 2016 Jul 7; 128(1): 72-81.

3. Lenalidomide treatment promotes alloproliferation of $CD8^+$ T cells that have a unique gene expression profile and enriches for an effector memory phenotype with enhanced polyfunctional cytokine secretion capacity.

   Besley C, Kotsiou E, Petty R, Sangaralingam A, Le Dieu R, Gribben J, Davies J,. Blood. 2014; 124(21): 1103. Abstract.

4. Lenalidomide potentiates human T Cell alloresponses by selectively increasing proliferation of alloreactive $CD8^+$ cells which exhibit a novel gene expression profile - implications for IMID therapy after allogeneic haematopoietic cell transplant.

   Besley C, Kotsiou E, Petty R, Sangaralingam A, LeDieu R, Gribben J, Davies J.

   British Journal of Haematology. 165, (Supp 1) 16. Abstract.

Abstract

Immunomodulatory drugs (IMiDs) could enhance both direct anti-tumour and graft-versus-tumour effects after allogeneic haematopoietic stem cell transplantation (AHSCT). However, clinical experience with IMiDs after AHSCT using adult peripheral blood (APB) as a stem-cell source has been limited by graft-versus-host disease. Characterization of the mechanisms by which IMIDs modulate alloresponses of T cells and identification of differential effects on T cells from different cell sources could facilitate more effective use of these drugs in the setting of AHSCT. Using *in vitro* modelling, multi-parameter flow cytometry and gene expression analysis, I have determined the impact of the widely used IMiD lenalidomide on alloresponses of APB and umbilical cord blood (UCB)-derived T cells.

Lenalidomide-treatment potentiates net alloproliferation of APB-derived T cells by selectively enhancing proliferation of $CD8^+$ T cells. These $CD8^+$ T cells have enhanced effector memory differentiation, are enriched for polyfunctional effectors, have enhanced direct-cytotoxicity against heamatopoietic target-cells and have a distinct gene expression profile with altered expression of key immunoregulatory-genes and depletion of cellular ikaros.

Importantly, while effects on $CD8^+$ T cells derived from UCB are similar, lenalidomide has contrasting effects on allospecific proliferation of APB and UCB-derived $CD4^+$ T cells. While lenalidomide-treatment has no effect on alloproliferation of APB-derived $CD4^+$ T cells, it reduces alloproliferation of UCB-derived $CD4^+$ T cells. The reduction in UCB-derived $CD4^+$ T cell alloproliferation is accompanied by selective expansion of $CD4^+CD25^+FOXP3^+$ regulatory T cells (Treg), resulting in an overall reduction in UCB-derived T cell alloproliferation.

These findings demonstrate that lenalidomide has a differential impact on alloresponses of T cells from different cell sources; alloresponses of APB-derived T cells are increased via selective expansion of polyfunctional $CD8^+$ effectors, while alloresponses of UCB-derived T cells are limited by expansion of tolerogenic Treg. These findings have important implications for the future use of IMiDs in the setting of AHSCT.

Dedication

This thesis is dedicated firstly to my parents, for always encouraging my insatiable need to 'know' everything and supporting my academic and non-academic endeavours. And to my wonderful Rowan, for moving cities, jobs and flats (more than once) so I could fulfil my ambitions, even though sometimes I think you wondered what all the fuss was about.

Thank you.

Acknowledgements

The work presented in this thesis would not have been possible without the help, support and encouragement of a number of people.

I would particularly like to thank my supervisor Dr Jeff Davies. I couldn't have wished for a better supervisor whose combination of encouragement and ability to see to the heart of the question always challenged me, and I believe helped me to become a better researcher and clinician.

I'd also like to thank Drs Robert Petty, Eleni Kotsiou and Jennifer Ball; my fantastic Post-docs who helped me learn my way around the lab and were so generous with their time, helping me think through experiments and results and come up with better solutions.

My thanks also to all the staff in the Barts Cancer Institute Core Flow Cytometry facility, particularly William Day and Dr Becki Pike for their help with technical issues during experiments and for help with flow sorting.

Dr Sameena Iqbal and her team at the Barts Cancer Institute tissue bank for their help with acquiring and processing samples from patients and volunteers.

Dr Essam Ghazaly and Miss Chat Gnanaranjan for their help with the MS/MS UHPLC experiments.

Dr Ajanthah Sangaralingam for her help with analysis of Affymetrix gene expression array data.

Thank you also to Professor John Gribben for being so encouraging when I took my first tentative steps along the path to a PhD and for assembling such a wonderful department at the Centre for Haemato-Oncology. It's been a fantastic place to learn and work.

I also want to mention Dr Lily Keane. Thank you for always being there, your boundless enthusiasm for science and for keeping me sane when the mountain seemed too high to climb.

And finally I'd like to acknowledge the healthy donors and patients who so generously provided blood and my funding bodies the MRC (Clinical Research Fellowship MR/M001733/1) and Barts and The London Charity (Clinical Research Fellowship 512/1737).

Table of Contents

List of Figures

List of Tables

Abbreviations

4-1BBL	4-1BB ligand
ADCC	Antibody dependent cellular cytotoxicity
Ag	Antigen
aGvHD	Acute GvHD
AHSCT	Allogeneic haematopoietic stem cell transplant
AML	Acute myeloid leukaemia
AP-1	Activating protein 1
APB	Adult peripheral blood
APC	Antigen presenting cell
APF	Alloproliferative precursor frequency
aRNA	Amplified RNA
ATG	Anti-thymocyte globulin
BM	Bone marrow
C	Celsius
CAR	Chimeric antigen receptor
CC	C Chemokine
CCL	C C motif ligand
CCR	C Chemokine receptor/s
CD	Cluster of differentiation
CD40L	CD40 ligand
cDNA	Complementary DNA
CFSE	Carboxyfluorescein succinimidyl ester
cGvHD	Chronic GvHD
ChIP	Chromosome immunoprecipitation
CLL	Chronic lymphocytic leuakaemia
CM	Central memory
CML	Chronic myelocytic leukaemia
CMV	Cytomegalovirus
CO_2	Carbon dioxide
Cr	Chromium
CRBN	Cereblon
C_T	Cycle threshold
CTL	Cytotoxic T lymphocyte
CTLA4	Cytotoxic T lymphocyte associated protein 4
DAMP	Damage associated molecular pattern
DAPI	4', 6-diamidino-2-phenylindole
DC	Dendritic cells
DLI	Donor lymphocyte infusion
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EM	Effector memory
FACS	Flourescence activated cell sorting or flow cytometry
FAIM3	Fas anti-apoptotic inhibitory molecule 3

FAM	Fluorescein amidite
FasL	Fas ligand
FBS	Fetal bovine serum
FcR	Fragment crystallisable receptor
FIC	Full Intensity conditioning
FITC	Fluorescein isothiocyanate
FMO	Fluorescence minus one
FOXP3	Forkhead box P3
FSC	Forward scatter
g	g-force
G-CSF	Granulocyte colony stimulating factor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCRMA	G C robust multi-array average
GM-CSF	Granulocyte macrophage colony stimulating factor
GvHD	Graft versus host disease
GvT	Graft versus tumour effect
Gy	Gray
HBSS	Hanks buffered basic salt solution
HDAC	Histone deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen
HSC	Haematopoietic stem cells
HSV-TK	Herpes simplex virus thymidine kinase
iCas9	Inducible caspase 9
IFNγ	Interferon gamma
Ig	Immunoglobulin
IGF1	Insulin like growth factor 1
IKZF1	Ikaros
IL	Interleukin
IMiD	Immunomodulatory drug
IRF4	Interferon regulatory factor 4
KIR	Killer immunoglobulin-like receptor
KO	Knockout
MACS	Magnetic cell sorting
MDS	Myelodysplastic syndrome
MDSC	Myeloid derived suppressor cells
mg	Milligrams
mHag	Minor histocompaibilty antigen
MHC	Major histocompatibility complex
mins	Minutes
ml	Milliliters
MM	Multiple myeloma
mM	Millimolar
mRNA	Messenger RNA
MS/MS UHPLC	Tandem mass spectrometry and ultra high performance liquid chromatography

mTOR	Mammalian target of rapamycin
MUD	Matched unrelated donor
NFAT	Nuclear factor of activated T cells
NFκB	Nuclear factor κB
ng	Nanograms
NK	Natural killer cell
NKT	Natural killer T cell
nm	Nanometers
NP	Non-alloproliferative
P	Alloproliferative
PAMP	Pathogen associated molecular pattern
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PD1	Programmed death 1
PE	Phycoerythrin
PFKFB4	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4
PMA	Phorbol 12-myristate 13-acetate
PMCH	Pro-melanin concentrating hormone
PMT	Photomultiplier tube
PNAd	Peripheral node addressin
PTCy	Post transplant cyclophosphamide
QC	Quality control
qRT PCR	Quantitative reverse transcriptase polymerase chain reaction
RIC	Reduced intensity conditioning
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RQ	Relative quantity
RT	Room temperature
S1P	Sphingosine 1 phosphate
SEE	Staphyloccocal enterotoxin e
sIL2 R	Soluble interleukin 2 receptor
siRNA	Small interfering RNA
SLO	Secondary lymphoid organ
SOCS2	Suppressor of cytokine signalling 2
SSC	Side scatter
T cell	T lymphocyte
TCD	T cell depletion
TCR	T cell receptor
Teff	T effector cells
TEMRA	Terminal effector memory re-expressing RA
Th1	T helper 1 lymphocyte
Th2	T helper 2 lymphocyte
TIM 3	T cell immunoglobulin domain and mucin domain 3
TNF	Tumour necrosis factor alpha
TNFR2	Tumour necrosis factor receptor 2
Treg	T regulatory cells

UCB	Umbilical cord blood
UCBT	Umbilical cord blood allogeneic haematopoietic stem cell transplant
VCAM 1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
WT1	Wilms tumour antigen 1

Chapter 1 - Introduction

1.1 Allogeneic haematopoietic stem cell transplantation

1.1.1 The process of allogeneic haematopoietic stem cell transplantation

Allogeneic haematopoietic stem cell transplant (AHSCT) is a potentially curative treatment for a range of haematological cancers that would otherwise not respond to chemotherapy or radiotherapy. Although there is a high degree of heterogeneity in how an individual AHSCT is performed, the success of the treatment in terms of tumour eradication is dependent on an immunological phenomenon known as the graft-versus-tumour effect (GvT). This beneficial GvT is intimately linked with pathological graft-versus-host disease (GvHD) and it has been the goal of basic and clinical AHSCT research for many decades to maximise GvT and minimise GvHD[1].

While the exact components of the transplant process may vary from patient to patient and centre to centre, the process can be broadly categorised as follows:

I. Pre-transplant conditioning
II. Infusion of donor cells collected from a different individual
III. Post-transplant immunoprophylaxis

Figure 1.1 Shows a schematic representation of AHSCT

Pre-transplant conditioning serves a number of purposes; firstly it may contribute to the eradication of residual tumour, secondly it serves to suppress the immune system of the transplant recipient in order to prevent rejection of the donor graft and thirdly it may also contribute to GvHD prevention by modulation and depletion of antigen presenting cells (APC)[2]. A combination of chemotherapy and radiotherapy may be used as conditioning and there are myriad protocols used depending on patient characteristics, disease characteristics and physician/transplant centre preference[3].

Figure 1.1

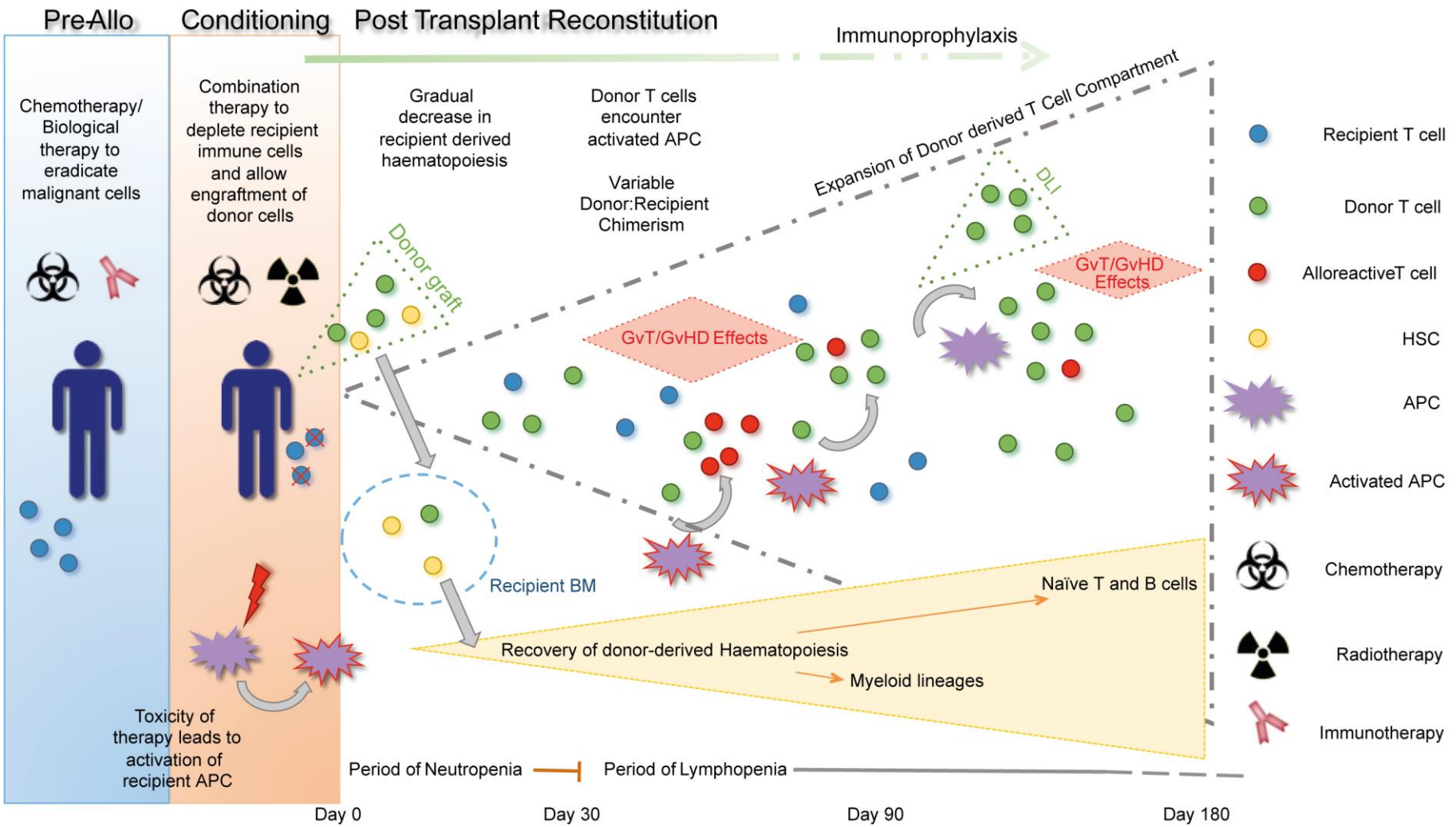

Pre-Allo
Conditioning
Post Transplant Reconstitution
Immunoprophylaxis
Chemotherapy/ Biological therapy to eradicate malignant cells
Combination therapy to deplete recipient immune cells and allow engraftment of donor cells
Gradual decrease in recipient derived haematopoiesis
Donor T cells encounter activated APC
Variable Donor:Recipient Chimerism
Expansion of Donor derived T Cell Compartment
DLI
Donor graft
GvT/GvHD Effects
GvT/GvHD Effects
Recipient BM
Naïve T and B cells
Recovery of donor-derived Haematopoiesis
Myeloid lineages
Toxicity of therapy leads to activation of recipient APC
Period of Neutropenia
Period of Lymphopenia
Day 0
Day 30
Day 90
Day 180
Recipient T cell
Donor T cell
AlloreactiveT cell
HSC
APC
Activated APC
Chemotherapy
Radiotherapy
Immunotherapy

Figure 1.1 Schematic representation of allogeneic haematopoietic stem cell transplantation (AHSCT)

This figure depicts the major stages of AHSCT. Patients undergo treatment for their underlying haematological malignancy to induce remission, conditioning therapy then prepares the patient for the donor graft. Once the donor cells have been infused there is a gradual recovery of haematopoiesis with potential for allo-activation of infused donor T cells that may exert graft-versus-host disease (GvHD) or graft-versus-tumour effects (GvT). Donor lymphocyte infusions (DLI) may be administered after AHSCT to augment the alloresponse. Haematopoietic stem cells in the graft home to the bone marrow and give rise to de novo myeloid and then lymphoid lineages.

Adapted from Krenger *et al.* 2011 [4]

APC = Antigen presenting cell, HSC = Haematopoietic stem cell, BM = Bone Marrow, DLI = Donor lymphocyte infusion

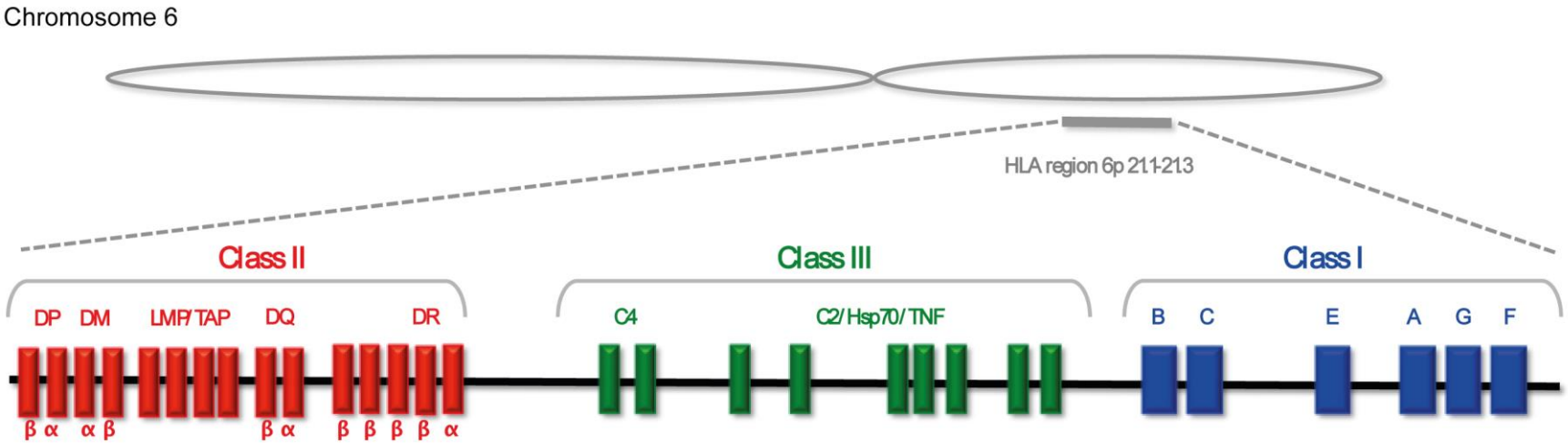
Conditioning protocols can be categorised as full intensity conditioning (FIC) or reduced intensity conditioning (RIC). FIC protocols aim to maximise tumour eradication, but as a consequence completely eradicate the recipient's bone marrow function and allow for full donor engraftment. Reduced intensity conditioning (RIC) aims for sufficient immunoablation to allow engraftment of donor cells but is less toxic and commonly results in the formation of a bone marrow chimera, with recovery of haematopoiesis arising from both residual recipient and donor cells (**Figure 1.1**) [3].

The source, composition and degree of tissue-type-matching (with the recipient) of donor cells can vary widely. Historically one of the major barriers to the success of AHSCT was a lack of understanding of the fundamental biology of self/non-self recognition, leading to frequent graft rejection. One of the most important leaps forward in AHSCT was the characterisation of the Human Leukocyte Antigen (HLA) system and the genes of the Major Histocompatibility Complex (MHC) that encode this highly polymorphic group of cellular antigens (**Figure 1.2**) [5]. This enabled donors to be HLA-matched with recipients. As HLA-typing techniques have improved, donors and recipients can now be matched at an allele level, leading to improved AHSCT outcomes [6].

Donor cells may now be sourced from an HLA-identical sibling, an HLA-matched unrelated donor (MUD), an HLA-mismatched unrelated donor, an HLA-haploidentical donor (usually a parent) or from HLA-matched or mismatched umbilical cord blood (UCB) [7]. In addition where the graft is collected from a related or unrelated donor the collection methods may vary. Haematopoietic stem cells, progenitor cells and mature cells may be harvested from the bone marrow directly, or from the peripheral blood following stem-cell-mobilisation using agents such as granulocyte colony stimulating factor (G-CSF) and corticosteroids. The cellular composition of the graft can be affected by the method of collection itself [8] or due to manipulation of the cellular components of the graft after collection. Particular cell types, for example T cells may be removed from the graft completely, or in some cases removed engineered and then returned to the graft [9].

Figure 1.2

A MHC locus Structure



B

Gene	DP	DM	DQ	DR	B	C	A
No alleles	693	20	980	2025	4242	295	3399
No Prots	550	11	655	1472	3131	208	2396

Figure 1.2 Structure of the human major histocompatibility (MHC) locus

(A) Arrangement of MHC Class I, II and III alleles on chromosome 6. Due to the arrangement on one chromosome human leucocyte antigen (HLA) haplotypes are inherited en-bloc from each parent, with each sibling having a 25% probability of being HLA-identical.

(B) Due to the highly polymorphic nature of the MHC locus, the probability of finding an unrelated HLA-matched donor for the purposes of allogeneic haematopoietic stem cell transplantation (AHSCT) is many magnitudes lower than for a sibling. This has made matching of donor and recipient for rare HLA alleles a significant challenge for AHSCT.

Adapted from Erlich *et al* 2001 [5]

Prots = proteins

Following infusion of donor cells, haematopoietic stem cells and precursors are known to home to the bone marrow where they can begin to reconstitute normal haematopoiesis, this process is known as engraftment. During this time immunosuppression is vital to prevent graft rejection and also to reduce the risk of GvHD [10].

1.1.2 The alloresponse – Graft-versus-Tumour effects and Graft-versus-Host Disease

Pioneering work by Rainer Storb and Hans Kolb in the 1960s and 1970s identified that the 'secondary disease' observed in mouse models of AHSCT, which came to be known as GvHD was mediated by the T lymphocyte (T cell) component of the donor graft [11-15]. It is now understood that both GvHD and GvT are a consequence of the immunological processes of allo-(non-self) recognition and alloreactivity. This is predominantly but not exclusively a T cell mediated phenomenon. Other specialised immune and in some cases non-immune cells may also contribute to the immunobiology of the alloresponse (reviewed by Blazar *et al* [16]).

The presence of endogenous antigens presented in the context of unique MHC class I molecules on almost all cells in the human body, and MHC class II on specialised haematopoietic cells mark them out as 'self' to the autologous immune system. When immune cells from a genetically non-identical individual are introduced, as in AHSCT, these cells can recognise differences in the MHC itself, in the endogenous peptides (minor histocompatibility antigens or mHags) or a combination of both (**Figure 1.3**). This process is known as allorecognition and if the stimulus to the donor immune cells is sufficient can result in activation and proliferation of the alloresponsive cells leading to alloreactivity [17].

Alloreactivity against non-self MHC, mHag or tumour antigens in the recipient may be directed against residual tumour cells and thus lead to GvT. In some cases this same process is directed against healthy non-tumour tissues; in particular towards the skin, liver or gut causing harmful acute GvHD [16].

Figure 1.3

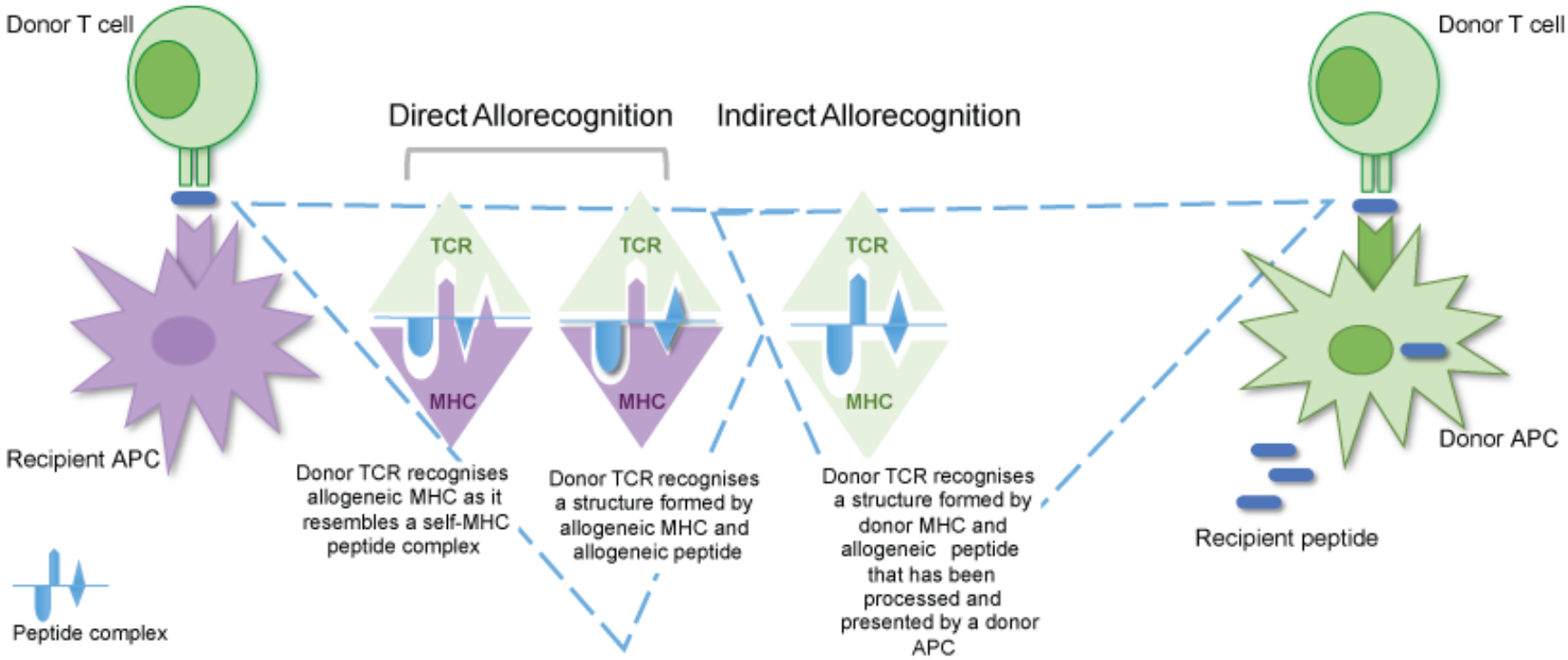

Donor T cell
Direct Allorecognition
Indirect Allorecognition
Donor T cell
TCR
TCR
TCR
MHC
MHC
MHC
Recipient APC
Donor APC
Donor TCR recognises allogeneic MHC as it resembles a self-MHC peptide complex
Donor TCR recognises a structure formed by allogeneic MHC and allogeneic peptide
Donor TCR recognises a structure formed by donor MHC and allogeneic peptide that has been processed and presented by a donor APC
Recipient peptide
Peptide complex

Figure 1.3 Direct and indirect pathways of allo-antigen presentation and T cell receptor (TCR) allo-activation

Allogeneic donor T cells are capable of directly recognising foreign major histocompatibilty (MHC) molecules on recipient antigen presenting cells (APC) either due to polymorphic epitopes on the MHC itself or due to a complex of recipient MHC and recipient-derived self-peptide. This process is known as direct-allorecognition.

Donor APC, either infused with the donor graft itself or arising later after transplant as a result of donor haematopoiesis can engulf and process recipient derived proteins and present non-self peptide in the context of self-MHC to also stimulate donor T cell alloresponses. This process is known as indirect-allorecognition.

Adapted from Abbas *et al.* 2014[18]

TCR= T cell receptor

GvHD was historically categorised as acute or chronic. Acute GvHD (aGVHD) was previously defined as occurring before day 100 after infusion of the donor graft and presents clinically as an acute inflammatory syndrome typically affecting the skin, gut and liver. Chronic GvHD (cGvHD) was defined as occurring after day 100 and presenting with clinical features more typical of autoimmune disease. Due to changes in transplant practice, including RIC and donor lymphocyte infusions (DLI) these arbitrary time-points are now felt to be unhelpful in regards to classifying GvHD and the clinical features, regardless of time from transplant are used to make a diagnosis[19].

Despite the harmful effects of GvHD numerous studies have shown that it is often associated with GvT, with individuals who experience GvHD having lower rates of relapse of their primary tumours. In fact, this observation was partly responsible for the recognition that a GvT effect existed. However, while there is a close link between GvT and GvHD, the occurrence of GvHD is not absolutely necessary for cure, and neither does it guarantee cure after AHSCT[1].

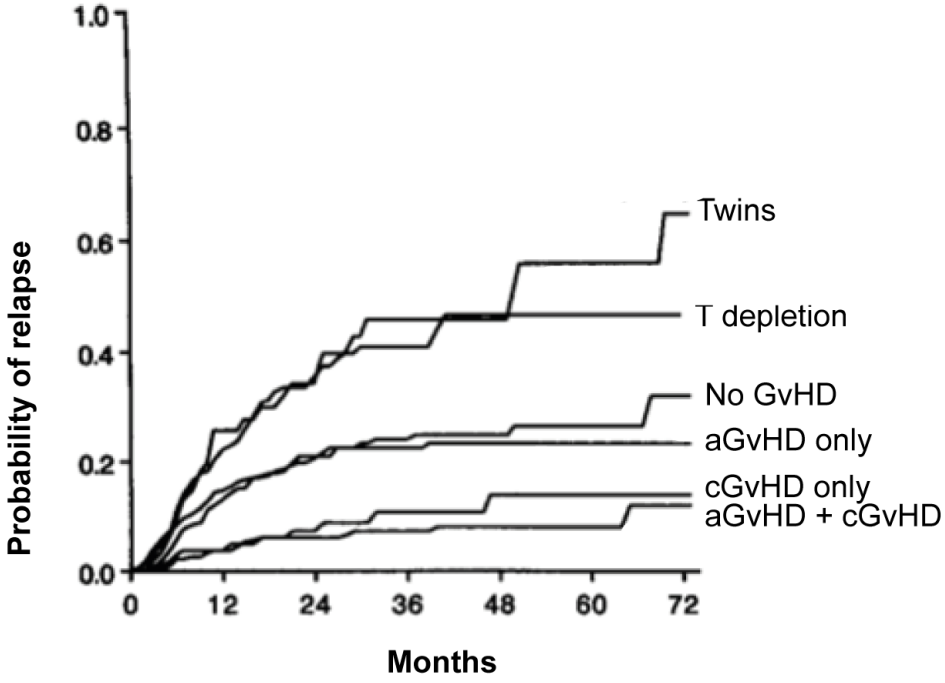
1.1.3 Evidence for GvT

In the early days of AHSCT the purpose of the donor graft was largely to provide a source of cells that could re-populate the bone marrow and rescue the recipient from the effects of myeloablative doses of chemotherapy and radiotherapy. Allogeneic cells also had the benefit of being guaranteed to be free of tumour cells and thus superior to an autologous graft. Only subsequently was it observed that the introduction of another individual's immune cells had an effect on reducing relapse over and above facilitating higher doses of chemotherapy and radiotherapy[1].

Initial observations were that recipients of grafts from identical twins, while not suffering the harmful effects of GvHD, experienced the highest rates of relapse compared with other graft sources and that those recipients who experienced GvHD had lower rates of relapse than those who did not. This suggested that a degree of immunological disparity resulting in GvHD also facilitated recognition and control of residual tumour (**Figure 1.4**)[1].

Figure 1.4

A


1.0
0.8
0.6
0.4
0.2
0.0
Probability of relapse
0
12
24
36
48
60
72
Months
Twins
T depletion
No GvHD
aGvHD only
cGvHD only
aGvHD + cGvHD

B

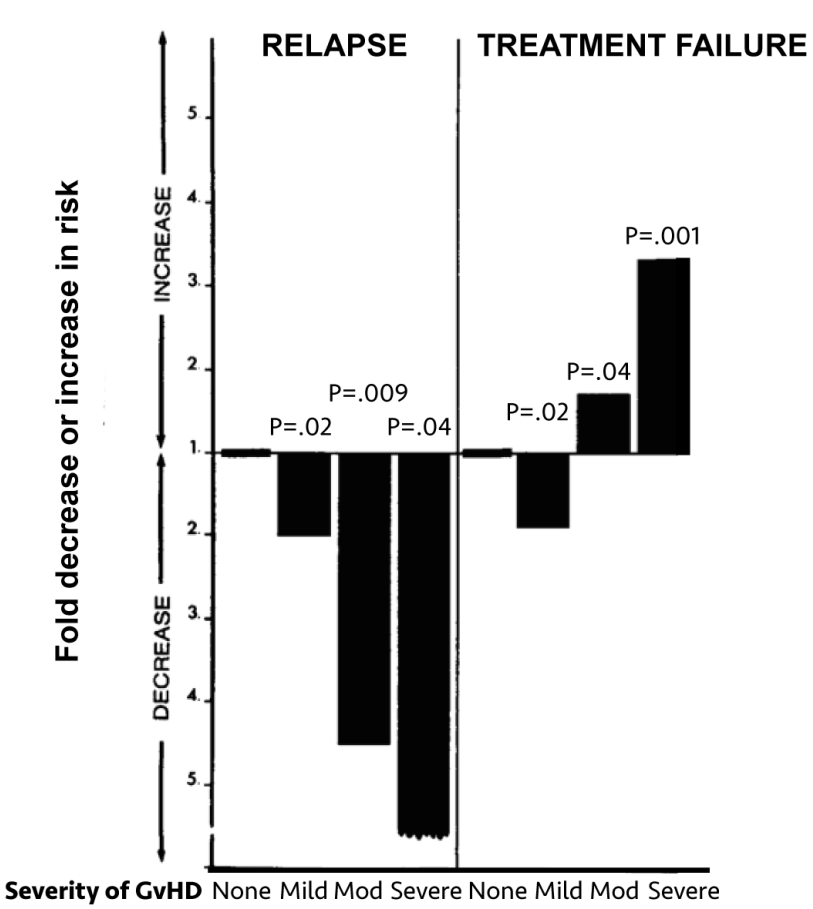

RELAPSE
TREATMENT FAILURE
Fold decrease or increase in risk
INCREASE
DECREASE
5
4
3
2
1
2
3
4
5
P=.02
P=.009
P=.04
P=.02
P=.04
P=.001
Severity of GvHD None Mild Mod Severe None Mild Mod Severe

Figure 1.4 Effect of graft-versus-host disease (GvHD) on probability of relapse after allogeneic haematopoietic stem cell transplant (AHSCT)

(A) Actuarial probability of relapse after AHSCT for leukaemia according to type of graft and development of GvHD.

(B) Fold increase and decrease in risk of relapse and treatment failure after AHSCT for leukaemia among patients with both acute and chronic GvHD as compared to patients without GvHD. Because no patients with severe GvHD relapsed the fold decrease could not be accurately estimated.

Reproduced from Horowitz *et al.* [1]. (T depletion= T cell depletion, aGvHD= acute GvHD, cGvHD= chronic GvHD).

Following the discovery that the T cell component of the graft is important in the development of GvHD there was a movement to T cell deplete grafts prior to infusion [20]. While effective at reducing the incidence of GvHD it was quickly recognised that this approach also led to increased rates of relapse, proving that T cells are important for long-term disease control [21-23].

Further evidence for the importance of T cells for disease control after AHSCT was provided by the fact that delayed infusions of T cells, so called donor lymphocyte infusions (DLI) after AHSCT are able to re-induce remission in individuals with evidence of disease relapse [24-27] and the finding that donor tumour-antigen-specific T cells can be identified in the peripheral blood of AHSCT and DLI recipients [28,29].

Although in the majority of AHSCT T cells are the dominant cells in GvT reactions, it has now been shown that they are not the only immune cells capable of exerting GvT. In the context of haploidentical-AHSCT where rigorous T cell depletion (TCD) is necessary to prevent severe aGvHD (due to the 50% HLA disparity between donor and recipient), natural killer (NK) cells can provide tumour control [30].

NK cells are a subset of lymphocytes important in the innate immune system recognition of virally infected cells. NK cells have surface killer immunoglobulin-like receptors (KIR) that recognise specific MHC class I molecules. When there is a mismatch or a loss of cognate MHC sequences NK cells no longer receive inhibitory signals and a change in the balance of activating and inhibitory KIR signalling leads to killing of cells with missing-self via the perforin/granzyme pathway [31].

In vitro studies have shown that inhibitory KIR mismatch with MHC class I molecules on tumour cells leads to tumour cell killing, and recipients of haploidentical KIR-mismatched AHSCT have lower rates of relapse compared to KIR-matched. Interestingly there is no evidence, either in animal models or in clinical trials that NK cells cause aGvHD. In fact they seem to be protective against aGvHD. This is thought to be due to the missing-self effect being restricted to the haematopoietic compartment and to NK cell killing of recipient APC, thus preventing their interaction with and activation of donor T cells [32-34].

1.1.4 Immunobiology of GvHD & GvT

In 1966 Billingham described 3 criteria necessary for the development of GvHD [35]. These were:

- The graft must contain immunocompetent cells.
- The recipient must express tissue antigens that are not present in the donor.
- The recipient must be incapable of mounting an effective response to eliminate the transplanted cells.

Once these criteria are fulfilled the immunobiological processes that lead to tissue damage can be split into 3 phases: the initiation phase, T cell recruitment and activation phase and tissue damage phase. The tissue damage that results leads to further release of immunoactive substances that lead to further activation and proliferation of T cells and further tissue damage. Thus aGvHD is often described a vicious cycle (reviewed by Ferrara *et al.* [36], **Figure 1.5**).

Active research into each step in the process of aGvHD has aimed to ameliorate or prevent tissue damage. Much effort has focused on the T cells, however T cells are also vital for GvT effects. Other important factors influencing the T cell alloresponse include: activation of APC, the cytokine milieu, T cell trafficking to target organs and immunoregulatory mechanisms.

The immunobiology of cGvHD is distinct from that of acute. It is felt that cGvHD arises due to a failure of thymic selection of emerging naïve T cells derived from donor HSC, whereas aGvHD is due to alloreactivity of T cells that are infused with the donor graft (reviewed in Jamil *et al.* [37]). As investigation of metrics relating to cGvHD is outside of the scope of this thesis from this point on GvHD refers to aGvHD alone.

Figure 1.5

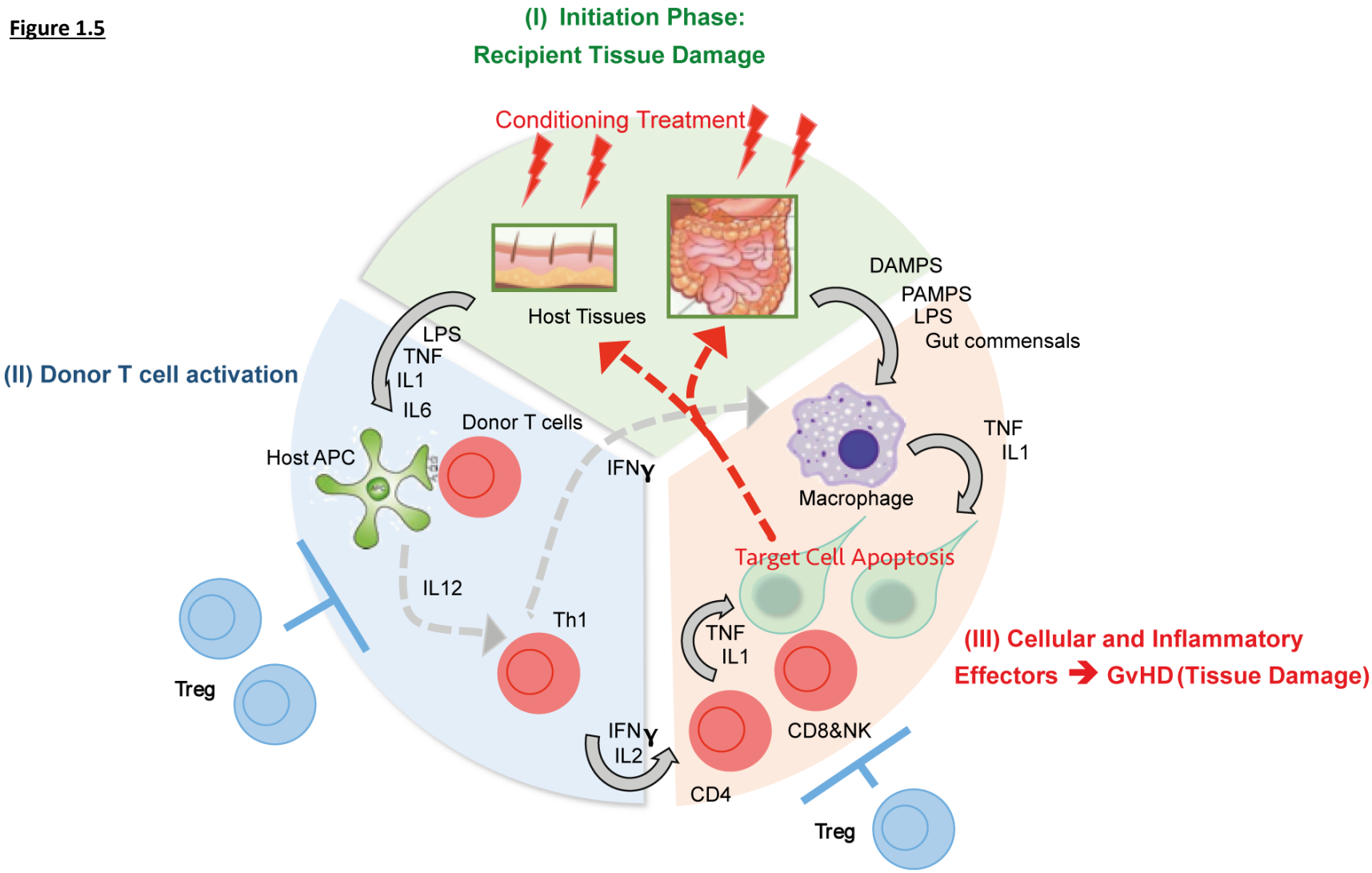

(I) Initiation Phase:
Recipient Tissue Damage
Conditioning Treatment
Host Tissues
DAMPS
PAMPS
LPS
Gut commensals
LPS
TNF
IL1
IL6
(II) Donor T cell activation
Donor T cells
Host APC
IFNγ
TNF
IL1
Macrophage
Target Cell Apoptosis
IL12
Th1
TNF
IL1
(III) Cellular and Inflammatory
Effectors → GvHD (Tissue Damage)
Treg
IFNγ
IL2
CD8&NK
CD4
Treg

Figure 1.5 Schematic representation of the cycle of aGvHD

Damage to recipient tissues leads to release of pro-inflammatory mediators such as damage associated molecular patterns (DAMPS), pathogen associated molecular patterns (PAMPS) and cytokines (interleukin (IL) 1 and 6), as well as inducing translocation of gut commensals and lipopolysaccharide (LPS). These activate recipient antigen presenting cells (APC) and cells of the innate immune system that in turn secrete pro-inflammatory cytokines such as IL1 and tumour necrosis factor (TNF) that attract donor T cells and lead to donor T cell alloreactivity.

Alloreactive donor T cells and activated cells of the innate immune system then exert tissue damage inducing target cell apoptosis both via direct and indirect mechanisms (interferon gamma (IFNγ), TNF and IL1). Further tissue damage leads to further release of PAMPS, DAMPS and pro-inflammatory cytokines and further activation of donor T cells. Thus leading to a 'vicious cycle'.

Adapted from the classic model of immune-pathogenesis of GvHD originally proposed by J. Ferrara [36].

NK = Natural killer cell, Treg = T regulatory cell, Th1 = T helper 1 cell.

1.1.5 T cell subsets: GvT & GvHD

There is ongoing debate over the relative contributions of $CD4^+$ versus $CD8^+$ T cells to GvHD [38]. Although $CD8^+$ T cells are generally held to be responsible for causing tissue damage, due to their MHC class I restriction and capacity for cell killing (which is also likely to be important for GvT) this may be an oversimplified view [39,40]. $CD4^+$ T cells play an important role in the initiation of GvHD by providing help to $CD8^+$ T cells and may also exert tissue damage [41-43]. Some investigators have attempted to resolve this issue by separating the two subsets. A strategy of $CD8^+$ T cell depleted DLI has been shown to result in reduced rates of post-DLI GvHD [44,45], whether a GvT effect is retained is less clear [46]. Evidence from animal models suggests that depleting $CD4^+$ T cells may diminish GvT [47].

Within the $CD4^+$ T cell population there are further subdivisions including T helper type 1 (Th1) and T helper type 2 (Th2) cells. It would appear that Th1 responses, characterised by production of interleukin 2 (IL2) and gamma interferon (IFNγ) are the more important in the pathogenesis of aGvHD [48,49], while Th2 are less important. However a clinical study of Th2-polarised DLI did not show a significant reduction in GvHD [50], in line with other work suggesting that both Th1 and Th2 cells are important [51].

T cells also vary in their degree of antigen experience and therefore how rapidly and vigorously they can respond to antigenic stimulation. Naïve T cells are antigen inexperienced and require a greater degree of T cell receptor (TCR) and co-stimulatory signalling than memory T cells, which are 'primed' for rapid recall responses [52]. There is evidence that T cells responsible for GvHD reside predominantly in the naïve T cell compartment and that memory T cells, due to their existing specificity for pathogens do not cause GvHD [53], but do contribute to immune reconstitution and may exert GvT effects by cross-reactivity with tumour antigens [54]. However concern has been raised that clinical strategies using selected memory T cells may result in compromised GvT effects as naïve T cells also exert more potent GvT [55]. In the setting of AHSCT for immunodeficiency where GvT is not required, depletion of naïve cells from HLA-mismatched grafts has successfully resulted in low rates of GvHD [56]. A clinical trial in the US is currently evaluating the maximal tolerated dose of naïve T cell depleted DLI after AHSCT for haematological malignancy (NCT01627275).

The other major T cell subset that has been recently recognised as playing an important role in AHSCT is the $CD4^+$ T regulatory cell (Treg). It has been shown that the relative proportion of Treg and T effector (Teff) cells is important in determining the risk of GvHD, with increased numbers of Treg relative to Teff in both the donor graft [57-59], in recipient peripheral blood soon after transplant [60] and in DLI [61] being associated with a lower risk of GvHD [62].

1.1.6 Antigen presenting cells and the alloresponse

To become activated T cells require 2 signals, signal 1 occurs on engagement of the TCR by cognate antigen in the context of MHC, and signal 2 is provided by the activation of co-stimulatory receptors such as CD28 by their cognate ligands. In order to appropriately restrict T cell responses to prevent the development of autoimmunity, the ability to deliver these signals to T cells is largely restricted to specialised 'professional' APC such as dendritic cells (DC), although there are other cell types that can take on an APC-like role given appropriate conditions (endothelial cells, lymph node stromal cells and epithelial cells, as reviewed by Kambayashi and Laufer [63]).

APC play an important role as the afferent arm of the alloresponse, as while $CD8^+$ T cells can directly recognise tumour cells, the efficiency of anti-tumour responses is much improved if tumour associated antigens are presented to both $CD4^+$ and $CD8^+$ T cells by professional APC. The relative contribution of recipient and donor derived APC to the alloresponse after AHSCT has been the subject of a number of studies. Experiments in mice have shown that certain recipient APC are rapidly depleted after conditioning; (particularly FIC) such as circulating DC and those in lymphoid organs, while tissue resident DC such as Langerhan's cells are more resistant to chemo and radiotherapy [64,65]. Conditioning leads to the activation of any remaining recipient APC, priming them to activate donor T cell alloresponses. Therefore complete removal of recipient APC would seem a good strategy for prevention of GvHD. In support of this, Merad *et al.* demonstrated that if recipient DC in the skin are eradicated prior to transplant, skin GvHD is almost completely prevented [65].

In contrast, Reddy *et al.* have highlighted the importance of recipient APC for GvT. This group demonstrated that recipient APC are better at stimulating GvT responses than donor APC, indicating that direct allorecognition after AHSCT is important for anti-tumour effects. This is also supported by the observation that more potent GvT effects are exerted when DLI are given to patients with mixed donor chimerism (indicating that recipient cells persist) than full donor

chimerism. When tumour burden is low or the tumour slowly progressive processing of tumour antigen and presentation by donor APC may make a more important contribution to GvT [66].

Counter-intuitively it appears that *in vivo* post-AHSCT the onset of severe acute GvHD is associated with low numbers of circulating DC in peripheral blood [67], this may be due to increased retention in inflamed tissues. However this also highlights a potential limitation of many studies of GvHD. While it is practically easier to examine changes in peripheral blood, this does not necessarily inform as to changes at the tissue level where T cell priming and end-organ damage actually takes place.

1.1.7 The role of cytokines in GvT & GvHD

Cytokines play an important role in the pathogenesis of GvHD and as such have been the focus of many studies and therapeutic strategies. The cytokines widely accepted to be most important will be briefly considered here:

Tumour necrosis factor alpha

Tumour necrosis factor alpha (TNF) is released in large amounts by activated APC during the initiation phase of GvHD, co-stimulating donor T cells that in turn secrete further TNF that mediates target-cell apoptosis. A study by Middleton *et al.* examined genetic polymorphisms in the *TNF* gene of AHSCT recipients and determined that the d3 homozygous allele of the *TNFd* microsatellite was associated with severe GvHD [68]. This suggests that recipients carrying this variant of the *TNF* gene may be more susceptible to cytokine activation during pre-transplant conditioning. Serum levels of TNF after AHSCT also correlate with likelihood of and severity of GvHD [69]. For these reasons anti-TNF antibody therapy has been trialled as an option for treatment of steroid refractory aGvHD, with some success [70].

Interferon gamma

IFNγ is known to promote donor T cell activation and differentiation. High levels of IFNγ after AHSCT are generally felt to be detrimental, skewing the donor T cell response towards a Th1 phenotype and driving aGvHD [71]. IFNγ has been shown to exacerbate gut GvHD in particular, by increasing production of other inflammatory cytokines in the gut [72].

However there is evidence that also supports a role for IFNγ in preventing GvHD by promoting contraction of donor effector T cells, modulation of expression of immunoregulatory molecules on recipient tissues and generation of Treg. In support of this, IFNγ knockout (KO) murine models have shown that in the absence of IFNγ severe and lethal GvHD still occurs (reviewed in Wang *et al.* [73]).

IFNγ also has important roles in GvT, acting to promote the proliferation and cytotoxic potential of donor $CD8^+$ T cells as well as via an independent direct anti-tumour effect [73].

Interleukin 1

Interleukin 1 (IL1) is an important inflammatory cytokine that has a role in a range of inflammatory disorders including GvHD. One of the roles of IL1 is to increase the expression of a number of genes implicated in the pathogenesis of GvHD including: TNF, interleukin 6 (IL6), interleukin 12 (IL12) and the receptor for interleukin 2 (reviewed in Dinarello 1996 [74]). Interestingly, although IL1 would seem to make a good target for GvHD treatment a phase III study of IL1 blockade failed to show a benefit [75].

Interleukin 6

IL6 is known to play a role in inflammatory responses of B cells and T cells. The role of IL6 in GvHD is less clear, but there is evidence from mouse models that IL6 may exacerbate GvHD [76]. Human studies also suggest a role for this cytokine as IL6 levels in serum have been found to be increased in AHSCT recipients who experience GvHD [77]. Based on these findings the humanised IL6 receptor blocking antibody tocilizumab has been used to treat severe steroid refractory GvHD with some patients appearing to respond, although not durably [78].

Interleukin 12

IL12 is produced by activated APC and promotes Th1 differentiation of $CD4^+$ T cells and proliferation of $CD8^+$ T cells [79]. This may be expected to promote GvHD, but studies have shown no association between IL12 levels and human GvHD. Furthermore, high levels of IL12 in the serum are in fact associated with reduced risk of relapse [80]. A murine study of exogenous IL12 administration has also demonstrated a protective effect of IL12 against GvHD, but unfortunately associated with significant toxicity [81]. This toxicity has also been observed in human studies in other settings [82], which has dampened enthusiasm for use of IL12 after AHSCT.

Interleukin 2

IL2 is the major autocrine cytokine for activated T cells, supporting proliferation and survival and thus an important cytokine in the immunobiology of both GvHD and GvT. In a study by Tanaka *et al.* cytokine gene expression levels, including IL1β, IL2, IL6, IFNγ and TNF in mixed lymphocyte cultures were assessed for their predictive value on AHSCT outcome. Increased IL2 in culture media predicted severe GvHD [83], leading the authors to conclude that this *in vitro* assay could be useful in determining the prognosis of AHSCT or in selecting donors.

Given the importance of IL2 for donor T cells, anti-IL2 receptor antibodies have been of interest in the treatment of GvHD. The advantage of targeting the receptor, rather than the cytokine is that this should eliminate only alloreactive T cells (that will have upregulated the IL2 receptor) rather than the whole T cell compartment. One of these antibodies, basiliximab has been used relatively successfully in a phase II study for the treatment of steroid refractory GvHD [84].

Importantly, IL2 also plays a critical role in the expansion of Treg that help to control GvHD. For this reason some groups have explored the administration of low doses of recombinant IL2 in conjunction with donor lymphocytes, reporting successful expansion of Treg with no excess toxicity from GvHD [85].

1.1.8 Role of T cell trafficking in GvT & GvHD

In the normal immune system T cells re-circulate between the blood and the secondary lymphoid organs (SLO) where they are brought into contact with APC. APC interact with naïve and central memory T cells in the T cell zone of the lymph node. If a T cell with the cognate TCR for antigen presented is encountered the T cell will become activated, proliferate and differentiate, leave the lymph node and migrate to the site of infection or inflammation. Tissue resident effector memory T cells that have previously encountered antigen may respond to tissue resident APC and become activated locally to the site of infection, leading to a more rapid immune response [86].

It has been recognised that in order to exert GvT or GvHD, donor T cells must be able to migrate to APC for priming and also migrate to target organs. This has led T cell migration to become regarded as the '4th Billingham Criterion', necessary for the pathogenesis of GvHD [87].

Investigators have begun to consider whether factors involved in T cell migration could therefore provide a therapeutic target to separate GvT and GvHD effects, especially in view of the fact that sites of GvT and GvHD are usually anatomically distinct. There are 3 major classes of migratory receptors that influence T cell movements and could be targeted: selectins, integrins and chemokine receptors (**Figure 1.6**).

Broadly speaking selectins are critical for the tethering and rolling of T cells along endothelium that facilitates the egress of T cells into tissues, while integrins are expressed at endothelial cell junctions and on extracellular matrix and are felt to play a role in directing T cells through endothelial junctions and tissues. These molecules tend not to display particular tissue specificity and therefore do not present an attractive therapeutic target for the prevention of GvHD (reviewed in Marelli-Berg *et al.* [88]).

In contrast the chemokine – chemokine receptor axis that is involved in directing T cells towards the anatomical site of infection or inflammation presents a more attractive therapeutic target as certain chemokines (CC) and their receptors such as c-chemokine receptor 9 (CCR9) direct T cells in an organ specific manner, in this case to the gut [89].

Further evidence that the CC-CCR axis could be used to modulate the alloresponse is provided by the observation that naïve T cells, thought to be the primary mediators of GvHD are c-chemokine receptor 7 (CCR7) positive, whereas their memory counterparts, thought not to cause GvHD are CCR7-negative [55]. However it has been shown experimentally that CCR7 KO naïve T cells retained the ability to cause GvHD and effector memory cells with enforced lymph node homing (due to enforced CD62-ligand expression) remained unable to cause GvHD. As CCR7 directs migration to secondary lymphoid organs (SLO), this indicates that priming of alloreactive T cells can occur outside of the SLO [90]. In addition a major disadvantage of restricting donor T cell migration through SLO (and therefore access to professional APC) may be that GvT responses could be detrimentally affected.

Good targets for manipulation of the CC-CCR axis might be CCR2, CCR5, CCR9 and CCR10. There is some evidence for the involvement of each of these receptors in the pathogenesis of GvHD.

Figure 1.6

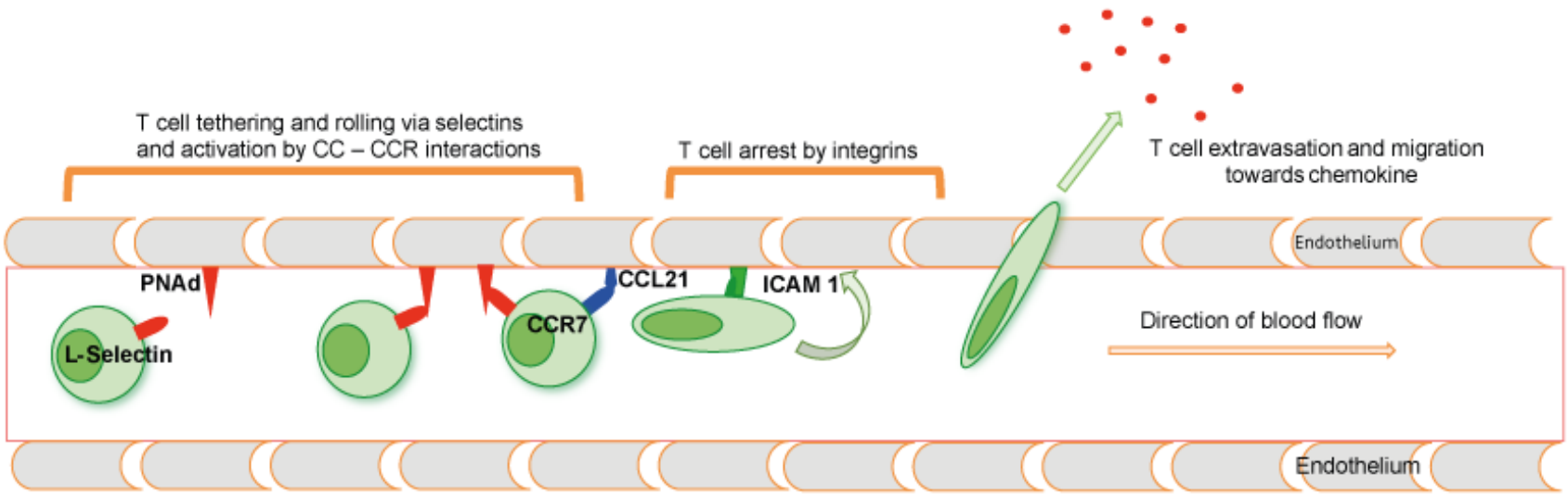

T cell tethering and rolling via selectins
and activation by CC – CCR interactions
T cell arrest by integrins
T cell extravasation and migration
towards chemokine
Endothelium
PNAd
CCL21
ICAM 1
L-Selectin
CCR7
Direction of blood flow
Endothelium

Figure 1.6 Mechanisms of T cell migration

Schematic depicting processes involved in T cell migration from the circulation into tissues. T cells are induced to tether and roll on the endothelial surface by interaction of endothelial adhesion molecules such as peripheral node addressin (PNAd) with selectins on T cells. Activation of migratory receptors occurs due to interactions of endothelial bound chemokines such as CC type chemokine ligand 21 (CCL21) with their cognate receptors on T cells (CC chemokine receptor 7). This activation leads to up-regulation and activation of integrins on the T cell such as leucocyte functional antigen 1 (LFA1) that binds to intercellular adhesion molecule 1 (ICAM1) on endothelial cell junctions and promotes T cell extravasation. Chemokine gradients both inside the vessels and outside guide T cells to sites of inflammation.

Adapted from Masopust *et al.* [91].

CCR2

CCR2 is the receptor for CCL2 (previously known as monocyte chemo-attractant protein 1, MCP1), a chemokine released from inflamed tissues in a non-tissue specific manner, and known to be released in tissues affected by GvHD [92]. CCR2 KO $CD8^+$ T cells have been shown to be less able to cause GvHD, while retaining GvT effects in a murine model [93].

CCR5

CCR5 is the receptor for CCL5 (previously known as RANTES), another chemokine released from inflamed tissues in a non-tissue specific manner and implicated in the pathogenesis of GvHD. It has been demonstrated that recipients of AHSCT with deletional-mutations in CCR5 (leading to defective CCR5) have a significantly reduced risk of GvHD [94]. A study in mice has shown that recipients of CCR5 KO donor T cells had reduced rates of GvHD, although the effect appeared to be dependent on the conditioning used prior to adoptive transfer [95]. In addition a Phase I/II study of an oral CCR5 antagonist, maraviroc in combination with standard GvHD prophylaxis in high-risk AHSCT recipients has shown promising results in terms of GvHD prevention, without any apparent impact on relapse rates at 1 year [96].

CCR9

CCR9 and its ligand CCL25 have been shown to be specifically and highly expressed in small intestinal T cells and small intestinal mucosa respectively, with almost no expression in other tissues [89]. Recently the retinoic-acid-signalling pathway that leads to the up-regulation of CCR9 on T cells has been implicated in the pathogenesis of gastro-intestinal GvHD in murine models [97,98]. At present the role of CCR9 T cells and retinoic acid in human GvHD is unknown, however the possibility of targeting CCR9 is appealing as specific oral inhibitors to CCR9 are already in clinical trials for the treatment of inflammatory bowel disease and have shown promising results [99].

CCR10

A number of signals have been implicated in driving migration of T cells into the skin including: cutaneous lymphocyte-associated antigen, CCR4, CCR10 and CCR10 ligand CCL27 [100]. Studies support a role for CCR10 in the pathogenesis of aGvHD. Elevated levels of CCL27 were found in paediatric patients suffering from acute skin GvHD and correlated with elevated numbers of CCR10-positive T cells in both the skin and peripheral blood. Importantly infiltration of these T cells was restricted to the skin and not found at other sites of GvHD [101].

1.1.9 Prevention of GvHD

Significant improvements in the prophylaxis and management of acute GvHD have been made since the early years of transplant when GvHD seemed an almost insurmountable barrier to the field. A huge leap forward was made when the HLA-MHC system was recognised and classified, allowing for the first time for matching of donors and recipients [102]. Since that time improvements have been more incremental, and clinicians remain better at preventing GvHD than treating it when it occurs, however an increased understanding of the immunobiology of GvHD has provided some new avenues for investigation.

1.1.9.1. Immunoprophylaxis

The second major breakthrough in the prevention of GvHD, following the implementation of HLA-typing was the use of potent immunosuppressant drugs prior to, during and for variable lengths of time following AHSCT with the aim of suppressing donor T cell activation. As with other indiscriminate anti-T cell therapies an undesirable consequence of this may be blunting of GvT, in support of this it has been shown that in patients who relapse after AHSCT a proportion will respond to withdrawal of immunosuppression [103].

Pharmacological agents used alone or in combination as immunoprophylaxis are summarised in **Table 1.1** based on the review by Gatza *et al.* [104].

Table 1.1 Agents used as immunoprophylaxis following AHSCT

Drug		Target
In routine clinical use	Ciclosporin	Inhibits T cells: Cyclophilin-calcineurin
	Methotrexate	Inhibits T cells: Dihydrofolate reductase
	Mycophenolate mofetil	Inhibits T cells: Inosine monophosphate dehydrogenase
	Tacrolimus	Inhibits T cells: FKBP12-calcineurin
	Sirolimus	Inhibits T cells: FKBP12- mammalian target of rapamycin (mTOR)
Developmental	Pentostatin	Inhibits lymphocytes: Adenosine deaminase
	Bortezomib	Inhibits lymphocytes: 26s proteasome
	Vorinostat	APC (reducing pro-inflammatory cytokines) and enhances Treg: Histone deacetylases
	Atorvastatin	Modulation of T cells and APC: HMG Co-A reductase

1.1.9.2 Use of reduced intensity conditioning

The realisation that GvT leads to long-term control of primary tumours caused a paradigm shift in the approach to AHSCT. The ability to use lower doses of immunosuppressive rather than myelosuppressive chemotherapy has made AHSCT a treatment option that can now be offered to older and frailer patients. A major advantage of RIC AHSCT regimens is that as a consequence of reduced tissue damage and inflammation caused by conditioning, the activation of host APC and release of DAMPs and PAMPs that can drive donor T cell activation is also reduced. This has resulted in RIC leading to reduced rates of severe GvHD [105].

1.1.9.3 Selective depletion of alloreactive cells

***Ex vivo* depletion based on activation markers**

One desirable strategy for controlling and eliminating GvHD is the selective depletion of only alloreactive cells. This would leave pathogen-specific immunity intact and could potentially allow reductions in immunosuppressive prophylaxis. At present a technology to identify alloreactive cells prior to allogeneic stimulus has not been developed, therefore donor T cells must be allostimulated and then subsequently identified based on either their expression of activation induced surface proteins or by their proliferation which can be assessed by various methods.

For this reason an important consideration in the *ex vivo* approaches to alloreactive T cell depletion has been selection of appropriate stimulator cells; these must be capable of initiating a robust response and they must not express tumour associated antigens, as the loss of T cells responding to these would negatively impact GvT. Both haematopoietic and non-haematopoietic cells such as skin fibroblasts have been previously used as stimulator cells [106,107].

Removal of donor T cells that express CD25 (the alpha subunit of the IL2 receptor) following allostimulation has been explored by a number of groups using a variety of techniques. *In vitro* data was supportive of a significant reduction in alloreactive T cells with retained pathogen and tumour immunity [108] and a Phase I/II study of CD25 allodepleted DLI following haploidentical-AHSCT has shown promise [109]. A potential disadvantage of CD25 depletion strategies is that these will also deplete Treg, as Treg constitutively express high levels of CD25 [110].

An alternative target, CD69 has also been assessed as a potential marker of alloreactive T cells for depletion. A study by Hartwig *et al.* demonstrated that depletion of CD69 positive responder

T cells following allostimulation could not completely abrogate secondary alloresponses. However this strategy did spare Treg that do not up-regulate CD69 on activation [111,112].

***In vivo* depletion using high dose cyclophosphamide**

Cyclophosphamide is an alkylating agent that has particular activity in rapidly proliferating cells. Importantly HSC and Treg contain high levels of aldehyde dehydrogenase that converts the active metabolite of cyclophosphamide, phosphoramide mustard to an inactive form making them resistant to the drug. Cyclophosphamide is therefore attractive as a therapy that can lead to selective apoptosis of alloreactive donor T cells proliferating early after AHSCT without impairing haematopoietic reconstitution. This strategy may also allow reductions in, or avoidance of long-term immunosuppression (reviewed by Al-Homsi *et al.* [113]). The question remains as to whether donor T cells that would exert GvT are also deleted at this point [114].

The majority of clinical trials utilising this approach have been in the haploidentical-AHSCT setting, where as previously mentioned the risk of GvHD is high if TCD is not undertaken. A large retrospective multi-centre study from an Italian collaboration found that post-transplant cyclophosphamide (PTCy) in combination with two other immunosuppressive drugs could provide good protection from GvHD, with a 22% incidence of grades II-IV aGvHD. However the cumulative incidence of relapse of 24% at 1 year made relapse the main cause of death [115].

A similar study of haploidentical-AHSCT in an adult cohort showed very similar results. In this case in addition to PTCy the recipients received single agent long-term immunoprophylaxis. The incidence of grade II-IV aGvHD was 24% and the risk of relapse-related death at a median follow up of 313 days was 23% [116].

A phase II study of PTCy as single agent GvHD prophylaxis in an adult RIC-AHSCT cohort with, in the majority of cases a HLA-matched donor the results were more disappointing. Incidence of grade II-IV aGvHD was 45% and relapse-free-survival at 2 years only 34% [117]. A further multi-centre study in a similar adult cohort following FIC resulted in rates of grade II-IV aGvHD of 51% and disease free survival at 2 years of 62% [118]. These studies indicate that single agent PTCy may not be adequate to control GvHD and may negatively impact on GvT, especially in the RIC setting.

'Suicide' T cells

An exciting approach pioneered at the San Raffaele Scientific Institute has been the engineering of donor T cells to express an inducible suicide-switch. The insertion of a herpes simplex virus thymidine kinase (HSV-TK) suicide gene into the donor T cells confers sensitivity to the anti-viral drug ganciclovir, leading to DNA damage and cell death. If following infusion of HSV-TK T cells the patient develops signs of acute GvHD these cells can be reliably and quickly eliminated by administration of intravenous ganciclovir [119].

HSV-TK T cells administered as DLI to patients suffering relapsed disease after AHSCT have been shown to expand and persist in recipients. In 35% of recipients administration of HSV-TK DLI led to complete remission from relapsed disease, with the best anti-tumour responses achieved in cases where the greatest expansions of cells were seen. GvHD developed in 25% of recipients and was successful treated by ganciclovir [120].

A phase I/II multi-centre study used HSV-TK T cells as pre-emptive DLI following haploidentical-AHSCT for high-risk leukaemia. In this study 79% of recipients obtained HSV-TK T cell engraftment and 45% developed GvHD requiring ganciclovir treatment. Although in all patients GvHD resolved following ganciclovir, 6 of these 10 patients required additional immunosuppression with steroids [121]. At present the long-term impact of deletion of HSV-TK cells on GvT responses is unclear.

There are some disadvantages of the HSV-TK T cell approach. A major disadvantage is the use of ganciclovir as a trigger for the suicide-switch as ganciclovir is often used for the treatment of cytomegalovirus (CMV) infection, which is a common complication of AHSCT [122]. It is therefore likely that patients who do not have GvHD but are suffering from CMV will receive ganciclovir and as a consequence also undergo depletion of their HSV-TK T cells, potentially negatively impacting on GvT. Another disadvantage is that these cells are also potentially immunogenic as they contain viral elements [123].

T cells engineered with an alternative suicide-switch based on inducible caspase 9 (iCas9) have also been tested in a phase I study. This switch is activated by the administration of an otherwise bio-inert small molecule AP103 that causes dimerisation and activation of the inducible caspase 9 leading to apoptotic cell death. Following haploidentical-AHSCT, DLI of iCas9 T cells led to GvHD in 30% of recipients. Administration of AP103 was able to successfully deplete circulating T cells

leading to resolution of symptoms [124], however long-term follow-up has shown a high rate of relapse in patients who received AP103, suggesting this approach has a detrimental effect on GvT effects [125].

Alloanergisation

Another strategy to deplete alloresponsive T cells while sparing pathogen-specific and tumour immunity takes advantage of a fundamental principle of T cell biology; the requirement for co-stimulatory signalling in combination with specific TCR-MHC interaction to achieve activation of T cells. This co-stimulatory signalling is often referred to as 'signal 2' and it is accepted that after binding of MHC and cognate antigen to the TCR initiating 'signal 1' a lack of 'signal 2' results in T cell anergy. This state of anergy means that the T cell will fail to respond to re-stimulation with the same antigen even when co-stimulatory signals are present [126].

Allostimulation in the presence of co-stimulatory blockade in the form of anti-CD80 and anti-CD86 antibodies was shown to induce allo-anergy; significantly reducing alloproliferative responses to re-stimulation with alloantigen while retaining alloproliferative responses to viral pathogens and tumour associated antigens [127]. In a phase I study of haploidentical-AHSCT using allo-anergised BM grafts, 38% of recipients developed clinical evidence of GvHD that responded to treatment, while cumulative incidence of relapse was 17% at 10 years; indicating that this strategy for alloanergisation could not completely prevent GvHD, but that the manipulated T cells retained GvT activity [128].

1.1.9.4 $CD4^+$ T regulatory cells

Treg are known to play an important role in controlling immune responses (reviewed in Roncarolo *et al.*[129]). In murine models of AHSCT Treg have been shown to be protective against lethal GvHD while preserving GvT, even in MHC-mismatched mice [130] [62]. Studies in human AHSCT have shown that increasing proportions of Treg in the donor graft [57] or following AHSCT [131] are associated with reduced risk of GvHD. In a small study of patients who received Treg DLI following UCB-AHSCT (UCBT) rates of GvHD were reduced compared to historical controls [132,133]. However the effect of Treg DLI on GvT remains unclear, one study by Hicheri *et al.* raised concern that DLI with higher proportions of Treg were associated with increased risk of relapse, indicating that high frequencies of Treg in DLI may compromise the GvT effect [61].

The use of low doses of IL2 *in vivo* has also been proposed as an alternative to the *ex vivo* expansion of Treg. This strategy capitalises on the fact that Treg constitutively express high levels of the high affinity IL2 receptor α subunit (CD25) and therefore will be preferentially stimulated by low doses of IL2 when compared to Teff. In a phase II clinical trial aimed at treatment of cGvHD (rather than aGvHD), the administration of low dose IL2 daily for 12 weeks led to improvement in cGvHD in 61% of patients and was associated with significant expansion of Treg in peripheral blood [134]. Whether this approach could be used in the treatment or prevention of aGvHD remains unknown.

1.1.10 Strategies to augment GvT

Strategies to maximise beneficial GvT have been and remain an active area of AHSCT research, with a particular focus on therapies that can separate this effect from GvHD. Most of the clinically relevant methods proposed are based on adoptive cellular therapies that select cellular subsets with well-defined roles in anti-tumour responses or those that can promote *in vivo* expansions of these subsets.

1.1.10.1 T cell manipulation

T cells are simultaneously, the most beneficial cells and the cells that pose the greatest risk in AHSCT, mediating both beneficial GvT and harmful GvHD. The first attempts at T cell manipulation involved simply removing them, either using polyclonal anti thymocyte globulin (ATG) or more recently monoclonal antibodies [135,136] or magnetic bead technology [137]. The major barrier to the success of T cell depletion (TCD) is that this strategy also negatively impacts on GvT [21,22,138] as well as increasing the risk of post-AHSCT infective complications [139].

In order to maintain GvT while reducing GvHD various other strategies to redirect or refine the T cell alloresponse have been attempted. Most of these rely on *ex vivo* expansion and manipulation of donor T cells to generate tumour-antigen-specific T cells. This can be achieved by two approaches; either by stimulating donor T cells with leukaemia associated antigens or recipient derived tumour cells in the presence of optimal conditions to promote proliferation of a large number of cells that can then be re-infused for a specific patient [140], or by directly genetically modifying the donor T cells to confer specificity [141]. The first approach is laborious and limited by the fact that not all recipients' tumours express antigens capable of eliciting a robust T cell response.

Genetic modification may provide a more widely applicable approach to generate tumour-specific T cells. Two main strategies exist, the first is the transfer of a high affinity tumour antigen specific TCR into donor T cells, an approach that has proven effective in some solid cancers [142], but requires complex processes to silence the endogenous TCR to prevent immune cross-reactions [143]. The second is the generation of T cells containing a chimeric-antigen-receptor (CAR) that confers new specificity, independent of HLA-restriction and with higher affinity and avidity than the endogenous TCR. These cells are known as CAR T cells (reviewed in Maus *et al.* [141]).

An alternative method to boost tumour antigen-specific donor T cell responses *in vivo* is to use cancer vaccines after AHSCT to enhance tumour antigen presentation by host or donor APC and thus GvT but not GvHD [144]. Animal models have demonstrated that vaccination of donors prior to stem-cell collection results in expanded populations of tumour-antigen specific T cells that can be infused as part of the donor graft and exert GvT in recipients. The ethics of this approach in human volunteer donors might be problematic [145,146]. However, even without donor vaccination a small study of recipient vaccination with Wilms tumour antigens following AHSCT demonstrated that antigen-specific T cell responses can be induced, although as yet there is no evidence of a significant improvement in patient outcome [147,148].

1.1.10.2 Donor lymphocyte infusions (DLI)

DLI describes the delayed infusion of un-manipulated or manipulated T cells after AHSCT. This delayed dose of beneficial donor T cells is particularly important for those patients who have received TCD-AHSCT. The greatest successes of DLI have come in a subgroup of recipients who have undergone AHSCT as treatment for chronic myelocytic leukaemia (CML). In CML, DLI have been associated with regression of minimal residual disease and cure from relapsing disease [25]. Unfortunately results in other settings have not been as good. It has become apparent that a number of variables influence the success of DLI, these include: the timing of DLI, the underlying tumour and the relative donor-recipient chimerism status.

Timing of DLI can be vitally important; too soon after transplant and there is an increased risk of DLI-induced GvHD due to an ongoing inflammatory milieu, too late and co-stimulatory cytokines required for optimal APC-T cell interactions are reduced resulting in sub-optimal GvT effects. The amount of tumour present is also likely to increase with time if not adequately controlled in the immediate post-AHSCT period. In most cases DLI cannot control overt relapse of disease,

necessitating further chemotherapy prior to DLI. While chemotherapy prior to DLI can increase the efficacy of DLI, this also leads to release of inflammatory mediators that will again increase the risk of GvHD [26].

Donor-recipient chimerism provides a measure of the degree to which haematopoiesis is derived from the recipient's own remaining HSC and how much from donor-derived HSC, and is usually measured in nucleated-cells derived from the peripheral blood or bone marrow. In recipients of T cell-replete grafts and/or those who have converted to full donor-chimerism, DLI have been shown to be less effective than following TCD-AHSCT or in recipients with mixed-chimerism [9]. This may be due to either of two factors; insufficient antigen-presentation and/or allostimulation to elicit a donor T cell response, or failure of the donor T cells to respond to allostimulation.

Insufficient allostimulation in the context of full donor-chimerism may be due to the conversion of tissue-resident APC to donor-origin. Evidence suggests that indirect antigen-presentation by donor-derived APC is less efficient at stimulating donor T cell responses [149]. Therefore where relapse occurs due to failure of donor T activation by donor-derived APC, further donor T cells alone are likely to be ineffective [150].

A study by Porter *et al.* explored *ex vivo* activation of DLI with anti-CD3 and anti-CD28-coated beads as a strategy to overcome failure of donor T cell responses to allostimulation. Patients with evidence of disease-relapse following AHSCT and standard-DLI, received escalating doses of *ex vivo* activated-DLI. While it appeared that GvHD occurred earlier after activated-DLI than conventional DLI, this was not felt to be a limiting toxicity and 8 of 18 recipients achieved a complete response. Unfortunately this response was not durable in 50% of patients [151].

1.1.10.3 Natural killer cell therapies

Natural killer (NK) cells are the first lymphocyte population to reconstitute following AHSCT [152] and have well described roles in both pathogen and tumour immunity (reviewed in Della Chiesa *et al.* and Choi *et al.* [31,153]). Importantly there is evidence *in vivo* that NK cells can exert GvT after AHSCT without contributing to GvHD [154,155]. Findings from animal experiments also indicate that protection from GvHD can be mediated by NK cell lysis of host APC [156] and NK cells may also contribute to control of alloreactive donor T cells [157].

The process of allorecognition in NK cells is distinct from that of T cells and relies on recognition of 'missing self' via a number of surface activatory and inhibitory receptors that bind to polymorphic determinants of MHC class I such as HLA-Cw1. The major families of receptors are those characterised by lectin-like heterodimers such as CD94-NKG2A and those composed of immunoglobulin-like (Ig) domains known as killer cell inhibitory receptors (KIR). As KIR genes in particular vary between individuals this leads to the likelihood that in AHSCT donor and recipient may be KIR-mismatched [31,158]. (**Figure 1.7**)

The benefits of NK cell alloreactivity have been best realised in the setting of haploidentical-AHSCT. In this instance stringent TCD required due to the high degree of MHC-mismatch and risk of graft rejection is overcome by administering very high doses of HSC [159]. Rates of GvHD are low but consequently risk of relapse is high and outcomes are best when there has been demonstrated to be an inhibitory KIR mismatch between recipient and donor [160-162].

A further strategy to improve outcome in the haploidentical-AHSCT setting has been the infusion of NK cell DLI to augment the NK GvT effect, at present there is no evidence to show these significantly improve outcome [163]. In an effort to improve the activity of NK cell DLI several groups have attempted to augment NK cell activity using IL15 (an important homeostatic cytokine for NK cells) and the co-stimulatory molecule 4-1BBL (4-1BB ligand). Unfortunately this strategy was associated with acute-severe GvHD [164]. A similar phase I/II study of IL15 stimulated NK cells without the addition of 4-1BBL following haploidentical-AHSCT for solid tumours did not observe an excess of GvHD, which may implicate the 4-1BBL as the cause of this toxicity [165].

There remains some uncertainty regarding the relative contribution of NK cell-mediated GvT outside of the haploidentical-AHSCT setting, where the immunodominant effect of the T cell response may overshadow that of the NK alloresponse. Some authors argue that NK cell KIR-mismatch may confer additional benefit [166], while others argue that while NK cell numbers in donor grafts can improve outcomes, KIR-mismatch has no additional impact. [167]. It has also been argued that mismatches in activating KIR could lead to increased rates of GvHD in this setting [168,169].

Figure 1.7

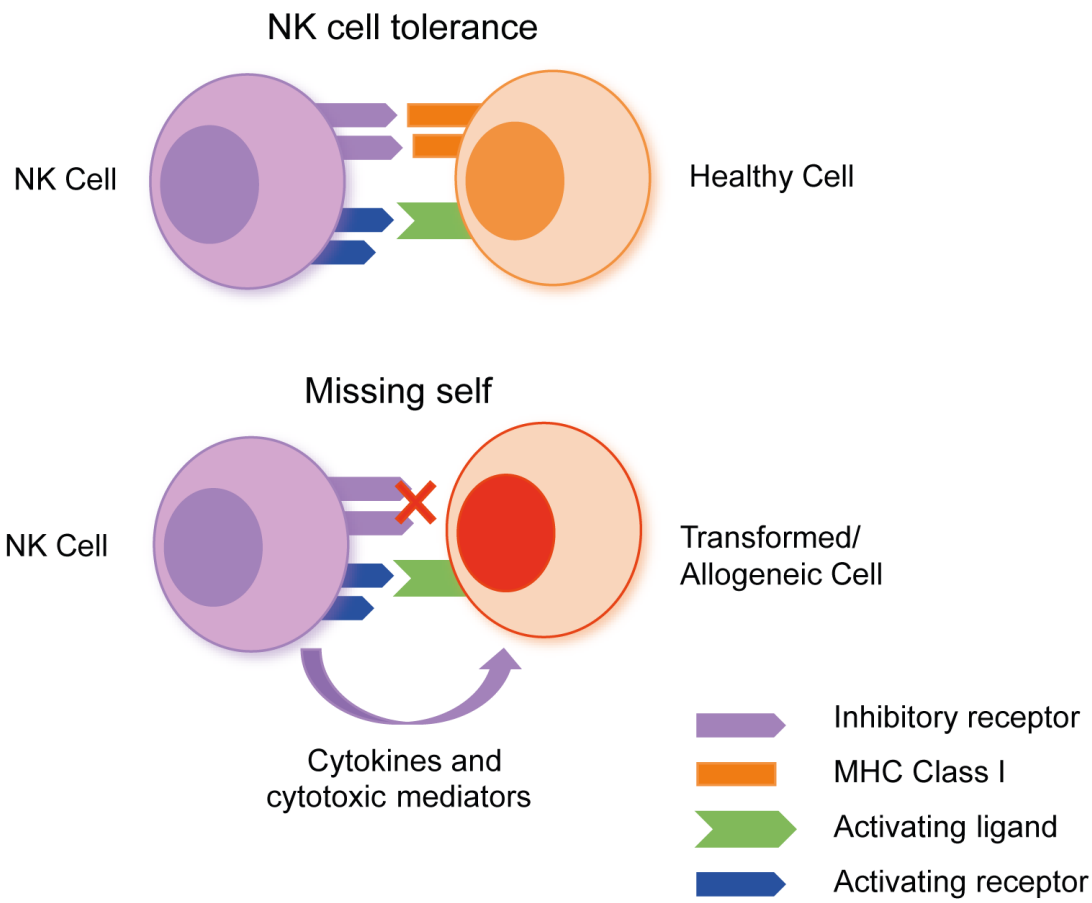


Figure 1.7 Natural killer cell receptors and activation

Natural killer (NK) cells can directly recognise and destroy target cells such as virally transformed and malignant cells. Activation versus tolerance of NK cells is induced by the balance of activating and inhibitory signals delivered via activating and inhibitory NK cell receptors.

Classically, in the alloresponse recipient cell incompatibility for major histocompatibility (MHC) molecules recognised by inhibitory receptors on NK cells, leads to loss of inhibitory signalling and the cell is destroyed as if it were virally-transfomed.

Based on Della Chiesa *et al.* [31]

1.1.10.4 Immune checkpoint blockade

The recent success of immune checkpoint blockade in the treatment of advanced solid tumours has fuelled enthusiasm for the use of these agents in the treatment of haematological malignancy. By blocking inhibitory signalling by tumour cells to T cells the immune response can be re-engaged against the tumour. The two targets that have at present been explored in clinical trials are cytotoxic T lymphocyte associated protein 4 (CTLA4) and programmed death 1 (PD1).

There is some evidence that T cell exhaustion, characterised by increasing expression of PD1 in conjunction with other exhaustion related markers such as T cell immunoglobulin domain and mucin domain 3 (TIM3) is associated with relapse of leukaemia after AHSCT [170]. This is likely due to a late failure of donor mediated GvT. Evidence that reversing this pattern could result in improvements in GvT is provided by the observation that DLI can reduce the expression of exhaustion related genes such as *PD1* in patients who respond to favourably to DLI [171]. There of course remains a concern as to whether removing the 'brakes' from the donor T cell alloresponse could result in GvHD as well as GvT.

A recent publication by Davids *et al.* has shown that ipilimumab, an anti-CTLA4 monoclonal antibody could be delivered safely after AHSCT, although some GvHD was observed. The authors report durable disease responses in a proportion of the patients [172]. The anti-PD1 monoclonal antibody, nivolumab has also been used successfully in at least one case of relapsed Hodgkin lymphoma following AHSCT [173]. Trials of ipilimumab (NCT00060372/NCT01919619) and nivolumab alone or in combination (NCT01822509) after AHSCT are ongoing.

1.2 Lenalidomide

Lenalidomide (Revlimid® Celgene) was initially developed following the success of its parent compound, thalidomide in the treatment of myeloma. Subsequently the immunomodulatory properties of thalidomide and its derivatives have broadened the potential uses of these agents and there is considerable interest in whether they could be employed to augment immunotherapy approaches such as AHSCT.

1.2.1 Lenalidomide: Structure and pharmacokinetics.

Lenalidomide (3-(4'-amino-1 oxo 1,3-dihydro-2H-isoindol-2-yl) piperidine-2,6-dione) is a derivative of thalidomide with anti-neoplastic, anti-angiogenic and immunomodulatory properties. The chemical structure of lenalidomide ($C_{13}H_{13}N_3O_3$) differs only slightly from the parent compound by the addition of an amine-group and the loss of an oxo-group from its phthaloyl ring, but this modification confers hundred-several thousand-fold more potent immunomodulatory properties than observed with thalidomide [174,175].

In healthy individuals and those with normal renal function lenalidomide displays linear pharmacokinetics, with plasma levels in proportion to the amount administered up to an oral dose of 400mg. Lenalidomide is well absorbed orally (≥90% of dose) and maximum plasma levels are achieved within 1 hour of administration [176,177]. Approximately 84% of the oral dose is excreted unchanged in the urine within 12 hours and the drug does not accumulate with multiple dosing. The rapid speed of renal elimination of the drug is felt to indicate that both active and passive renal clearance mechanisms are involved, however a candidate renal transporter has not yet been identified [178]. The mean terminal half-life *in vivo* has been demonstrated to be 3 to 4 hours in the clinically relevant dose range (5-50mg) and is increased by 6-12 hours in varying degrees of renal impairment [179].

Lenalidomide distributes equally between the intracellular and plasma components of the blood. The drug has low plasma protein binding (≤40%) with a small percentage of the drug being metabolised to 5-hydroxy-Lenalidomide (approximately 3%) and N-acetyle-Lenalidomide (approximately 4%) by plasma enzymes. Both of these metabolites are also eliminated in the urine. An estimate of the possible *in vitro* half-life has been given as 8 hours [176,179].

1.2.2 Anti-neoplastic properties of lenalidomide

An in depth discussion of the anti-neoplastic actions of lenalidomide is beyond the scope of this thesis, however they will be outlined in brief.

Following the observations that the bone marrow of myeloma patients displays a high degree of neo-vascularisation and that thalidomide has potent anti-angiogenic properties, trials of thalidomide in refractory and multiply relapsed myeloma were undertaken and have demonstrated thalidomide has potent anti-myeloma activity [180,181]. However the effects of the drug were found to be more complex than initially expected. In fact treatment with thalidomide did not appear to have a significant effect on the vascularity of the tumour [180] and has since been found to exert an anti-tumour effect via a number of different immunomodulatory and molecular mechanisms [181].

Lenalidomide is a member of a class of immunomodulatory drugs (IMiDs®, Celgene) that were developed from the parent compound thalidomide to have enhanced anti-neoplastic actions with decreased toxicity. Lenalidomide has been shown to have activity not only against multiple myeloma [177,181] but also against a range of other haematological malignancies including: 5q- myelodysplastic syndromes (MDS) [182], chronic lymphocytic leukaemia (CLL) [183-185] , B cell non Hodgkin lymphomas [186-188], mantle cell lymphoma [189,190] and T cell lymphoma [191,192] as well as some solid cancers such as melanoma [193,194] and prostate cancer [195,196].

The mechanisms underlying the direct cytotoxicity of lenalidomide on myeloma cells have been well described and include: inhibition of secretion of IL6, insulin like growth factor 1 (IGF1) and vascular endothelial growth factor (VEGF) as well as generation of pro-apoptotic signals by up-regulation of caspase 8 and down-regulation of apoptosis inhibitors ((cellular inhibitor of apoptosis protein 2, FLICE inhibitory protein, interferon regulatory factor 4 (IRF4) and nuclear factor-κB (NFκB)) [181,197,198].

IMiDs have also been shown to disrupt support from the bone marrow microenvironment to myeloma cells. One interesting feature of this disruption is the down-regulation of cell surface adhesion molecules such as intercellular adhesion molecule 1 (ICAM1), vascular cell adhesion protein 1 (VCAM1) and E-selectin [199]. Importantly despite this effect on the bone marrow stroma, lenalidomide has not been demonstrated to have deleterious effects on the growth and

survival of normal bone marrow progenitors and $CD34^+$ HSC, in fact one study has reported a dose dependent increase in total numbers of $CD34^+$ cells exposed to lenalidomide following 6 days of culture [200].

1.2.3 Immunomodulatory properties of lenalidomide

The first described immunomodulatory use of IMiDs was the use of thalidomide to treat erythema nodosum leprosum patients, where it was found that treatment led to decreased levels of TNF in the plasma and healing of painful skin lesions. The mechanism underlying this effect is common to all IMiDs and is inhibition of TNF production by monocytes and macrophages [174,175,201]. The investigation of IMiDs in other inflammatory and immunological disorders has gone on to reveal a range of other immunomodulatory properties including effects on T cells, NK cells, B cells and APC.

1.2.3.1 Lenalidomide effects on T cells

A number of studies have now described the effects of lenalidomide on mitogen-stimulated T cell function *in vitro* (summarised in **Table 1.2**). These include: increased activation (as measured by CD25, HLA DR or CD40 ligand expression), proliferation, immune synapse formation, secretion of pro-inflammatory cytokines (IL2, TNF, IFNγ,) chemotaxis (stimulated by S1P and CCL21) and cytotoxicity (measured by expression of CD107a and perforin as well as apoptosis of target cells) by healthy $CD4^+$ and $CD8^+$ T cells [174,202-206]. In addition lenalidomide exposure decreases the amount of antigenic peptide required to elicit a T cell response [207], reduces T cell expression of PD1 [208] and increases 'repair' of T cell defects due to aging, HIV or tumour-induced inhibition of function [186,206,209-211].

Lenalidomide-treatment of myeloma, solid tumour and CLL patients has also been demonstrated to have effects on T cells *in vivo* including: expansion of $CD8^+$ T cells [212,213], particularly those with a central memory (CM) and effector memory (EM) phenotype [214,215], increased numbers of circulating activated T cells (identified by HLA DR or CD69 expression) [215,216], increases in inflammatory cytokines in plasma (IL2, TNF, IFNγ and IL6)[193,217] and increases in both $CD4^+$ and $CD8^+$ cytokine producing T cells at early time-points following initiation of treatment (IL2, IFNγ and TNF) [212].

The effects of lenalidomide on Treg are less clear. *In vitro* studies using IL2 to expand Treg from PBMC in culture suggest that the addition of lenalidomide significantly reduces the expansion of Treg compared to addition of IL2 alone, with no effect on cell death [218]. However Grygorowitz *et al.* report that despite *in vitro* down-regulation of Treg proliferation lenalidomide enhanced the suppressive capacity of the cells [219]. The results from limited *in vivo* data are also conflicting, the majority of papers suggest that lenalidomide treatment is associated with a decrease in circulating Treg [212,213,216,220] while one group report an increase [221].

1.2.3.2 Lenalidomide effects on the T cell alloresponse

Thalidomide has been shown to potentiate the human $CD8^+$ T cell alloresponse to purified allogeneic DC in the absence of $CD4^+$ T cell help [222]. The effect of newer IMiDs, such as lenalidomide on the T cell alloresponse is unknown. One study by Luptakova *et al.* examined the effect of lenalidomide on proliferative T cell responses of 3 unique donors to purified allogeneic DC, demonstrating an increase in T cell alloproliferation above control. Further characterisation of the lenalidomide-treated alloresponse was not undertaken and therefore the effect on specific T cell subsets and their functions remains unknown [208].

Table 1.2 Effects of lenalidomide on immune cell subsets

Cell Type	Source	Stimuli Used	Dose of Lena	Effect of Lenalidomide treatment	Ref
T cell PBMC	Healthy human PBMC	LPS aCD3 Ab	0.01-100μM	1. Decreased TNF production from LPS stimulated PBMC 2. Increased proliferation of aCD3 Ab stimulated T cells (H-thymidine) 3. Decreased IL-1β, IL12 and IL6 production by LPS stimulated PBMC (ELISA) 4. Increased IL10 production by LPS stimulated PBMC (ELISA) 5. Increased IFNγ production by aCD3 Ab stimulated T cells (ELISA) 6. Increased IL2 production by aCD3 Ab stimulated PBMC (ELISA) 7. Increased CD40L expression on aCD3 Ab stimulated T cells	174
T cells	Healthy PBMC Patient serum (receiving Lenalidomide treatment)	aCD3 Ab LPS	10μg/ml	1. Decreased expression of TNFR2 on aCD3 Ab stimulated $CD4^+$ and $CD8^+$ T cells 2. Increased expression of CD25 on aCD3 Ab stimulated $CD4^+$ and $CD8^+$ T cells 3. Increased production of IL2 and sIL2 R by aCD3 Ab stimulated PBMC 4. Increased proliferation of aCD3 Ab stimulated PBMC (H-thymidine) 5. Increased IL2 and sIL2 R in serum of patients receiving Lenalidomide treatment	202
T cells	Healthy PBMC HIV patient PBMC	Ag pulsed autologous DC	1μM	1. Increased IFNγ, IL2 and TNF positive $CD8^+$ T cells from both healthy and HIV infected patients (intracellular FACS) 2. Increased $CD8^+$ T cell lysis of Ag pulsed DC (Cr release) 3. Effect on $CD8^+$ cells was independent of $CD4^+$ cells	223
T cells	Healthy PBMC Jurkat T cells	aCD3 Ab SEE	0.001-10 μM	1. Increased IL2 production by aCD3 Ab stimulated $CD4^+$ and $CD8^+$ T cells (ELISA and mRNA) 2. Increased IL2 and IFNγ production by aCD3 Ab stimulated Th1 T cells and IL5 and IL10 by Th2 T cells 3. Increased IL2 production by SEE stimulated Jurkat cells	203

T cells	Healthy PBMC	aCD3 Ab	10 µM	1. Increased IFNγ production by aCD3 Ab stimulated $CD4^+$ and $CD8^+$ T cells (cytokine capture) 2. Increased G1-S phase transition in aCD3 Ab stimulated T cells 3. Increased T cell proliferation in response to aCD3 Ab, immature and mature DC 4. Overcomes B7-CD28 blockade by inducing CD28 phosphorylation and NFκB activation	204
T cells	Healthy PBMC CLL patient PBMC	sAg	0.5µM	1. Increased immune synapse formation between autologous T cells and tumour cells	209
T cells	Healthy PBMC	PMA + Ionomycin	0.001-10 µM	1. Increased IL2 production by PBMC	205
T cells	Healthy PBMC	aCD3 Ab & aCD28 Ab	0.01 - 100 µM	1. Increased IFNγ, IL2 2. Decreased IL17 3. Increased proliferation 4. Increased chemotaxis	206
T cells	Healthy PBMC	Ag pulsed autologous DC	10 µM	1. Increased IFNγ production and granzyme B expression of $CD8^+$ T cells (ELISA) 2. Increased expansion of Ag specific CD 8^+ T cells 3. Decreased CD45RA expression on $CD4^+$ and $CD8^+$ T cells	224
T cells	HIV patient PBMC	Ag pulsed autologous DC	0.1 & 0.5 µM	1. Increased IFNγ and TNF production by CD8 T^+ cells (intracellular cytokine staining) 2. Increased proliferation of $CD8^+$ T cells (intracellular cytokine staining) 3. Increased perforin and CD107a expression by $CD8^+$ T cells (intracellular cytokine staining) 4. Increased $CD8^+$ T cells with polyfunctional cytokine capacity (intracellular cytokine staining) 5. Increased breadth of antigen recognition and responses at lower peptide concentrations by $CD8^+$ T cells	207
T cells	Healthy PBMC & HIV patient PBMC	aCD3 Ab & aCD28 Ab	0.01 - 1 µM	1. Increased IFNγ & IL2 production (intracellular cytokine staining) 2. Increased chemotaxis (to S1P & CCL21) 3. Restoration of HIV T cell function to level seen in healthy control T cells	210

T cells	Healthy PBMC	aCD3 & aCD28 Ab	1 or 10 µM	1. Decreased TNFR2 expressing $CD4^+$ T cells	220
T cells	MDS patient PBMC	aCD3 Ab & aCD12 Ab PMA & Ionomycin	5 µM	1. Increased proliferation on $CD4^+$ and $CD8^+$ cells (BrdU) 2. Increased Th1 cytokine production (intracellular cytokine staining) 3. Decreased IL4 production (intracellular cytokine staining) 4. *In vivo* increased in naïve and CM $CD8^+$ T cells with decreased EM $CD8^+$ T cells in PB of MDS patients following treatment	225
T cells	Myeloma patients	Pneumococcal vaccination	25mg/day	1. Increase in T cell vaccine responses in patients receiving treatment at time of vaccination compared with those receiving vaccine prior to treatment	226
T cells	Relapsed Colonic Cancer patients PBMC		25mg/day	1. Decreased % naïve $CD4^+$ T cells 2. Increased % activated (HLA DR+) $CD4^+$ T cells 3. Increased % activated (HLA DR+) $CD8^+$ T cells 4. Decreased EM $CD4^+$ T cells 5. Decreased EM $CD8^+$ T cells 6. Decreased Treg	215
T cells	Healthy PBMC & Myeloma patient PBMC	Autologous DC/MM fusion Allogeneic DC aCD3 Ab & aCD28 Ab	1 µM	1. Increased IFNγ production by T cells (aCD3/28 Ab) 2. Decreased expansion of Treg (aCD3/28 Ab) 3. Decreased PD1 expression on healthy and myeloma T cells 4. Increased proliferation on allogeneic DC stimulation 5. Increased IFNγ production on autologous DC/MM stimulation 6. Decreased Treg expansion on autologous DC/MM stimulation 7. Increased MM cell lysis by CTL following DC/MM stimulation (granzyme B)	208
Treg	Healthy PBMC	IL2	10 µM	1. Decreased expansion and proliferation of Treg from activated PBMC (flow $CD4^+CD25^{high}$CTLA-4^+ cells) 2. Inhibition of suppressive capacity 3. Decreased FOXP3 expression 4. Decreased OX40 expression	218

T cells DC	Murine splenocytes and bone marrow	Autologous Ag pulsed DC	10 µM	1. Increased endocytic activity of DC (fluorescent bead uptake) 2. Increased expression of MHC I and CD86 on DC 3. Increased TNF and MIP1α expression on DC 4. Greater proliferative responses of $CD8^+$ T cells stimulated by treated DC than untreated 5. Greater production of IFNγ and perforin by $CD8^+$ T cells stimulated with treated DC than untreated (intracellular cytokine staining)	227
T cells NK cells MDSC	Murine A20 lymphoma model	Idiotype vaccine against A20 murine lymphoma	5mg/kg	1. Improved outcome response to vaccine (decreased tumour bulk, increased OS) 2. Increased IFNγ producing $CD8^+$ T cells 3. Improved response to vaccine is mainly $CD8^+$ T cell dependent 4. Decreased splenic MDSC 5. Increased splenic NK cell numbers	228
T cells NK cells	Healthy human PBMC Myeloma patient PBMC	aCD3 Ab Autologous DC	0.1 – 10µg/ml	1. Increased proliferation of patient and healthy aCD3 Ab stimulated T cells (H-thymidine) 2. Increased IFNγ and IL2 production by aCD3 Ab stimulated T cells (ELISA) 3. Increased lysis of MM cell lines (HS Sultan, Raji and K562) by healthy PBMC (Cr release) 4. Increased lysis of autologous myeloma cells by patient PBMC (when IL2 also added) (Cr release)	229
T cells NK Cells	Healthy PBMC	K652, MM.1S, U266, ARH-77 cell lines	.25 to 5 µmol/L	1. Increased PBMC cytotoxicity against target cell lines (Cr release) 2. Increased ADCC of PBMC against target cells (Cr release) 3. Cytotoxicity and ADCC were significantly reduced with IL2 R blockade or CD56 depletion 4. Increased binding of NFAT and AP-1 to IL-1 promoter in T cells	230
NK cells	Murine NK cells	Lymphoma model	2.5 - 40 µg/ml	1. Increased circulating NK cells 2. Depletion of NK cells abrogated anti-tumour effect of treatment	231
NK cells	Healthy PBMC	IL2, IL12 and IL18	5 µM	1. Decreased IFNγ production 2. Increased CD56 expression 3. Decreased inhibitory KIR expression	232

NK Cells	Healthy PBMC	IL2	0.1 – 10 µM	1. Increased ADCC against multiple solid tumour cell lines	[233]
NK cells DC	Murine NK cells	Murine DC	5µg/ml	1. Increase in generation of murine DC from bone marrow cells in *in vitro* culture 2. Increased CD11c and MHC class II expression 3. Increased production of IFNγ, TNF and MCP1 by DC 4. Increased NK ADCC 5. Increased NK cell infiltration of tumour bed in tumour bearing mice	[234]
NK cells Monocyte	Healthy PBMC	IgG and IL2	0.0032 - 25 µM	1. Increased IFNγ production by NK cells (ELISA) 2. Increased ADCC of NK and Monocytes against B cell lymphoma cell lines (Nawalma, Farage, Raji) 3. Increased NK cell production of chemokines including IL6, IL8, RANTES, MIP1α & β, MCP1 and GM-CSF (ELISA) 4. Increased expression of FasL on NK cells	[235]
NKT cells	Healthy PBMC Myeloma and MDS 5q- patient PBMC	Ag pulsed autologous DC	1µM	1. Increased expansion of NKT cells 2. Increased numbers of IFNγ positive NKT cells (intracellular cytokine staining) 3. Increased numbers of circulating NKT cells found in peripheral blood of patients receiving treatment	[236]
NKT cells	Healthy PBMC Myeloma patient PBMC	Ag pulsed autologous DC	2 µM/L	1. Increased INFγ, IL2 (ELISA) of both healthy and patient NKT cells 2. Decreased IL4 (ELISA) of both healthy and patient NKT cells	[237]
Myeloid NLC	PBMC from CLL patients	Autologous CLL cells	10 µM	1. Decreased CLL survival due to decreased support from NLC due to: Increased secretion of IL10 and decreased HLA DR expression	[238]

Ref = reference, aCD3 Ab = anti-CD3 antibody, LPS = lipopolysaccharide, TNFR2 = Tumour necrosis factor receptor 2, IL2 R = interleukin 2 receptor, sIL2 R = soluble interleukin 2 receptor, IL = interleukin, IFN = interferon, TNF = tumour necrosis factor, SEE = staphylococcal enterotoxin e, Ag = antigen, DC = dendritic cells, CLL = chronic lymphocytic leukaemia, S1P = sphingosine 1 phosphate, MDS = myelodysplasia, CCL = chemokine c ligand, CM = central memory, EM = effector memory, Treg = T regulatory cells, MM = multiple myeloma, CTL = cytotoxic T lymphocytes, MDSC = myeloid derived suppressor cells, ADCC = antibody dependent cellular cytotoxicity, NLC = Nurse like cells, Lena = Lenalidomide, HIV = Human immunodeficiency virus, ELISA = Enzyme linked immunosorbent assay, NKT = Natural killer T cell, GM-CSF = granulocyte macrophage colony stimulating factor, MCP-1 = monocyte chemoattractant protein 1, MIP 1= macrophage inflammatory protein 1, Cr = chromium, AP-1 = activating protein 1, NFAT = Nuclear factor of activated T cells, PB = peripheral blood, KIR = killer immunoglobulin-like receptor.

1.2.3.3 Lenalidomide effects on APC

There are a small number of *in vitro* studies that have examined the effect of lenalidomide on the afferent arm of the T cell response. These indicate that lenalidomide potentiates APC function in a number of ways including: increased expression of MHC Class II, CD86 and CD11c, increased secretion of the pro-inflammatory cytokines (IFNγ, TNF and CCL2 by murine DC) [234] as well as increased endocytic capacity [227]. Henry *et al.* also report that lenalidomide-treated DC were more efficient initiators of $CD8^+$ T cell responses in a murine ovalbumin-specific model [227], this may be due to the fact that lenalidomide has been shown to enhance immune synapse formation [186,209].

1.2.3.4 Lenalidomide effects on NK cells

Lenalidomide-induced augmentation of NK cell activity is believed to be responsible for some of the anti-neoplastic action of lenalidomide. Evidence for this comes from *in vitro* studies showing that exposure to lenalidomide leads to: expansion of NK cells in cultured PBMC, enhanced expression of activating receptors (NKp30 and NKp46), reduced expression of inhibitory KIR (NKAT2 and NKB1) [232], increased production of cytokines and chemokines (TNF, CCL2 and CCL5)[233], enhanced antibody dependent cellular cytotoxicity (ADCC) [214,235,239] as well as direct cellular cytotoxicity against cancer cell lines [230]. These effects on NK cells appear to be as a consequence of enhanced cytokine production (particularly IL2) from T cells also present in the cultures rather than by cell-intrinsic effects as it can be abrogated by anti-IL2 receptor antibodies or by purification of NK cells [230,232,239].

In vivo data support the *in vitro* data showing expansion of NK cell numbers in peripheral blood of patients treated with lenalidomide for solid cancer, [215] myeloma [229] and CLL where this was associated with improved response to therapy [240]. In a murine model of B cell lymphoma IMiD treatment of mice also resulted in increased NK cell infiltration of tumours [234].

1.2.4 Molecular mechanism underlying the actions of lenalidomide

IMiDs had long entered routine clinical use before the molecular mechanisms underlying their action were fully understood. In a landmark paper in Science, Ito *et al.* identified that the intra-cellular target of thalidomide was cereblon. Binding of thalidomide to this protein, which forms the substrate receptor of an E3 ubiquitin ligase was found to mediate the teratogenic effects that thalidomide can cause in embryos [241]. Further *in vitro* studies have shown that the presence of cereblon is required for the anti-myeloma activity of thalidomide and lenalidomide [242], for increased TNF and IL2 production by T cells [243] and for improved immune synapse formation [211]. *In vivo* studies support these *in vitro* findings; high gene expression of *cereblon* is associated with good response to IMiD therapy and good prognosis in myeloma [244,245]. Conversely the acquisition of inactivating mutations in *cereblon* results in acquired drug resistance [246].

In the last 2 years it has become apparent that the binding of IMiDs to cereblon enhances the activity of the E3 ubiquitin ligase, resulting in greater ubiquitination and thus degradation of selected substrates (**Figure 1.8**). In particular the transcription factor ikaros has been shown to become depleted via this mechanism in myeloma cell lines [247] and T cells [248]. Low levels of *ikaros* gene expression in patients have also been demonstrated to result in poorer responses to IMiD treatment [249].

Ikaros is member of a family of zinc finger endo-nucleases, known as the Ikaros transcription factors including aiolos, helios, ios and pegasus. The best described of these is ikaros, which has roles as both a tumour-suppressor gene and master regulator of lymphocyte differentiation. The important role of ikaros in lymphocyte development is underlined by the finding that *ikaros*-null mice fail to develop B cells or NK cells and have an abnormal $CD4^+$-skewed T cell repertoire [250]. Binding of ikaros to CD8 regulatory elements leading to activation of the gene may explain why CD8 expression is selectively lost in the absence of *ikaros* [251].

In mature T cells ikaros has been reported to have a number of roles. Mouse models with heterozygous deletion of *ikaros* have been found to invariably develop T cell malignancy. This is likely due to a lower threshold for entry in to the cell cycle in response to TCR engagement and accelerated G1-S phase transition in response to signalling via the IL2 receptor [252]. During T cell activation and TCR signalling ikaros is seen to localise in toroids in the nucleus that co-localise with areas of DNA replication and cyclin proteins, this change in the nuclear staining pattern is

blocked by inhibitors of TCR signalling, (including inhibitors of Lck, Fyn, protein kinase C and phosphoinositide 3 kinase and mTOR) which demonstrates that this change is TCR dependent [252].

As well as influencing the strength of signalling through the IL2 receptor ikaros has been shown to play an important role as a repressor of IL2 gene transcription with a role in anergy induction. Investigators have used chromosome immunoprecipitation (ChIP) to demonstrate ikaros binding to the IL2 promoter and small interfering RNA (siRNA) for *ikaros* RNA to show that in the absence of ikaros activity IL2 levels were increased [253,254]. Naïve T cells were shown to have an increased susceptibility to ikaros due to the distal region of the IL2 promoter already having an open chromatin conformation facilitating ikaros binding compared to other subsets. Finally ikaros loss can release T cells from their dependence on co-stimulatory signalling via CD28, whereas under normal conditions the loss of signal 2 would result in T cell anergy [254]. This effect on co-stimulation is in agreement with a paper by LeBlanc *et al.* who demonstrated that lenalidomide treatment could overcome co-stimulatory blockade with CTLA4-chimeric antibodies [204].

Ikaros also plays an important role in recruiting histone deacetylases (HDAC) and in mediating chromatin remodelling of discreet regions of DNA that promote differentiation to a Th2 phenotype (including transcription factors GATA3, cmaf, Tbet and STAT1), with loss of ikaros leading to secretion of Th1 cytokines even when cells are cultured in Th2 skewing conditions [255]. The downstream effects of ikaros depletion described could explain the increased activation and proliferation of T cells demonstrated *in vitro*.

Figure 1.8

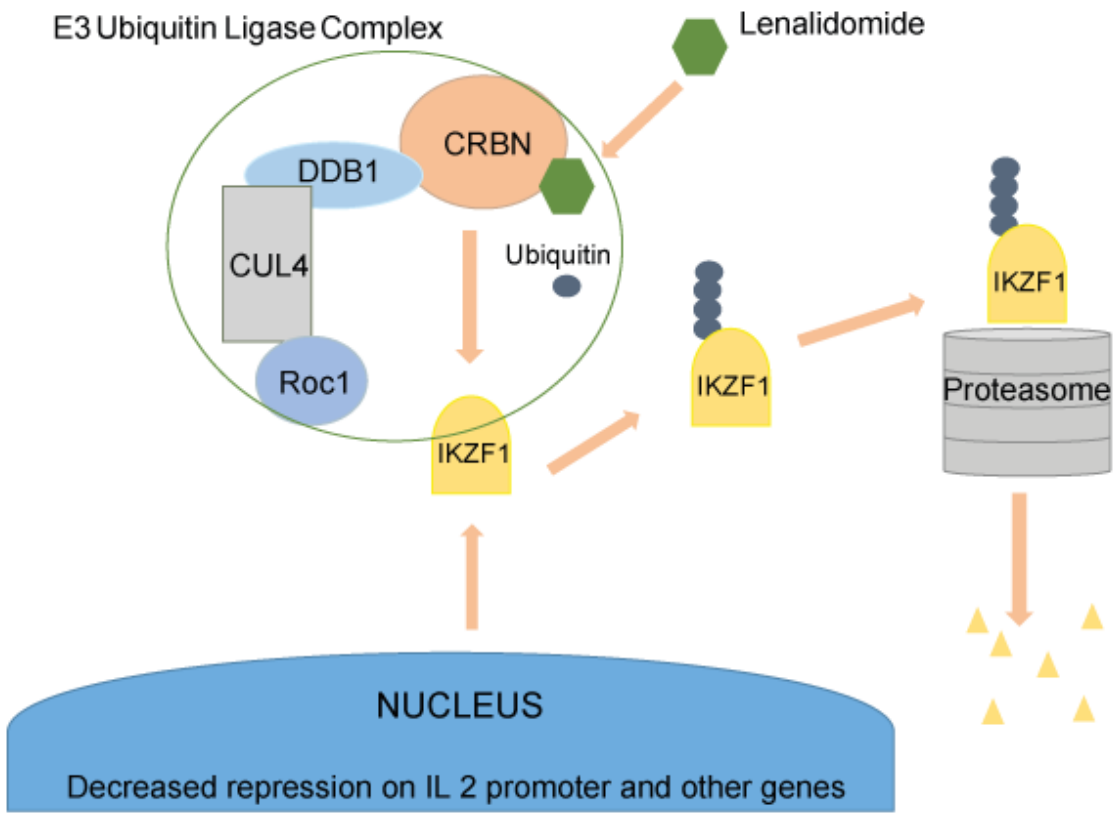


Figure 1.8 Schematic representation of lenalidomide modulation of cereblon activity

Lenalidomide binds to cereblon (CRBN), which acts as the substrate receptor for a cullin-ring E3 ubiquitin ligase complex (other parts of the complex include ring box protein 1 (Roc1), DNA damage binding protein 1 (DDB1) and cullin 4 (CUL4)). Binding of lenalidomide enhances the activity of cereblon leading to increased ubiquitination of targets (including Ikaros (IKZF1)) resulting in their increased degradation at the proteasome and releasing transcriptional repression on target genes such as the interleukin 2 (IL2) promoter

Based on findings of Gandhi *et al.* [248]

1.2.5 Clinical trials of lenalidomide after AHSCT

Following on from *in vitro* and *in vivo* data demonstrating lenalidomide's potential to improve T cell responses to tumour in the autologous setting a number of small clinical trials have provided some insight into the effect of the drug in the allogeneic setting. As the most extensive use of lenalidomide has been in myeloma, it has been logical that the majority of these trials have sought to exploit both the anti-myeloma cytotoxic effect of lenalidomide to eradicate minimal residual disease after AHSCT as well as possible T cell allostimulatory effects to boost GvT (Trials are summarised in **Table 1.3**). A common concern of these trials has been an association between the administration of lenalidomide and the onset or severity of GvHD observed [256,257].

Drawing firm conclusions from the results of these studies is difficult due to the heterogeneity of the patient population, the differences in exclusion criteria, the differing treatment regimes for lenalidomide administered (some including dexamethasone and in combination with DLI), the different timing after AHSCT of commencement of lenalidomide-treatment and the small numbers of patients enrolled. However it would appear that the risk of GvHD is higher in those studies where lenalidomide-treatment was started within 6 months of AHSCT. In the study by Coman *et al.* the authors reported that the risk of GvHD was higher in patients who had previously experienced GvHD, had active GvHD at time of initiation of lenalidomide, who received lenalidomide as their first salvage therapy after AHSCT and in those who had recently stopped immunosuppression [258].

Limited data from concurrent peripheral blood immunomonitoring of patients in 3 trials indicates that lenalidomide was associated with increased HLA DR expression on $CD4^+$ and $CD8^+$ T cells (particularly $CD8^+$ T cells) as well as an increase in the proportion of activated (NKp44/NKp30 positive) NK and $CD56^{dim}$ NK cells that may represent a shift towards a more cytotoxic phenotype. However there was no significant increase in IFNγ producing cells and no change in Treg at any early time-point, although in those patients who continued therapy there was a significant late increase in Treg [256,259,260]. In the study by Wolschke *et al.,* NK cells harvested from the peripheral blood of AHSCT recipients treated with lenalidomide demonstrated increased lytic activity against a myeloma cell line *ex vivo*. However in none of these studies were the authors able to demonstrate an association between any of these immune parameters and outcome.

Due to the small numbers enrolled, lack of a control cohort and the relatively short follow up of many of these trials, it is difficult to assess the contribution of lenalidomide to GvT, especially in view of the direct cytotoxicity of lenalidomide against myeloma. However, good response in terms of eradication of minimal residual disease after lenalidomide therapy was often associated with improvement in donor chimerism [261] and in the study by Coman *et al.* there was an association between occurrence of GvHD and overall response rate (hazard ratio 2.33, p 0.03) [258].

There are also case reports in the literature describing cases of lenalidomide-induced remission of relapsed haematological malignancy after AHSCT. These include: complete cytogenetic remission of relapsed 5q- AML following 3-6 cycles of 10mg of lenalidomide, with a concurrent increase to 87% donor chimerism in bone marrow in one case [262,263], complete remission of relapsed plasma cell leukaemia after AHSCT associated with conversion to full donor chimerism [264], complete remission of relapsed CLL after AHSCT (in association with development of GvHD) [265] and partial response of relapse Hodgkin lymphoma relapsing following AHSCT [266]. The last 2 cases are particularly interesting as single agent lenalidomide might not be expected to have as strong a direct cytotoxic effect on CLL and Hodgkin lymphoma as in 5q- syndromes and plasma cell derived malignancies.

There is very little *in vitro* or *in vivo* data to indicate the mechanisms by which lenalidomide exerts effects after AHSCT. In most cases the rationale for the use of the drug in this setting has been for its direct anti-tumour effect or extrapolated from data in artificially stimulated T cells. There has been a recent cooling of enthusiasm for further studies in this area due to the perceived risk of GvHD, but a deeper understanding of the immunological changes induced may present new strategies allowing safer and more effective use of IMiDs after AHSCT.

Table 1.3 Clinical trials of lenalidomide after AHSCT

Study	Design	No Pts	Dose (mg)	+ Dex	DLI	Timing of Lena (mo)	Median No cycles	Response	OS/PFS	GvHD	Organ GvHD	Reason for discont
Alsina 2014 [267]	P, M, R, Ph IIa, maint	30	10	N	N	3	Max 12, 46% discont after 2	33% ORR	PFS 63% OS 78% 18mo	47% Gd II-IV 30% Gd III-IV 17%	Skin Gut	37% GvHD 11% Neutropenia 5% infection 5% rash
Kroger 2009 [268]	P, S, maint	32 (2 Lena)	15	N	Y (2)	NG	4	59% CR	NG	43%	NG	NG
Kroger 2013 [269]	P, S, rel/ref	33	5	N	Y	5.5	6	46% CR 48% PR	PFS 52% OS 79% 3yr est	34% Gd II-III	NG	46% PD 23% GvHD 31% Other
Kneppers 2011 [256]	P, M, maint	30	10	N	N	3	3	37% ORR	OS 94% 61% PFS at 2 yrs	37% Gd II-IV	Skin Liver Gut	17% PD 43% GvHD 17% Other
Wolscke 2013 [260]	P, S, Ph I/II, maint	24	5 (75%) 10 (15%)	N	N	4.5	NG	ORR 42%	OS 79% PFS 61% At 2yrs	38% Gd I-IV	Liver Gut	29% GvHD
Sockel 2012 [257]	P, M, Ph II, maint	10	10	N	N	2.5	5	CR 40%	OS 50% at 331 days	60% Gd III-IV	Skin Gut	40% PD 40%GvHD
Bensinger 2014 [270]	P, S, rel	18	25	1	N	12	12	ORR 56% CR 28%	OS 56% at 35mo	11%	NG	56% PD 11% GvHD 6% Other

El-Cheikh 2012 [271]	P, S, rel/ref	12	10 ref 25 rel	N	Y	6	5	75% ORR 25% CR	OS 69% PFS 50% at 2yrs	8% Gd II	Skin Gut	17% PD 8% GvHD
Lioznov 2010 [259]	R, S, rel	24	25 (83%) 15 (17%)	Y	Y (18)	11	5	66%ORR 8%CR	Med PFS 9.7months	16% Gd II	Skin	NG
Coman 2013 [258]	R, M, rel	52	25 (79%)	77%	19%	24	6	83% ORR (29% CR)	Med PFS 18months	58%	NG	29% PD 15% GvHD 22% Other
Blum 2010 [261]	P, S, Ph I, rel	31 (7 post allo for AML)	50 MTD	N	N	NG	1	16% CR	NG	100% in responders	Skin	43% PD 29% GvHD
Mimmema 2009 [272]	P, S, rel	16	25	Y (8 pts)	Y (11 pts)	>3	6	ORR 46% 87% +Dex	Median 0S 395 days PFS 328 days	31% Gd II-IV	NG	NG

S = single centre, M = Multi-centre, P = Prospective, R = Retrospective, PH = Phase, maint = maintenance, ORR = overall response rate, PFS = progression free survival, OS = overall survival, Gd = Grade, NG = Not given, CR = complete response, PR = partial response, VGPR = very good partial response, Y = Yes, N = No, PD = progressive disease, Lena = lenalidomide, mo = months, pts = patients, rel = relapse, ref = refractory, GvHD = graft versus host disease, Allo = allogeneic haematopoietic stem cell transplant, AML = acute myeloid leukaemia, Max = maximum, Discont = discontinued, MTD = maximum tolerated dose.

1.3 Rationale

The outcome for patients who relapse following AHSCT is poor. Data from St Bartholomew's Hospital shows that 80% of patients transplanted for acute myeloid leukaemia/MDS with evidence of failed alloreactivity will relapse within 2 years of AHSCT [273].

Cellular therapies such as DLI are available to some patients following AHSCT, but in many cases it is not possible to re-access donors, this is particularly true in cases of UCBT.

Alternative therapies that can enhance donor T cell alloresponses are urgently required for these patients. IMiDs such as lenalidomide may be useful in this scenario, however in order to use these drugs safely and effectively a greater understanding of the effect of lenalidomide on human T cell alloresponses is required.

1.4 Hypothesis

Characterisation of the cellular and sub-cellular mechanisms by which lenalidomide can modulate the human T cell alloresponse may facilitate more effective use of lenalidomide and other IMiDs to improve the outcome of AHSCT.

1.5 Aims

1. To comprehensively characterise changes in the *in vitro* human T cell alloresponse caused by lenalidomide exposure during allostimulation including:
    - Identifying which subsets of T cells are affected
    - Phenotypic changes of alloreactive T cells that may facilitate identification *in vivo*
    - Changes in cytokine production and effector/regulatory cell ratios of alloproliferative cells

2. To determine whether lenalidomide exposure causes differential effects on *in vitro* alloresponses of human T cells from different graft sources:
    - To compare alloresponses of APB and UCB-derived T cells exposed to lenalidomide

3. To determine whether lenalidomide exposure has any differential effects that could predict selective GvHD and/or GvT responses:
    - Potential of T cells to migrate to target organs of GvT/GvHD
    - Cytotoxicity against haematopoietic target cells

4. To identify sub-cellular mechanisms underlying the effect of lenalidomide on the human T cell alloresponse:
    - Identify whether different exposure strategies alter effects of lenalidomide on the alloresponse
    - Identify whether mechanisms underlying known actions of lenalidomide in mitogen-stimulated T cell responses are also involved in modulation of the alloresponse after lenalidomide exposure
    - Identify any additional mechanisms unique to the alloresponse

Chapter 2 - General materials and methods

2.1 Human cells

PBMC from leucocyte cones from healthy adult blood donors, and UCB mononuclear cells from donated units were isolated by density-gradient centrifugation. Additional PBMC samples from HLA-matched allogeneic transplant recipients and their sibling donors were sourced from the tissue bank maintained by the Centre for Haematology-Oncology, Barts Cancer Institute, London, UK. The study was approved by the London Research Ethical Committee (05/Q0605/140 and 06/Q0604/110) and was conducted in accordance with the Declaration of Helsinki.

2.2 Isolation and cryopreservation of PBMC

Leucocyte cones were obtained from NHS Blood and Transplant. These contain 10-20 millilitres (ml) of leucocyte rich blood from healthy donors collected as a waste product during platelet-pheresis. Contents were washed from the cone using phosphate buffered saline (PBS) via a wide bore (16 gauge) sterile needle and 20ml sterile syringe into a sterile 50ml falcon tube and made up to a total volume of 50ml with PBS. Ten ml of the cell suspension was then layered over 5ml Lymphoprep^{TM} (Fresenius-Kabi) and the PBMC separated by density gradient centrifugation as per the manufacturers protocol.

The PBMC layer was extracted from the sample-Lymphoprep^{TM} interface using a Pasteur pipette and transferred to a fresh sterile 50ml falcon tube. PBMC were washed twice (with PBS after centrifugation at first 350g and then 500g for 5 minutes (mins) at room temperature (RT) to pellet with supernatant discarded) and residual red cells removed by incubation for 7 mins in 10ml of red cell lysis buffer (Biolegend). PBMC were then washed (as above with centrifugation step at 500g) and counted (using a Vicell-XR^{TM} cell viability analyser, Beckman-Coulter) before being resuspended in supplemented media (RPMI 1640 Aq media containing 10% fetal bovine serum (FBS) and 1% penicillin streptomycin) at a concentration of $10x10^7$ cells per ml. UCB cells from collections that would otherwise be discarded were processed in the same way as leukocyte cones. Volume of UCB received varied from 20ml to 200ml.

A 20% dimethyl sulfoxide (DMSO) freeze-mix was made up using DMSO (Fisher Scientific) and foetal bovine serum (FBS). Five hundred µl of freeze-mix and 500µl cell suspension were pipetted into 1.8ml cryovials (Corning) to give a final concentration of 10% DMSO and $5x10^7$cells/ml. Cryovials were then transferred to a CoolCell® and frozen down at approximately -1°C/min to -80°C before transfer to liquid nitrogen storage.

2.3 Lenalidomide stock solution preparation

Lenalidomide powder was provided as a gift by Celgene. A 10mM stock solution was made by dissolving lenalidomide powder in DMSO and vortex mixing. A small amount of lenalidomide powder was weighed out and volume of DMSO was calculated using the equation:

Volume (L) = Mass (g) / (Molar Mass (g/mol) x Molarity (mol/L).

The molar mass of Lenalidomide is 259.6g/mol.

Fifty µl aliquots of the solution were pipetted into 500µl sterile eppendorf tubes, frozen down and stored at -80°C.

2.4 Detection and absolute quantification of lenalidomide in biological samples

2.4.1 Validation of stock solution and standard curve

My thanks to Dr Essam Ghazaly and Miss Chathunissa Gnanarajan for help with the design, performance and analysis of these tandem mass spectrometry and ultra-high performance liquid chromatography (MS/MS UHPLC) experiments. The protocol used was adapted from Iqbal *et al.* [274].

A 50µl aliquot of Lenalidomide stock solution was thawed and diluted in 30% Acentonitryl and 1% Formic Acid solution to give a 1µM solution. One hundred µl of this solution and a 500ng/ml solution of carbamazepine (the internal standard) was then analysed using an ultra-performance liquid chromatography system (Accela UPLC, Thermo Scientific, UK) equipped with an Acquity UPLC BEH C18, 1.7 µm, 100 × 2.1 mm column (Waters) and a mobile phase consisting of a mixture of water containing 0.1% formic acid (A), and acetonitrile containing 0.1% formic acid (B). The mobile phase gradient was employed, comprising: buffer A = 90% at 0 - 1 min, from

90 to 20% over 2 minutes, held at 20% for 2 mins, from 20 to 90% over 0.1 mins, ending with 90% for 2.9 minutes, all at a flow rate of 250 µl/min.

Eluting compounds of interest were detected using triple stage quadrupole Vantage mass spectrometry system (Thermo Scientific) equipped with an electrospray ion source. Samples were analysed in the Multiple Reaction Monitoring (MRM), positive ion modes at a spray voltage of 3500 V. Nitrogen was used as sheath and auxiliary gas at a flow rate of 30 and 10 arbitrary units, respectively. Argon was used as collision gas with pressure of 1.5 mTorr. The optimum transitional daughter ions mass and collision energy of each analyte were as follows: Lenalidomide 260.0 $\rightarrow$ 149.2 (collision energy 16 V) and Carbamazepine 237.1 $\rightarrow$ 194.3 (collision energy 20 V) (**Figure 2.1**).

Fresh calibration standards (0-10 µg/ml) were prepared by diluting the stock solution (10 µg/ml) in 10% acetonitrile. High and low control samples (5 and 0.5 µg/ml) were also prepared. The standard curve generated using linear regression analysis (by plotting lenalidomide concentration versus area ratio (lenalidomide area divided by the internal standard area) could then be used to calculate lenalidomide concentration in culture media and cells.

2.4.2 Preparation of media and cells for MS/MS UHPLC analysis

One hundred µl of medium was taken from experimental flasks and transferred to an eppendorf tube. Three hundred µl of ice-cold methanol (containing 500ng/ml of the internal standard) was added to the tube and vortex mixed for 1 min. The tube was then kept on ice for 30 mins, centrifuged at 750g for 10 mins at 4°C and the supernatant then transferred to a fresh eppendorf tube. After methanolic evaporation to dryness, tubes were stored at -80°C until analysis, at which time dried extracts were reconstituted in 10% acetonitrile solution and injected into the UHPLC-MS/MS system.

Cells taken from experimental flasks were counted and an aliquot containing approximately 1 million cells was transferred to an eppendorf tube. Tubes were centrifuged at 750g for 5 mins at 4°C to pellet cells. Supernatant was completely removed using a pipette. The cell pellets were then stored at -80°C. On thawing 300µl of ice cold methanol was added to the cell lysates and extraction proceeded as above. Tubes were again stored at -80°C until analysis.

Figure 2.1

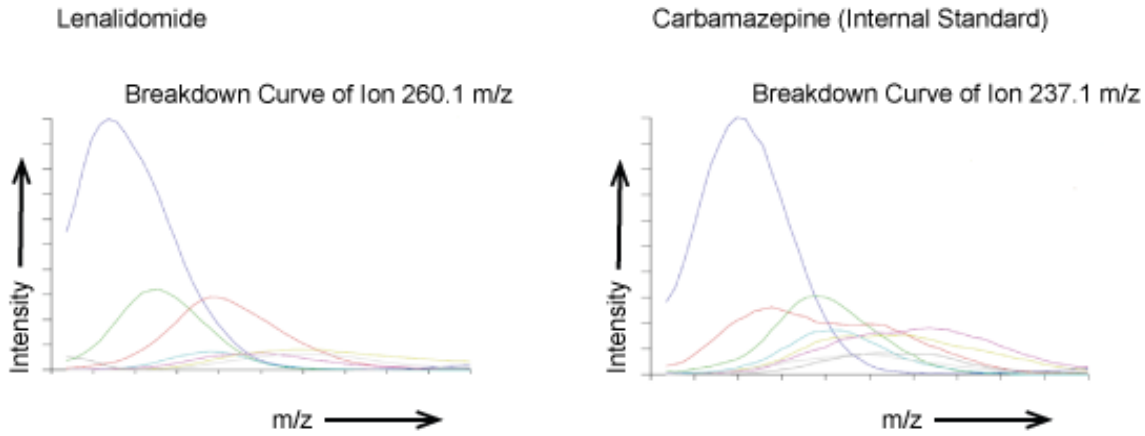


Figure 2.1 Identification of Lenalidomide by MS/MS UHPLC

Lenalidomide and the Internal Standard (carbamazepine) were identified based on time of flight and characteristic fragmentation pattern of ions. Coloured lines represent abundance of product ions of fragmentation. The blue line represents the main breakdown product.

2.5 One-way primary allogeneic co-cultures

One way primary allogeneic co-culture (otherwise known as primary mixed lymphocyte culture/reaction) allows assessment of *in vitro* alloresponses. In this model, stimulator PBMC are inactivated, by either irradiation or mitomycin C, allowing only the responder lymphocytes to proliferate in response to foreign histocompatibility antigens. Historically incorporation of radio-labelled thymidine was used as a measure of responder proliferation, more recently carboxyfluorescein diacetate succinimidyl ester (CFSE) and novel cell-tracker dyes have allowed more sensitive assessment of proliferation and cell subset-specific kinetics of cell division [275-278]. Although this model has been criticised in the past for the variability of responses [279] and cannot be used clinically to predict GvHD, it has shown some correlation with graft-rejection and other clinical outcomes [280-282] and is the most widely used *in vitro* model to study alloresponses (reviewed in Mehrotra *et al.*[283]).

Healthy PBMC were selected from liquid nitrogen storage and defrosted in a 37°C water bath, transferred to a 50ml sterile tube with a sterile Pasteur pipette and washed twice with PBS (centrifugation at 500g for 5 mins at RT to pellet with supernatant discarded) before counting (Vicell-XR^{TM}), resuspension in supplemented RPMI at a concentration of 2x10^6 cells per ml and resting overnight (12-16 hours).

The following day one healthy donor was assigned as responder, PBMC were counted and stained with CFSE (as described below). Remaining individual donor stimulator PBMC were counted and transferred to 50ml sterile falcon tubes, pelletted by centrifugation at 500g for 5 mins at RT, supernatant discarded and resuspended in warmed supplemented media at 10x10^6 cells/ml. Falcon tubes were then immediately irradiated at 40Gray (Gy) using a RS2000 biological irradiator (RadSource Technologies).

Allogeneic responder PBMC and single donor stimulator PBMC were then mixed at a 1:1 ratio in paired upright 25cm^2 sterile cell culture flasks (as previously described [127]) as depicted in **Figure 2.2**. Three biological replicates (unique donor-responder pairs) plus an autologous co-culture as an internal negative control were set-up simultaneously.

Figure 2.2

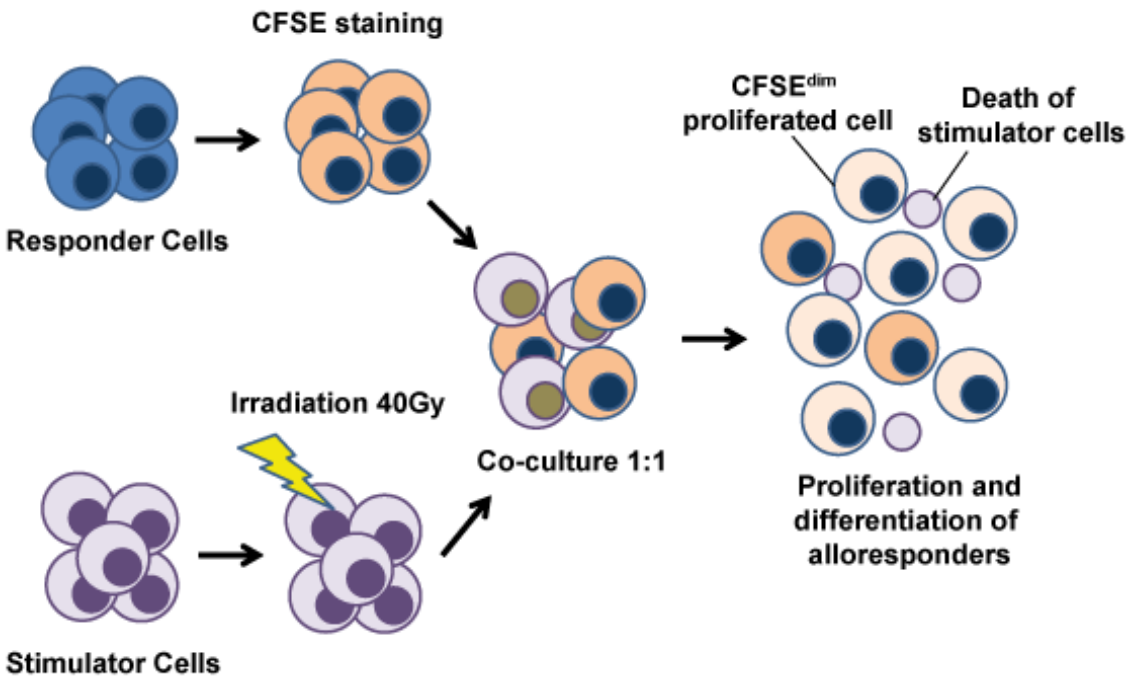


Figure 2.2 Schematic of allogeneic co-culture

Randomly assigned responder PBMC are stained with CFSE, while allogeneic and autologous stimulator PBMC are irradiated at 40 Gray (Gy). Responders and stimulators are co-cultured in a 1:1 ratio and alloresponses measured at a pre-defined time-point.

Lenalidomide at a final concentration of 1µM, or 1µl/10ml vehicle control (DMSO) was added to one flask of each paired allogeneic and autologous co-culture. Flasks were then incubated at 37°C in humidified air with 5% carbon dioxide (CO_2) in sterile incubators for 7-11 days. A flask of unstained responder cells and CFSE labelled responder cells was also kept and incubated.

2.5.1 Proliferation dye labelling of responder PBMC

To allow assessment of proliferation, responder cells were labelled with proliferation dyes: CFSE (or for a limited number of experiments Cell Tracker Violet) (both Invitrogen). Cell Tracker Violet staining was as per manufacturer's protocol. CFSE staining was adapted from the protocol and therefore is detailed below.

Responder PBMC were washed (after centrifugation at 500g for 5 mins at RT to pellet with supernatant discarded) and resuspended in PBS in a 15ml sterile tube at a concentration of $10x10^6$ cells per 250µl. A working dilution of 1µM CFSE was made by diluting a 1mM stock solution (CellTrace™, Invitrogen) 1:1000 in PBS. Working dilution CFSE was then added in 1:1 volume to cell suspension to give a final concentration of 0.5µM CFSE. The tube containing the cells was then incubated at RT in the dark for 8 mins with brief vortex mixes approximately every 90 seconds. Staining was quenched by adding an equal volume of ice-cold FBS and incubating at RT for 4 mins. Cells were then washed with supplemented media twice (after centrifugation at 500g for 5 mins at RT to pellet with supernatant discarded) before counting and resuspension in supplemented media at a concentration of $1x10^6$/ml.

2.6 Magnetic cell separation

In some experiments specific subsets of PBMC were isolated either prior to, or after allogeneic co-culture by magnetic bead-based sorting, using Miltenyi technology. Negative magnetic cell separation (MACS) allowed isolation of untouched target cells by depletion of non-target cells, minimising activation of target cells caused by the separation process. Non-target cells are labelled with a biotin-conjugated monoclonal antibody cocktail and then bound by anti-biotin antibody coated micro-beads. Cells coated with micro-beads are then depleted by retaining them within a MACS® column in the magnetic field of a MACS separator. All reagents and consumables for separation were purchased from MACS® Miltenyi Biotec.

Cells for separation were prepared as per the manufacturers protocol. Briefly cells were washed with PBS (as above) passed through a cell strainer (Fisher Scientific) to remove clumps of cells, centrifuged at 500g for 10 mins at RT to pellet cells and supernatant completely aspirated using a pipette. The cell pellet was resuspended in cold MACS buffer at a concentration of 40μl buffer per 10^7 cells. Biotin antibody cocktail (10μl per 10^7 cells) was then added and cells incubated for 5 mins at 4°C. Following this 30μl cold MACS buffer per 10^7 cells was added, followed by micro-bead cocktail (20μl per 10^7 cells) and cells incubated for a further 10 mins at 4°C.

During this time cell separation columns were attached to a QuadroMACS® magnetic separator and 3ml of cold MACS buffer added to each column. Flow though was collected into 15ml sterile falcon tubes. After incubation cell suspension was added to the columns and flow through collected in the same 15ml falcon tubes. After flow though of cell suspension the columns were washed with a further 3ml of cold MACS buffer. Falcon tubes were then centrifuged at 500g for 10 mins at 4°C, supernatant discarded and washed with PBS (as above). Cells were then counted, an aliquot reserved for evaluation of purity and cells used for further experiments.

For experiments where large numbers of samples required MACS separation the AutoMACS automatic cell separator was used. Cells were prepared as above prior to labelling and then loaded into the AutoMACS machine along with required reagents. The machine then performs automatic labelling and separation as per manufacturers pre-defined protocol. Separated cells from the AutoMACS were then processed in the same way as following manual separation.

2.7 Flow cytometric assessment of alloresponses

Flow cytometry, also referred to as FACS (Fluorescence activated cell sorting, FACS®, Beckton Dickinson) is a method that can be used to identify, characterise and isolate populations of cells. FACS is based on principles of light scattering by particles (in this case cells) passing across a beam of light and/or laser, in combination with additional information about the particles obtained using fluorochromes attached to specific cellular target molecules and induced to emit fluorescence.

Cells in suspension, that have been labelled with fluorochrome-conjugated monoclonal antibodies are aspirated into the flow cytometer and forced into a single cell stream. This stream then passes the cells individually through beams of light and lasers. As the cell passes through

the beam of light, light scattered in the forward direction and sideways is captured and focused to a detector which converts this information into a digital readout of that particular 'event' (i.e. cell) for the parameters known as forward scatter (FSC) and side scatter (SSC). FSC gives information as to cell size, while SSC gives information as to the granularity of the cell. Using these parameters alone it is possible to distinguish different populations of PBMC.

As cells continue pass to through laser beams, certain fluorochromes will become excited and emit light at a particular wavelength. This emitted fluorescence is captured and split into specific colours by optical filters and directed to photomultiplier tubes (PMT) where the signals are converted to digital readouts that are displayed as events. The software displays cumulative data for a number of events as dot-plots or histograms.

Depending on the numbers of lasers and optical filters a flow cytometer contains, multiple combinations of fluorochromes can be used to identify co-expression of a number of cellular targets simultaneously and therefore to identify rare subsets of cells (reviewed in Bendall *et al.*[284]).

It is also possible to sort individual cells into separate populations, based on pre-defined sort criteria using electrostatic charge. Application of charge to form droplets within the cell stream encapsulates target cells that can then be deflected from the main stream and collected.

2.7.1 Surface marker labelling of responder cells for flow cytometry and flow cytometric sorting

Cells of interest were transferred to 5ml round bottom tubes or 96 well u-bottom/v-bottom sterile culture plates, centrifuged at 500g for 5 mins at RT to pellet with supernatant discarded and washed in FACS buffer.

A master mix of fluorochrome-conjugated monoclonal antibodies (fluorescent antibodies) was made up for surface markers of interest in FACS buffer or fixable viability dye where necessary (see below for fixable viability dye).

Master mix was added to samples at 100µl/million cells and incubated in the dark at RT for 15 mins. Additional single stained samples were prepared as compensation controls using UltraComp eBeads™ (eBioscience) as well as single CFSE and viability dye-stained responder cells and unstained responder cells.

Following incubation cells were washed with FACS buffer (as above) and resuspended in 1:2000 4', 6-diamidino-2-phenylindole (DAPI) in FACS buffer for viability staining (provided fixable viability dye had not been previously added) and kept on ice before acquisition on a LSR Fortessa Analyser (Beckton Dickinson).

For flow cytometric sorting cells were incubated in the dark at 4°C for 30 mins after addition of master mix, with intermittent vortex mixing every 10 mins. Sorting was performed in liaison with core flow facility staff at Barts Cancer Institute on a FACS Aria Fusion cell sorter (Beckton Dickinson). Sorted cells were collected into sterile 20% FBS in PBS.

Fluorescent antibodies used for FACS analysis and sorting are listed in Table 1A Appendix A.

2.7.2 CD107a and intracellular target labelling for flow cytometry

CD107a or lysosomal-associated membrane protein 1 (LAMP1) can be used as a surrogate marker of cytotoxicity. This protein is expressed on the cell surface as lysosomes containing granzymes and perforin fuse with the cell membrane and release their contents. CD107a expressing cells have been shown to mediate cytotoxicity in an antigen specific manner (reviewed in Zaritskaya *et al.* [285]).

Cellular production of cytokines can be assessed following inhibition of protein secretion by cells to allow cytokines to accumulate within the cell and therefore enable detection. Cells are then fixed and permeabilised to allow fluorescent antibodies to access intracellular proteins [286]. Similarly fixation and permeabilisation of cells allows fluorescent antibodies to bind to other intracellular targets such as transcription factors and signalling molecules.

Cells were counted before transfer to 5ml round bottom tubes or 96 well u-bottom/v-bottom sterile culture plates. Anti-CD107a fluorescent antibody and protein transport inhibitor (containing Brefeldin A and Monensin, Invitrogen) was added to the cells (as per manufacturer's

protocol) and cells were incubated at 37°C in humidified air with 5% CO_2 for 6 hours. An additional positive control was set up using cells from the autologous untreated co-culture in the same way but in addition to the protein inhibitor cocktail cell stimulation cocktail (containing PMA and Ionomycin, eBioscience) was added prior to incubation.

Following incubation cells were centrifuged at 500g for 5 mins at RT to pellet with supernatant discarded, washed with PBS to remove residual protein transport inhibitor, and resuspended in 1:500 diluted fixable viability dye (Zombie yellow, Biolegend) containing fluorescent antibody master mix (as above). Cells were then washed in FACS buffer (as above), fixed and permeabilised using Fixation and Permeabilisation Buffers (Biolegend) as per manufacturers protocol.

A master mix of fluorescent antibodies was made up for intracellular cytokines of interest in permeabilisation buffer. Single stains were also prepared (as above) as were fluorescence minus one (FMO) controls using cells from a lenalidomide-treated allogeneic co-culture. Cells were then incubated in the dark at RT for 30 mins, before washing twice in permeabilisation buffer (after centrifugation at 350g for 5mins at RT with supernatant discarded) and acquisition on the flow cytometer.

Nuclear factor staining was carried out in the same way but using nuclear factor fixation and permeabilisation buffer (Biolegend) as per manufacturers protocol. Isotype controls were also prepared.

2.7.3 Flow cytometry data analysis

Each individual fluorochrome will be excited by and emit fluorescence over a range of wavelengths of light. This means that spectral overlap or spillover can occur when multiple fluorochromes are used simultaneously in one experiment. To adjust for this compensation is applied to remove signal from a given fluorochrome from all neighbouring channels where it is also detected (**Figure 2.3**). The compensation values detected can then be applied to all the data.

Compensation for spectral overlap was performed using single stained UltraComp eBeads (eBioscience) or single stained cells using BD FACSDiva^{TM} automated compensation software. PMT voltages were optimised and compensation performed prior to acquisition of experimental samples for each experiment to ensure that settings were optimal for that experiment. Compensated flow cytometry standard (FCS) files were then loaded into FlowJo version 7/10 (Treestar) software for further analysis.

Figure 2.3

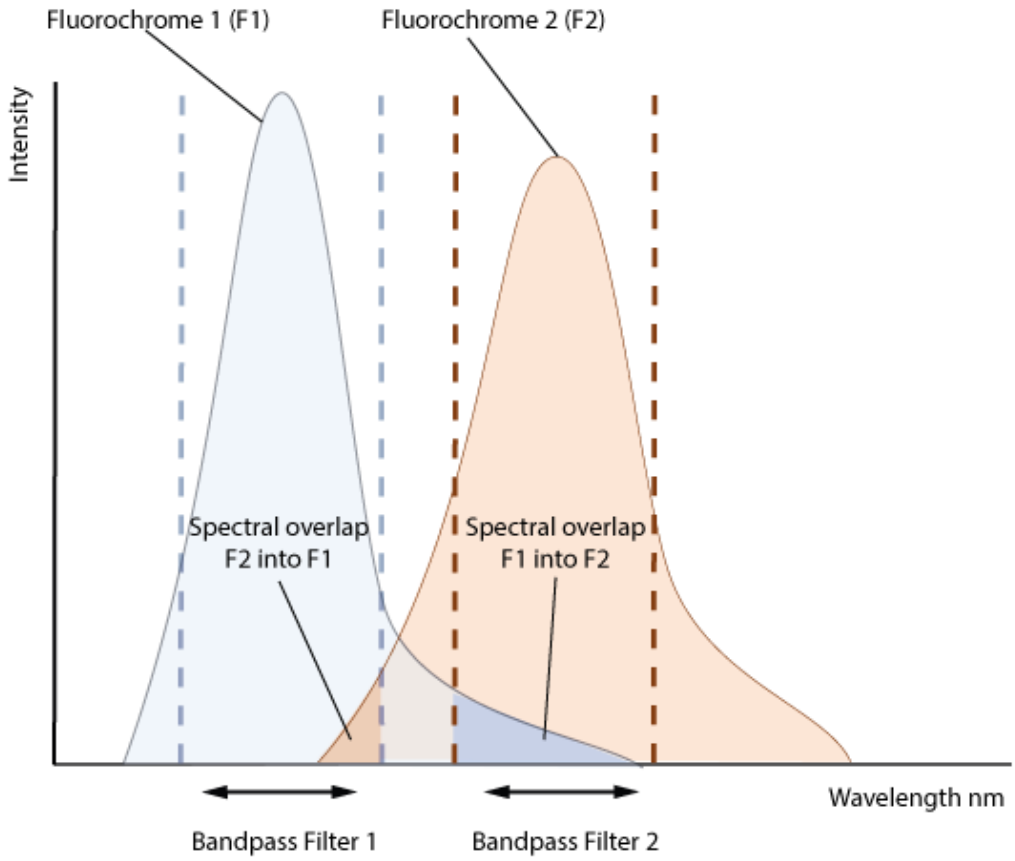


Figure 2.3 Compensation for spectral overlap of two fluorochromes

Bandpass filters capture and modulate the emitted pulse of fluorescent light from fluorochromes to minimize signal to noise. Despite this some fluorescence from fluorochromes with overlapping emission spectra is detected by 'adjacent' filters. The intensity of this pulse is lower than that of the 'target' and therefore compensation allows subtraction of this overlap while retaining the 'signal' for target events.

2.7.4 Cell phenotype analysis

Cellular subsets of interest within the total population can be resolved based on characteristic FSC and SSC as well as expression of surface markers and/or intracellular markers. For identification of important alloresponder cell subsets the following algorithm was applied to all samples: A region of interest was assigned based on the low FSC and SSC characteristics of lymphocytes. Boolean gating was then used to refine this population to single cells and live cells (based on DAPI or Zombie Yellow negativity). Following this further Boolean gates were applied to identify particular T cell or NK cell subsets, an example for $CD4^+$ and $CD8^+$ T cells is shown in **Figure 2.4**. **Table 2.1** Shows gates applied to identify T cell and NK cell subsets of interest.

Where isotype and FMO controls were included to aid assessment of expression of markers with continuous patterns of expression (i.e. ikaros), these were used to set gates (**Figure 2.5**).

2.7 5 Allospecific cytokine production

To determine the allospecific production of cytokines from background the following formula was used:

% cytokine-positive cells in allogeneic co-culture - % cytokine-positive cells in corresponding autologous co-culture

2.7.6 Rare-event analysis

Intracellular cytokine producing cells were rare-events in T cell populations of interest, occurring at a frequency of <5% of the total population. In order to minimise errors in detection a minimum of 50 positive events were acquired to give a coefficient of variance of <14% based on the calculation described by Allan and Keeney 2010 [287]:

$$CV = 100/\sqrt{n}$$

Where n=the number of positive events

Table 2.1 Markers used to identify lymphocyte subsets

Cell subset	Cell surface markers
T helper	$CD3^+CD4^+$
T cytotoxic	$CD3^+CD8^+$
T regulatory	$CD3^+CD4^+CD25^{high}FOXP3^+$ ($CD127^-$)
T effector	$CD3^+CD4^+CD25^+FOXP3^-$ or $CD3^+CD8^+CD25^+FOXP3^-$
Naïve	$CD3^+CD4^+/CD8^+CCR7^+CD45RA^+$
Central Memory	$CD3^+CD4^+/CD8^+CCR7^+CD45RA^-$
Effector Memory	$CD3^+CD4^+/CD8^+CCR7^-CD45RA^-$
TEMRA	$CD3^+CD8^+CCR7^-CD45RA^+$
NK	$CD3^-CD56^+$
Cytotoxic NK	$CD3^-CD16^+CD56^+$

Figure 2.4

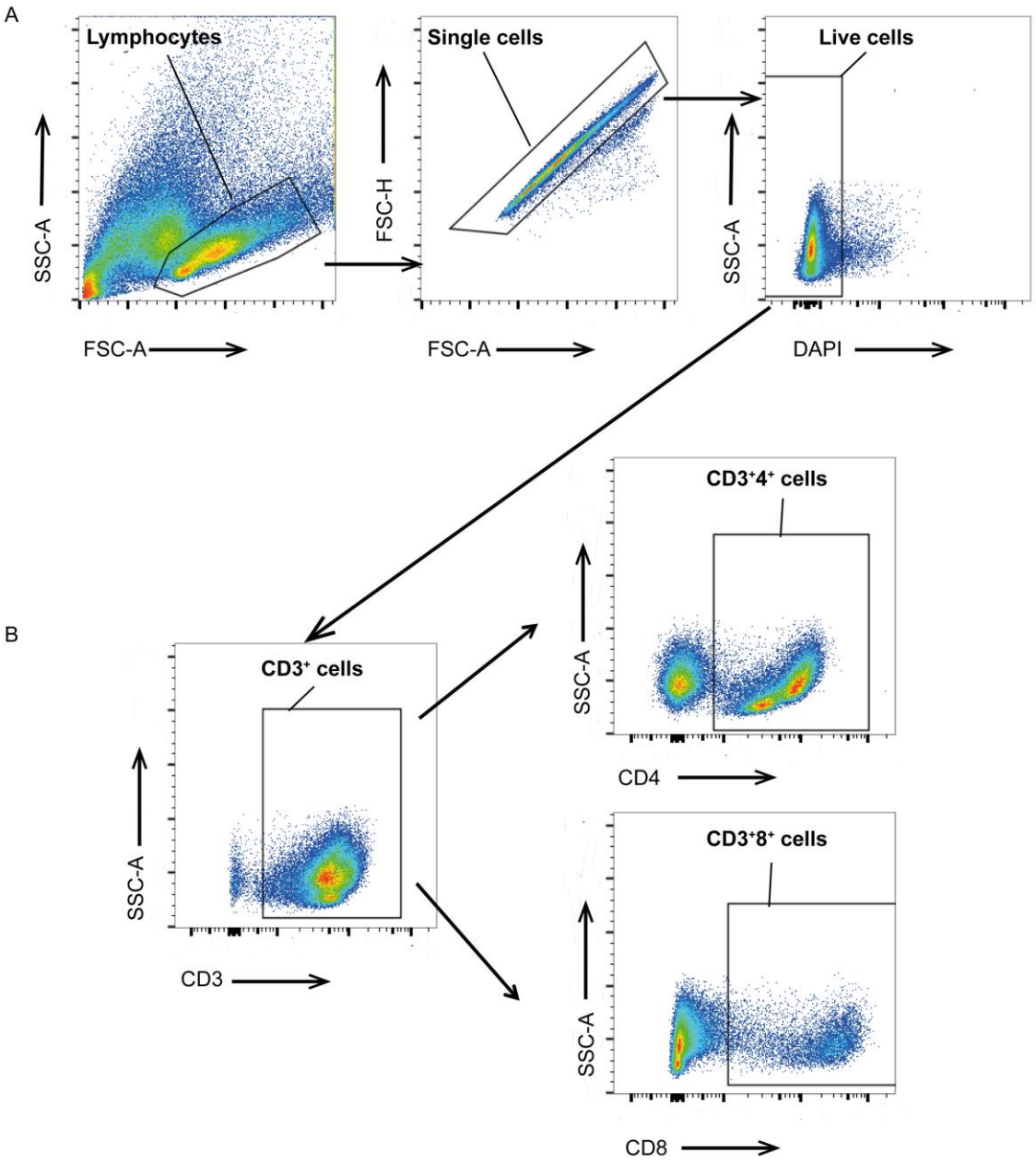


Figure 2.4 Gating strategy for analysis of flow cytometric data

(**A**) Lymphocytes were identified based on characteristic forward scatter area (FSC-A) and side scatter area (SSC-A). Doublets and dead cells were excluded.

(**B**) T cells were then identified as $CD3^+$ cells, which were then divided into $CD4^+$ and $CD8^+$ subsets by positivity for CD3 and CD4/8.

Figure 2.5

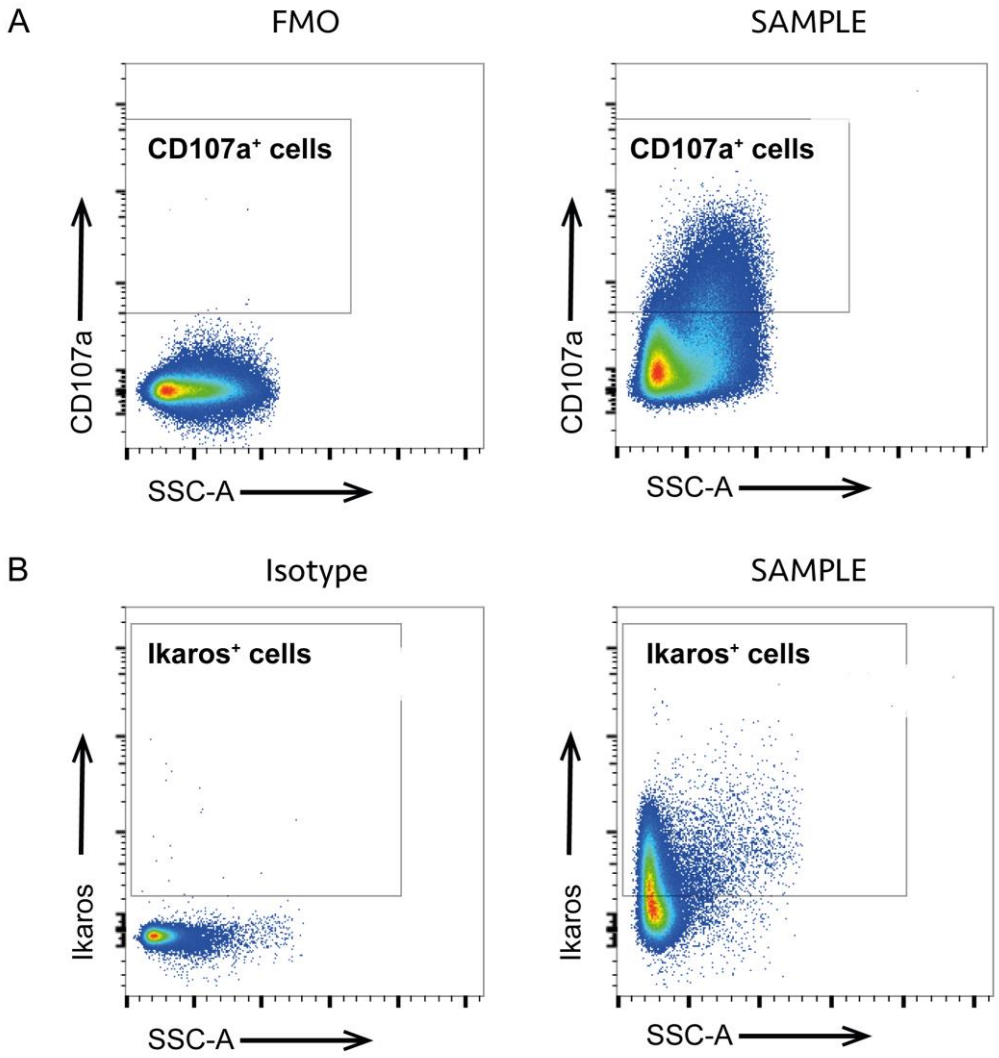


Figure 2.5 Gating based on FMO or isotype controls

For markers with continuous expression on cells, positive populations were defined according to fluorescence minus one (FMO) controls **(A)** or Isotype controls (Isotype) **(B)**.

2.7.7 Dye dilution for assessment of proliferation by flow cytometry

Proliferation dyes bind to cytosolic components within cells in a non-toxic manner. Each time the cell divides the fluorescent dye is apportioned equally between the daughter cells and thus the fluorescence intensity halves. This allows identification of cells that have divided (in this case alloproliferative cells), as well as cells that have not responded to stimuli and divided. It is also possible to resolve serial rounds of cell division and calculate the proportion of cells that have been stimulated to divide (for my experiments the "alloproliferative precursor frequency") from a given starting population [288-290].

FlowJo version 7 (Treestar) software contains a mathematical model that utilises fluorescence histograms to perform a 'best fit' analysis of number of rounds of cell division a population of cells has undergone. The model is also able to determine the alloproliferative precursor frequency for a population of cells (**Figure 2.6**). This analysis was performed and produced comparable values to the formula described by Martins *et al.* to manually mathematically calculate these values:

$$APF\ (\%) = 100c/(2^n+c)-(2^n xc)$$

where c = number $CFSE^{dim/neg}$ responders / number $CFSE^{dim/neg}$ responders + number $CFSE^{bright}$ responders) and $n = log_2[b/a]$, with a= median fluorescence intensity $CFSE^{dim/neg}$ responders and b = median fluorescence intensity $CFSE^{bright}$ responders

In order to be consistent with published literature the calculation method from Martins *et al.* was used throughout data presented in this thesis. The same formula was applied for Cell Tracker Violet.

2.7.8 Calculation of alloproliferative fraction

To determine allospecific proliferation from background the following formula was used:

% $CFSE^{dim}$ cells in allogeneic co-culture - % $CFSE^{dim}$ cells in corresponding autologous co-culture

Figure 2.6

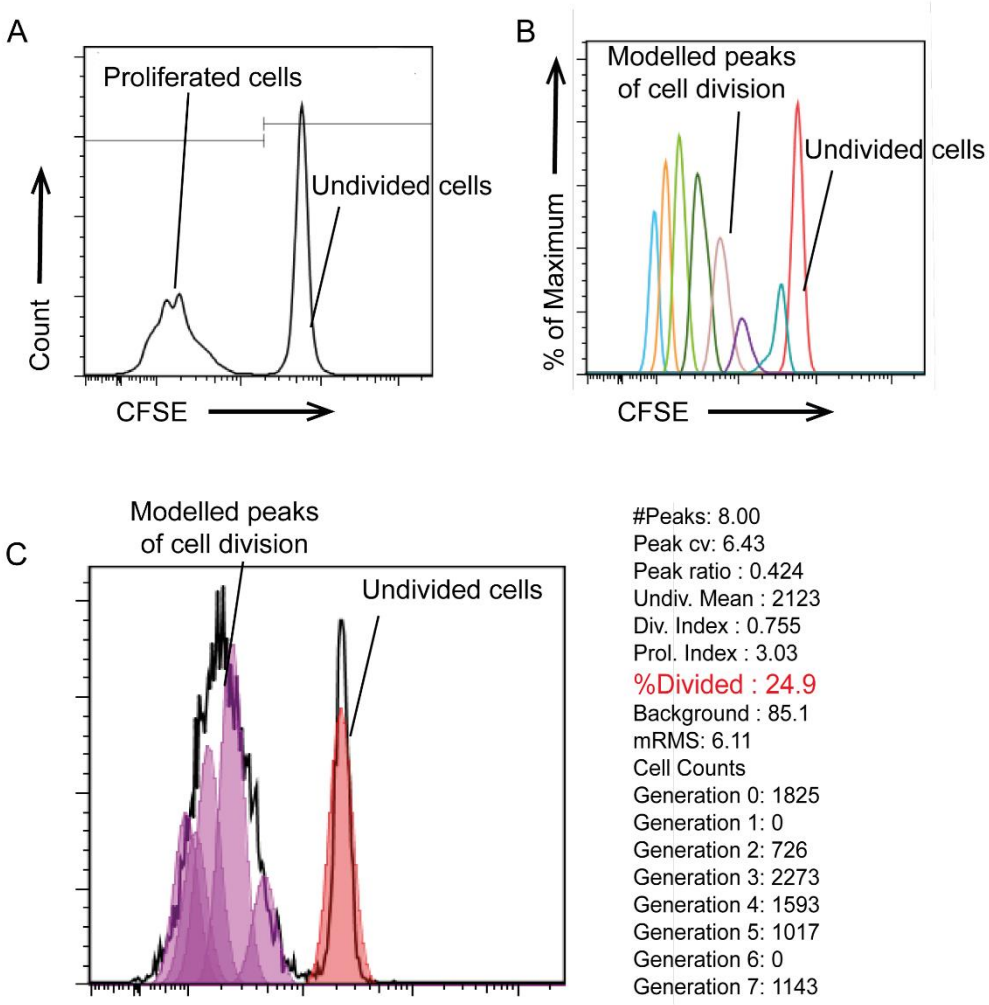


Figure 2.6 CFSE dye dilution assessment of proliferation

(A) Undivided or non-alloproliferative $CFSE^{+}$ cells are identified by retained bright fluorescence while divided or alloproliferative cells are identified by dim CFSE fluorescence.

(B) FlowJo software models rounds of cell division based on CFSE fluorescence histograms.

(C) Proliferation kinetic analysis allows calculation of alloproliferative precursor frequency (here described as % divided) based on proportions of cells in each round of cell division and numbers of undivided cells.

2.8 General statistical analysis

With the exception of qRT-PCR and GeneChip® data all other statistical analysis was conducted using Graphpad Prism version 7 (Graphpad Software). The D'Agostino-Pearson test of normalilty was applied and data presented in this thesis assessed to be non-normally distributed. Therefore non-parametric statistical tests were applied in all cases.

Statistical significance of differences in responses between untreated and lenalidomide-treated cells were determined using the Wilcoxon matched pairs signed rank test in all cases where paired samples were available and Mann Whitney test where samples were not paired or paired samples were missing for technical reasons. Where comparison between groups or serial time-points was made the Kruskal-Wallis test was used, with Dunn's post-test correction for multiple comparison.

2.9 General reagents

Table 2.2 Details of general reagents

General Reagent	Details & Manufacturer
FBS	Fetal Bovine Serum (Heat Inactivated),Gibco® by Life technologies™
RPMI	Roswell Park memorial Institute (RPMI) 1640 AQMedia™, Sigma® Life Sciences
PBS	Dulbecco's Phosphate Buffered Saline, Sigma® Life Sciences
Penicillin Streptomycin	10,000 units Penicillin and 10mg Streptomycin per ml, Sigma® Life sciences
Supplemented media	RPMI with the addition of 10% FBS and 1% Penicillin Streptomycin
FACS buffer	1ml FBS added to 49ml PBS
MACS buffer	PBS with the addition of 0.5% FBS and 2mM EDTA
Nuclease free water	Nuclease free water (not DEPC treated), Ambion by Life technologies

Chapter 3 – The impact of lenalidomide on cellular T cell alloresponses of adult peripheral blood-derived T cells

3.1 Introduction

The T cell alloresponse is absolutely critical to the success or failure of AHSCT. By exerting GvT effects donor T cells provide effective and long lasting immune control of haematological malignancy. There is ample evidence that T cells are required for the GvT effect: the efficacy of DLI in CML, the fact that T cell depletion of donor grafts results in increased relapse and that withdrawal of immunosuppression can result in re-induction of remission. The precise immunobiology of GvT responses is still unclear and is likely to involve complex interactions between multiple cell types as well as cytokine and chemokine signals, not to mention the influence of the tumour cells.

Evidence from experimental animal models has provided evidence that both $CD4^+$ and $CD8^+$ T cell subsets are required for optimal GvT [47] and that naive T cells can give rise to more potent GvT than their memory counterparts [55]. T cells mediate GvT via a number of mechanisms including: cytokine mediated cytotoxicity, Fas:Fas-ligand (FasL) induced apoptosis or perforin and granzyme cytotoxicity. It appears that $CD4^+$ T cells contribute to GvT mainly by secretion of cytokines while $CD8^+$ T cells contribute more by perforin and granzyme direct cell cytotoxicity [291].

The immunomodulatory drug lenalidomide has been demonstrated both *in vitro* and *in vivo* to enhance T cell responses to synthetic stimuli and to be associated with the clinical onset of GvHD when used early following AHSCT. Given the close link between GvHD and GvT this could also lead to reductions in relapse. However the exact mechanisms by which lenalidomide influences the human alloresponse remain unknown and this gap in knowledge has limited safe and effective clinical use of the drug in the setting of AHSCT.

3.2 Aim

To characterise the effect of lenalidomide exposure on cellular components of the human adult peripheral blood-derived T cell alloresponse.

3.3 Specific materials and methods

3.3.1 TCR Vβ subfamily distribution analysis by flow cytometry

The human T cell repertoire can be divided into 2 subpopulations based on the type of T cell receptor (TCR) they express. Ninety to 99% of T cells express αβ and the remainder express γδ TCRs [292]. The αβ heterodimers form the antigen recognising portion of the TCR, for this reason a high degree of diversity in these proteins is generated by somatic genetic recombination during thymic maturation of T cells. Part of this recombination involves the 65 described variable (V) β segments of the gene; these can be grouped into 25 sub-families based on a high degree of homology at the nucleotide level [293,294].

In a normal healthy individual one can therefore expect a 'normal' distribution of Vβ subfamilies in peripheral blood T cells. In cases where an antigenic stimulus has been experienced and T cells with the TCR corresponding to that specific antigen have responded there will be a clonal expansion of T cells with that specific TCR resulting in skewing of the Vβ subfamily distribution [295].

The IOTest® Beta Mark kit (Beckman Coulter) can identify T cells bearing 70% of normal TCR Vβ subtypes. This kit uses combinations of fluorescein isothiocyanate (FITC) and phycoerythrin (PE) labelled monoclonal antibodies to identify TCR Vβ subfamilies. The specificities of the antibody mixes supplied are shown in **Table 3.1.**

Table 3.1 Fluorochrome combinations IOT Beta Mark kit

Vial	TCR Vβ Subfamily	Fluorochrome
A	5.3 7.1 3	PE PE+FITC FITC
B	9 17 16	PE PE+FITC FITC
C	18 5.1 20	PE PE+FITC FITC
D	13.1 13.6 8	PE PE+FITC FITC
E	5.2 2 12	PE PE+FITC FITC
F	23 1 21.3	PE PE+FITC FITC
G	11 22 14	PE PE+FITC FITC
H	13.2 4 7.2	PE PE+FITC FITC

Cells from unstained responder PBMC (as a baseline), in addition to cells from corresponding standard Cell Tracker Violet labelled lenalidomide-treated (1μM) and untreated co-cultures were transferred to 5ml round bottom tubes and prepared for surface labelling (as per Chapter 2). In addition to labelling for surface T cell markers CD4, CD8 and viability staining with Zombie Yellow each tube was labelled with monoclonal antibody mix from one of the vials provided in the IOTest® Beta Mark kit and incubated in the dark at RT for 20 mins, as per the manufacturers recommendations. An unstained control was included as were single stained controls.

Following acquisition on the BD Fortessa II flow cytometer and analysis using FlowJo V10.1 (Treestar) software (as per Chapter 2) it was possible to assign the Vβ subfamily distribution for the $CD4^+$ and $CD8^+$ T cells. It was then possible to compare the Vβ subfamily distribution of responder cells at baseline and following 9 days of allostimulation in the presence or absence of lenalidomide. Representative flow plots are shown in **Figure 3.3**.

3.3.2 Allogeneic co-cultures with purified $CD4^+$ and $CD8^+$ T cell subsets

$CD4^+$ and $CD8^+$ T cells from CFSE-labelled responder PBMC were isolated using negative magnetic selection (CD4 T cell and CD8 T cell isolation kits, Miltenyi biotec) as described in Chapter 2.

Following isolation CFSE-labelled purified $CD4^+$ or $CD8^+$ T cells were co-cultured in a 1:1 ratio with allogeneic irradiated stimulators for 9 days in the presence or absence of 1μM lenalidomide to examine cell subset intrinsic effects of lenalidomide. In addition $CD4^+$ cells were mixed with $CD8^+$ T cells in a 2:1 ratio, to recapitulate the normal CD4:CD8 T cell ratio in healthy peripheral blood and co-cultured with allogeneic stimulators as above. Finally a standard co-culture (as per Chapter 2) using un-sorted PBMC from corresponding responders was also included to allow comparison of responder T cell proliferation in each scenario. A schematic of the experimental design is depicted in **Figure 3.1**.

Figure 3.1

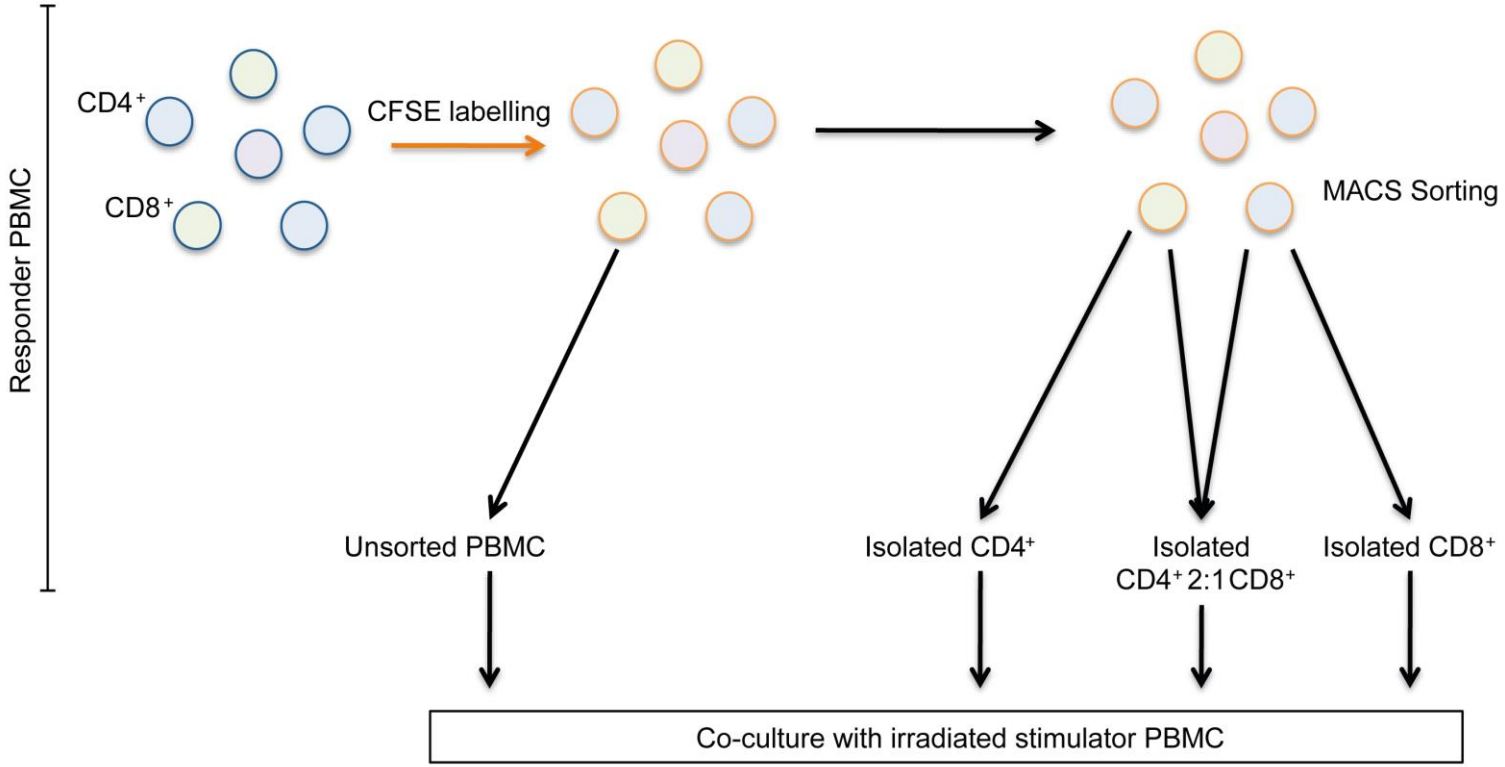

Responder PBMC
CD4+
CD8+
CFSE labelling
MACS Sorting
Unsorted PBMC
Isolated CD4+
Isolated CD4+ 2:1 CD8+
Isolated CD8+
Co-culture with irradiated stimulator PBMC

Figure 3.1 Schematic of allogeneic co-cultures using purified T cell subsets as allogeneic responders

Responder PBMC were labelled with CFSE and then either co-cultured with irradiated allogeneic stimulator PBMC in a 1:1 ratio or magnetically sorted to isolate pure populations of $CD4^+$ and $CD8^+$ T cells which were then also co-cultured with the same stimulator PBMC in a 1:1 ratio. Cells from isolated $CD4^+$ and 8^+ T cell populations were mixed in a 2:1 ratio and then also co-cultured with the same stimulator in a 1:1 ratio. In each case the number of cells in the co-culture was kept equal.

3.4 Results

3.4.1 Lenalidomide potentiates adult peripheral blood derived T cell alloresponses by selectively increasing the proliferation of allospecific $CD8^+$ T cells

I first examined the effect of lenalidomide on allospecific proliferation. Using an *in vitro* model of HLA-mismatched stimulator and responder PBMC from healthy adult donors I measured responder proliferation by CFSE dye dilution at serial time points in untreated allogeneic co-culture. The proportion of $CFSE^{dim}$ proliferative cells increased over the time course with a maximum proliferation seen at day 9 of co-culture (median 22% compared to 72%, $p<0.001$ **Figure 3.2, A**). This was also true when allospecific proliferation was calculated as % $CFSE^{dim}$ responders (allogeneic co-culture) - % $CFSE^{dim}$ responders (autologous co-culture) (median 17% compared to 47%, $p<0.01$) as shown in **Figure 3.2, A**. Addition of 1μM lenalidomide on day 0 of allogeneic co-culture led to a greater increase ($p<0.05$) in allospecific proliferation compared to untreated co-cultures on day 9 (**Figure 3.2, B**). The time point of day 9 was therefore chosen for all further experiments.

I then went on to assess T cell proliferation. The addition of lenalidomide (1μM) to allogeneic co-cultures resulted in significantly greater responder $CD3^+$ T cell proliferation than untreated co-cultures (median 47% compared to 34%, $p<0.001$, **Figure 3.2, C**). This effect was due to a selective increase in proliferation of $CD8^+$ T cells (median 43% (untreated) versus 58% (lenalidomide-treated) $p<0.001$) (**Figure 3.2, D**). In contrast, lenalidomide had no significant effect on proliferation of $CD4^+$ T cells (median 30% (untreated) versus 34% (lenalidomide-treated) (**Figure 3.2, E**).

The effect of lenalidomide on $CD8^+$ T cell alloproliferation was dose-dependent with a plateau in proliferation seen at concentrations of $\geq$1μM (**Figure 3.2, F**). A 1μM concentration corresponds to plasma levels in patients after oral doses of approximately 7.5mg/day [176]. This dose is comparable to doses used in clinical trials of lenalidomide after AHSCT, therefore a lenalidomide concentration of 1μM of was used for all subsequent experiments.

Figure 3.2

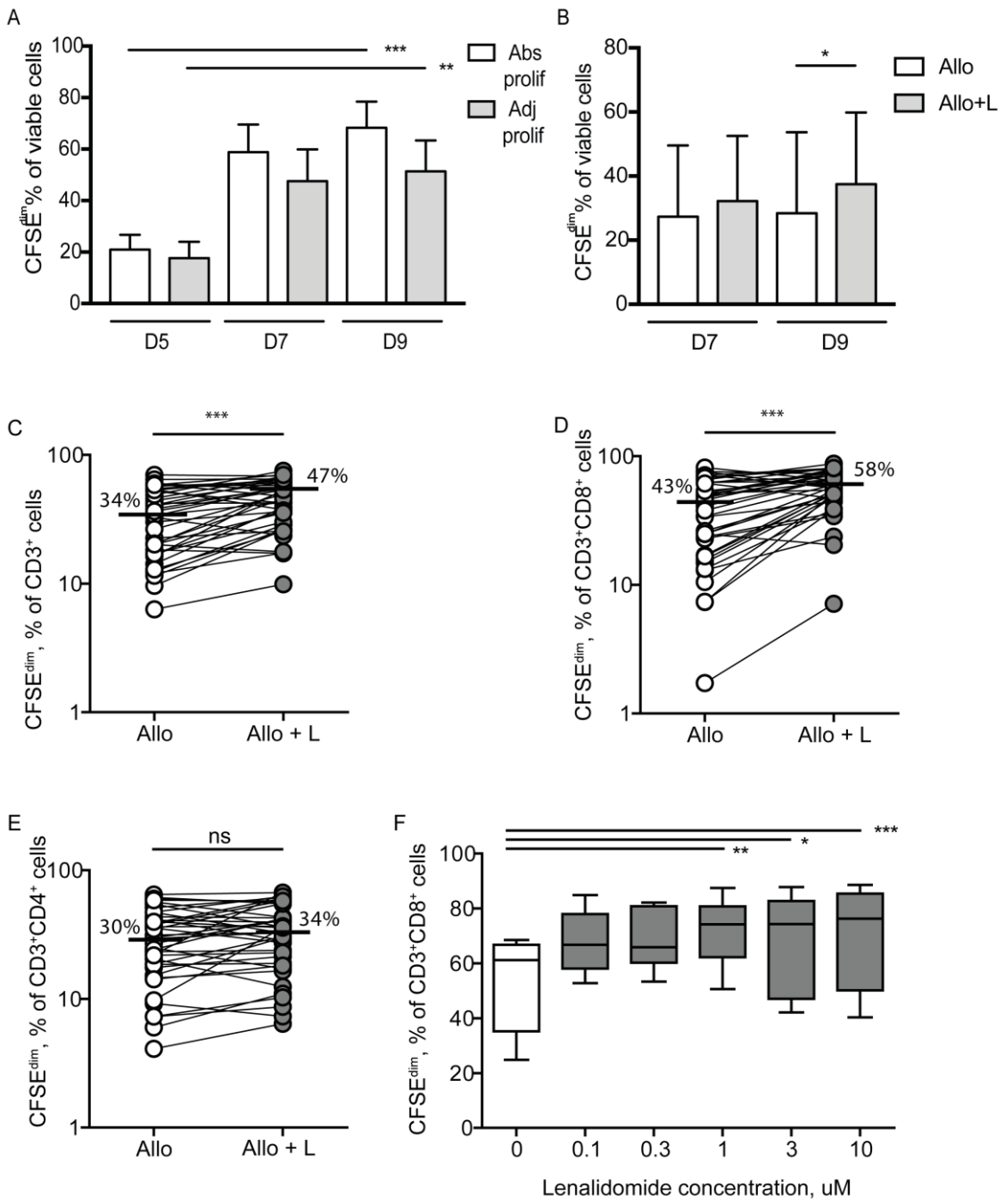

A
CFSE^dim % of viable cells
100
80
60
40
20
0
***
**
Abs prolif
Adj prolif
D5
D7
D9
B
CFSE^dim % of viable cells
80
60
40
20
0
*
Allo
Allo+L
D7
D9
C
CFSE^dim, % of CD3+ cells
100
10
1
***
34%
47%
Allo
Allo + L
D
CFSE^dim, % of CD3+CD8+ cells
100
10
1
***
43%
58%
Allo
Allo + L
E
CFSE^dim, % of CD3+CD4+ cells
100
10
1
ns
30%
34%
Allo
Allo + L
F
CFSE^dim, % of CD3+CD8+ cells
100
80
60
40
20
0
***
*
**
0
0.1
0.3
1
3
10
Lenalidomide concentration, uM

Figure 3.2 Lenalidomide enhances $CD8^+$ T cell alloresponses

(A) Mean (+/- SD) alloproliferation (%$CFSE^{dim}$) at sequential time-points of primary allogeneic co-culture of mononuclear cells. Results are shown for 6 unique donor-responder pairs. ** = P<0.01, *** = P<0.001. Abs prolif = absolute proliferation, Adj prolif = adjusted proliferation.
(B) Mean (+/- SD) alloproliferation (%$CFSE^{dim}$) at sequential time-points of primary allogeneic co-culture of mononuclear cells in the absence (Allo) or presence of lenalidomide (Allo + L). Results are shown for 9 unique donor-responder pairs. * P<0.05.
(C) $CD3^+$ T cell proliferation (% $CFSE^{dim}$) following primary allogeneic co-culture of mononuclear cells in the absence (Allo) or presence of lenalidomide (Allo + L). Horizontal lines and adjacent numbers are medians. Results are shown for 40 unique donor-responder pairs. *** = P<0.001.
(D) $CD8^+$ T cell proliferation following primary allogeneic co-culture in the absence (Allo) or presence of lenalidomide (Allo + L). Horizontal lines and adjacent numbers are medians. Results are shown for 40 unique donor-responder pairs. *** = P<0.001.
(E) $CD4^+$ T cell proliferation following primary allogeneic co-culture in the absence (Allo) or presence of lenalidomide (Allo + L). Horizontal lines and adjacent numbers are medians. Results are shown for 40 unique donor-responder pairs. ns = not significant.
(F) APB $CD8^+$ T cell proliferation following primary allogeneic co-culture with increasing starting concentrations of lenalidomide. Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 9 unique donor-responder pairs. * = P<0.05, ** = P<0.01, *** = P<0.001.

Lenalidomide increases alloproliferation of CD8$^+$ T cells by increasing the alloproliferative precursor frequency without affecting the specificity of the alloresponse

Having demonstrated that lenalidomide increases the proportion of responder CD8$^+$ T cells that have divided after a given time in allogeneic co-cultures, I next sought to identify whether this was a result of more rapid alloreactive T cell division or recruitment of greater numbers of alloreactive T cells to proliferate. I used proliferation kinetic analysis of CFSE-labelled responder cells to determine the number of cell divisions and the proportion of starting populations entering cell division (the alloprecursor frequency, APF).

Alloproliferative CD8$^+$ T cells had undergone a median of 4 rounds of cell division by day 9 in both untreated and lenalidomide treated allogeneic co-cultures (**Figure 3.3, A**). In contrast the CD8$^+$ T cell APF in lenalidomide treated co-cultures increased nearly three-fold (from median 6% (untreated) to 17% (lenalidomide-treated), $p<0.0001$), consistent with lenalidomide recruiting a greater proportion of the responder CD8$^+$ T cell pool to divide during allostimulation (**Figure 3.3, B**). In agreement with there being no significant increase in CD4$^+$ T cell proliferation in lenalidomide treated co-cultures, there was no effect on the CD4$^+$ APF (**Figure 3.3, C**).

As lenalidomide treatment recruited a larger proportion of CD8$^+$ T cells to proliferate after allostimulation, I next asked whether lenalidomide had an effect on the specificity of alloproliferative CD8$^+$ T cells. To examine this I determined the TCR Vβ subfamily distribution of alloproliferative CD8$^+$ T cells after allostimulation with and without lenalidomide. In untreated allogeneic co-cultures alloproliferative CD8$^+$ T cells demonstrated oligoclonal expansion of several TCR Vβ subfamilies when compared to subfamily distribution of baseline CD8$^+$ T cells. Importantly, lenalidomide exposure resulted in further expansions of the same TCR Vβ subfamilies rather than expansions of additional TCR Vβ subfamilies (**Figure 3.3, D-E**).

Figure 3.3

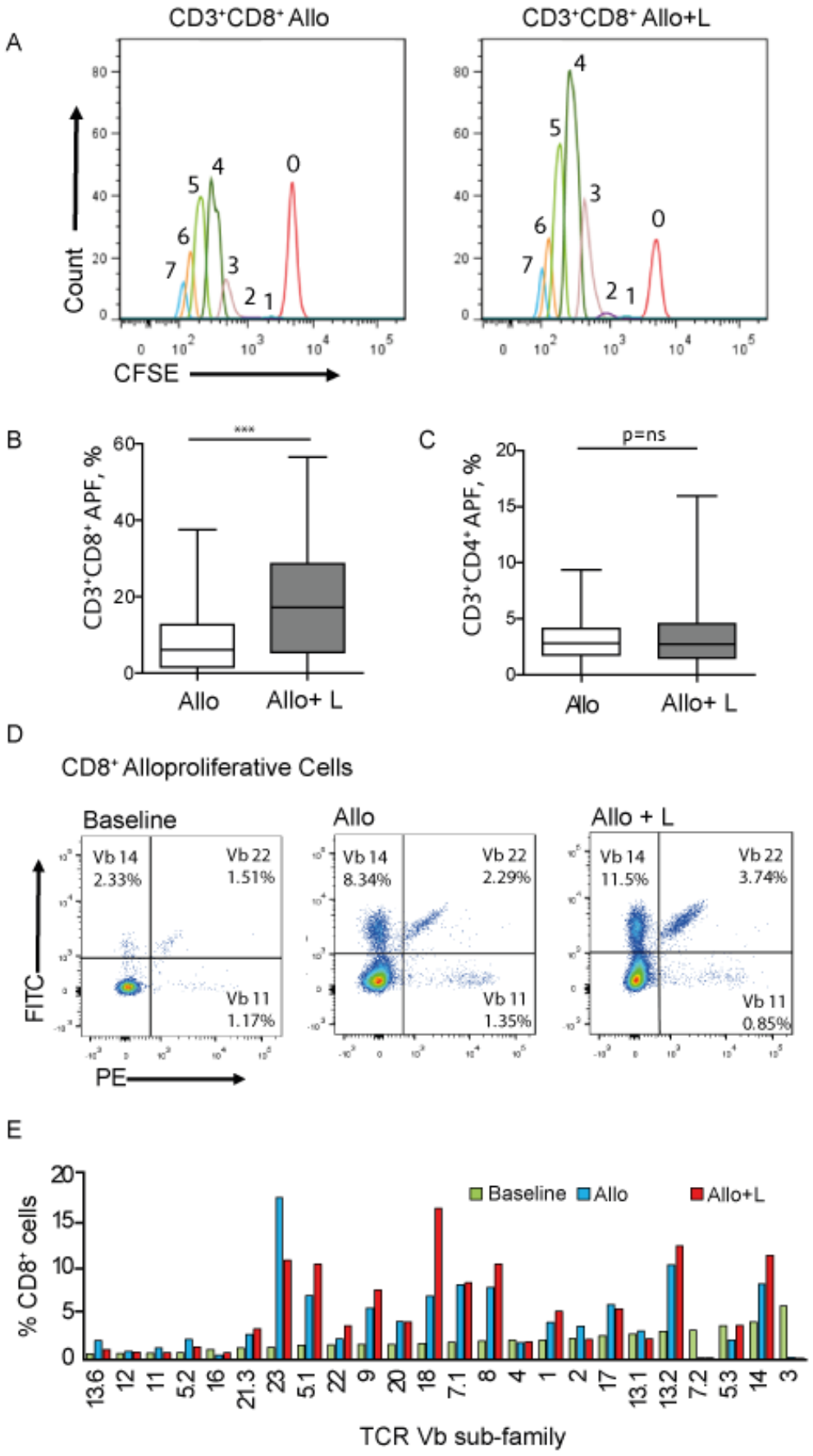

A
CD3+CD8+ Allo
CD3+CD8+ Allo+L
Count
CFSE
B
CD3+CD8+ APF, %
***
Allo
Allo+ L
C
CD3+CD4+ APF, %
p=ns
Allo
Allo+ L
D
CD8+ Alloproliferative Cells
Baseline
Allo
Allo + L
Vb 14 2.33%
Vb 22 1.51%
Vb 11 1.17%
Vb 14 8.34%
Vb 22 2.29%
Vb 11 1.35%
Vb 14 11.5%
Vb 22 3.74%
Vb 11 0.85%
FITC
PE
E
% CD8+ cells
Baseline
Allo
Allo+L
13.6 12 11 5.2 16 21.3 23 5.1 22 9 20 18 7.1 8 4 1 2 17 13.1 13.2 7.2 5.3 14 3
TCR Vb sub-family

Figure 3.3 Lenalidomide increases alloproliferation of $CD8^+$ T cells by increasing the alloproliferative precursor frequency without affecting the specificity of the alloresponse

(A) Histograms depicting APB $CD8^+$ T cells after allostimulation in the absence (Allo) or presence of lenalidomide (Allo+L). Undivided cells and populations of cells that have undergone one or more cell divisions are resolved based on CFSE dye dilution. Numbers above peaks represent the hierarchical number of cell divisions each peak has undergone. Representative data is shown from one of 37 unique allogeneic co-cultures.

(B) $CD8^+$ T cell alloproliferative precursor frequency (APF) in allogeneic co-cultures in the absence or presence of lenalidomide. Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 37 unique donor-responder pairs. *** = $P<0.001$.

(C) $CD4^+$ T cell alloproliferative precursor frequency (APF) in allogeneic co-cultures in the absence or presence of lenalidomide. Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 37 unique donor-responder pairs. ns = not significant.

(D) Representative flow plots depict flow cytometric assessment of TCR Vβ repertoire at baseline, after allogeneic co-culture (Allo) and lenalidomide-treated allogeneic co-culture (Allo + L) showing expansion of $CD3^+CD8^+$ T cells within TCR subfamily Vβ 14.

(E) Histogram depicting overall TCR Vβ subfamily distribution of $CD8^+$ T cells at baseline and after allogeneic co-culture (Allo) and lenalidomide-treated allogeneic (Allo + L) co-culture. Result of one representative experiment (of 3) is depicted.

3.4.2 Lenalidomide exposure during antigen-priming is sufficient to potentiate the alloresponse

In the allogeneic co-culture system used, the majority of irradiated stimulator PBMC are lost from the co-culture by day 3 (**Figure 3.4, A**), during this time the stimulator PBMC interact with responder PBMC via direct alloantigen presentation to elicit an alloresponse. After day 3 allospecific responder T cells begin to proliferate and differentiate. I first aimed to determine how long lenalidomide, added at initiation of the co-culture was detectable in co-culture media and cells. This would determine whether drug was present during the antigen priming and/or the expansion phases of the T cell alloresponse. Therefore informing whether the changes in T cell phenotype observed at day 7 and day 9 were potentially due to an early effect of the drug, that altered T cell differentiation programs or due to potentiation of later expansion of specific subsets during alloresponder proliferation.

Using tandem mass-spectrometry and ultra-high performance liquid chromatography (MS/MS-UHPLC) I determined that lenalidomide was detectable in allogeneic co-culture media and cells at high levels until 48 hours of co-culture following which levels declined rapidly (**Figure 3.4, B**). This suggests that lenalidomide acted early during the priming phase of the alloresponse and is in agreement with my earlier data demonstrating that the $CD8^+$ T cell alloproliferative precursor frequency is increased in lenalidomide treated co-cultures. Therefore it is likely that lenalidomide acts to decrease the threshold for allospecific T cell recruitment, rather than by augmented proliferation of T cells already involved in alloproliferative responses.

3.4.3 Lenalidomide is required during allostimulation for optimal potentiation of $CD8^+$ T cell alloproliferation

Having determined that the downstream effects on alloproliferative T cell phenotype induced by lenalidomide were due to changes induced during antigen priming I next asked whether this was due to actions on the stimulator cells or the responder cells, or both.

Most of the available *in vitro* data would suggest the effect is likely to be due changes in the responder T cells, as responses to synthetic T cell stimuli are enhanced by lenalidomide. However our group has previously shown that pre-treatment of both the T cells and tumour cells with lenalidomide is required to enhance formation of immune synapses between autologous T

cells and tumour cells [209], and there is evidence that demonstrates enhanced *in vitro* APC function after lenalidomide treatment [227].

I therefore went on to perform allogeneic co-cultures using stimulator and responder APB-derived PBMC that had been pre-treated for 24 hours alongside lenalidomide treated and untreated co-cultures, as previously described. Pre-treatment of stimulator or responder cells with lenalidomide for 24 hours had no effect on $CD8^+$ T cell alloproliferation, whereas pre-treatment of both modestly increased alloproliferation of $CD8^+$ T cells compared to untreated co-cultures, although this did not reach statistical significance (**Figure 3.4, C**).

Figure 3.4

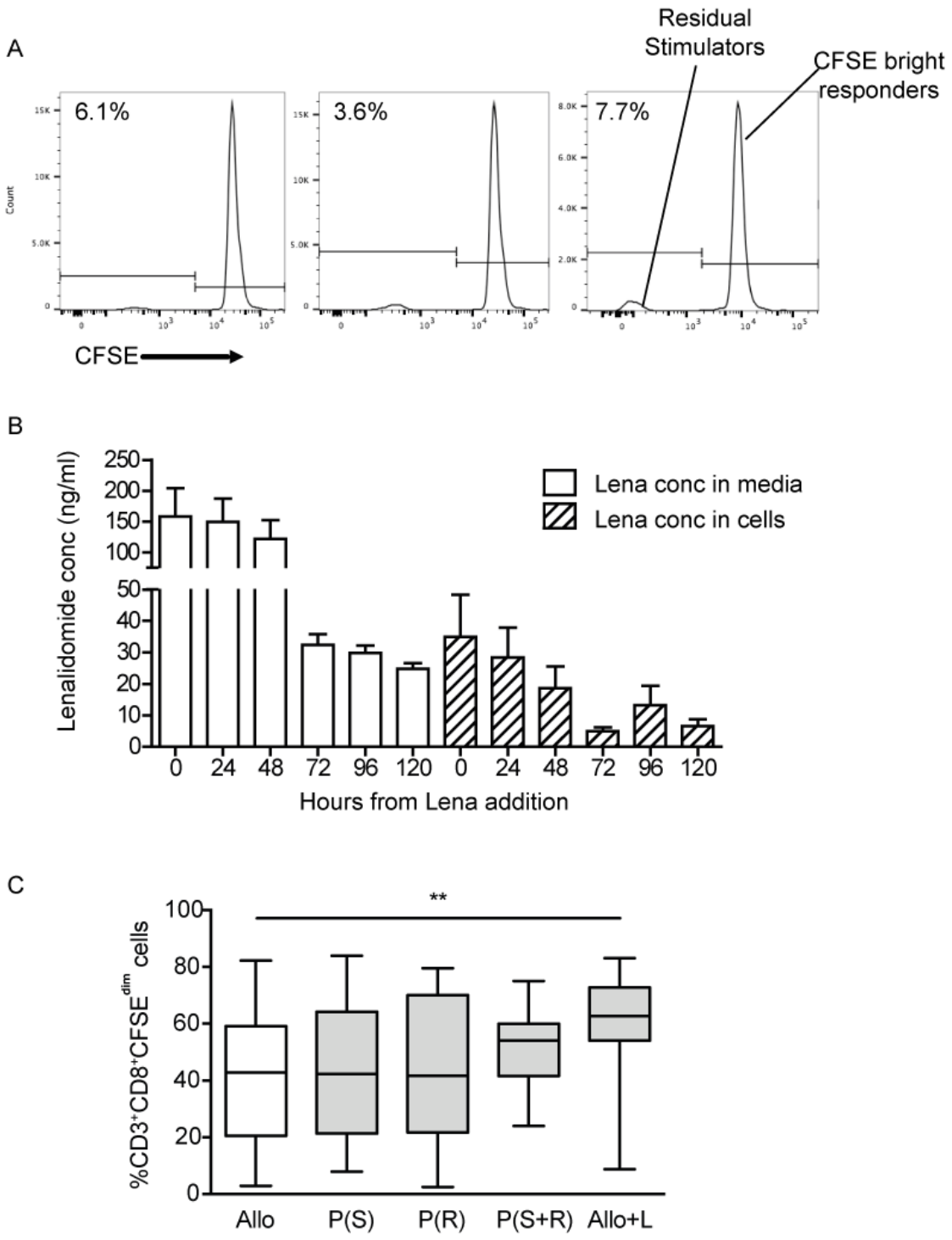

A
Residual Stimulators
CFSE bright responders
6.1%
3.6%
7.7%
Count
CFSE
B
Lena conc in media
Lena conc in cells
Lenalidomide conc (ng/ml)
Hours from Lena addition
C
**
%CD3+CD8+CFSEdim cells
Allo
P(S)
P(R)
P(S+R)
Allo+L

Figure 3.4 Lenalidomide acts early during antigen-priming of $CD8^+$ T cell alloresponses

(A) Histograms depicting APB PBMC on day 3 of allogeneic co-culture, percentages of remaining viable stimulator cells are indicated based on negativity for CFSE fluorescence. Representative data is shown from 3 unique allogeneic co-cultures.

(B) Mean (+/- SD) of lenalidomide (Lena) concentration (conc) in media and cells at serial time-points during allogeneic co-culture. Results are shown for 4 unique donor-responder pairs.

(C) APB $CD8^+$ T cell proliferation following primary allogeneic co-culture (Allo); with 24 hours of pre-treatment (P) of stimulators (S), responders (R) or both or addition of 1μM of lenalidomide to co-cultures (Allo+L). Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 7 unique donor-responder pairs. ** = $P<0.01$.

3.4.4 Allostimulation in the presence of lenalidomide results in expansion of CD8$^+$ effector T cells

I next further characterised the effect of lenalidomide during allostimulation by assessing the proportions of CD4$^+$ and 8$^+$ responder T cells with naïve, central memory (CM), effector memory (EM), or T effector memory cells re-expressing RA (T_{EMRA}) phenotype identified by co-expression patterns of CCR7 and CD45RA (as per Sallusto *et al.*[296])(**Figure 3.5, A**). Allostimulation without lenalidomide resulted in an increase in the proportion of CD8$^+$ T cells with an effector memory phenotype compared to baseline frequencies (median 48% versus 20%). Lenalidomide exposure during allostimulation resulted in a significant further increase in the proportion of responder CD8$^+$ T cells with an effector memory phenotype (median of 59% (lenalidomide-treated) versus 48% (untreated), $p<0.001$). This was accompanied by a significant decrease in the proportion of CD8$^+$ T cells with naïve and central memory phenotypes, consistent with lenalidomide promoting differentiation of alloreactive CD8$^+$ T cells from naïve and central memory cells to effector cells (**Figure 3.5, B**). Similarly, allostimulation led to an increase in CD4$^+$ cells with an EM phenotype and a decrease in naïve phenotype. However, in contrast with CD8$^+$ T cells, there was no additional effect on the proportion of CD4$^+$ T cells with a naïve, CM or EM phenotype following lenalidomide exposure (**Figure 3.5, C**).

The surface expression of CD25 (the IL2 receptor alpha subunit (IL2 Rα)) is a well-accepted marker of activated T cells (reviewed in [297]), additionally co-expression of CD25 and CD127 (the IL7 receptor alpha subunit (IL7Rα)) can distinguish between clonally expanded short-lived CD8$^+$ effector populations and CD8$^+$ T cells that will go on to develop a memory phenotype [298]. I therefore went on to measure the expression of CD25 and CD127 on T cells allostimulated in the presence or absence of lenalidomide.

Figure 3.5

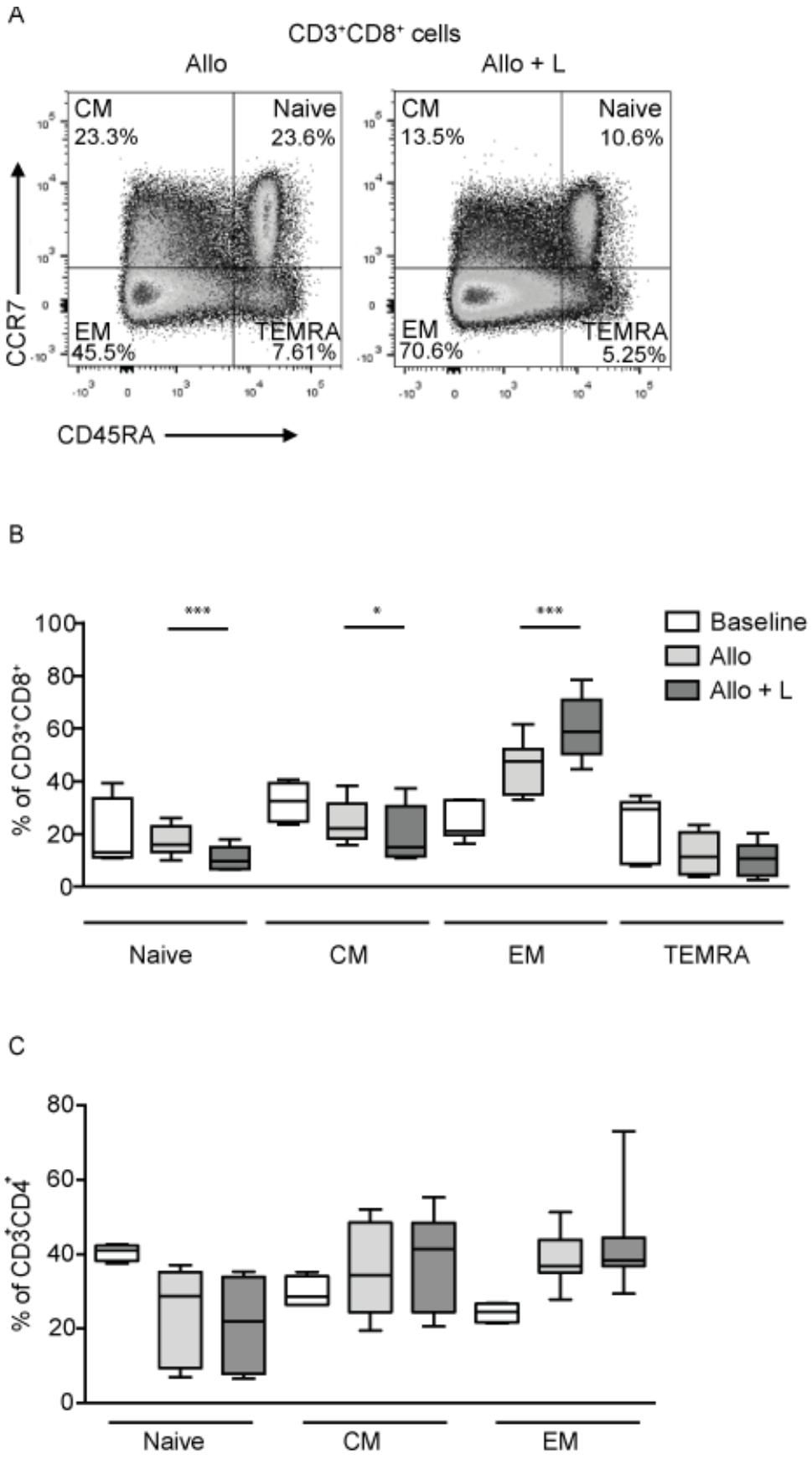

A
CD3+CD8+ cells
Allo
Allo + L
CM
23.3%
Naive
23.6%
EM
45.5%
TEMRA
7.61%
CM
13.5%
Naive
10.6%
EM
70.6%
TEMRA
5.25%
CCR7
CD45RA
B
% of CD3+CD8+
100
80
60
40
20
0
***
*
***
Baseline
Allo
Allo + L
Naive
CM
EM
TEMRA
C
% of CD3+CD4+
80
60
40
20
0
Naive
CM
EM

Figure 3.5 Allostimulation in the presence of lenalidomide expands $CD8^+$ effector memory T cells

(A) Representative plots depicting C-C chemokine receptor 7 (CCR7) and CD45RA co-expression patterns of $CD8^+$ T cells after allogeneic co-culture in the absence (Allo) or presence of lenalidomide (Allo+L). Percentages of naïve, central memory (CM), effector memory (EM) and T-effector memory cells re-expressing RA (TEMRA) subsets are indicated.
(B) Percentages of naïve, CM, EM and TEMRA cells subsets within $CD8^+$ T cells in baseline cells and after allogeneic co-culture in the absence or presence of lenalidomide. Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 11 unique donor-responder pairs. * = $P<0.05$, *** = $P<0.001$.
(C) Percentages of naïve, CM, EM and TEMRA cells subsets within $CD4^+$ T cells in baseline cells and after allogeneic co-culture in the absence or presence of lenalidomide. Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 11 unique donor-responder pairs. ns = not significant.

In agreement with the enhanced $CD8^+$ T cell effector-differentiation after allostimulation with lenalidomide, there was a significant increase in the proportion of alloproliferative $CD8^+$ T cells expressing CD25 compared to untreated (from median 20% (untreated) to 38% (lenalidomide-treated), $p<0.01$). Within the $CD8^+CD25^+$ alloproliferative subset there was also a significant decrease in the proportion of $CD127^{neg}$ cells (which were also FOXP3 negative, and therefore not $CD8^+$ Treg) due to an increase in the proportion of $CD127^+FOXP3^{neg}$ cells (**Figure 3.6, B**). This indicates that lenalidomide exposure during allostimulation results not only in an expansion of effector $CD8^+$ T cells, but that it confers a greater potential on the cells to retain immunological memory.

Figure 3.6

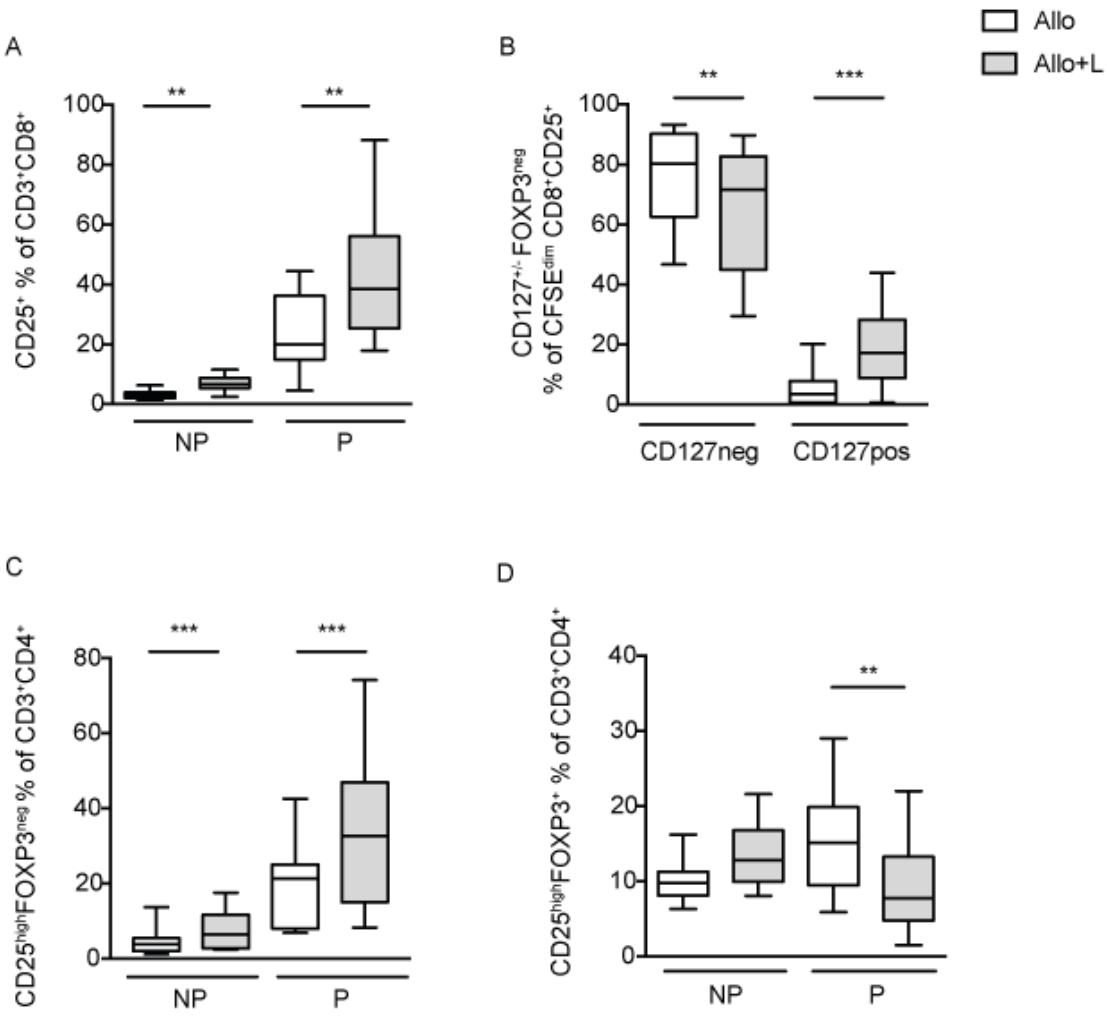

A
B
C
D
Allo
Allo+L
CD25+ % of CD3+CD8+
CD127+/- FOXP3neg % of CFSEdim CD8+CD25+
CD25highFOXP3neg % of CD3+CD4+
CD25highFOXP3+ % of CD3+CD4+
NP
P
CD127neg
CD127pos
**
***

Figure 3.6 Allostimulation in the presence of lenalidomide increases activation markers and expands memory precursors of $CD8^+$ T cells while reducing $CD4^+$ T regulatory cells

(A) Percentage of $CD25^+$ $CD8^+$ T cells in non-alloproliferative (NP) and alloproliferative (P) fractions after allogeneic co-cultures in the absence (Allo) or presence (Allo+L) of lenalidomide. Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 12 unique donor-responder pairs. ** = P<0.01.

(B) Percentage of alloproliferative $CD25^+$ $CD8^+$ T cells with CD127+/- $FOXP3^{neg}$ phenotype. Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 15 unique donor-responder pairs. **= P<0.01, ***+P<0.001.

(C) Percentage of $CD25^+$ $CD4^+$ T cells in non-alloproliferative (NP) and alloproliferative (P) fractions from allogeneic co-cultures in the absence or presence of lenalidomide. Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 12 unique donor-responder pairs. *** = P<0.001.are shown.

(D) Proportion of $CD4^+$ T cells with a Treg phenotype in non-alloproliferative (NP) and alloproliferative (P) fractions after allogeneic co-culture in the absence or presence of lenalidomide. Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 18 unique donor-responder pairs. ** = P<0.01.

3.4.5 Lenalidomide increases the proportion of alloproliferative CD8$^+$ T cells with a polyfunctional effector phenotype

To determine whether the increased proliferation and effector differentiation after lenalidomide exposure was accompanied by an increase in effector function, I next measured cellular production of pro-inflammatory cytokines and expression of the lysosomal-associated membrane protein CD107a on alloproliferative CD4$^+$ and CD8$^+$ T cells on day 7 of allogeneic co-culture (**Figure 3.7, A**). Day 7 was chosen (rather than day 9) based on existing data demonstrating the kinetics of cytokine production by responder cells in allogeneic co-culture from *Martins* et al. [290].

Following lenalidomide exposure, significantly greater proportions of alloproliferative CD8$^+$ T cells had capacity to secrete IFNγ, TNF, and IL2 and expressed the surrogate marker of degranulation CD107a (**Figure 3.7, B**). Despite no significant change in CD4$^+$ T cell proliferation, within the proliferative fraction of CD4$^+$ T cells there was a significant increase in cells with the capacity to secrete IFNγ, TNF, and IL2, as well as an increase in cells expressing CD107a (**Figure 3.7, C**). CD4$^+$ T cells with this cytotoxic phenotype have been described a playing important roles in responses to chronic viral infection [299,300]. In the context of the T cell alloresponse it is possible that exposure to lenalidomide, while not increasing alloproliferation of CD4$^+$ T cells could confer an enhanced helper and cytotoxic functional phenotype to this cell subset.

Importantly, lenalidomide also significantly increased the percentage of alloproliferative CD8$^+$ T cells with a polyfunctional effector phenotype (those with capacity to up-regulate two or more of these effector molecules) (**Figure 3.7, D-F**). Cells with this phenotype have been attributed with potent cytotoxic capacity [301] and shown to be important in improved anti-tumour responses to solid cancers [302].

Figure 3.7

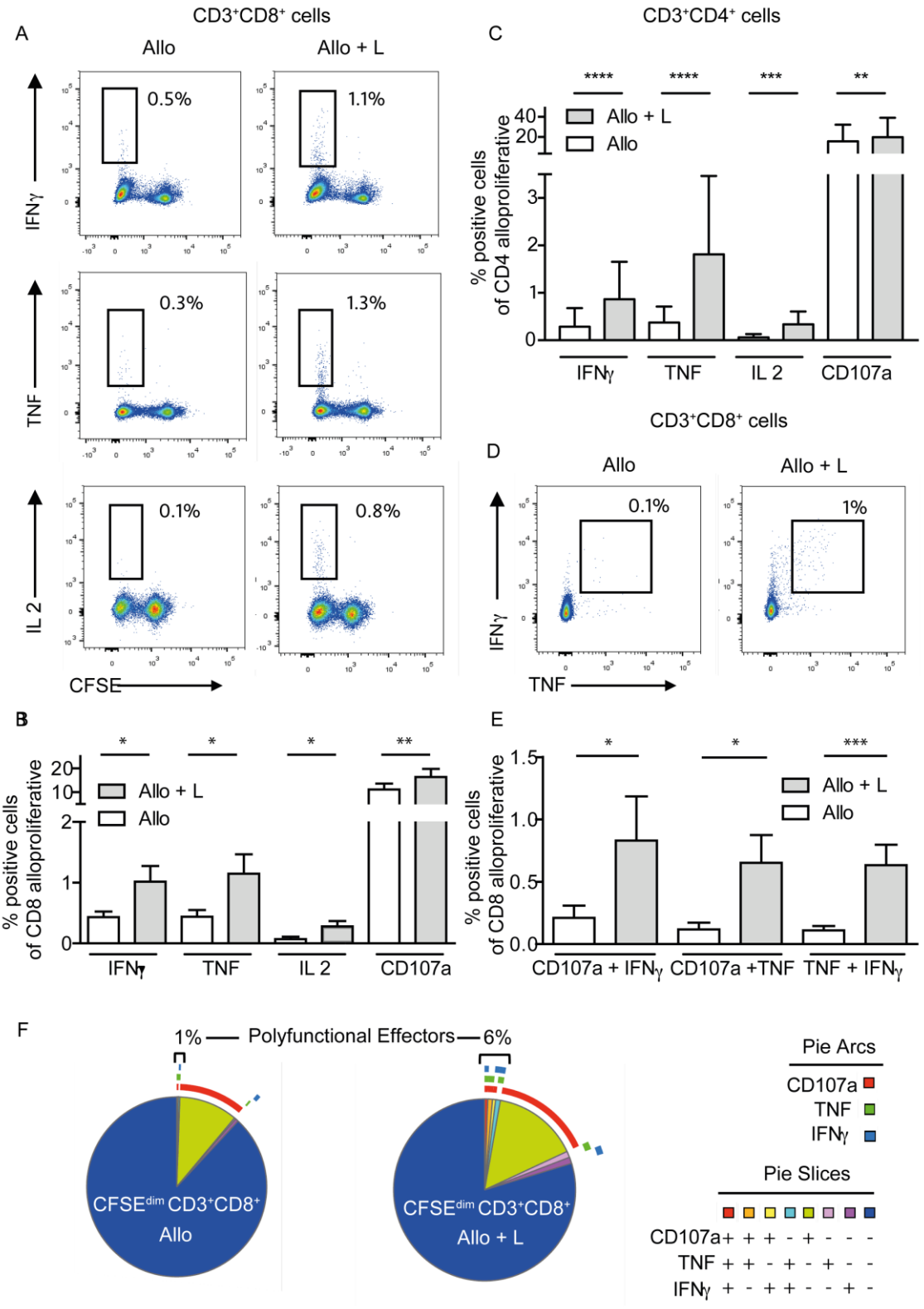

A
CD3+CD8+ cells
Allo
Allo + L
0.5%
1.1%
IFNγ
0.3%
1.3%
TNF
0.1%
0.8%
IL 2
CFSE
C
CD3+CD4+ cells
****
****
***
**
% positive cells of CD4 alloproliferative
Allo + L
Allo
IFNγ
TNF
IL 2
CD107a
D
CD3+CD8+ cells
Allo
Allo + L
0.1%
1%
IFNγ
TNF
B
*
*
*
**
% positive cells of CD8 alloproliferative
Allo + L
Allo
IFNγ
TNF
IL 2
CD107a
E
*
*
***
% positive cells of CD8 alloproliferative
Allo + L
Allo
CD107a + IFNγ
CD107a +TNF
TNF + IFNγ
F
1%
Polyfunctional Effectors
6%
CFSEdim CD3+CD8+ Allo
CFSEdim CD3+CD8+ Allo + L
Pie Arcs
CD107a
TNF
IFNγ
Pie Slices
CD107a+ + + - + - - -
TNF + + - + - + - -
IFNγ + - + + - - + -

Figure 3.7 Lenalidomide exposure during allostimulation increases the proportion of $CD8^+$ T cell with polyfunctional cytokine capacity

(A) Representative dot plots depict cytokine-accumulating cells after allogeneic co-culture in the absence (Allo) or presence of lenalidomide (Allo + L).

(B) Mean (+/- SD) frequencies of cytokine-accumulating and CD107a-expressing cells within $CD8^+$ $CFSE^{dim}$ T cells after allogeneic co-culture in the absence or presence of lenalidomide. Results are shown for 12 (IL2), 18 (CD107a) and 21 (TNF and IFNγ) unique donor-responder pairs * = P<0.05, ** = P<0.001.

(C) Mean (+/- SD) frequencies of cytokine-accumulating and CD107a-expressing cells within $CD4^+$ $CFSE^{dim}$ T cells after allogeneic co-culture in the absence or presence of lenalidomide. Results are shown for 12 (IL2), 18 (CD107a) and 21 (TNF and IFNγ) unique donor-responder pairs ** = P<0.01. *** = P<0.001, **** = P<0.0001.

(D) Representative dot plots depict dual positive cytokine-accumulating cells after allogeneic co-culture in the absence (Allo) or presence of lenalidomide (Allo + L).

(E) Mean (+/- SD) frequencies of polyfunctional cytokine-accumulating and CD107a-expressing cells within $CD8^+$ $CFSE^{dim}$ T cells after allogeneic co-culture in the absence or presence of lenalidomide. Results are shown for 12 (IFNγ/TNF + CD107a) 21 (TNF + IFNγ) unique donor-responder pairs. ** = P<0.01. *** = P<0.001.

(F) SPICE (simplified presentation of incredibly complex evaluations) charts depict different effector cell populations within $CFSE^{dim}$ $CD8^+$ T cells after allogeneic co-culture in the absence or presence of lenalidomide. Aggregate data from 21 unique donor-responder pairs are depicted.

3.4.6 Lenalidomide can influence $CD8^+$ T cell alloresponses in the absence of $CD4^+$ T cell help.

In order to better understand whether the effects of lenalidomide observed on $CD4^+$ and $CD8^+$ T cell subsets were due to cell-intrinsic effects, I went on to perform allogeneic co-cultures using sorted $CD4^+$ and $CD8^+$ responder populations to assess whether the effect on alloproliferation observed in a mixed co-culture could be recapitulated. Interestingly although the magnitude of $CD8^+$ T cell alloproliferation was decreased when $CD4^+$ responders were removed, exposure to lenalidomide still significantly increased the proportion of alloproliferative $CD8^+$ T cells in the absence of $CD4^+$ cells (**Figure 3.8**). This is evidence that a $CD8^+$ subset specific effect exists independently and in addition to enhanced $CD4^+$ T cell 'help' by augmented production of Th1 type cytokines. This also supports an effect on $CD8^+$ T cells that is, at least in part independent of responder APC.

Figure 3.8

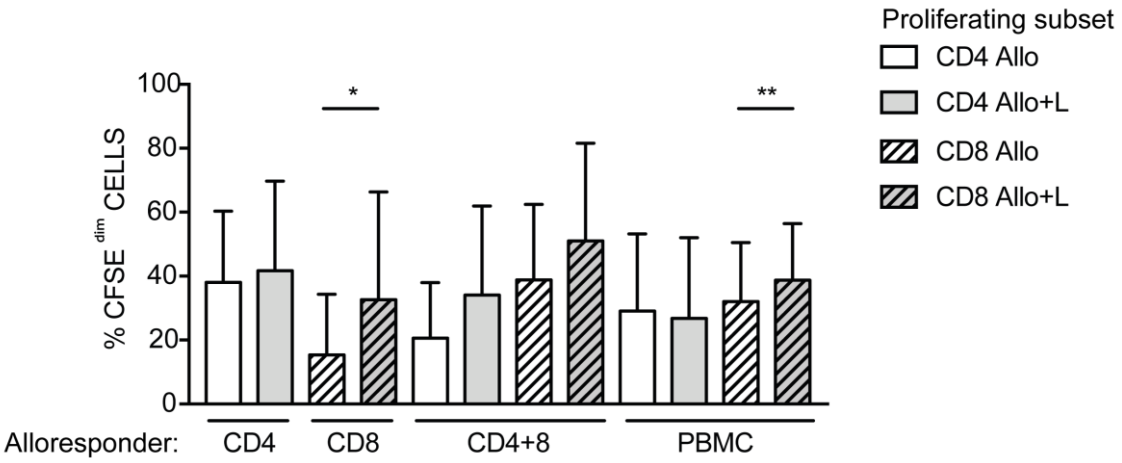


Figure 3.8 Lenalidomide exposure during allostimulation increased CD8$^+$ T cell alloproliferation in the absence of CD4$^+$ T cell help.

Mean (=/- SD) proportion of alloproliferative (CFSE^{dim}) cells in untreated (Allo) or lenalidomide treated (Allo+L) allogeneic co-cultures using sorted CD4$^+$, CD8$^+$, CD4+CD8 T cells (in a 2:1 ratio) and unsorted PBMC as responders. *=P<0.05, **=P<0.01

3.4.7 Allostimulation in the presence of lenalidomide results in an increase in the CD4$^+$ T effector cell to regulatory cell ratio

Studies have demonstrated that the net outcome of T cell alloresponses are influenced by the relative proportions of functionally distinct Teff and Treg CD4$^+$ T cells [133,303]. Although earlier experiments showed lenalidomide exposure did not significantly impact on alloproliferation of CD4$^+$ T cells, I therefore chose to also characterise the effect of lenalidomide exposure during allostimulation on Teff and Treg subsets within the responder CD4$^+$ T cell pool. CD4$^+$ Teff cells were defined as CD4$^+$CD25$^+$FOXP3^{neg} and Treg as CD4$^+$CD25$^+$FOXP3$^+$ (as per Rezvani *et al.*[57]). Cells with a Treg phenotype were also assessed for expression of CD127, to ensure low or absent expression (data not shown for simplicity).

Allostimulation in the presence of lenalidomide increased the proportion of CD4$^+$CD25$^+$FOXP3^{neg} Teff and decreased the proportion of CD4$^+$CD25$^+$FOXP3$^+$ Treg, resulting in a significant increase in the Teff:Treg ratio (**Figure 3.9, B-C**). This was due to both a significant increase in the proportion of alloproliferative CD4$^+$ T cells with an effector phenotype as well as a significant decrease in the proportion of alloproliferative CD4$^+$ T cells with a Treg phenotype (**Figure 3.9, C-D**). Importantly, these results demonstrate that although lenalidomide does not impact on net alloproliferation of CD4$^+$ T cells, it has a qualitative effect on the CD4$^+$ T cell alloresponse in addition to increasing proliferation of alloreactive CD8$^+$ T cells.

Figure 3.9

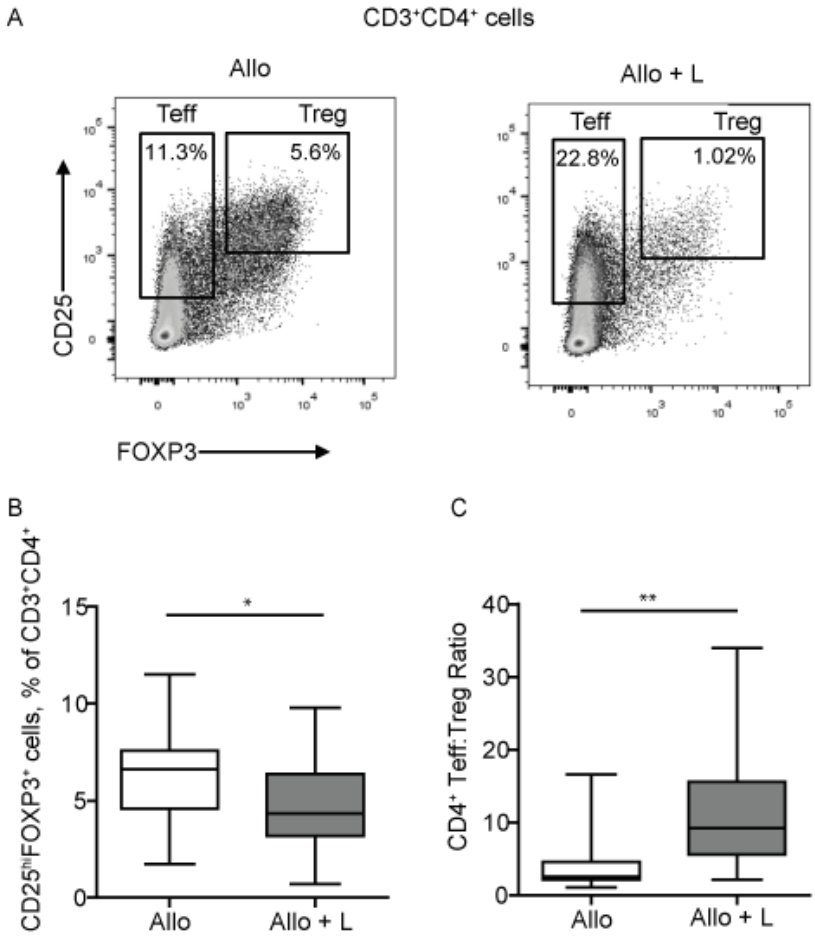

A
CD3⁺CD4⁺ cells
Allo
Allo + L
Teff
Treg
11.3%
5.6%
22.8%
1.02%
CD25
FOXP3
B
CD25ʰⁱFOXP3⁺ cells, % of CD3⁺CD4⁺
*
Allo
Allo + L
C
CD4⁺ Teff:Treg Ratio
**
Allo
Allo + L

Figure 3.9 Allostimulation in the presence of lenalidomide increases the $CD4^+$ T effector to T regulatory cell ratio

(A) Co-expression patterns of CD25 and FOXP3 were used to identify $CD25^{hi}FOXP3^+$ $CD3^+CD4^+$ T regulatory (Treg) and $CD25^{hi}FOXP3^{neg}$ T effector (Teff) cells. Representative dot plots depict cells after allogeneic co-culture in the absence (Allo) or presence of lenalidomide (Allo+L).
(B) Proportion of $CD4^+$ T cells with a Treg phenotype after allogeneic co-culture in the absence or presence of lenalidomide. Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 18 unique donor-responder pairs. * = $P<0.05$.
(C) Ratio of $CD4^+$ Treg and Teff cells after allogeneic co-culture in the absence or presence of lenalidomide. Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 18 unique donor-responder pairs. ** = $P<0.01$.

3.4.8 Lenalidomide exposure increases alloproliferation of NK cells

While the focus of this thesis is the characterisation of the impact of lenalidomide on the human T cell alloresponse, there are other cell types that can contribute to human alloresponses. NK cells have the capacity to exert GvT effects, particularly in the setting of haploidentical-AHSCT, without causing GvHD.

There is evidence that lenalidomide has immunostimulatory effects on NK cells *in vitro* [230,232] and *in vivo* [229] from a number of studies in the autologous setting. Therefore it is important to assess the effect of lenalidomide on NK cell alloresponses that could lead to enhanced GvT effects.

Conventionally $CD56^+$ NK cells are categorized as $CD56^{bright}$ and $CD56^{dim}$, with $CD56^{bright}$ NK cells regarded as cells with poor cytotoxic ability and enhanced cytokine production compared to $CD56^{dim}$ for which the converse is true. *In vivo* $CD56^{bright}$ NK cells also generally lack expression of CD16, the fragment crystallisable (Fc) gamma receptor III (FCγRIII) (reviewed in Bjorkstrom *et al* [304]) that enables NK cells to exert ADCC.

Using the same allogeneic co-culture model and CFSE dye-dilution I examined the proliferation and phenotype of NK cells. Proliferation of $CD3^{neg}CD56^+$ NK cells in lenalidomide treated co-cultures was significantly increased after 9 days of allogeneic co-culture (median of 58% (untreated) to 77% (lenalidomide treated) $p<0.0001$) (**Figure 3.10, A**). This was accompanied by an increase in $CD56^+16^+$ cells (**Figure 3.10, B**) and a significant increase in levels of CD56 expression, as shown by a significant increase in the median fluorescence intensity (MFI) of CD56 (**Figure 3.10, D**).

$CD16^+$ NK cells from allogeneic co-cultures had the expected dim-intermediate pattern of CD56 expression, while those from lenalidomide treated cultures showed up-regulation of CD56 on $CD16^+$ NK cells (**Figure 3.10, C**). This acquisition of CD16 by $CD56^{bright}$ NK cells has been previously described as being inducible *in vitro* by stimulation of NK cells with IL2 [305]. Therefore it is likely that both the increase in proliferation and the phenotypic changes observed in NK cells in my allogeneic co-cultures are a secondary phenomenon induced by the increased production of IL2 by alloproliferative $CD8^+$ and $CD4^+$ T cells.

Figure 3.10

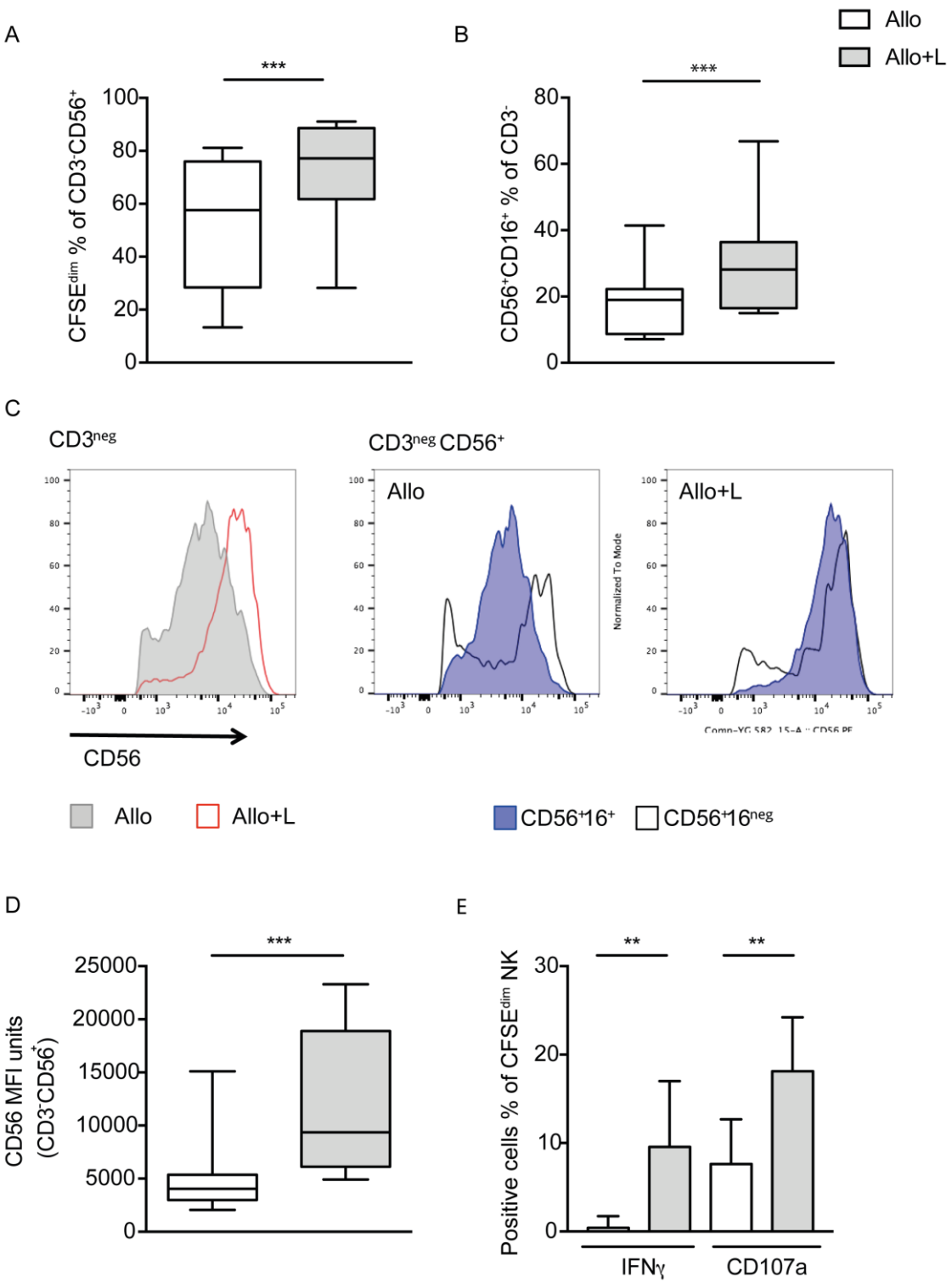

A
B
Allo
Allo+L
***
***
CFSE^dim % of CD3^-CD56^+
CD56^+CD16^+ % of CD3^-
C
CD3^neg
CD3^neg CD56^+
Allo
Allo+L
Normalized To Mode
CD56
Allo
Allo+L
CD56^+16^+
CD56^+16^neg
D
E
***
**
**
CD56 MFI units (CD3^-CD56^+)
Positive cells % of CFSE^dim NK
IFNγ
CD107a

Figure 3.10 Secondary expansion of NK cell subsets in allogeneic co-cultures treated with lenalidomide

(A) $CD3^-CD56^+$ NK cell proliferation (% $CFSE^{dim}$) following primary allogeneic co-culture of adult peripheral blood (APB) mononuclear cells in the absence (Allo) or presence of lenalidomide (Allo+L). Horizontal lines and adjacent numbers are medians. Results are shown for 16 unique donor-responder pairs. *** = P<0.001.

(B) Proportion of $CD3^-$ NK cells co-staining for CD56 and CD16 following primary allogeneic co-culture in the absence (Allo) or presence of lenalidomide (Allo+L). Horizontal lines and adjacent numbers are medians. Results are shown for 16 unique donor-responder pairs. *** = P<0.001.

(C) Representative histograms depicting CD56 expression on $CD3^-$ NK cell subsets after allostimulation in the absence or presence of lenalidomide.

(D) Relative expression of CD56 following primary allogeneic co-culture of mononuclear cells in the absence (Allo) or presence of lenalidomide (Allo+L) measured by median fluorescence intensity (MFI). Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 13 unique donor-responder pairs. *** = P<0.001.

(E) Mean (+/- SD) frequencies of cytokine-accumulating and CD107a-expressing cells within $CD3^-CD56^+CFSE^{dim}$ NK cells after allogeneic co-culture in the absence or presence of lenalidomide. Results are shown for 10 unique donor-responder pairs ** = P<0.01.

However interestingly Romagnani *et al.* describe that $CD56^{bright}CD16^{+}$ NK cells can be isolated *in vivo* from efferent lymph suggesting that in lymph nodes where local IL2 levels may be increased the same phenomenon could occur.

Finally I examined IFNγ production and CD107a expression on proliferative $CD3^{neg}CD56^{+}$NK cells from treated and untreated allogeneic co-cultures. In the majority of allogeneic co-cultures IFNγ-producing cells were below the level of detection, however in treated co-cultures there was a significant increase in both IFNγ and CD107a expressing cells. Again these results must be viewed in the context of the mixed co-culture system employed. *In vitro,*. IL2 dependent increases in both IFNγ and CD107a production by NK cells have been described in the literature [306,307].

3.4.9 Lenalidomide increases alloproliferation of T cells in HLA-matched allogeneic co-cultures

In the majority of AHSCT the donor and recipient are HLA-matched. The first choice donor will often be a HLA-identical sibling if available and if not a 10/10 HLA-matched unrelated donor, denoting matching at the allele level for HLA-A, B, C, DRB1and DQ will be sought. Therefore I went on to examine the effect of lenalidomide exposure during allostimulation using samples taken from patients about to undergo AHSCT at St Bartholomew's Hospital as stimulator cells and cells from their HLA-identical sibling AHSCT donors as responders.

I chose to use the standard allogeneic co-culture (as described in Chapter 2) without cytokine modification for these experiments. This ensured consistency between the experiments in the HLA-mismatched and HLA-matched setting. The limitation of this approach is that HLA-matched allogeneic co-cultures often give no read-out of alloproliferation. However I was interested to determine whether addition of lenalidomide to the co-cultures could increase responder proliferation above the threshold of detection.

Three matched donor and recipient pairs were tested and in each case there was a small increase in overall T cell proliferation in the presence of lenalidomide compared to control (**Figure 3.11, A**). Interestingly in these three samples there also appeared to be a small increase in $CD4^+$ T cell proliferation in lenalidomide treated co-cultures (**Figure 3.11, B**). I also examined the effect on T cell subsets expressing CCR7 and CD45RA but was unable to detect any changes in these three pairs (**Figure 3.11, C-D**).

Figure 3.11

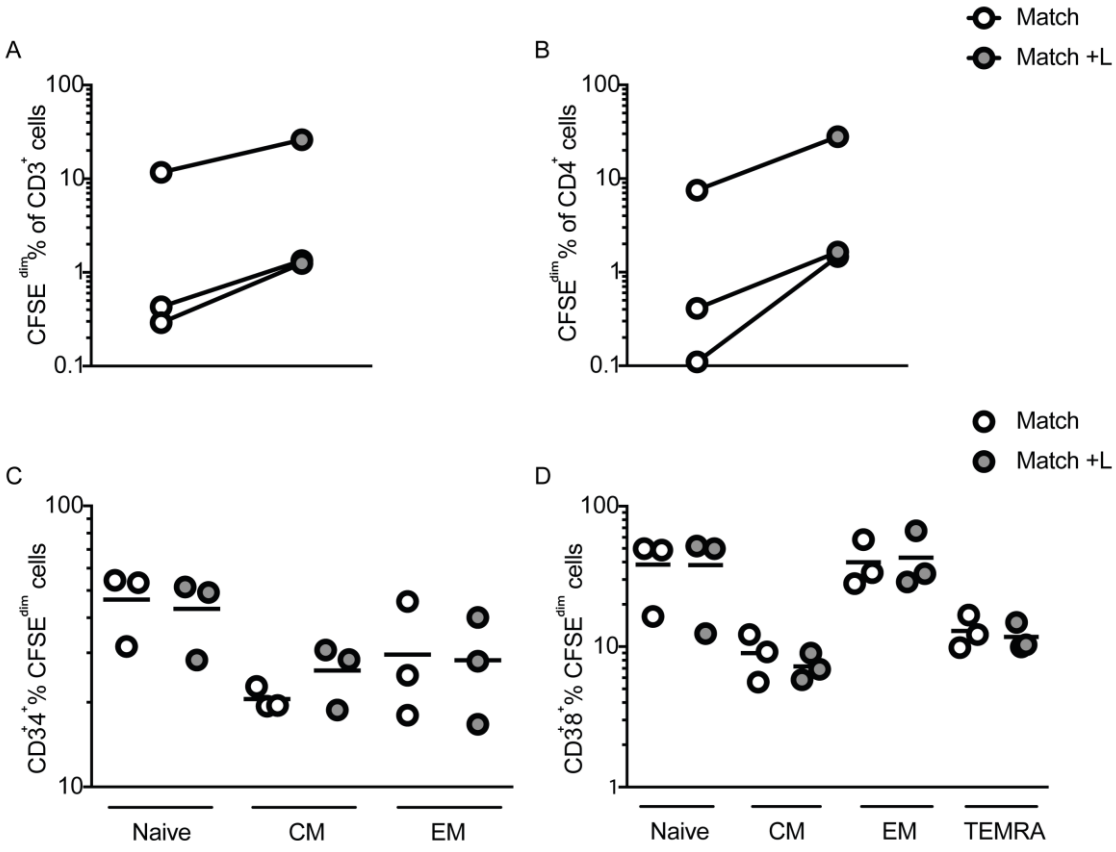


Figure 3.11 Lenalidomide increased T cell alloproliferation in HLA-matched allogeneic co-cultures

(A) $CD3^+$ T-cell proliferation (% $CFSE^{dim}$) following primary allogeneic co-culture of HLA-matched mononuclear cells in the absence (Match) or presence of lenalidomide (Match+L). Results are shown for 3 unique donor-responder pairs.

(B) $CD4^+$ T-cell proliferation (% $CFSE^{dim}$) following primary allogeneic co-culture of HLA-matched mononuclear cells in the absence (Match) or presence of lenalidomide (Match+L). Results are shown for 3 unique donor-responder pairs.

(C) Percentages of naïve, CM and EM cell subsets within $CD4^+$ T cells after HLA-matched allogeneic co-culture in the absence or presence of lenalidomide. Results are shown for 3 unique donor-responder pairs.

(D) Percentages of naïve, CM, EM and TEMRA cell subsets within $CD8^+$ T cells after HLA-matched allogeneic co-culture in the absence or presence of lenalidomide. Results are shown

3.5 Discussion

Using an HLA-mismatched *in vitro* model of AHSCT I have demonstrated that the addition of a clinically relevant concentration of the immunomodulatory drug lenalidomide results in significant alterations in the human T cell alloresponse. Addition of lenalidomide to allogeneic co-cultures resulted in a selective increase in proliferation of $CD8^+$ T cells. These cells retained a similar TCR Vβ subfamily distribution compared to untreated cells, consistent with lenalidomide lowering the threshold for $CD8^+$ T cell activation and recruitment of alloreactive cells with lower avidity TCRs to similar immunodominant alloantigens. These expanded allospecific $CD8^+$ T cells had an effector memory phenotype and were capable of enhanced polyfunctional cytokine production. Importantly these cells would be predicted to have increased capacity to destroy cellular targets and may explain the induction of GvHD [256,257] and potential GvT [265,266] observed in the clinical literature. I also observed an increase in allospecific $CD8^+$ effectors that had a memory precursor ($CD25^+CD127^+$) phenotype after lenalidomide treatment, which could confer long-term immunosurveillance important for maintaining GvT.

This effect on $CD8^+$ T cell proliferation is in keeping with increased $CD8^+$ T cell counts observed in the peripheral blood of patients receiving lenalidomide for haematological and solid malignancy [212], including increases in T cells with an EM phenotype [215]. In addition the increased expression of CD25 is in agreement with the increased expression of an alternative marker of T cell activation, HLA DR observed in peripheral blood of patients who have received lenalidomide following AHSCT [259].

I have shown using MS/MS-UHPLC that lenalidomide exposure early in the alloresponse results in the expansion of APB-derived $CD8^+$ T cells by enhancing priming of allospecific T cells early in the process of allostimulation, rather than by augmenting the expansion of alloreactive T cells at a later stage. However as the allogeneic co-cultures were not re-dosed with lenalidomide over the course of the co-culture an additional effect on proliferating cells cannot be excluded. This would be an interesting additional experiment that could be conducted, in view of the fact that in the clinic patients generally receive several weeks of continuous lenalidomide treatment.

Pre-treatment of stimulator and responder cells prior to allogeneic co-culture has provided interesting insight into the cellular mechanisms that may be involved in the lenalidomide alloresponse. The data may also help to inform clinical strategies as the experiment also models

(*in vitro*) the scenario of pre-treatment of a patient prior to transplant/DLI (pre-treatment of stimulators) and pre-treatment of donor cells (i.e. DLI) prior to adoptive transfer.

Given that pre-treatment of stimulators alone did not result in increased $CD8^+$ T cell proliferation this suggests that potentiation of direct antigen presentation by stimulator APC alone is not sufficient for the effect of lenalidomide on alloproliferation, and that pre-treatment of patients prior to T cell adoptive therapy may not be effective. However as pre-treatment of responder cells was also unable to recapitulate the alloproliferative response, this suggests that in the absence of TCR stimulation effects on responder T cells are also not sufficient for the optimal effect of lenalidomide.

Interestingly, although the result did not reach statistical significance it appears that pre-treatment of both stimulators and responders did result in increased alloproliferation of $CD8^+$ T cells over control, suggesting that lenalidomide can have effects on both APC and T cells that contribute to the alloresponse, but that contact between these cells in the presence of the drug is required for the optimal effect of lenalidomide.

However other possible reasons, inherent in the experimental design that could explain why pre-treatment of stimulator and responder cells did not lead to any significant effect should be considered. It is possible that either a longer period of pre-treatment, or a higher dose of lenalidomide is necessary, however I think this is unlikely based on other published data. This period of pre-treatment was chosen based on the findings of Ramsay *et al.*, who showed a pre-treatment period of 24 hours was sufficient to induce improvements in immune synapse formation [209]. This was further supported by data from Gandhi *et al.* who demonstrated that ikaros levels drop significantly within 3 hours of lenalidomide treatment, with a significant reduction observed at 6 hours even at low concentrations such as 0.1μM.

I have also observed a decrease in the frequency of cells with a $CD4^+$ Treg phenotype in allogeneic co-cultures treated with lenalidomide. This is in contrast to the only data available concerning $CD4^+$ Treg counts in patients treated with lenalidomide after AHSCT [259,260] which suggests that Treg numbers in peripheral blood rise, but only several weeks following initiation of treatment. Given my results were from a day 9 time-point, this earlier fall in Treg frequency may not be captured in the clinical data. The late rise in Treg observed *in vivo* may reflect a counter-regulatory response to the expansion of $CD8^+$ 'enhanced effectors' I have described.

Unfortunately the *in vitro* model I employed cannot be used to assess late outcomes, such as those that occur during immune reconstitution following AHSCT. This is a major limitation of currently available *in vitro* experimental models.

The differentiation of 'enhanced $CD8^+$ effectors' following lenalidomide exposure from the naïve $CD8^+$ compartment is consistent with potentiation of the alloresponse *in vivo*, as alloreactive cells that contribute to GvHD and GvT are known to reside in this compartment [53,55]. In order to definitively prove that the increase in EM cells observed is due to differentiation of naïve $CD8^+$ T cells (rather than from proliferation of existing EM cells) it would be necessary to isolate naïve, CM, EM and T_{EMRA} cells and use these as responders in co-culture with allogeneic stimulators. Unfortunately when I attempted these experiments it proved difficult to isolate sufficient numbers of cells and I was unable to maintain the viability of the sorted cells over the course of the co-culture. This may be due to the small number of cells and degree of processing, or due to the lack of immunostimulatory and immunoregulatory signals that these cells would normally receive when in a mixed cell culture.

The allogeneic co-culture model presented used healthy *steady state* T cells as responder cells. However the cells that make up an allogeneic donor graft are usually G-CSF mobilised PBMC. G-CSF mobilisation has been shown to impact on the phenotype of T cells contained in the graft, skewing the cells towards a Th2 phenotype [308], reducing cytokine production [309] and expanding populations of Treg. [310]. A case could therefore be made for G-CSF mobilised peripheral blood as a source of T cells for my experiments. However there is good evidence to show that the effect of G-CSF on the genotype and phenotype of T cells is transient [311,312] and likely to have reversed by the time lenalidomide would be administered after AHSCT (earliest 4-6 weeks in published studies). On this basis, I chose to use *steady state* T cells rather than G-CSF mobilized PBMC as my primary responder cells. The use of *steady state* healthy APB-derived T cells for assessment of human alloresponses, and as a comparator to alternative graft sources [313-316] is also predominant in the literature and therefore allows me to more easily contextualise my data within the broader field of AHSCT research.

The allogeneic co-cultures in these experiments were likely to be highly HLA-mismatched. This has the advantage of stimulating both $CD4^+$ and $CD8^+$ T cell responses, as mismatch would be expected in both class I and class II MHC in the majority of donor-recipient pairs randomly chosen from healthy blood donors. Additionally differences in alloproliferation and alloreactive

phenotype can be more easily detected due to the magnitude of the mismatched T cell alloresponse. However, as discussed previously the majority of clinical AHSCT is performed in the HLA-matched setting. In order to address this some HLA-matched co-cultures were performed, however due to the small number of paired donor and recipient samples available the number of experiments was limited.

A limitation of HLA-matched co-cultures is that by their very nature these co-cultures rarely result in any significant alloproliferation and therefore cytokines are often required to make alloresponses detectable [280]. This was particularly evident when trying to quantify $CD8^+$ T cell proliferation, as the frequency of alloproliferative $CD8^+$ T cells was below the threshold of confidence for detection. I was reluctant to alter the experimental design of the HLA-matched co-cultures from that of the mismatched by the addition of cytokines, as this would be a confounding factor when trying to determine the effect of lenalidomide. Collection of matched donor and recipient samples is ongoing and further HLA-matched co-cultures could be conducted, with or without cytokine augmentation. An alternative approach to examine the effect of lenalidomide in a HLA-matched setting would be to examine the phenotype of reconstituting T cells in sibling-transplant recipients treated with lenalidomide, to determine whether the phenotype described in lenalidomide-treated HLA-mismatched co-cultures is recapitulated *in vivo*.

Finally, while the experiments detailed here were not designed to examine NK cell alloresponses I have found proliferation, IFNγ production and CD107a expression of NK cells to be increased in lenalidomide treated co-cultures. It is likely that these changes are secondary to the effect on T cell alloresponses via increased IL2 production. However given NK cells are known to exert GvT without GvHD, and therefore strategies to optimise NK cell activity following AHSCT would be clinically desirable, it would be very interesting to examine the effect of lenalidomide on purified NK cells used as alloresponders in allogeneic co-culture.

As NK cells form a very small proportion of lymphoid cells in the peripheral blood, cell numbers may limit these experiments. It is also true that in the absence of T cells it may be difficult to detect NK cell alloresponses [317]. Evidence from patients who have received lenalidomide after AHSCT has demonstrated increased expression of activating receptors on NK cells [260], indicating that effects on NK cells occur, but whether these occur via T cells *in vivo* as well as *in vitro* is unclear. While studying individual cell types in isolation can provide insight into the mechanisms

of action of a drug at the cellular and molecular level, when considering alloresponses it is likely that the net outcome is due to a complex interaction between multiple cell types. Therefore mixed-cell culture models may still provide relevant insight and information.

In summary, I have shown that lenalidomide exposure potentiates human alloresponses by selectively expanding alloreactive $CD8^+$ effector T cells. These findings are consistent with the *in vivo* effects of lenalidomide observed in clinical trials after AHSCT. Furthermore, these findings may also provide a phenotype to track lenalidomide responses *in vivo* as well as informing future strategies to use IMiDs to strengthen anti-tumour responses in the setting of AHSCT.

Chapter 4 – The impact of lenalidomide on cellular alloresponses of umbilical cord blood-derived T cells

4.1 Introduction

The last 10 years have seen a rise in the numbers of AHSCT carried out using umbilical cord blood (UCB) as a graft source. The first choice donor remains a HLA-identical sibling, but as 70% of patients lack a suitable sibling donor, and 50% will also lack a suitable matched unrelated donor (MUD), alternative transplant sources such as UCB are increasingly important. This is particularly true for patients of mixed ethnic backgrounds, for whom in only 10% of cases a MUD can be identified [318,319].

UCB-AHSCT (UCBT) has been particularly successful in the treatment of children with haematological malignancy or bone marrow failure syndromes [320]. However the low cell doses in UCB grafts mean that only 25% of adults will have an UCB unit of sufficient cell dose available. The recent development of 'double-cord' UCBT has extended the opportunity for adult patients to access a UCB graft and research continues into *ex vivo* strategies to expand UCB units for clinical use.

UCB has some important advantages as a graft source. Rates of both acute and chronic GvHD in children and adults undergoing UCBT are lower than for recipients of peripheral blood or bone marrow derived grafts, as is the severity of acute GvHD even when UCB units are only partially matched (4/6 HLA match at HLA-A, B and DRB1). Despite this relapse rates do not appear to be increased after UCBT compared to other transplants and overall survival is comparable. The major disadvantages of UCBT are a greater risk of graft rejection and slower haematopoietic recovery than is seen for other graft sources, which means that for 4/6 (or less) HLA-matched grafts there is an increase in transplant related mortality (TRM) after UCBT [320,321]. Important differences in UCB composition are likely to underpin the reduced rates of GvHD seen after UCBT even in cases of HLA-mismatch.

UCB T cells are almost entirely found within the naïve compartment [322,323]. Although this might be expected to increase rates of GvHD, as evidence from APB suggests GvHD inducing cells reside in this subset [53] it appears that the same is not true of UCB. Whether this is due to cell

intrinsic differences or secondary effects due to differences in other cells making up UCB grafts is unclear.

Despite their naivety data from a number of publications show that UCB T cells are as capable, if not better at responding to allogeneic stimuli than APB-derived T cells in terms of alloproliferation [324,325]. However UCB-derived T cells have reduced capacity to produce and secrete cytokines [325,326] and have reduced lytic capacity against allogeneic targets [324] when compared to APB. Interestingly *in vivo* data from murine models suggests that UCB-derived T cells may mediate superior GvT effects than APB-derived T cells despite these differences [314,327]. Differences between APB-derived and UCB-derived T and NK cells are summarised in **Table 4.1**.

As yet the effects of immunomodulatory drugs, like lenalidomide on UCB-derived T cell alloresponses are unknown. A clinical trial of pre-treatment with lenalidomide given on days -8 to -2 prior to UCBT +/- NK cell infusion on Day -2 is currently recruiting (NCT01619761). There have not yet been any clinical trials using lenalidomide or other IMiDs after UCBT. Given the differences in T cell phenotype and function between UCB and APB-derived T cells lenalidomide may have differential effects on T cell alloresponses from these two graft sources. Investigation of differential effects will inform future clinical trials and strategies for use of IMiDs in the setting of UCBT.

Table 4.1 Differences in UCB and APB-derived T & NK cells

Characteristic	UCB	APB
T cell response to mitogens	Modest	Vigorous
T cell response to alloantigens	Vigorous	Vigorous
Proportion of naïve T cells	Majority	Fewer
Production of IL2, IFNγ and TNF from activated T cells	Low	High
Susceptibility of T cells to apotosis	Increased compared to APB	Decreased compared to UCB
Perforin expression by cytotoxic T cells	Low	High
Allogeneic cytotoxicity of activated T cells	Low	High
Treg phenotype	Predominantly naïve	Predominantly memory
Treg expansion on in vitro stimulation	High	Lower
IL10 production by Treg	High	Lower
NK cell phenotype	Increase in precursor cells	Increased mature cells
NK cell activity	Comparable	

Adapted from Brown and Bousiottis 2008 and Lucchini *et al.* [328,329]

4.2 Aim

To characterise the effect of lenalidomide exposure on the human UCB-derived T cell alloresponse and compare this with the effect observed on steady state APB-derived T cells alloresponses.

4.3 Results

4.3.1 Net alloresponses of UCB-derived T cells are reduced following allostimulation in the presence of lenalidomide, although a selective increase in the proliferation of allospecific effector CD8$^+$ T cells remains

Firstly I measured the effect of lenalidomide exposure on alloproliferation of UCB-derived T cells using the allogeneic co-culture model (as described in Chapter 2). Consistent with previous studies I found that UCB-derived T cells exhibited greater alloproliferative responses than APB-derived T cells [324,325]. Importantly, in contrast to my findings in APB T cells, lenalidomide exposure significantly **reduced** the net alloproliferation of responder CD3$^+$ T cells from UCB (median 58% (untreated) compared to 41% (lenalidomide-treated), $P<0.05$, **Figure 4.1, A**).

I therefore next examined T cell subset specific alloproliferation. I found that lenalidomide exposure during allostimulation increased alloproliferation of CD8$^+$ T cells derived from UCB (median 53% (untreated) versus 61% (lenalidomide-treated), $P<0.05$) as it had for APB-derived T cells (**Figure 4.1, B**). Similarly, as with APB lenalidomide treatment increased the alloproliferative precursor frequency (APF) of UCB CD8$^+$ T cells (median 14% (untreated) to 24% (lenalidomide-treated) $P<0.001$, **Figure 4.1, C**), with no change in the median number of rounds of cell division (**Figure 4.1, D**).

Figure 4.1

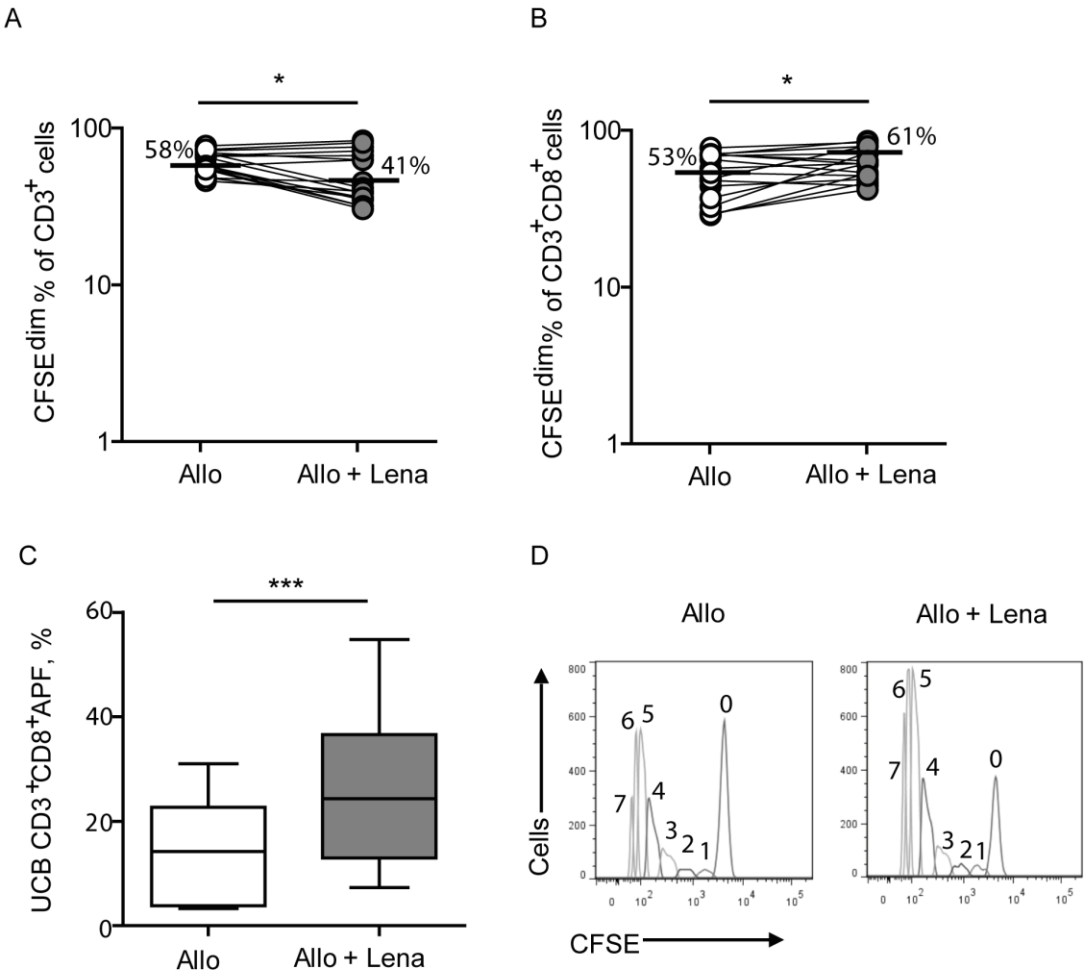

A
*
100
10
1
58%
41%
CFSE dim % of CD3+ cells
Allo
Allo + Lena
B
*
100
10
1
53%
61%
CFSE dim % of CD3+CD8+ cells
Allo
Allo + Lena
C
***
60
40
20
0
UCB CD3+CD8+APF, %
Allo
Allo + Lena
D
Allo
Allo + Lena
Cells
CFSE
0
1
2
3
4
5
6
7

Figure 4.1 Net alloproliferation of UCB T cells is reduced following exposure to lenalidomidewhile

(A) $CD3^+$ T cell proliferation (% $CFSE^{dim}$) following primary allogeneic co-culture of umbilical cord blood (UCB) mononuclear cells in the absence or presence of lenalidomide. Horizontal lines and adjacent numbers are medians. Results are shown for 17 unique donor-responder pairs. * = P<0.05.

(B) UCB $CD8^+$ T cell proliferation following primary allogeneic co-culture of in the absence or presence of lenalidomide. Horizontal lines and adjacent numbers are medians. Results are shown for 17 unique donor-responder pairs. * = P<0.05.

(C) UCB $CD8^+$ T cell alloproliferative precursor frequency (APF) in allogeneic co-cultures in the absence or presence of lenalidomide exposure. Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 17 unique donor-responder pairs. *** = P<0.001.

(D) Histograms depicting UCB $CD8^+$ T cells after allostimulation in the absence or presence of lenalidomide exposure. Undivided cells and populations of cells that have undergone one or more cell divisions are resolved based on CFSE dye dilution. Numbers above peaks represent the hierarchical number of cell divisions each peak has undergone. Representative data is shown from one of 17 unique allogeneic co-cultures.

4.3.2 Allostimulation in the presence of lenalidomide expands CD8$^+$ effector T cells from UCB

Consistent with the effect observed on APB-derived T cells, lenalidomide exposure during allostimulation of UCB-derived T cells resulted in a significant increase in CD8$^+$ T cells with an EM phenotype (24% (untreated) versus 43% (lenalidomide-treated) P<0.001) accompanied by a significant decrease in cells with a naive phenotype, consistent with lenalidomide promoting differentiation of naïve UCB-derived T cells to EM cells (**Figure 4.2, F**).

Although frequencies of allospecific cytokine-secreting CD8$^+$ T cells from UCB were significantly lower than in APB T cells (consistent with previous published studies [325]) lenalidomide exposure also increased the proportion of alloproliferative UCB-derived CD8$^+$ T cells accumulating either IFNγ or TNF. Interestingly, the proportion of cells expressing CD107a was decreased (**Figure 4.3**).

Figure 4.2

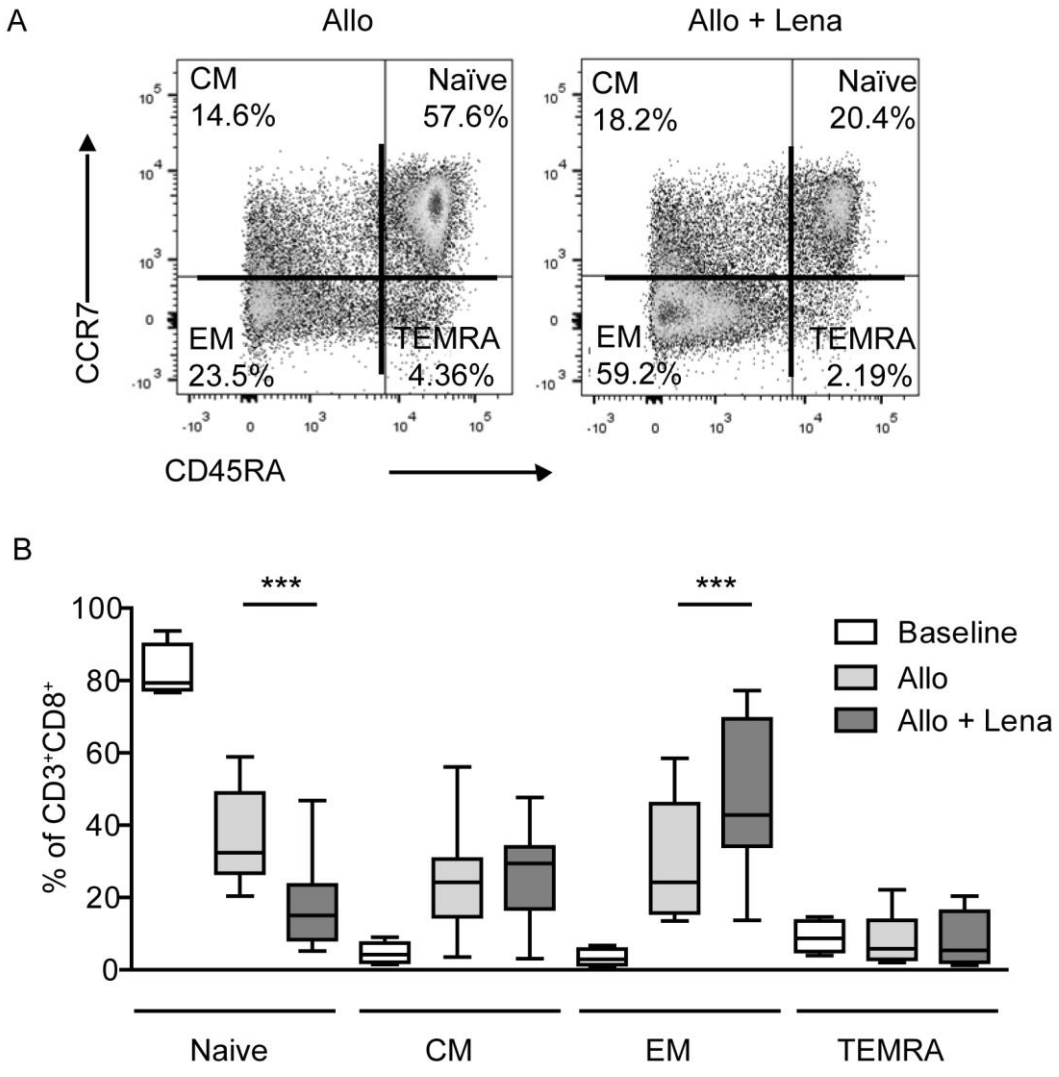

A
Allo
Allo + Lena
CM
14.6%
Naïve
57.6%
EM
23.5%
TEMRA
4.36%
CM
18.2%
Naïve
20.4%
EM
59.2%
TEMRA
2.19%
CCR7
CD45RA
B
***
***
% of CD3+CD8+
100
80
60
40
20
0
Baseline
Allo
Allo + Lena
Naive
CM
EM
TEMRA

Figure 4.2 Lenalidomide increases effector differentiation of UCB CD8$^+$ T cells.

(A) Representative plots depicting C-C chemokine receptor 7 (CCR7) and CD45RA co-expression patterns of UCB-derived CD8$^+$ T-cells after allogeneic co-culture in the absence or presence of lenalidomide. Percentages of naïve, central memory (CM), effector memory (EM) and T-effector memory cells re-expressing RA (TEMRA) subsets are indicated.
(B) Percentages of naïve, CM, EM and TEMRA cell subsets within UCB CD8$^+$ T cells in baseline cells and after allogeneic co-culture in the absence or presence of lenalidomide. Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 17 unique donor-responder pairs. *** = P<0.001.

Figure 4.3

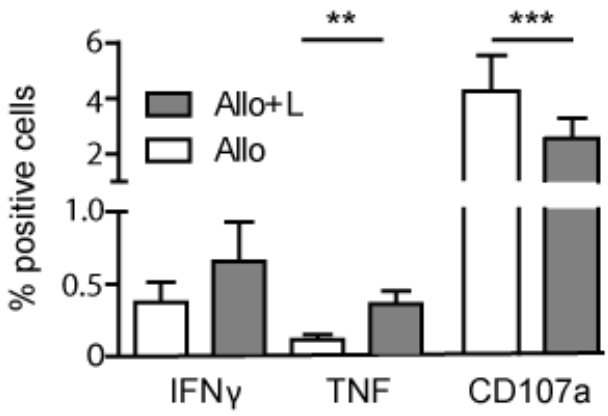


Figure 4.3 Allostimulation in the presence of lenalidomide increases cytokine production of UCB CD8$^+$ T cells but decreases CD107a up-regulation

Mean (+/- SD) frequencies of cytokine-accumulating and CD107a-expressing cells within UCB-derived CD8$^+$ CFSEdim T cells after allogeneic co-culture in the absence or presence of lenalidomide. Results are shown for 12 unique donor-responder pairs. ** = P<0.01, *** = P<0.001

4.3.3 The differential effect of lenalidomide on alloproliferation of APB and UCB CD3$^+$ T cells is due to a selective reduction in proliferation of UCB CD4$^+$ T cells

My findings that lenalidomide had opposing effects on alloproliferation of CD3$^+$ T cells from APB and UCB despite a common effect of potentiating alloproliferation of CD8$^+$ T cells suggested that lenalidomide may have differential effects on CD4$^+$ T cell alloresponses of these different cell sources. I therefore determined the impact of lenalidomide exposure on APB and UCB-derived CD4$^+$ T cell alloresponses.

Lenalidomide exposure during allostimulation resulted in a significant reduction in alloproliferation of UCB-derived CD4$^+$ T cells (median 58% (untreated) versus 41% (lenalidomide-treated, $p<0.01$ **Figure 4.4, A**). This effect was not due to a reduction in recruitment of alloreactive CD4$^+$ UCB-derived T cells, as the CD4$^+$ APF was not significantly reduced after lenalidomide exposure (**Figure 4.4, B**).

4.3.4 The reduction in alloproliferation of UCB CD4$^+$ T cells after lenalidomide exposure is accompanied by a selective expansion of CD4$^+$ regulatory T cells

Given the finding that lenalidomide exposure resulted in significantly decreased alloproliferation of CD4$^+$ T cells derived from UCB, I next determined the effect of lenalidomide on the proportions of CD4$^+$ T cells with Teff or Treg phenotypes.

Lenalidomide exposure during allostimulation resulted in a significantly increased frequency of UCB-derived CD4$^+$ T cells with a Treg phenotype (**Figure 4.4, C**-**D**) and a significant reduction in the Teff:Treg ratio compared to untreated allogeneic co-cultures (**Figure 4.4, E)**. As CD4$^+$ Treg can potently suppress the proliferation of Teff, an expansion of Treg after lenalidomide exposure may provide a mechanism for the reduction in CD4$^+$ alloproliferation observed in UCB T cells. Consistent with this mechanism, the fold-change in frequency of UCB-derived CD4$^+$ Treg after lenalidomide exposure was significantly correlated with the resulting reduction in overall allospecific CD4$^+$ T cell proliferation **(Figure 4.4, F).** These Treg were predominantly found in the CFSE^{dim} fraction, indicating that they arise from either proliferation of Treg in the starting CD4$^+$ population or by trans-differentiation from alloproliferative CD4$^+$ cells.

I then directly compared the impact of lenalidomide on $CD4^+$ Treg and Teff frequencies within the alloresponder $CD4^+$ T cell population after allostimulation of APB and UCB T cells. Lenalidomide exposure resulted in a median 1.8-fold increase in the frequency of $CD4^+$ Treg after allostimulation of UCB-derived T cells, compared to a median 0.6-fold decrease in the frequency of $CD4^+$ Treg after allostimulation of APB-derived T-cells ($p<0.01$). Importantly $CD4^+$ Treg frequencies were significantly greater in UCB T cells than APB T cells after allostimulation in the presence of lenalidomide (**Figure 4.4, G**).

Figure 4.4

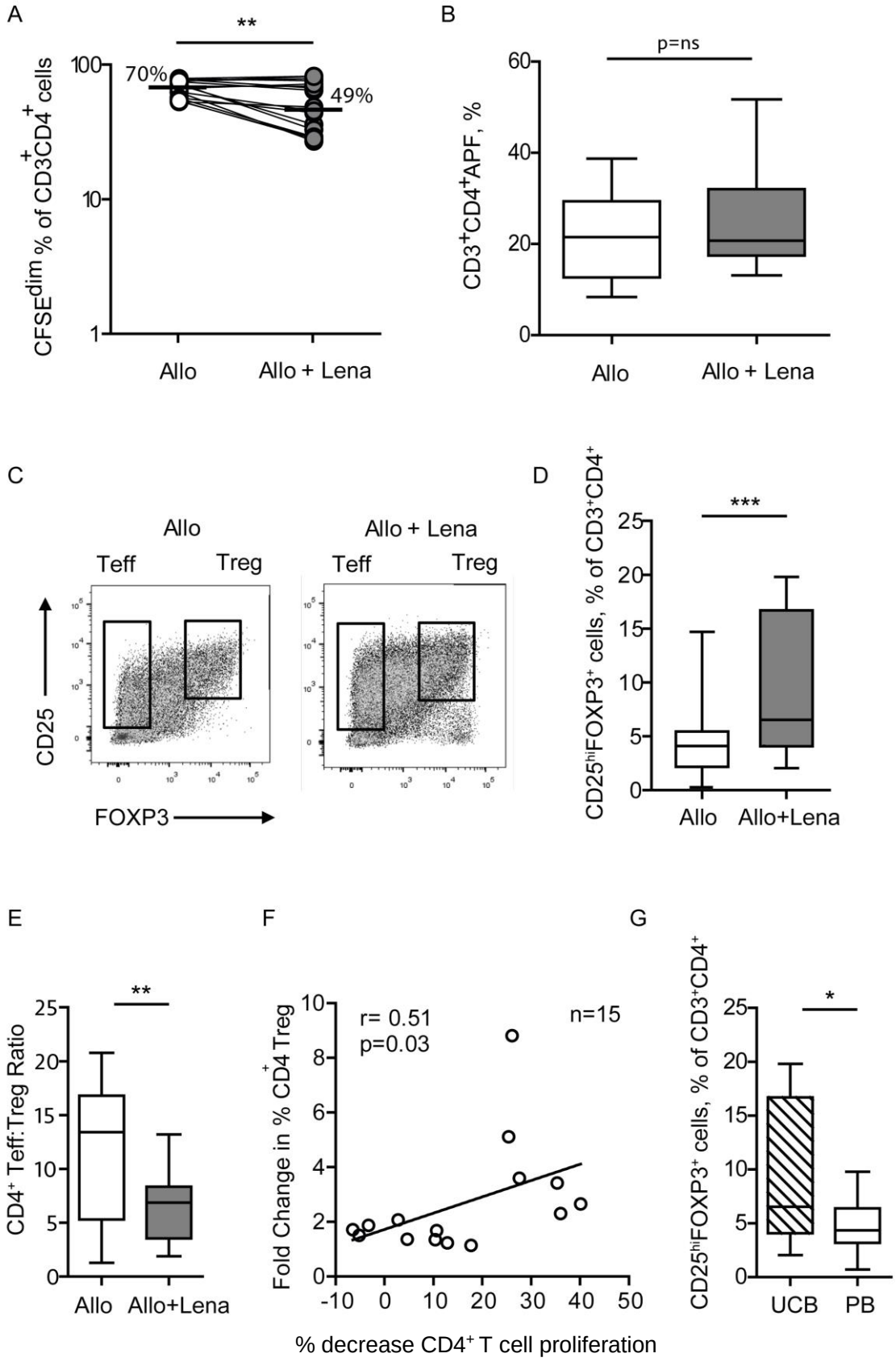

A
**
100
10
1
70%
49%
CFSE^dim % of CD3+CD4+ cells
Allo
Allo + Lena
B
p=ns
60
40
20
0
CD3+CD4+APF, %
Allo
Allo + Lena
C
Allo
Allo + Lena
Teff
Treg
Teff
Treg
CD25
FOXP3
D
***
25
20
15
10
5
0
CD25hiFOXP3+ cells, % of CD3+CD4+
Allo
Allo+Lena
E
**
25
20
15
10
5
0
CD4+ Teff:Treg Ratio
Allo
Allo+Lena
F
r= 0.51
p=0.03
n=15
10
8
6
4
2
0
-10
0
10
20
30
40
50
Fold Change in % CD4+ Treg
% decrease CD4+ T cell proliferation
G
*
25
20
15
10
5
0
CD25hiFOXP3+ cells, % of CD3+CD4+
UCB
PB

Figure 4.4 Lenalidomide exposure causes a reduction in UCB CD4$^+$ T cell alloproliferation while leading to a selective expansion of CD4$^+$ regulatory T cells

(A) UCB CD4$^+$ T cell proliferation following primary allogeneic co-culture in the absence or presence of lenalidomide. Horizontal lines and adjacent numbers are medians. Results are shown for 17 unique donor-responder pairs. ** = P<0.01.

(B) UCB CD4$^+$ T cell alloproliferative precursor frequency (APF) in allogeneic co-cultures in the absence or presence of lenalidomide exposure. Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 17 unique donor-responder pairs. ns = not significant.

(C) Co-expression patterns of CD25 and forkhead box p3 (FOXP3) were used to identify UCB-derived CD25hiFOXP3$^+$ CD3$^+$CD4$^+$ T-regulatory (Treg) and CD25^{hi}FOXP3^{neg} T-effector (Teff) cells. Representative dot plots depict cells after allogeneic co-culture in the absence or presence of lenalidomide.

(D) Proportion of UCB CD4$^+$ T cells with a Treg phenotype after allogeneic co-culture in the absence or presence of lenalidomide. Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 12 unique donor-responder pairs. *** = P<0.001.

(E) Ratio of UCB CD4$^+$ T-regulatory (Treg) and CD25^{hi}FOXP3^{neg} T-effector (Teff) cells after allogeneic co-culture in the absence or presence of lenalidomide. Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 12 unique donor-responder pairs. ** = P<0.01.

(F) Correlation of fold-change of UCB CD4$^+$ Treg versus % reduction in CD4$^+$ T cell proliferation after allogeneic co-culture in the presence of lenalidomide. Results are shown for 15 unique donor-responder pairs. r=spearman's rank correlation coefficient.

(G) Frequencies of CD4$^+$ T cells with a Treg phenotype after allogeneic co-culture in the presence of lenalidomide of UCB and APB T (PB) cells. Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 12 UCB and 18 APB unique donor-responder pairs. * = P<0.05.

4.3.5 Lenalidomide treatment of UCB PBMC increases alloproliferation of UCB-derived NK cells

Having determined that lenalidomide exposure during allostimulation increased $CD8^+$ T cell alloproliferation but decreased $CD4^+$ T cell alloproliferation, leading to a net reduction in $CD3^+$ T cell alloresponses of UCB-derived T cells. I finally went on to examine if alloresponses of NK cells derived from UCB were affected by lenalidomide treatment.

Consistent with the effect of lenalidomide on APB-derived NK cells, proliferation of UCB-derived $CD3^{neg}CD56^+$ NK cells exposed to lenalidomide was significantly increased after 9 days of allogeneic co-culture (median of 71% (untreated) to 84% (lenalidomide treated) $p<0.001$, **Figure 4.5, A**). In contrast the up-regulation of CD56 expression on NK cells observed in APB was not recapitulated in UCB-derived NK cells (**Figure 4.5, B**).

Figure 4.5

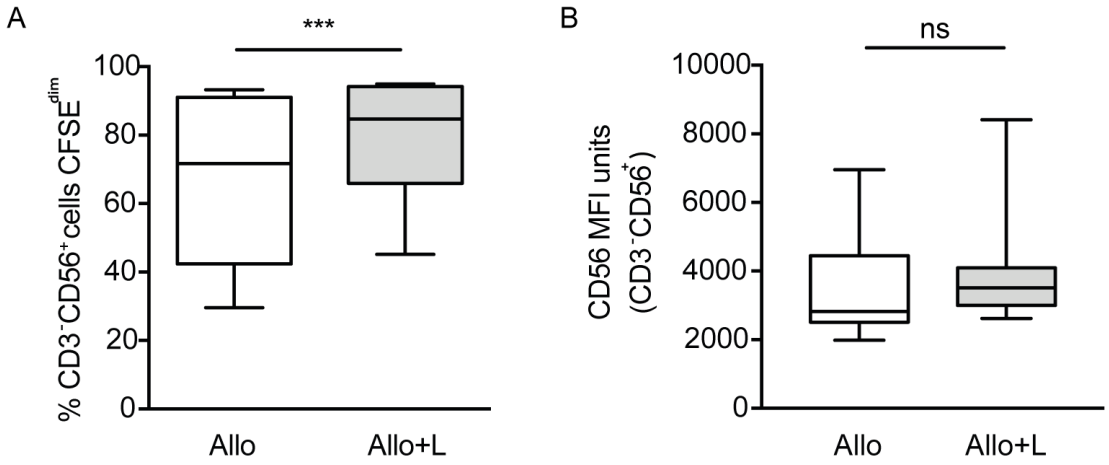


Figure 4.5 Secondary expansion of NK cells in allogeneic co-cultures treated with lenalidomide

(A) $CD3^{-}CD56^{+}$ NK cell proliferation (% $CFSE^{dim}$) following primary allogeneic co-culture of UCB peripheral blood mononuclear cells in the absence (Allo) or presence of lenalidomide (Allo + L). Horizontal lines and adjacent numbers are medians. Results are shown for 15 unique donor-responder pairs. *** = P<0.001.

(B) Relative expression of CD56 following primary allogeneic co-culture of UCB mononuclear cells in the absence (Allo) or presence of lenalidomide (Allo + L) measured by median fluorescence intensity (MFI). Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 12 unique donor-responder pairs. ns = not significant.

4.4 Discussion

The number of AHSCT utilising UCB as a cell source increases year on year (www.cibmtr.org). I therefore chose to examine alloresponses of UCB-derived PBMC in the presence and absence of lenalidomide to determine whether the phenotypic and functional changes observed after lenalidomide treatment of APB-derived PBMC could be recapitulated in this alternative cell source.

Unexpectedly I found that while $CD8^+$ T cell alloresponses of UCB recapitulate those of APB, the $CD4^+$ alloresponse is significantly differentially impacted, with a reduction in net $CD4^+$ T cell alloproliferation. This reduction in overall $CD4^+$ T cell alloproliferation is associated with a subset specific expansion in $CD4^+$ cells with a Treg phenotype. This is in contrast with the decrease in $CD4^+$ Treg seen in allogeneic co-cultures using APB-derived PBMC treated with lenalidomide.

This difference in $CD4^+$ Treg alloreponses is likely explained by differences in the starting phenotype of the $CD4^+$ Treg themselves. UCB Treg have a naive phenotype compared to APB Treg that are composed of both naïve and memory phenotypes [330]. As alloresponses of APB-derived $CD8^+$ T cells appeared to be enhanced due to increased recruitment of naïve $CD8^+$ cells, a similar naïve subset selective effect on Treg may occur. Proliferation of naïve Treg in UCB would be expected to result in a greater shift in the Treg:Teff ratio than in APB due to a proportional increase in naïve Treg in the starting population.

Interestingly Treg from UCB are also easier to isolate than APB Treg. Single-step magnetic isolation of $CD25^+$ T cells can reliably purify cells with suppressive capacity from cord blood. Cells isolated this way have uniform high expression of CD25 compared to APB cells isolated in the same way, where a broad spectrum of CD25 expression is seen. In agreement with this, following expansion with anti-CD3 and anti-CD28 beads in the presence of IL2, UCB-derived Treg exhibit potent suppression of third party alloresponses while those derived from APB are weakly and variably suppressive [330,331]. Assessment of FOXP3 expression of $CD25^+$ UCB-derived Treg determined that these cells expressed high levels of *FOXP3* mRNA and protein, both immediately after isolation and after expansion. Corresponding $CD25^{neg}$ cells did not express *FOXP3* immediately after isolation, and although mRNA levels increased after expansion, FOXP3 protein levels remained low. Indicating that UCB-derived Treg may have a more stable phenotype than

APB-derived Treg. This may be due to the predominantly naïve phenotype of UCB Treg as *FOXP3* expression is thought to be more stable in CD45RA$^+$ Treg [330,332].

One limitation of the experiments presented is that I have not proven the suppressive capacity of the UCB-derived cells I have identified as CD4$^+$ Treg. However based on the findings of both Godfrey *et al.* and Lin *et al.* [330,331] I have actually been more stringent in my identification of Treg (including CD4, CD25 and FOXP3 in my panel). Identification of UCB Treg based on CD25 expression alone has proven sufficient for isolation and subsequent adoptive transfer of UCB Treg to patients. Therefore it is highly likely that the cells I have identified as Treg have suppressive capacity. However to confirm this I could isolate these cells and assess their capacity to suppress proliferative T cell responses in allogeneic co-culture.

The question remains as to why, if the expanded Treg population in lenalidomide treated co-cultures is responsible for limiting CD4$^+$ T cell alloproliferation so significantly, why the increase in CD8$^+$ T cell alloproliferation remains preserved. It is however interesting to note that the magnitude of the increase in lenalidomide treated CD8$^+$ T cell alloproliferation was reduced in UCB allogeneic co-cultures (median 15% increase) compared with APB (median 33% increase) and expression of CD107a was reduced in UCB-derived T cells, which may indicate a degree of suppression of CD8$^+$ T cell proliferation and cytotoxic capacity.

NK cell alloresponses were found to be increased after lenalidomide treatment of UCB allogeneic co-cultures as seen in APB co-cultures. However the same up-regulation of CD56 expression on NK cells was not observed. This may be because of differences in UCB NK cells compared to APB. UCB contains more NK cells than APB, with a greater proportion of those NK cells expressing high levels of CD56 but with a reduced capacity to produce cytokines and lyse tumour cells than APB derived NK cells [333]. Despite these differences there is significant interest in utilising NK cells expanded from UCB units as NK-cell DLI after AHSCT.

In summary this data suggests that the use of lenalidomide following UCBT could provide direct anti-tumour activity and potentiate $CD8^+$ and NK cell driven GvT. Importantly, the potentially tolerogenic effect of increased Treg after lenalidomide treatment could also cause less potential for induction of harmful GvHD. This supports a potentially advantageous immunomodulatory effect of lenalidomide after UCBT and is worthy of further investigation and potential clinical application.

Chapter 5 – Characterisation of the molecular mechanisms underlying the effect of lenalidomide on the $CD8^+$ T cell alloresponse

5.1 Introduction

Until recently the mechanism by which lenalidomide (and other thalidomide derivatives) exerted immunomodulatory effects on mitogen stimulated T cell responses was unknown. A landmark paper by Ito *et al.* [241] demonstrated that cereblon, a protein that acts as the substrate receptor for an E3-ubiquitin ligase complex is an important target of thalidomide. Further work by Zhu *et al.* [242] and Lopez-Girona *et al.* [243] showed that functional cereblon is required for the anti-myeloma activity and T cell immunomodulatory effects of lenalidomide respectively. But it was not until 2014 that members of the ikaros transcription factor family were identified as substrates of cereblon that undergo increased degradation at the proteasome in the presence of lenaldomide [247,249,334].

As is the case with the phenotypic and functional characterisation of the alloresponse after lenalidomide exposure, there is no published data regarding the molecular mechanism of action of lenalidomide in the alloresponse. Having identified the major changes in the *in vitro* T cell alloresponse after lenalidomide treatment and that lenalidomide acts during the antigen-priming phase of the alloresponse, I next aimed to determine the molecular mechanisms by which lenalidomide caused these changes. I examined whether the mechanisms described in other settings involving cereblon and ikaros play a role, as well as exploring whether additional pathways may be involved.

Given the finding that lenalidomide potentiates $CD8^+$ T cell alloresponse of both steady state APB-derived T cells and UCB-derived T cells I focused on the effect of lenalidomide on $CD8^+$ T cells on the molecular level.

5.2 Aim

To determine the molecular mechanisms by which lenalidomide potentiates $CD8^+$ T cell alloresponses of APB and UCB.

5.3 Specific material and methods

5.3.1 RNA extraction

Cell fractions isolated by FACS/MACS were washed in PBS (as previously described), counted and transferred to 2ml RNAse and DNAse free, sterile eppendorf tubes. Cells were centrifuged at 500g at 4°C for 5 mins to pellet and supernatant completely removed with a pipette taking care not to disturb cell pellets. 700µl of TRIzol® (Life technologies) was added to the tube and cell pellet homogenised in the solution by pipetting up and down gently. Homogenised samples were then transferred to a -80°C freezer for storage.

Batched RNA samples in TRIzol® were thawed on ice and RNA was extracted from homogenised samples using the Direct-zol™ RNA Mini-prep kit as per manufacturers protocol, including an in column DNAse treatment step to minimise genomic DNA contamination. Eluted RNA was dissolved in 25µl of RNAse free water and transferred to a -80°C freezer for storage.

5.3.2 RNA assessment

Nanodrop

RNA samples were assessed for concentration and quality using a Nanodrop 1000 spectrophotometer (ND-1000, Nanodrop). RNA quantity is assessed by ultraviolet light absorbance at 260nm (1 optical density (OD) unit = 50µg/µl) and quality by the absorbance ratios at 260/280nm and 260/230nm. A 260/280 ratio of approximately 2 is taken to indicate good quality RNA and >1.8 acceptable quality. Lower 260/280 values indicate sample contamination by protein. All APB-derived samples were within 1.84-2.11, UCB-derived samples were within 1.64-2.05. Reduction in 260/230 ratios indicates other contaminants: salts, carbohydrates or phenols (usually guanidium thiocyanate).

Agilent bioanalyser

RNA degradation was assessed using the Agilent RNA 6000 Nano kit on the Agilent Bioanalyser 2100 (both Agilent Technologies) as per manufacturers protocol, for all samples prior to array analysis and for samples prior to quantitative reverse-transcription polymerase chain reaction (qRT-PCR) if there was sufficient RNA. Degradation is assessed by the ratio of 18s and 28s ribosomal RNA bands in the total RNA as assessed by capillary electrophoresis (**Figure 5.1**). In intact RNA the ratio should be 2:1, 28s:18s. The Bioanalyser software generates an RNA integrity

numbers (RIN) for each sample based on the electropherogram, with 10 being completely intact and 1 completely degraded. RINs for evaluable samples were >8.6 and 28:18s ratios 1.7-2.3. (Methods for assessment of RNA are reviewed extensively in Fleige and Pfaffl 2006 [335]).

Figure 5.1

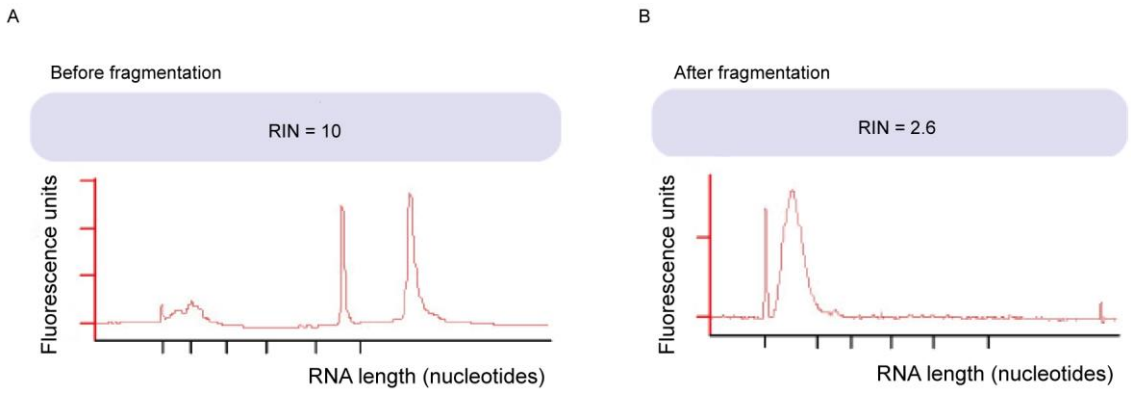


Figure 5.1 Assessment of RNA integrity

(**A**) Representative electropherogram for an RNA sample used for gene expression analysis. 18s and 28s peaks are visible. The ratio of 18s to 28s in this case was 2 corresponding to an RNA integrity number (RIN) of 10, indicating good RNA integrity.

(**B**) Representative electropherogram for an aRNA sample used for gene expression following RNA fragmentation. The reaction should produce a distribution of 35-200nt aRNA fragments with a peak at 100-120nt as seen.

5.3.3 Gene expression profiling using GeneChip® Human Genome U133 Plus 2.0 arrays

Cells from allogeneic co-cultures were MACS sorted negatively isolating $CD8^+$ T cells and then further purified by FACS sorting into $CFSE^{bright}$ non-alloproliferative and $CFSE^{dim}$ alloproliferative $CD8^+$ cells (**Figure 5.2-5.3**). Purity for all samples was >95%. RNA extracted from these cells was then used for gene expression profiling.

5.3.3.1 Sample preparation

RNA extracted from experimental samples was thawed at 4°C and then prepared for gene expression analysis using 3' IVT Express Kit (Affymetrix). One hundred ng of RNA from experimental samples was input (as per manufacturers recommendation). RNA was first amplified and transcribed to complementary DNA (cDNA) using a 16 hour incubation time appropriate for the amount of input RNA. Generated cDNA was then *in vitro* reverse-transcribed and labelled with biotin. Generated amplified RNA (aRNA) was purified using manufacturers RNA binding beads and wash buffers prior to quantitation (by Nanodrop as above) and fragmentation (all as per manufacturers protocol). Fragmentation of the RNA is required for optimal assay sensitivity and was assessed by Agilent Bioanalyser with satisfactory distribution of RNA fragments for all samples (**Figure 5.1**)

Fragmented aRNA was then hybridised to the GeneChips® using the GeneChip® hybridisation oven 640 (Affymetrix). At this point amplified labelled aRNA binds to complementary sequences (probes) immobilised on the microarray. Following hybridisation chips were transferred to the GeneChip® fluidics station 400 (Affymetrix) for staining. Anti-biotin antibodies conjugated to fluorophores then bind to biotin labelled array-bound aRNA. Following staining chips were transferred to the GeneChip® Scanner 3000 7G (Affymetrix) to be read.

Thanks to Tracy Chaplin-Perkins for assistance with the hybridisation, staining and reading of the GeneChips®.

Figure 5.2

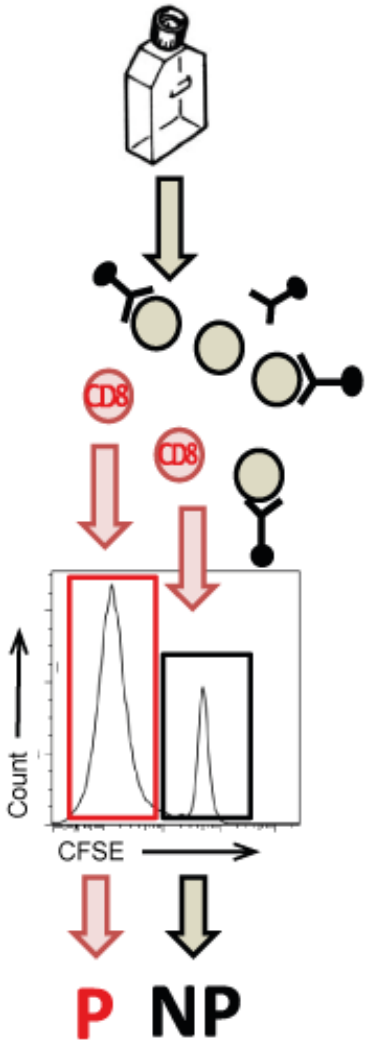


Figure 5.2 Sorting strategy for isolation of cells for gene expression analysis

Alloproliferative (P) and non-alloproliferative (NP) $CD8^+$ T cells were purified from allogeneic co-cultures first by negative MACS isolation of $CD8^+$ T cells and then subsequent FACS sorting to fractionate alloproliferative and non-alloproliferative cells based on CFSE fluorescence.

Figure 5.3

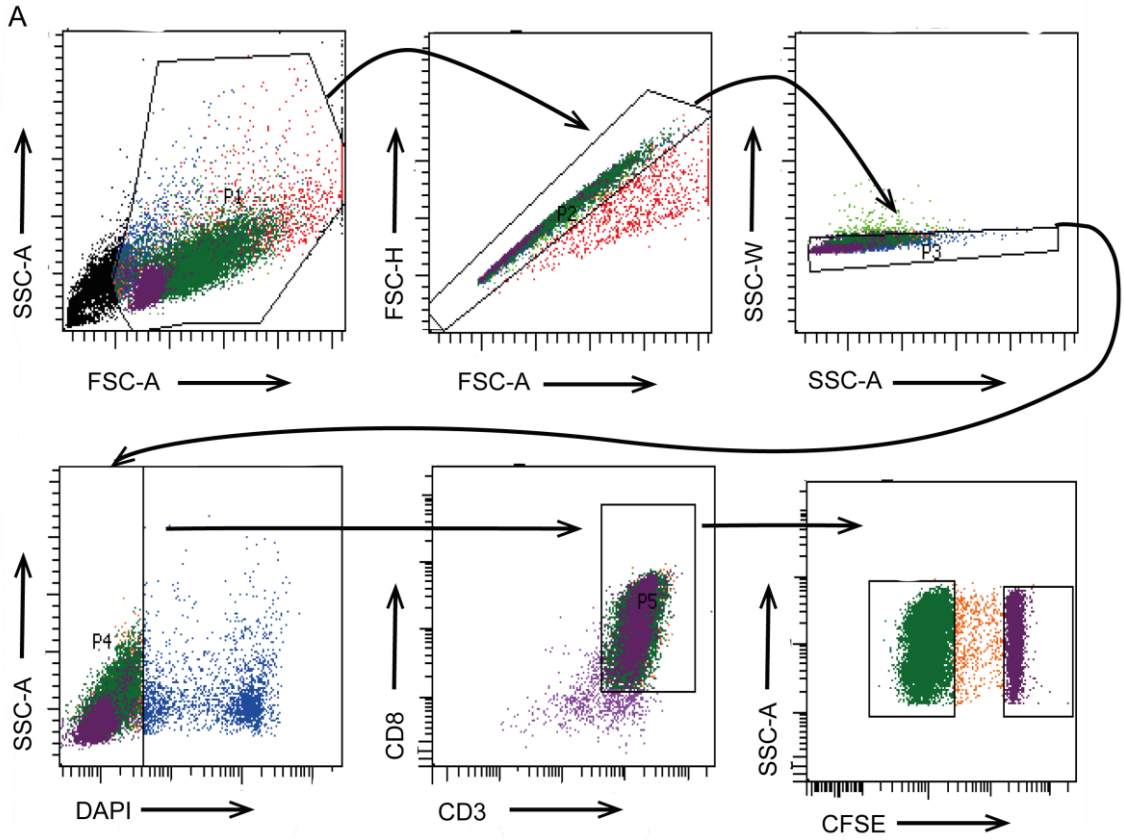

A
SSC-A
FSC-A
P1
FSC-H
FSC-A
P2
SSC-W
SSC-A
P3
SSC-A
DAPI
P4
CD8
CD3
P5
SSC-A
CFSE

Figure 5.3

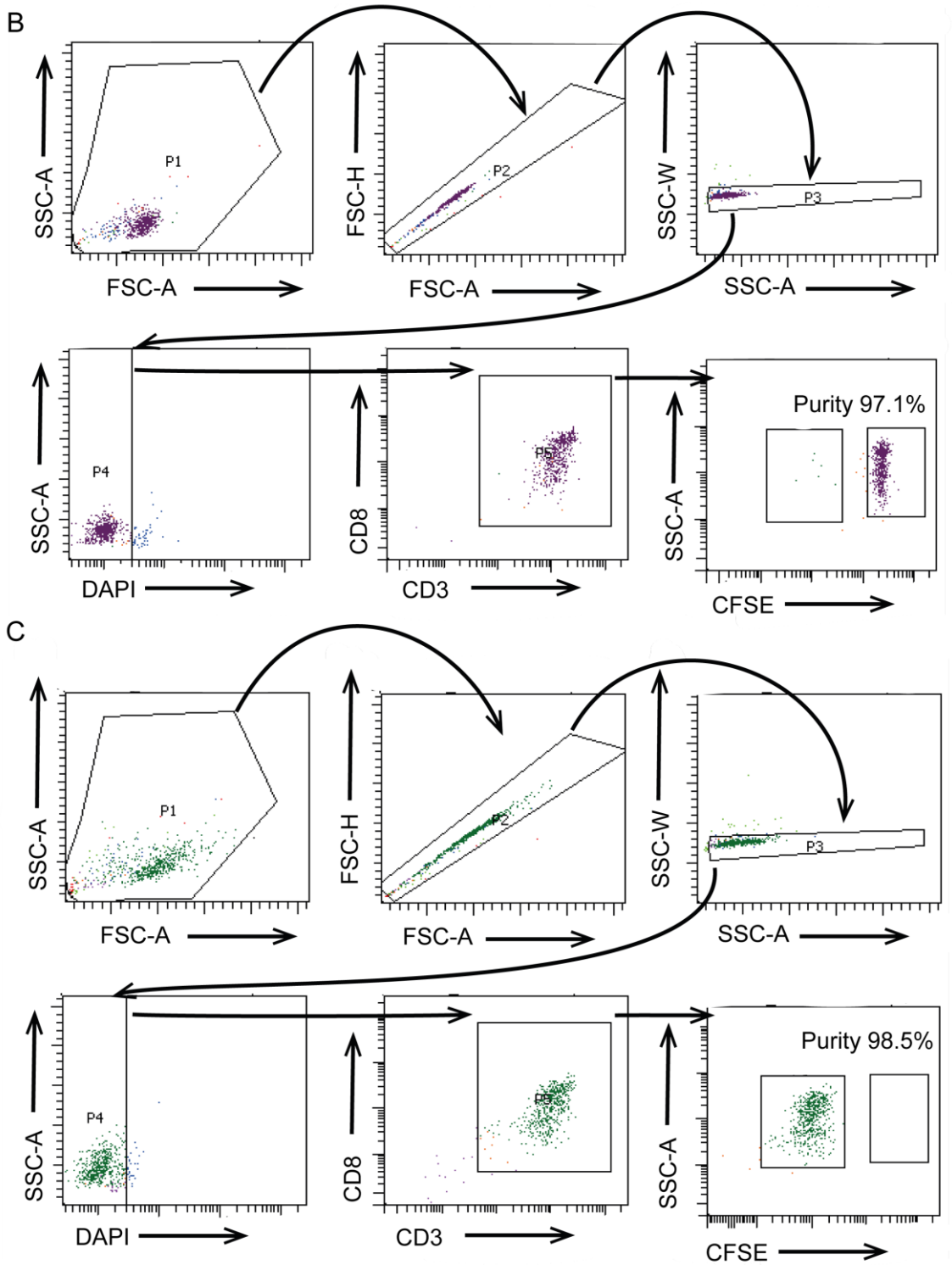

B
SSC-A
FSC-A
P1
FSC-H
FSC-A
P2
SSC-W
SSC-A
P3
SSC-A
DAPI
P4
CD8
CD3
P5
SSC-A
CFSE
Purity 97.1%
C
SSC-A
FSC-A
P1
FSC-H
FSC-A
P2
SSC-W
SSC-A
P3
SSC-A
DAPI
P4
CD8
CD3
P5
SSC-A
CFSE
Purity 98.5%

Figure 5.3 Region and gating strategy for FACS purification of non-alloproliferative and proliferative fractions of $CD8^+$ alloresponder T cells

(A) $CD8^+$ T cells isolated from allogeneic co-cultures using negative MACS selection were subsequently FACS sorted. Events in the lymphocyte regions were selected and doublets excluded. Dead cells were excluded based on positive staining for DAPI. Events in the $CD3^+CD8^+$ gate were then isolated based on bright or dim fluorescence for CFSE.

(**B-C**) Purity checks on isolated $CFSE^{bright}$ and $CFSE^{dim}$ cells (respectively) showed >95% pure populations.

5.3.4 Analysis of GeneChip® raw-data

Raw data from the GeneChip® scanner was loaded into R software version 3.1.0 (R Project for Statistical Computing, Wien, Austria) via Bioconductor packages (http://www.bioconductor.org) and arrayMvout used to perform quality control (QC) assessments of the data. This package generates measures of background fluorescence, scale factors (to correct for the percentage of absent/marginal/positive calls), median relative log expression (RLE), mean normalised unscaled standard error (NUSE), actin 3'/5' ratio, GAPDH 3'/5' ratio and a measure of RNA degradation. During RNA preparation for hybridisation various control RNAs are 'spiked' into the samples to aid with these quality control steps. Any arrays that do not meet pre-defined thresholds are excluded from analysis. All samples passed QC assessments.

Following QC the raw data files are normalised to remove systematic biases and intra-chip differences including: sample preparation, variability in hybridization, scanner settings and experimenter bias. This is done by GCRMA (G C robust multi-array average) algorithm that adjusts intensity as detected by the scanner for optical noise and non-specific binding, factoring in the individual probe sequences to determine expected probe affinities.

Data was then filtered to decrease the false discovery rate (FDR) by removing genes with low overall intensity and or high variability as these genes were felt to be unlikely to carry relevant information to the experimental question. Differential expression was then analysed using the LIMMA test (Bayesian regularised t-test) [336] and the Benjamini-Hochberg correction [337] for multiple testing applied to generate adjusted p values for each gene. P values <0.05 were taken as significant.

The R package gplots was also used to generate hierarchical clustering of the differentially expressed genes.

I am very grateful to Dr Ajanthah Sangaralingam who performed the statistical analysis from the raw data.

5.3.4.1 Over-representation analysis of differentially expressed genes to identify deregulated molecular pathways

Over-representation analysis was performed by entering relevant lists of differentially expressed genes into Ingenuity software version 26127183 (Qiagen) and ToppFunn (ToppGene Suite, Cincinnati Children's Hospital Medical Center). These software analyse whether the list supplied is significantly associated with a particular pathway or set of pathways, by comparing whether the genes in the list are more likely to appear than those from a randomly generated list of the same size, and therefore assign the association a p value.

5.3.5 qRT-PCR of gene expression targets

Genes that were identified as having roles in determining T cell phenotype and proliferation were chosen for further validation. APB $CD8^+$ T cells were isolated from allogeneic co-cultures by MACS negative isolation of $CD3^+$ T cells and then FACS sorting to isolate proliferative and non-proliferative fractions of $CD8^+$ T cells. RNA was extracted from sorted fractions using the same method as described above. In the case of UCB; MACS sorting alone was used to negatively isolate alloresponder $CD8^+$ T cells from allogeneic co-cultures, due to the low cell count in the co-cultures compared to APB that did not allow for such stringent fractionation.

5.3.5.1 Reverse transcription of RNA

RNA was converted to complementary DNA (cDNA) using the Improm-II® reverse transcription system (Promega) as per manufacturers protocol. Briefly, 250ng (APB-derived samples), or 100ng (for UCB-derived samples) RNA per reaction was incubated with random hexamers at 70°C to allow complementary binding. A reverse transcription (RT) master mix was made up containing nuclease-free water, RT buffer, magnesium chloride, deoxynucleotides (dNTPs), RNAse and Reverse Transcriptase enzyme and kept on ice. RT master mix was added to RNA mix samples on ice and the samples were transferred to a thermal cycler set to 25°C for 5 mins, 42°C for an hour and 70°C for 15 mins.

In parallel with the samples prepared as described a set of no-RT controls were prepared using the same protocol as above but with the omission of the reverse transcriptase enzyme, this was substituted with nuclease free water. Generated cDNA was stored at -20°C.

5.3.5.2 qRT-PCR sample preparation and analysis

cDNA was thawed at 4°C and an qRT-PCR master mix prepared for each sample as per manufacturers protocol and kept on ice. The master mix contained TaqMan® Fast Universal PCR Master Mix (2x), no AmpErase® UNG (Applied Biosytems®), nuclease free water and cDNA. In parallel mixes were also made for no-RT controls and no-template controls in which the cDNA component was replaced with nuclease free water. One no-template control was included for each batch of samples. The purpose of the no-RT controls was to detect any genomic DNA contamination and the no-template control to detect any contamination of reagents.

Master mixes were then pipetted into a 96 well optical plate (Applied Biostystems) with experimental samples in triplicate. Fluorescein amidite (FAM) labelled probe/primers for genes of interest (Table 2A, Appendix A) and housekeeping genes (GAPDH and 18s) were then added to appropriate wells and the plates sealed. Samples were then mixed and centrifuged briefly before loading into the 7900HT Fast Real Time PCR System (Applied Biosytems®). The qRT-PCR reaction settings were as per manufacturer's protocol: Initial HOLD at 95°C for 20 seconds followed by 40 cycles at: 95°C for 1 sec followed by 60°C for 30 sec.

5.3.6 Analysis of qRT-PCR data

Raw data from the qRT-PCR was entered into Microsoft Excel® (Microsoft). C_T (cycle threshold) values were taken as the number of cycles at which the fluorescence from the probe of interest reached a threshold that had been pre-defined. This threshold was set in the exponential phase of the reaction and was standardised for all probes and samples.

An average C_T value was generated from the replicates for each sample (3 unless excluded on the basis of a standard deviation of >0.5 from the other replicates). The C_T was then normalised against the expression of a housekeeping gene, which should have stable expression across all the samples. At this point GAPDH was excluded as a housekeeping gene as the C_T for GAPDH appeared to change in lenalidomide treated versus untreated samples. 18s was used for normalisation of all samples. This gave the ΔC_T.

To calculate the relative quantity the ΔC_T was then compared to an internal calibrator sample ($\Delta\Delta C_T$= ΔC_T test sample- ΔC_T calibrator sample). In this case samples from the untreated non-proliferative cells were used as the calibrator. For each sample set an average $\Delta\Delta C_T$ was

calculated, the $\log_2$ scaled data transformed to a linear scale and used to derive the relative quantity (RQ) using the equation $RQ = 2^{\Delta\Delta_{CT}}$.

Several comparisons were then possible based on the samples tested. Sample sets included untreated non-proliferative, untreated proliferative, treated non-proliferative and treated proliferative samples. Student paired t-test was used to test for statistically significant differences in fold-change between sample sets. Confidence intervals were calculated for the fold-change ratio's using Graphpad quickcalcs (www.graphpad.com/quickcalcs).

5.4 Results

5.4.1 Lenalidomide induces changes in the gene expression of alloproliferative CD8$^+$ T cells

Given my findings that lenalidomide potentiates CD8$^+$ T cell alloresponses, I next asked whether this was the result of quantitative or qualitative difference in the CD8$^+$ T cell alloresponse. In order to answer this question I examined the gene expression profiles of responder cells from lenalidomide treated and untreated co-cultures. I chose to focus on the CD8$^+$ T cells as this was the cell subset in which I had observed the most marked phenotypic changes and I chose the day 7 time-point, as at this time-point I had observed significant changes in CD8$^+$ T cell proliferation and cytokine production in both APB-derived and UCB-derived CD8$^+$ T cells.

Alloproliferative and non-proliferative responder CD8$^+$ T cells were isolated from lenalidomide-treated and untreated allogeneic co-cultures using a combination of magnetic-bead based and FACS sorting (**Figure 5.2**), resulting in 4 biologically-distinct alloresponder populations, as shown in **Figure 5.4**.

Figure 5.4

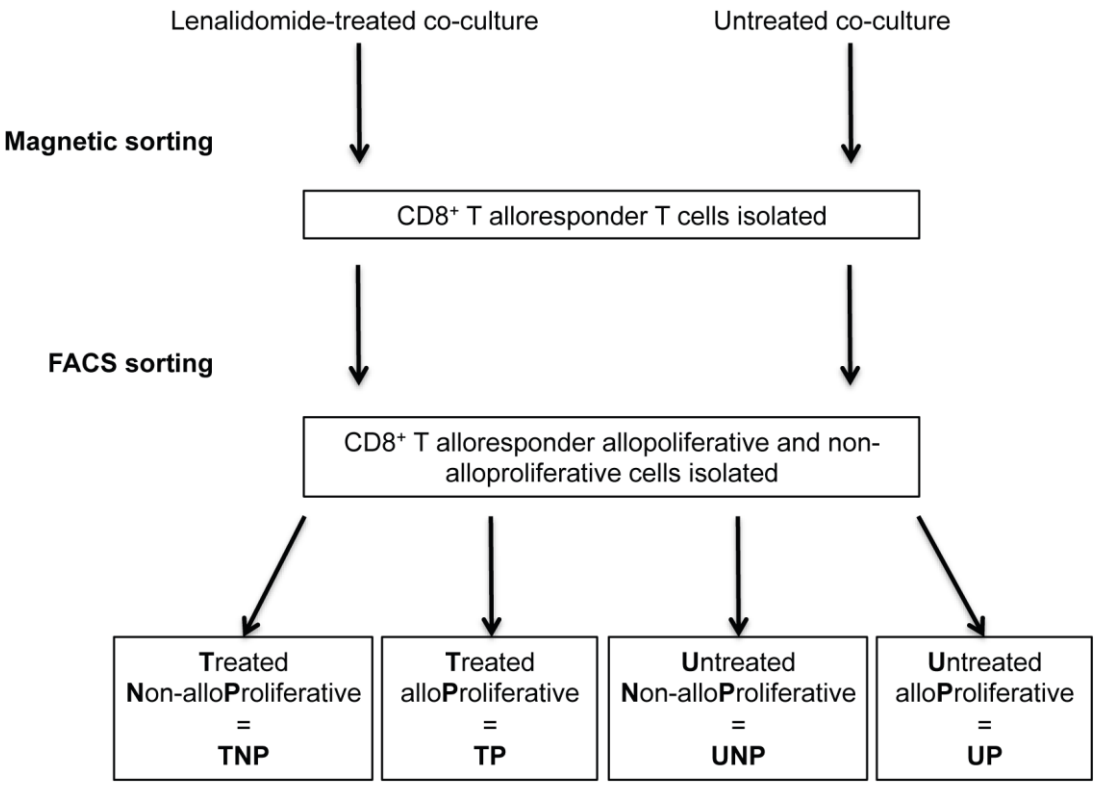


Figure 5.4 Schematic depicting alloresponder populations generated for gene expression profiling

Alloresponder CD8+ T cells were isolated from lenalidomide-treated and untreated allogeneic co-cultures by magnetic bead-based sorting. Purified $CD8^+$ alloresponder T cells were then sorted into alloproliferative and non-proliferative fractions by FACS.

Firstly I compared the gene expression of alloproliferative $CD8^+$ T cells against non-proliferative cells **not** exposed to lenalidomide (comparison UPvUNP) to determine the baseline gene expression changes that occur in the alloresponse. This revealed 2771 genes with statistically significantly (adjusted p value <0.05) altered gene expression. When the analysis was restricted to genes that showed a fold-change of ≥4 and are therefore more likely to be biologically relevant, this number of genes was reduced to 708 (full gene lists may be viewed at (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE84251). Of the 10 "most up-regulated" genes in this list, 9 have well described roles in promoting cellular proliferation and the cell cycle, which is as would be expected in a proliferative cell population (**Table 5.1**).

As expected a high proportion of these 708 highly differentially expressed genes (539/708) were also found to be altered in the comparison of lenalidomide treated alloproliferative versus lenalidomide treated non-proliferative cells (comparison TPvTNP). (**Figure 5.5, A-B**). Many of the genes with the most altered gene expression in this comparison were also found in the top gene list from the UPvUNP comparison. Interestingly a number of the unique top genes from this comparison were for genes that code for proteins that make up integrin subunits, have roles in the rearrangement of the cytoskeleton or are involved in actin polymerisation (**Table 5.2**) and would be predicted to be important for immune synapse function.

In order to understand whether lenalidomide was modulating the proliferative drive of these cells I then focused on a comparison of the **alloproliferative cells** from treated and untreated co-cultures (TPvUP comparison). As expected there was a greater overlap of genes with the TPvTNP comparison than the UPvUNP comparison, however there were also a number of genes with significantly altered expression that were unique to lenalidomide treated alloproliferative $CD8^+$ T cells (**Figure 5.5, C** and **Table 5.3**). A number of these genes have roles described in the modulation of T cell activation or differentiation. Importantly a comparison of TNPvUNP $CD8^+$ T cells revealed no significant changes in gene expression, consistent with the observation that the phenotypic and functional effects of lenalidomide observed have been confined to alloproliferative $CD8^+$ T cells.

Figure 5.5

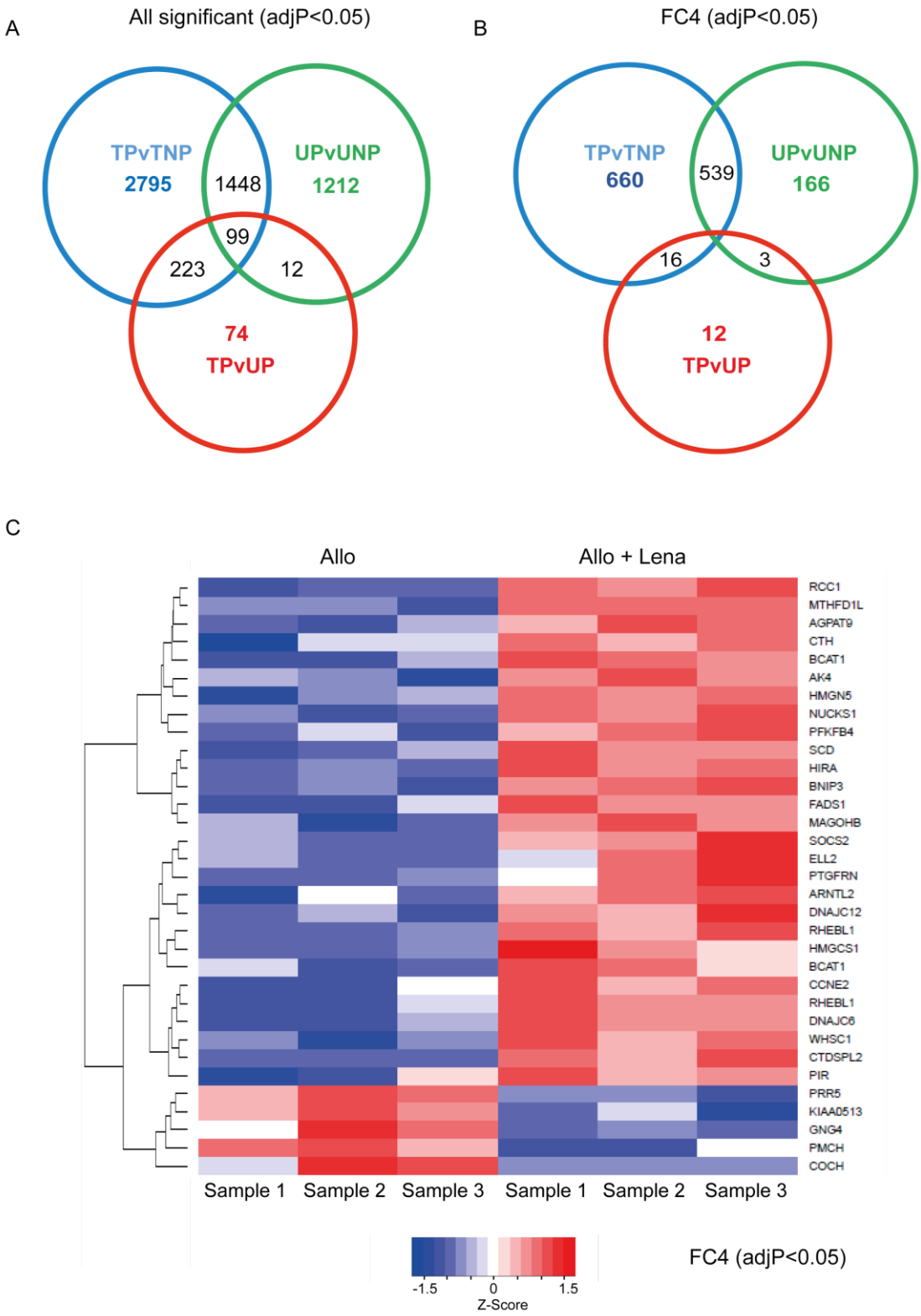

A
All significant (adjP<0.05)
TPvTNP
2795
1448
UPvUNP
1212
99
223
12
74
TPvUP
B
FC4 (adjP<0.05)
TPvTNP
660
539
UPvUNP
166
16
3
12
TPvUP
C
Allo
Allo + Lena
RCC1
MTHFD1L
AGPAT9
CTH
BCAT1
AK4
HMGN5
NUCKS1
PFKFB4
SCD
HIRA
BNIP3
FADS1
MAGOHB
SOCS2
ELL2
PTGFRN
ARNTL2
DNAJC12
RHEBL1
HMGCS1
BCAT1
CCNE2
RHEBL1
DNAJC6
WHSC1
CTDSPL2
PIR
PRR5
KIAA0513
GNG4
PMCH
COCH
Sample 1
Sample 2
Sample 3
Sample 1
Sample 2
Sample 3
-1.5
0
1.5
Z-Score
FC4 (adjP<0.05)

Figure 5.5 Lenalidomide exposure during allostimulation causes significant changes in gene expression of alloproliferative $CD8^+$ T cells

(A) Venn diagram showing overlap in gene expression changes between lenalidomide treated alloproliferative (TP), treated non-alloproliferative (TNP), untreated alloproliferative (UP) and untreated non-alloproliferative (UNP) $CD8^+$ T cells from allogeneic co-cultures. All genes with significantly altered gene expression (adjusted P value <0.05) are included. Data from 3 unique donor-responder pairs are shown.
(B) Venn diagram showing overlap in gene expression changes between TP, TNP, UP and UNP $CD8^+$ T cells from allogeneic co-cultures. All genes with fold-change (FC) ≥4 in gene expression (adjusted P value <0.05) are included. Data from 3 unique donor-responder pairs are shown.
(C) Heatmap depicting significantly dysregulated genes (adjusted P <0.05), with fold-change (FC) of ≥4 in high-stringency purified APB $CD3^+CD8^+CFSE^{dim}$ T cells from allogeneic co-cultures in the absence or presence of lenalidomide.

Table 5.1 Untreated Proliferative vs Untreated Non-proliferative comparison – Top genes

Gene Symbol	Gene Name	Log_2FC	Adj P Value
MS4A1	CD20	-6.8	<0.001
NR3C2	Mineralocorticoid Receptor Delta	-5.9	<0.0001
DKK3	Dickkopf WNT Signaling Pathway Inhibitor 3	-5.7	<0.0001
SCML1	Sex Comb On Midleg-Like 1 (Drosophila)	-5.6	<0.001
GPRASP1	G Protein-Coupled Receptor Associated Sorting Protein 1	-5.5	<0.0001
ENPP5	Ectonucleotide Pyrophosphatase/Phosphodiesterase 5 (Putative)	-5.5	<0.001
ZNF204P	Zinc Finger Protein 204, Pseudogene	-5.4	<0.0001
NOG	Noggin	-5.2	<0.05
SELM	Selenoprotein M	-5.1	<0.001
F2RL1	Coagulation Factor II (Thrombin) Receptor-Like 1	-5.0	<0.001
KIF14	Kinesin Family Member 14	6.0	<0.0001
ENTPD1	Lymphoid Cell Activation Antigen (CD39)	6.0	<0.0001
CDC20	Cell Division Cycle 20	6.0	<0.0001
MKI67	Marker Of Proliferation Ki-67	6.1	<0.0001
CDT1	Chromatin Licensing And DNA Replication Factor 1	6.1	<0.0001
CENPE	Centromere Protein E, 312kDa	6.1	<0.0001
E2F8	E2F Transcription Factor 8	6.2	<0.0001
KIF15	Kinesin Family Member 15	6.3	<0.0001
MCM10	Minichromosome Maintenance 10 Replication Initiation Factor	6.3	<0.0001
DEPDC1	DEP Domain Containing 1	6.5	<0.0001

Table 5.2 Treated Proliferative vs Treated Non-Proliferative comparison – Top genes

Gene Symbol	Gene Name	Log_2FC	Adj P Value
GPRASP1	G protein-coupled receptor associated sorting protein 1	-6.7	<0.0001
MS4A1	Membrane-spanning 4-domains, subfamily A, member 1	-6.1	<0.001
NR3C2	Nuclear receptor subfamily 3, group C, member 2	-6.0	<0.0001
DKK3	Dickkopf 3 homolog (Xenopus laevis)	-5.9	<0.0001
SCML1	Sex comb on midleg-like 1 (Drosophila)	-5.7	<0.001
ENPP5	Ectonucleotide pyrophosphatase/phosphodiesterase 5 (putative)	-5.7	<0.001
ZNF204P	Zinc finger protein 204, pseudogene	-5.6	<0.0001
F2RL1	Coagulation factor II (thrombin) receptor-like 1	-5.5	<0.0001
IL6R	Interleukin 6 receptor	-5.4	<0.001
ITGA6	Integrin, alpha 6	-5.2	<0.0001
ATP8B4	ATPase, class I, type 8B, member 4	5.1	<0.001
LMNB1	Lamin B1	5.1	<0.001
BCAT1	Branched chain amino-acid transaminase 1, cytosolic	5.1	<0.0001
ITGA2	Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	5.2	<0.0001
KIF2C	Kinesin family member 2C	5.2	<0.0001
ANLN	Anillin, actin binding protein	5.3	<0.0001
CKAP2L	Cytoskeleton associated protein 2-like	5.3	<0.0001
DEPDC1	DEP domain containing 1	5.3	<0.0001
KIFC1	Kinesin family member C1	5.4	<0.0001
ENTPD1	Lymphoid Cell Activation Antigen (CD39)	5.5	<0.001

(Red indicates genes shared with UPvUNP list)

Table 5.3 Treated proliferative vs Untreated Proliferative comparison – Top genes

Gene Symbol	Gene Name	Log_2FC	Adj P Value
GNG4	Guanine nucleotide binding protein (G protein), gamma 4	-3.4	<0.05
PMCH	Pro-melanin-concentrating hormone	-2.7	<0.05
KIAA0513	KIAA0513	-2.4	<0.05
COCH	Coagulation factor C homolog, cochlin (Limulus polyphemus)	-2.4	<0.05
PRR5	Proline rich 5 (renal)	-2.2	<0.05
KLHL28	Kelch-like 28 (Drosophila)	-2.0	<0.05
RRN3P1	RNA polymerase I transcription factor homolog (S. cerevisiae) pseudogene 1	-1.9	<0.05
IFNGR1	Interferon gamma receptor 1	-1.9	<0.05
ZBTB20	Zinc finger and BTB domain containing 20	-1.9	<0.05
SOX4	SRY (sex determining region Y)-box 4	-1.8	<0.01
FAIM3	Fas anti-apoptotic inhibitory molecule 3	-1.7	<0.05
RCC1	Regulator of chromosome condensation 1	2.4	<0.01
PTGFRN	Prostaglandin F2 receptor negative regulator	2.4	<0.05
RHEBL1	Ras homolog enriched in brain like 1	2.5	<0.01
CCNE2	Cyclin E2	2.4	<0.05
SOCS2	Suppressor of cytokine signaling 2	2.8	<0.01
CTDSPL2	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase like 2	2.8	<0.01
PFKFB4	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4	2.9	<0.01
PIR	Pirin (iron-binding nuclear protein)	3.1	<0.01
BCAT1	Branched chain amino-acid transaminase 1, cytosolic	3.1	<0.01
NUCKS1	Nuclear casein kinase and cyclin-dependent kinase substrate 1	3.9	<0.01

(Blue indicates genes with known immunomodulatory roles)

5.4.2 Lenalidomide alters expression of genes with known immunoregulatory roles in alloproliferative CD8$^+$ T cells

Genes with ≥four-fold up/down-regulated gene expression in TPvUP CD8$^+$ T cells with known immunomodulatory roles; *PMCH, FAIM3, SOCS2, PFKFB4* and *PIRIN* were chosen for validation in additional samples by qRT-PCR. The direction of the fold-change in mRNA between lenalidomide treated and untreated alloproliferative CD8$^+$ T cell samples was consistent with the array data and in the cases of *SOCS2, PFKFB4* and *FAIM3* reached statistical significance (**Figure 5.6, A**).

SOCS2 (suppressor of cytokine signalling 2) has been shown to play an important role in CD8$^+$ T cell differentiation and function [338,339]. It is thought to act indirectly via enhanced degradation of other SOCS family proteins SOCS1 and SOCS3 [340] leading to enhanced IL2 responsiveness. The mRNA levels of *SOCS2* were increased 6.8-fold in the array samples (array) and 2.3-fold in the qRT-PCR analysis (PCR) of lenalidomide treated alloproliferative (TP) CD8$^+$ T cells versus untreated alloproliferative (UP) CD8$^+$ T cells. This would be expected to lead to enhanced degradation of SOCS1 and SOCS3 and therefore increase CD8$^+$ differentiation to the effector memory phenotype observed.

PFKFB4 (6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4) is an enzyme component of the glycolytic pathway and expressed in activated T cells [341]. Alterations in metabolic pathways in T cells are well known to be important for T cell activation, proliferation and differentiation (reviewed in [342]), with a switch to glycolysis being important for effector CD8$^+$ T cell generation. An increase in mRNA of *PFKFB4* of 7.7 (array) and 3.3 (PCR) in TP versus UP cells would suggest that lenalidomide exposure during allostimulation results in up-regulation of metabolic pathways that are essential for CD8$^+$ effector differentiation.

Figure 5.6

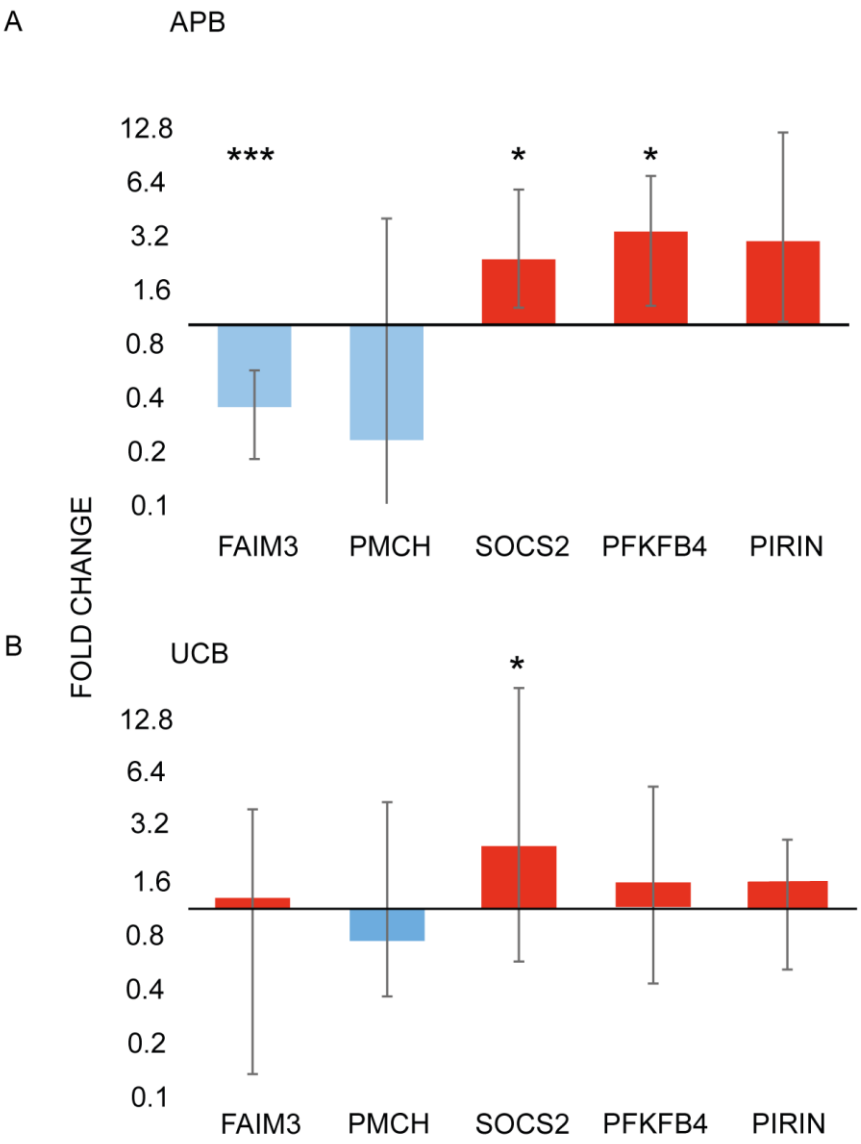


Figure 5.6 Lenalidomide alters expression of immunoregulatory genes in alloproliferative CD8$^+$ T cells from both APB and UCB

(**A**) Gene expression of immunoregulatory target genes was validated in purified APB-derived alloproliferative CD3$^+$CD8$^+$ T-cells from 6 unique donor-responder pairs by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). Bar chart depicts fold-change and 95% confidence intervals. * = P<0.05, *** = P<0.001. Solid line = no change (1 fold).

(**B**) Gene expression of immunoregulatory target genes was validated in purified UCB-derived CD3$^+$CD8$^+$ T-cells from 4 unique donor-responder pairs by qRT-PCR. Bar chart depicts fold-change and 95% confidence intervals. * = P<0.05. Solid line = no change (1 fold).

Less is known about the function of *FAIM3 (*fas anti-apoptotic inhibitory molecule 3*,* also known as *TOSO/FCMR)* in T cells. This molecule was first described as an inhibitor of Fas mediated apoptosis [343], however it has since been suggested that the results observed in previous publications may have been due to the IgM binding capacity of this molecule, hence it's renaming as FcμM Receptor. A recent paper has described that the levels of *FAIM3* mRNA decrease significantly on T cell activation and effector memory differentiation [344], although the authors were unable to draw a conclusion as to what role this gene plays in the process. In my dataset mRNA expression of *FAIM3* was decreased 3.2-fold (array) and 2.88-fold (PCR) in TP versus UP cells in line with the phenotypic shift towards effector memory cells.

Pirin is proposed as a redox-sensitive co-factor for NFκB activity by Liu *et al.* [345]. The authors describe that under conditions of cellular stress Pirin becomes oxidised to its active form, binds NFκB and promotes its actions. Others have described a role for reactive oxygen species (ROS) responsive proteins, such as Pirin in the differentiation of haematopoietic progenitor cells and in effector differentiation of $CD4^+$ T cells [346,347]. It is possible that alterations in *Pirin* reflect the up-regulation of genetic programmes that support greater expansion of effector $CD8^+$ T cells by protecting cells during cellular stress. The mRNA levels of *Pirin* were increased 8.6-fold (array) and 2.9-fold (PCR) in TP versus UP cells, however in the PCR validation cohort the fold-change failed to reach statistical significance (P=0.06).

mRNA levels of *PMCH* (pro-melanin concentrating hormone) were significantly decreased in TP compared to UP cells in my array dataset, with a decreased of 6.7-fold. This gene is of interest as previous work from our group has shown it plays a role in impaired T cell responses to tumour [348]. Although the PCR validation cohort showed a decrease of 4.4-fold in mRNA levels of PMCH in TP cells compared to UP this did not reach statistical significance (P=0.12).

The observation that the expression of many of these immunoregulatory genes was also altered to a similar degree in lenalidomide-treated UCB-derived $CD8^+$ T cells suggests a common molecular mechanism of lenalidomide action on $CD8^+$ T cell alloresponses of APB and UCB T cells (**Figure 5.6, B**).

5.4.3 Lenalidomide exposure during allostimulation modulates expression of genes involved in T cell metabolism

Gene enrichment analysis was used to further interrogate the results of the APB-derived $CD8^+$ T cell gene expression analysis. A list of genes that were significantly ($P \leq 0.05$) up or down-regulated expression and fold-change ≥ 2 from the TPvUP comparison was entered into a number of analysis-tools to determine cellular pathways and processes in the $CD8^+$ T cell alloresponse that may be modulated by lenalidomide exposure. Over-representation analysis using 3 independent tools, Ingenuity Pathway Analysis (IPA, Qiagen), ToppGene Suite (Biomedical Informatics, Cincinnati Childrens Hospital Medical Centre) and EnrichR (Data Coordination and Integration Centre, Mount Sinai) consistently showed that genes involved in the c-Myc pathway as well as metabolic pathways such as fatty acid metabolism and amino acid metabolism were over-represented in the gene list (**Figure 5.7, Table 5.4**).

IPA and EnrichR also include modules that predict top upstream regulators or transcription factors that target genes represented in the gene list entered. Genes with a fold-change ≥2 from the TPvUP list were entered. Interestingly IPA revealed ikaros (IKZF1) as a significant predicted upstream regulator (**Table 5.5**) as well as TGFB (transforming growth factor beta) family members. EnrichR also revealed Myc and ILF2 (interleukin enhancing binding factor 2) as significantly over represented transcription factors with multiple targets in the gene list (**Table 5.6**).

Table 5.4 Pathways implicated in action of lenalidomide on alloproliferative CD8$^+$ T cells

Pathway	Adj P value (Bonferroni/FDR B&H)	Genes in analysis	No genes in pathway
Validated targets of c-Myc transcriptional activity	<0.0001/<0.0001	13 (BAX, BCAT1, RCC1, NME1,HSPA4, HSPD1, SHMT1, BIRC5, MINA, EIF4A1, CDC25A, CDK4, ENO1)	81
Homologous recombination	<0.001/<0.001	7 (EME1, BRCA2, RAD51, RAD51C, RAD51D, POLD2, RAD50)	28
DNA damage response	<0.001/<0.0001	9 (BAX, BID, BRCA1, RAD51, RAD50, CCNE2, CDC25A, CDK2, CDK4)	67
Meiotic recombination	<0.01/<0.001	9 (BRCA1, BRCA2, HIST1H3H, PSMC3IP, RAD51, RAD50, DMC1, CDK2, CDK4)	87
Biosynthesis of amino acids	<0.01/<0.001	9 (MAT2A, PGK1, ALDOA, PSAT1, SHMT1, CTH, BCAT1, ENO1, TPI1)	73
Cholesterol biosynthetic	<0.01/<0.01	5 (ACAT2, HMGCS1, FDPS, NSDHL, DHCR7)	18
DNA repair	<0.05/<0.01	10 (BRIP1, ERCC8, FANCB, BRCA1, BRCA2, RAD51, POLD2, POLR2H, RFC2, RAD50)	113
Integrated Cancer pathway	<0.05/<0.001	6 (BAX, BRCA1, RAD50, CDC25A, CDK2, CDK4)	35
Fanconi anaemia pathway	<0.05/<0.01	7 (BRIP1, FANCB, EME1, BRCA1, BRCA2, RAD51, RAD51C)	53
E2F transcription factor network	<0.05/<0.01	8 (PRMT5, HBP1, BRCA1, TFDP1, CBX5, CCNE2, CDC25A, CDK2)	73

FDR = False discovery rate, B&H = Benjamini & Hochberg. Top 10 (most significant adjusted (adj) P value) pathways from an over-representation analysis (TOPPFUNN) based on ≥2-fold (adj P≤0.05) dys-regulated genes in gene expression comparison of CD8$^+$ alloproliferative cells from lenalidomide treated versus control allogeneic co-cultures.

Figure 5.7

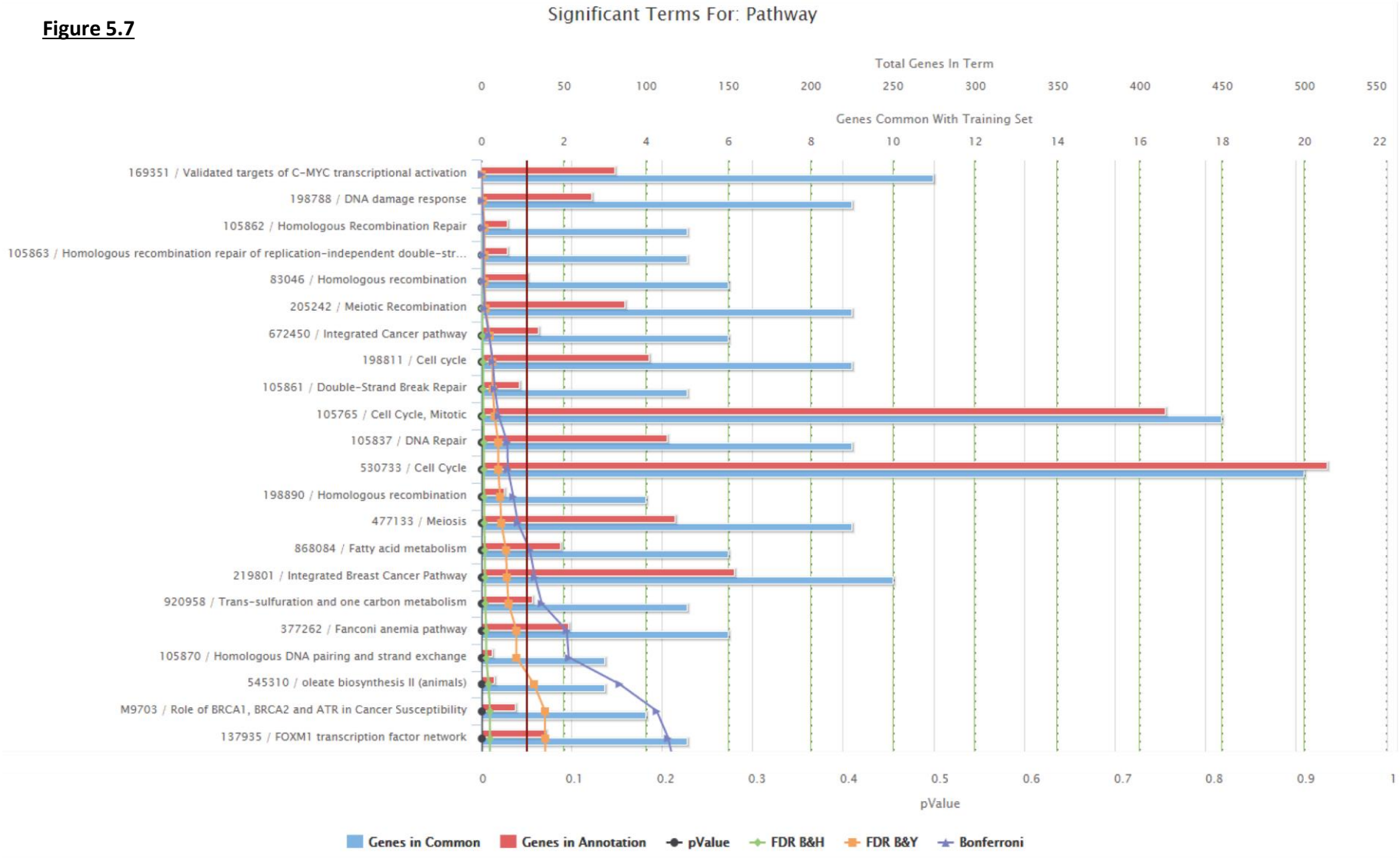

Significant Terms For: Pathway
Total Genes In Term
0 50 100 150 200 250 300 350 400 450 500 550
Genes Common With Training Set
0 2 4 6 8 10 12 14 16 18 20 22
169351 / Validated targets of C-MYC transcriptional activation
198788 / DNA damage response
105862 / Homologous Recombination Repair
105863 / Homologous recombination repair of replication-independent double-str...
83046 / Homologous recombination
205242 / Meiotic Recombination
672450 / Integrated Cancer pathway
198811 / Cell cycle
105861 / Double-Strand Break Repair
105765 / Cell Cycle, Mitotic
105837 / DNA Repair
530733 / Cell Cycle
198890 / Homologous recombination
477133 / Meiosis
868084 / Fatty acid metabolism
219801 / Integrated Breast Cancer Pathway
920958 / Trans-sulfuration and one carbon metabolism
377262 / Fanconi anemia pathway
105870 / Homologous DNA pairing and strand exchange
545310 / oleate biosynthesis II (animals)
M9703 / Role of BRCA1, BRCA2 and ATR in Cancer Susceptibility
137935 / FOXM1 transcription factor network
0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1
pValue
Genes in Common
Genes in Annotation
pValue
FDR B&H
FDR B&Y
Bonferroni

Figure 5.7 Lenalidomide exposure during allostimulation modulates expression of genes involved in multiple important cellular signalling pathways.

Graphical representation of over-representation analysis of differential gene expression of lenalidomide treated alloproliferative $CD8^+$ cells compared to untreated alloproliferative cells. Genes with significantly ($P<0.05$) altered gene expression with fold-change ≥2 were included in the analysis. Results are shown for 3 unique donor-responder pairs. Generated using TOPPFUNN. Red line = P 0.05.

Table 5.5 Potential mediators of lenalidomide actions

Upstream Regulator	Adj p value
TGFB3	<0.01
TGFB2	<0.01
CFTR	<0.01
IKZF1	<0.01
EPCAM	<0.01

Top Upstream Regulators identified on Ingenuity pathway analysis of genes with significantly altered (adjusted P<0.05) and ≥2-fold-change in gene expression in lenalidomide treated alloproliferative cells compared to untreated alloproliferative.

Table 5.6 Potential mediators of lenalidomide actions

Upstream Regulator	p value
CCNE1	<0.01
TP53	<0.01
ILF2	<0.01
MYC	<0.01
FOXP3	<0.01

Transcription factors - identified on EnrichR pathway analysis of genes with significantly altered (adjusted P<0.05) and ≥2-fold-change in gene expression in lenalidomide treated alloproliferative cells compared to untreated alloproliferative.

5.4.4 Lenalidomide exposure during allostimulation significantly reduced frequency of ikaros$^+$ CD8$^+$ T cells

Ikaros depletion via lenalidomide's action on cereblon is an important mechanism that contributes to the drug's anti-myeloma activity [247] and has also been shown to be necessary for immunomodulatory effects on autologous T cell responses [248]. The gene expression analysis suggested that ikaros also plays a role in the effect of lenalidomide on the alloresponse. I therefore next went on to confirm that lenalidomide exposure during allostimulation of T cells resulted in a decrease in ikaros protein expression using intracellular flow cytometry.

As predicted by the observations from my earlier MS/MS-HPLC experiments, at day 3 following exposure to high levels of lenalidomide in the co-culture media and cells there was a significant decrease in the proportion of APB-derived CD8$^+$ T cells positive for intracellular ikaros (**Figure 5.8, A**). Ikaros levels recovered to those of control co-cultures by day seven and day nine (**Figure 5.8, A**).

5.4.5 Lenalidomide exposure during allostimulation also significantly reduced frequency of ikaros in CD4$^+$ T cells

Given the observation that lenalidomide exposure during allostimulation increased alloproliferation of CD8$^+$ T cells but not CD4$^+$ T cells, I next went on to determine if this differential effect could be explained by differences in the expression of ikaros in these different T cell subsets.

Interestingly in the case of CD4$^+$ but not CD8$^+$ T cells the proportion of ikaros positive cells increased significantly (**Figure 5.8, B**) on allostimulation in the absence of lenalidomide, indicating that ikaros levels may be differentially regulated in CD4$^+$ and CD8$^+$ T cell subsets. However, consistent with the effect of lenalidomide on CD8$^+$ T cells, the frequency of ikaros positive CD4$^+$ T cells was reduced in lenalidomide-treated co-cultures (**Figure 5.8, B**).

In order to determine whether on a per-cell basis ikaros expression was altered by lenalidomide exposure, the median fluorescence intensity (MFI) for cells staining positive for ikaros was also assessed. In the case of both CD4$^+$ and CD8$^+$ T cells there did not appear to be any significant change in the level of ikaros protein expression (**Figure 5.8, C**).

Figure 5.8

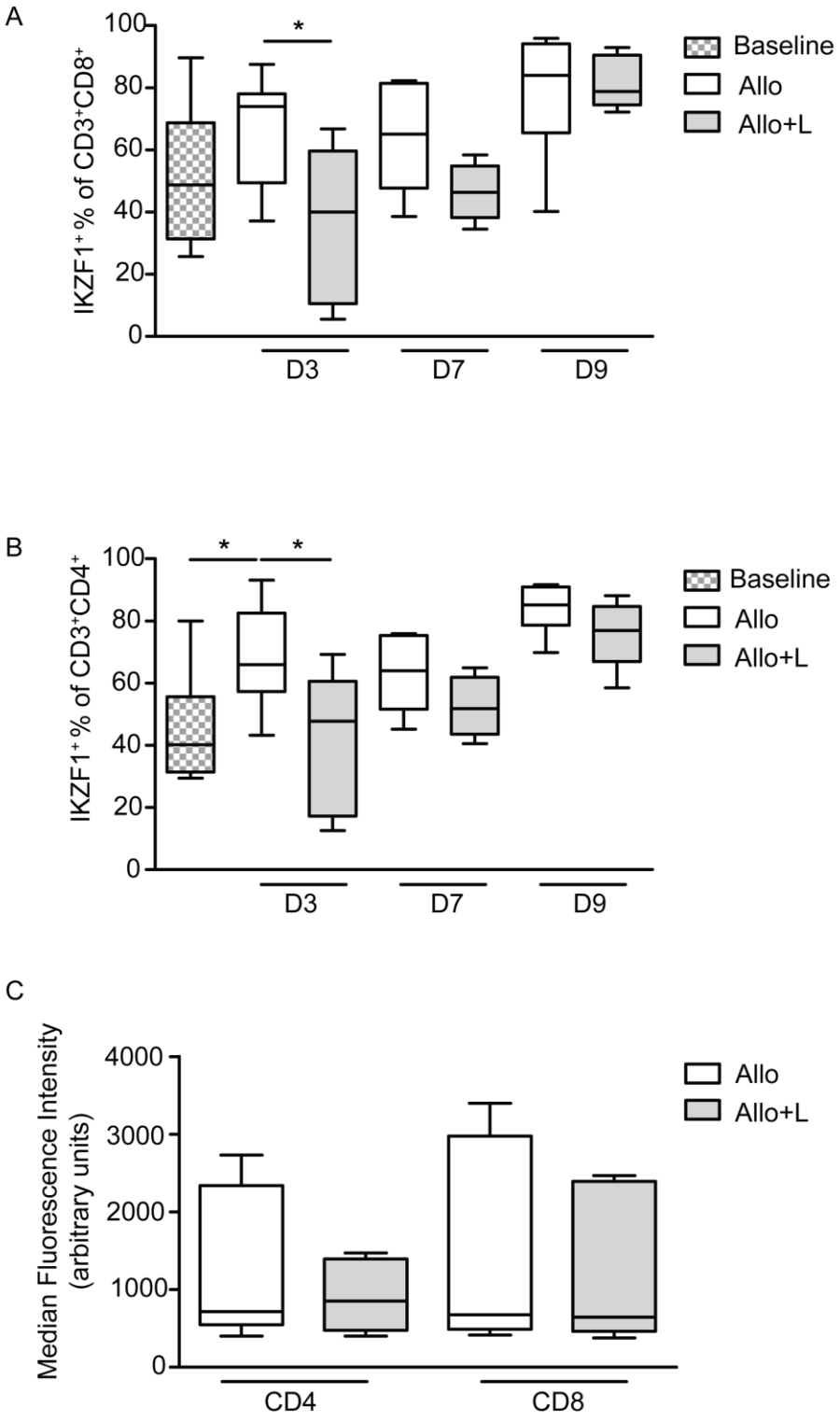

A
IKZF1+ % of CD3+CD8+
100
80
60
40
20
0
*
D3
D7
D9
Baseline
Allo
Allo+L
B
IKZF1+ % of CD3+CD4+
100
80
60
40
20
0
* *
D3
D7
D9
Baseline
Allo
Allo+L
C
Median Fluorescence Intensity (arbitrary units)
4000
3000
2000
1000
0
CD4
CD8
Allo
Allo+L

Figure 5.8 Lenalidomide exposure during allostimulation reduced the proportion of $CD4^+$ and $CD8^+$ T cells expressing ikaros

(**A**) Proportion of adult peripheral blood derived (APB) $CD8^+$ T cells expressing ikaros at serial time-points of allogeneic co-culture in the absence or presence of lenalidomide. Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 9 unique donor-responder pairs. * = $P<0.05$.

(**B**) Proportion of adult peripheral blood derived (APB) $CD4^+$ T cells expressing ikaros at serial time-points of allogeneic co-culture in the absence (Allo) or presence of lenalidomide (Allo+L). Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 9 unique donor-responder pairs. * = $P<0.05$.

(**C**) Relative expression of ikaros following primary allogeneic co-culture of APB derived PBMC in the absence or presence of lenalidomide measured by median fluorescence intensity. Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 9 unique donor-responder pairs.

5.4.6 Lenalidomide exposure during allostimulation reduced the frequency and cellular expression of ikaros in UCB-derived CD8$^+$ and CD4$^+$ T cells

Although no effect on APB-derived CD4$^+$ T cell alloproliferation was observed in lenalidomide-treated allogeneic co-cultures, a significant decrease in UCB-derived CD4$^+$ T cell alloproliferation was seen. This suggested that there could be a differential sensitivity of APB and UCB-derived CD4$^+$ T cells to lenalidomide, that could be explained by different frequencies of ikaros positive cells or altered intra-cellular expression of ikaros in these two cell sources. I therefore went on to determine ikaros expression in APB-derived T-cells.

A direct comparison of the levels of ikaros$^+$ CD4$^+$ and CD8$^+$ T cells derived from APB and UCB reveals that at baseline, a greater proportion of UCB-derived CD4$^+$ T cells express ikaros compared to APB-derived CD4$^+$ T cells, however by day 3 of allogeneic co-culture in the absence of lenalidomide frequencies of ikaros positive CD4$^+$ and CD8$^+$ T cells are similar in APB and UCB (**Figure 5.9, A**).

I next determined the effect of lenalidomide exposure on frequency of ikaros positive CD4$^+$ and CD8$^+$ T cells in allogeneic UCB co-cultures at day 3. Consistent with the effect observed in APB, there was a significant decrease in the proportion of CD4$^+$ and CD8$^+$ T cells positive for intracellular ikaros (**Figure 5.9, B**). However, in contrast to the findings in APB, there was a significant reduction in the level of cellular expression (measured by MFI) of ikaros in both UCB-derived CD4$^+$ and CD8$^+$ T cells on day 3 of allogeneic co-culture after lenalidomide exposure compared to controls (**Figure 5.9, C**). These data support a different level of sensitivity of UCB-derived CD4$^+$ and CD8$^+$ T cells to lenalidomide compared to APB, but cannot explain the differential effect on CD4$^+$ Treg.

One explanation for the differential effect on UCB-derived CD4$^+$ T cells compared to APB-derived CD4$^+$ T cells could be the fundamental immunological differences in the composition of APB and UCB. In particular UCB contains a much higher proportion of naïve T cells than APB [349]. Therefore I next proceeded to assess the proportion of cells expressing ikaros in naïve, EM, CM and TEMRA subsets. If naïve cells, for example, were more likely to express ikaros than CM this could significantly skew the comparison between APB and UCB ikaros. In fact, I observed a similar pattern of ikaros expression in CD4$^+$ T cell subsets derived from APB and UCB. The proportion of ikaros$^+$ cells decreased as cells moved from naïve to CM and EM phenotype, with a statistically

significant reduction in ikaros between naïve and EM subsets for both APB and UCB (**Figure 5.9, D**). The only observed difference between APB and UCB subsets was that UCB CM $CD4^+$ T cells appeared to contain a significantly higher proportion of $ikaros^+$ cells than APB CM $CD4^+$ T cells.

Figure 5.9

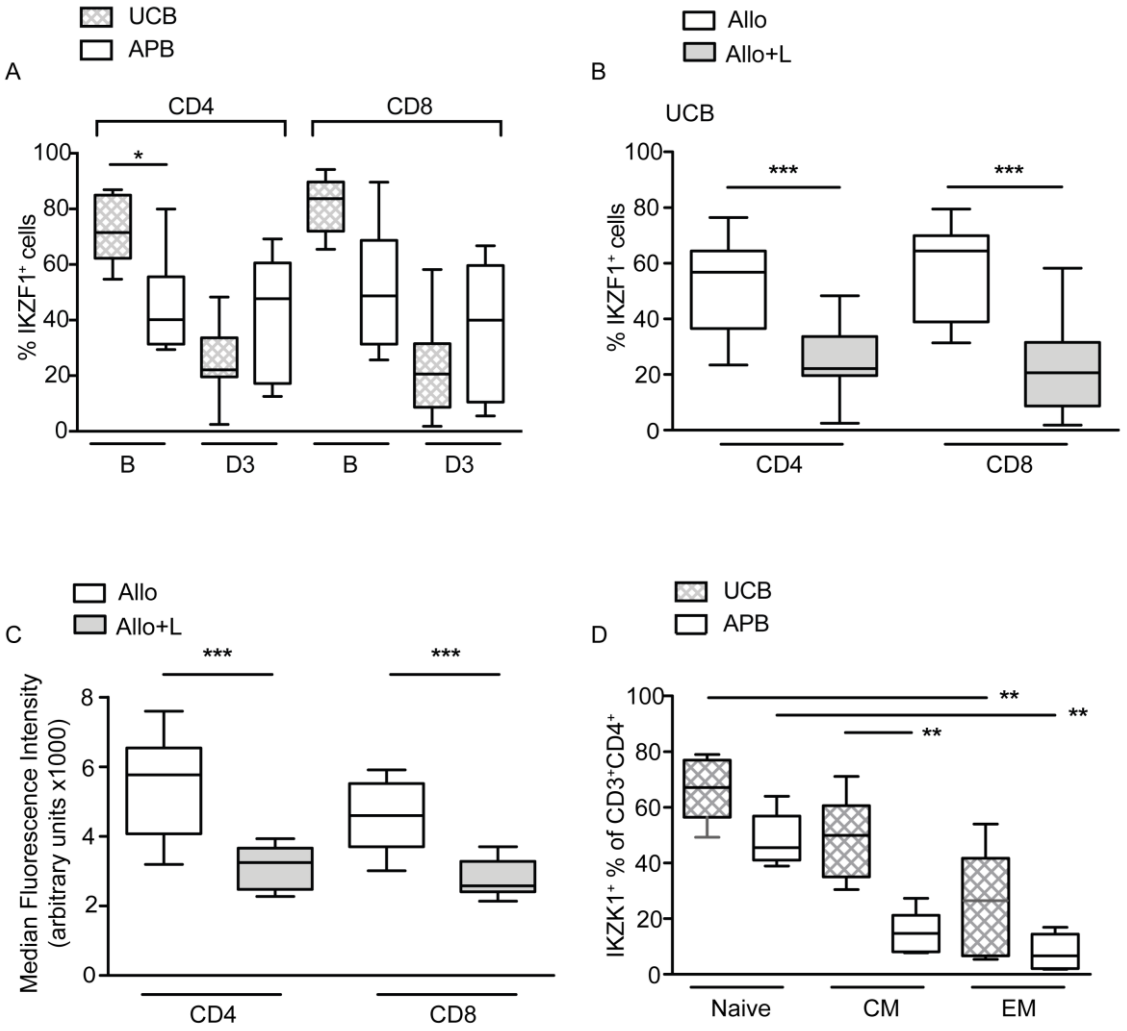

A
UCB
APB
CD4
CD8
*
% IKZF1+ cells
100
80
60
40
20
0
B
D3
B
D3
B
Allo
Allo+L
UCB
***
***
% IKZF1+ cells
CD4
CD8
C
Allo
Allo+L
***
***
Median Fluorescence Intensity (arbitrary units x1000)
8
6
4
2
0
CD4
CD8
D
UCB
APB
**
**
**
IKZK1+ % of CD3+CD4+
Naive
CM
EM

Figure 5.9 Differential ikaros expression in UCB-derived T cells does not alter lenalidomide dependent reduction in the proportion of UCB-derived $CD4^+$ and $CD8^+$ T cells expressing ikaros

(**A**) Proportion of APB and UCB-derived $CD4^+$ & $CD8^+$ T cells expressing ikaros in untreated allogeneic co-cultures. Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 9 unique donor-responder pairs. * = P<0.05

(**B**) Proportion of umbilical cord blood (UCB) $CD4^+$& $CD8^+$ T cells expressing ikaros after 3 days of allogeneic co-culture in the absence (Allo) or presence of lenalidomide (Allo+L). Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 11 unique donor-responder pairs. *** P<0.001.

(**C**) Relative expression of ikaros following primary allogeneic co-culture of UCB derived PBMC in the absence or presence of lenalidomide measured by median fluorescence intensity. Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 11 unique donor-responder pairs.

(**D**) Proportion of APB and UCB-derived $CD4^+$ T cells within naïve, central memory (CM) and effector memory (EM) subsets. Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 5 unique donor-responder pairs. *** = P<0.001.

5.4.7 Expression of cereblon is similar in APB and UCB

As the differential effect of lenalidomide on APB and UCB did not appear to be dependent on baseline differences in ikaros expression, I next examined expression of cereblon by qRT-PCR in $CD4^+$ and $CD8^+$ T cells isolated from healthy APB donors and UCB units using a magnetic bead-based technique (as described in Chapter 2). Increased degradation of ikaros is dependent on lenalidomide binding to cereblon and enhancing cereblon activity as a substrate receptor for an E3-ubiquitin ligase complex [248]. qRT-PCR for *cereblon* did not show any differences in mRNA levels between APB and UCB-derived $CD4^+$ or $CD8^+$ T cells (**Figure 5.10**).

Figure 5.10

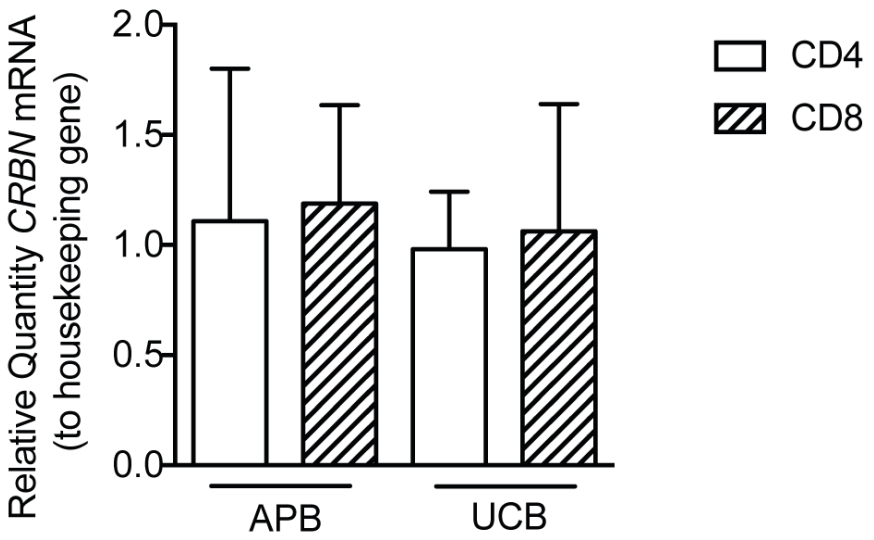


Figure 5.10 Cereblon expression is similar in APB and UCB

Mean (+/- SD) for relative quantity of *cereblon* (CRBN) mRNA in APB and UCB, $CD4^+$ and $CD8^+$ T cells (MACS sorted). Results are shown for 3 unique APB and 3 unique UCB donors.

5.5 Discussion

Having identified the major cellular changes in the *in vitro* T cell alloresponse after lenalidomide treatment, including the enhanced proliferation of effector $CD8^+$ T cells. I next determined that lenalidomide modulates both the cellular expression of ikaros in APB and UCB-derived $CD4^+$ and $CD8^+$ T cells and the gene expression of APB-derived $CD8^+$ T cells isolated from allogeneic co-cultures.

The depletion of intracellular ikaros observed in these experiments is consistent with the well-described cereblon-dependent mechanism of action of lenalidomide [243,334]. Therefore it is likely that this mechanism is at least in part responsible for both the changes I have described in T cell alloproliferation and phenotype *in vitro*, and for some of the clinical effects observed. Differential sensitivity or magnitude of ikaros depletion might explain differential effects on APB and UCB-derived $CD4^+$ T cells. However I have not been able to detect any significant differences in ikaros that would account for the decreased $CD4^+$ T cell alloproliferation or increase in Treg that I have seen after allostimulation of UCB-derived PBMC in the presence of lenalidomide. This may be because the effect on UCB-derived $CD4^+$ Treg is independent of ikaros and possibly cereblon, or due to differences in ikaros expression of UCB and APB-derived $CD4^+$ Treg that are not captured by examining the $CD4^+$ population as a whole.

A potential way to further dissect the role of ikaros in the lenalidomide-treated alloresponse would be to use responder cells in which cereblon had been knocked-down (cereblon-KO) using siRNA, and therefore unable to mediate the increased degradation of ikaros. Comparison of cereblon-KO T cell alloreponses with my current data could determine which effects were likely to be cereblon-ikaros dependent, and which were due to additional effects of lenalidomide. The caveat to this approach is that if some effects of lenalidomide on the alloresponse are due to enhanced degradation of other, as yet undetermined substrates of cereblon this will also result in loss of these effects.

Exploration of gene expression changes induced in alloproliferative cells by lenalidomide treatment may help to answer this interesting question of whether lenalidomide is modulating alloresponses by additional mechanisms and pathways to ikaros depletion. The finding that lenalidomide exposure during allostimulation made no difference to the gene expression of non-proliferative $CD8^+$ T cells reinforces the data I have presented demonstrating phenotypic and

functional changes only in alloproliferative cells. My findings also suggest that the effect of lenalidomide is dependent on TCR-engagement with cognate alloantigen and therefore results in an enhanced alloresponse that retains allospecificity.

Although, as expected ikaros was implicated as a top upstream regulator of differentially expressed genes in alloproliferative $CD8^+$ T cells treated with lenalidomide, TGFβ2 and 3 were also revealed as potential mediators of lenalidomide effects. This indicates that additional non-ikaros dependent mechanisms may be involved. TGFβ signalling has been shown to play an important role in controlling T cell activation, with a decrease in TGFβ resulting in enhanced naive T cell responses and effector differentiation, as well as a decrease in Treg (reviewed in Gorelik *et al.* [350]). Therefore, while TGFβ has not yet been described as a target of lenalidomide it is plausible that TGFβ could be involved in some of the changes I have described in T cell alloresponses. Quantification of TGFβ levels in lenalidomide-treated alloresponders may therefore be informative.

Over-representation analysis of differentially expressed genes also revealed that enhanced signalling via the Myc pathway may play a role in the effects of lenalidomide on the $CD8^+$ T cell alloresponse. Myc is thought to play a critical role in initiating metabolic reprogramming of T cells, leading to up-regulation of a number of genes involved in the glycolysis and glutaminolysis pathways that support T cell as they undergo rapid proliferation to effector cells (**Figure 5.11**) [351].

Fine-tuning of metabolic gene expression is also achieved by the transcription factors IRF4 (interferon regulatory factor 4) and AP4 (activating enhancer binding protein 4). Importantly the level of expression of these two transcription factors is fine-tuned by the strength of TCR signalling and levels of IL2, respectively [352,353]. As I have demonstrated that following lenalidomide treatment $CD8^+$ T cells appear to have a lowered threshold for TCR signalling and both $CD4^+$ and $CD8^+$ T cells have increased capability to produce IL2, this could influence the metabolic gene expression programmes of alloresponder T cells, impacting on functional activity or migratory capacity of cells [354]. Interestingly both Myc and IRF4 have been implicated in the action of lenalidomide on myeloma cells [197,355,356].

Figure 5.11

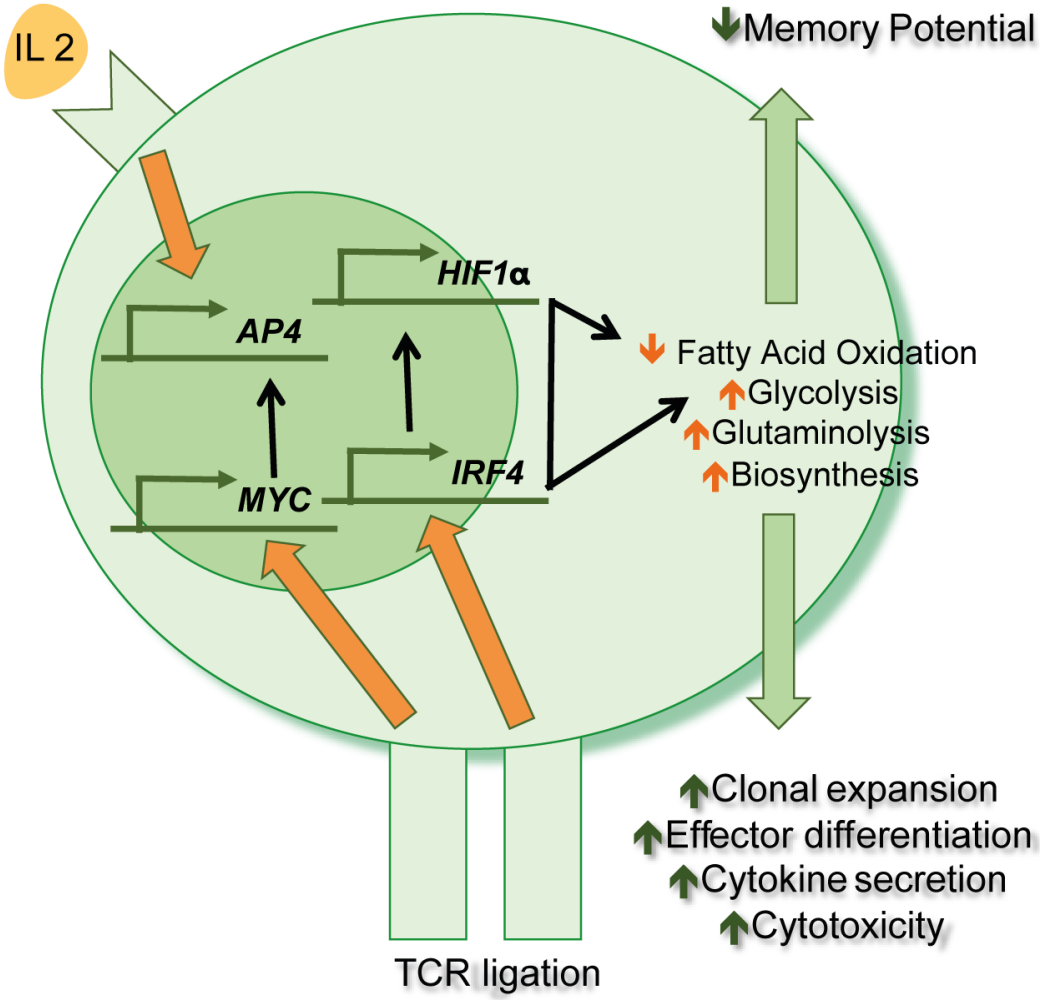


Figure 5.11 Metabolic regulation of T cell differentiation

Schematic representation of signalling events occurring on TCR (T cell receptor) and interleukin 2 (IL2) receptor ligation leading to metabolic reprogramming via changes in gene expression of key metabolic regulatory genes.

(Based on findings of multiple reviews [342,353,357])

I chose to examine the gene expression profile of $CD8^+$ T cells, as this was the T cell subset where I had seen the greatest phenotypic and functional changes following allostimulation in the presence of lenalidomide. However in view of the differential effect I have observed in UCB $CD4^+$ T cell alloresponses it would also be interesting to explore the gene expression changes caused by lenalidomide in allostimulated APB-derived $CD4^+$ T cells and to compare these to those in T cells derived from UCB. It may however be difficult to obtain adequate quantities of RNA from the small cell numbers obtained from UCB co-cultures to perform a similar experiment to that I have done with APB. An alternative approach that may be able to overcome this limitation could be to use RNA-sequencing.

A possible limitation of the gene expression experiment is the choice of the day 7 time-point. At day 7 the gene expression of the cells reflects the consequences of the action of lenalidomide on allostimulated cells several days before, rather than the direct effects of the drug. Day 7 was chosen as this was the first time-point at which a significant difference in $CD8^+$ T cell proliferation, CD107a expression and cytokine production was detectable. Therefore I felt that at this time the alloproliferative $CD8^+$ T cells from lenalidomide-treated cultures were qualitatively different from those from untreated. It was also technically possible at this time-point to purify sufficient numbers of both proliferative and non-proliferative responder cells. An alternative time-point that would be informative as to the direct effects of lenalidomide would be day 3, at which time lenalidomide is still present in the co-culture media. However it would be more difficult to identify and purify alloresponder and non-responder cells at this time, as it would be too early to expect a proliferative response. Alloresponder cells can be identified by the expression of activation markers such as CD25 or CD62L, however these have been shown to be imperfect methods for detection of alloresponsive cells with a high level of stringency.

In summary I have shown that the addition of lenalidomide to allogeneic co-culture results in enhanced $CD8^+$ T cell alloresponses by ikaros depletion. In addition lenalidomide appears to have profound and lasting effects on metabolic and immunoregulatory programmes within $CD8^+$ T cells resulting in a shift in the balance of $CD8^+$ T cell alloresponses towards a more potent effector phenotype.

Chapter 6 – Determining the impact of lenalidomide on T cell alloresponses that could selectively enhance GvT activity

6.1 Introduction

There is undoubtedly is a link between GvT and GvHD, with a wealth of evidence that the presence of GvHD predicts for a lower rate of relapse following AHSCT [1,273,358]. However the question of whether the immunological mechanisms underlying GvT and GvHD are fundamentally different is more difficult to answer. It could be argued that GvT effects merely represents a subset of GvHD reactions directed against alloantigens expressed on cells of the haemato-lymphoid compartment, rather than at other tissues. However there is increasing evidence that at an individual T cell level the cells that cause GvT and GvHD may not be the same [359]. There is also evidence that GvT can contribute to elimination of tumours that exist outside the haemato-lymphoid system, as in the case of regression of renal cell carcinoma following AHSCT [360].

Strategies to separate GvT and GvHD effects thus far have focused mainly on reducing the inflammatory drive that leads to indiscriminate T cell alloactivation, often by reducing the intensity of conditioning regimens [361]. Another approach more focused on GvT is increasing tumour antigen specificity by *ex vivo* manipulation of donor T cells [39,362,363] or cancer vaccine therapy [364]. However an alternative strategy might be to block the migration of alloreactive T cells to target organs of GvHD by blocking chemokine receptors or integrins [365-367], this would prevent damage to healthy tissue but not affect GvT responses. Early stage trials of drugs that can modulate T cell migration are ongoing [96].

Having previously found that lenalidomide treatment enhances alloproliferation and effector functions of alloproliferative $CD8^+$ T cells an important question remains; is there any differential effect on cellular parameters that might predict for differential GvHD versus GvT effects? As there is no single *in vitro* assay that can provide a simultaneous readout of GvT and GvHD a combination of assays was used, with the aim of comprehensively addressing this question.

6.2 Aim

Aim: To define the impact of lenalidomide treatment during allostimulation on human APB-derived alloresponder T cell migration and cytotoxicity against target-cells; to determine if treatment with lenalidomide could selectively enhance GvT or GvHD activity.

6.3 Specific materials and methods

6.3.1 Chemokine receptor expression

Chemokines are small (8-12kDa) secreted proteins, similar to cytokines that have an important role in cell trafficking. They can be subdivided into 4 families with the CXC or α subfamily (so called due to the first two cysteine residues being separated by another amino acid) and the CC or β subfamily (first two cysteines adjacent) most extensively characterised [368,369]. Chemokine receptors (CCR) are a subfamily of 7 transmembrane-spanning G-protein coupled receptors expressed on leucocytes. Some CCR are cell type specific, while others have a broader distribution and similarly some direct migration in a tissue specific manner, while others direct migration to a range of tissue types. Multiple chemokines may bind to the same CCR (detailed in **Table 6.1**).

The expression of chemokine receptors on responder T cells after allostimulation in the presence of lenalidomide was compared to that of untreated responder T cells to determine if lenalidomide treatment could alter the migratory capacity of alloresponsive T cells (cells were labelled and FACS analysis conducted as per the protocol detailed in Chapter 2). The Kruskal-Wallis test with Dunn's multiple comparisons test was used to compare chemokine expression at baseline and at serial time-points of allogeneic culture, Wilcoxon matched pairs signed rank test was used to compare treated with untreated cells, P values <0.05 were taken as significant.

Table 6.1 Characteristics and rationale for the choice of chemokine receptors examined.

CCR	Chemokines	Target Cell	Target Organ	Potential role in GvHD/GvT
CCR2	MCP 1 (CCL2) MCP 2 (CCL8) MCP 3 (CCL7) MCP 4 (CCL13) CCL16	Th1/T T_{EM} NK Basophils Monocytes	Sites of inflammation	Shown to play a role in liver and gut GvHD in murine models [93,370,371] SNPs in CCR2 gene associated with GvHD post-AHSCT [372]
CCR5	MIP 1α (CCL3) MIP 1β (CCL4) RANTES (CCL5) MCP 2 (CCL8) CCL11 CCL16	Th1 Activated-CD8 Treg NK DC Monocytes	Sites of inflammation	CCR5 positive cells found in human GvHD biopsies and on human alloreactive cells [373] CCR5 blocking drugs used in trials for GvHD prevention after AHSCT [96] CCR5 polymorphisms influence risk of GvHD [94]
CCR7	MIP 3β (CCL19) CCL21	Activated T cells DC	Secondary lymphoid organs (SLO)	Naïve T cells express CCR7 and are known to be important in GvHD [374]
CCR9	TECK (CCL25)	Activated T cells	Gut (small bowel)	Reducing CCR9 expression/inhibition of signalling pathway in mouse models reduced GvHD [97,98]
CCR10	CTACK (CCL27) MEC (CCL28)	Activated T cell Plasmablasts	Skin	Association between high numbers of $CCR10^+$ cells in PB and incidence of skin GvHD after AHSCT [101]
CXCR4	SDF 1 (CXCL12) MIG Ubiquitin	Activated T cells DC NK NKT	Bone marrow	Targeting of allogeneic cells to BM post-AHSCT [375]

Adapted from Luster 1998 and Zlotnik 2012 [368,369]

CCR = chemokine receptor, MCP = monocyte chemoattractant protein, MIP = macrophage inflammatory protein, RANTES = regulated on activation, normal T cell expressed and secreted, SDF = stromal-cell derived factor, MIG = monokine induced by gamma interferon, DC = dendritic cell, NK = natural killer cell, NKT = natural killer T cell, MEC = mucosae-associated epithelial chemokine, CTACK = Cutaneous T cell-attracting chemokine, TECK = Thymus-Expressed Chemokine, BM = bone marrow, PB = peripheral blood, SNPs = single nucleotide polymorphisms, T_{EM} = T effector memory, Th1 = T helper 1.

6.3.2 Transwell chemotaxis assays

The transwell assay used is based on that of the one designed by Boyden to analyse the chemotactic responses of leucocytes [376]. In this assay two medium containing chambers are separated by a porous membrane through which cells can transmigrate in a vertical direction into the lower chamber (usually containing a chemoattractant). Cells that have entered the lower chamber can then be isolated and counted by a number of techniques (reviewed in Kramer *et al.* [377]).

CFSE-labelled alloresponder PBMC or MACS purified alloresponder T cells and autologous control PBMC or T cells were isolated from untreated or lenalidomide treated allogeneic or autologous control co-cultures at day 9 (as per usual co-culture set up detailed in General methods). Cells were washed in PBS and then resuspended in RPMI 1640 Aq media (RPMI) at a concentration of $5x10^6$/ml.

A 96 well ChemoTx® microplate (Neuro Probe) with a 5μM pore size filter and 300μl capacity lower wells was prepared by pipetting 299μl of RPMI (no chemokine controls wells), RPMI containing CCL2 at a concentration of 100ng/ml, RPMI containing CXCL12 at a concentration of 100ng/ml (experimental wells) and CXCL12 at 2μg/ml (positive control wells) into designated wells. The framed filter was then applied over the lower chambers of the microplate ensuring that the liquid in the lower chamber made contact with the filter to form a seal (**Figure 6.1**).

Fifty μl of cell suspension was then applied to the top of the filter within the hydrophobic ring to form a hemispheric drop. Each sample was prepared in triplicate. Any wells in which the hemispheric drop appeared smaller was excluded from analysis as it was likely that the aqueous seal had been broken for these cells. The plate was then covered with a plastic lid and incubated for 4 hours at 37°C in humidified air with 5% CO_2.

Following incubation the filter was carefully removed from the microplate and discarded. After vigorous mixing 100μl was taken from each well of the microplate and transferred to a fresh 96 well culture plate. One hundred μl of Guava® ViaCount Flex (Merck Millipore) reagent was then added to each well and the plate acquired on a Guava® easyCyte Flow Cytometer. The remaining 200μl was used for FACS analysis of migrated cells. Cell were labelled for CD3, CD4 and CD8 as per protocol in Chapter 2.

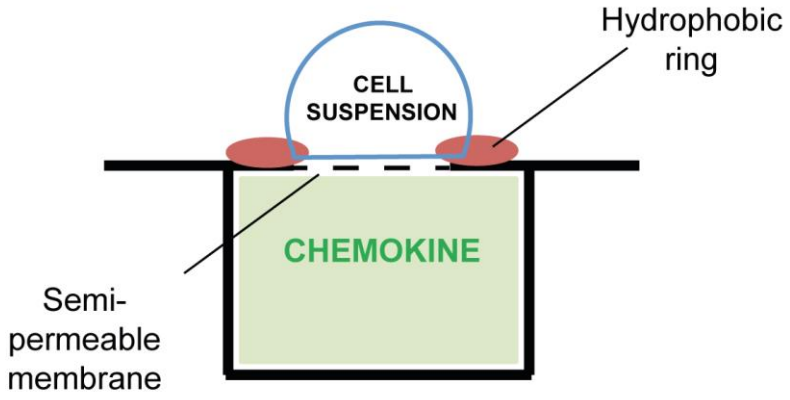
The ViaCount reagent is designed to enable counting and viability assessment of low-density samples on the EasyCyte flow cytometer. The cytometer was used in EasyFit mode to allow the software to automatically and independently assign live/dead status to cells and to provide an absolute cell count in each sample. Raw data was exported from the cytometer to Microsoft Excel (Microsoft). An average was taken of triplicate viable cell counts from each sample. This was then multiplied by 3 (to reflect cell count in total 300µl of original well) and expressed as a %migration based on the number of cells in the upper chamber being 250,000 (50µl of $5x10^6$/ml cell suspension). The Migration Index was then derived using the formula:

% cells migrated in experimental samples / % cells migrated in no chemokine control wells

Autologous samples were compared with autologous control and allogeneic samples with allogeneic controls. Wilcoxon matched pairs signed rank test was used to compare migratory index of untreated versus lenalidomide treated samples, P values <0.05 were taken as significant.

Figure 6.1

A



B

Figure 6.1 Transwell Migration Assay

(A) Schematic of upper chamber of transwell formed by drop of cell suspension, and lower chamber filled with media containing chemokine separated by semi-permeable membrane. (Adapted from http://www.neuroprobe.com/product/chemo_tx/)

(B) Photograph of ChemoTx plates used for experiments

(Reproduced from http://www.neuroprobe.com/product/chemo_tx/)

6.3.3 Time-lapse video microscopy chemokinesis assay

Using time-lapse imaging it is possible to assess movement of single cells in culture (reviewed in Kramer *et al.* [377]). The Nikon Biostation IM is a cell incubator with an integrated microscope that allows imaging of multiple samples simultaneously over time.

Firstly Nikon HiQ4 plates were coated with ICAM1-Fc chimera (Biolegend) prepared at 3μg/ml in sterile PBS and incubated overnight at 4°C. The next day ICAM1 solution was removed and the plates blocked with 2% bovine serum albumin (BSA) in sterile PBS for 1 hour at 4°C. The plates were then washed 3 times with warmed (37°C) hanks buffered basic salt solution (HBSS) containing 20mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (HBSS+20mM HEPES). Plates were kept in the incubator at 37°C containing the last wash of HBSS+20mM HEPES until cell solution was added.

Alloresponder T cells were MACS isolated from untreated or lenalidomide-treated allogeneic or autologous control co-cultures at day 9. Cells were washed in sterile PBS and counted before being resuspended in warmed HBSS+20mM HEPES containing either: no chemokine, CCL2 at 100ng/ml or CXCL12 at 100ng/ml at $1x10^6$cells/ml.

Plates were then loaded into the incubator chamber of the Nikon Biostation IM and incubated at 37°C with humidified air and 5% CO_2. Cells were allowed to settle onto the surface of the plate for 20 mins. Images were then recorded from each of the 4 wells of the plate at 30 second intervals for 30 mins at x20 magnification.

Images from the Nikon Biostation IM were imported into the NIS Elements Advanced Research software (Nikon) for analysis. Using the tracking module a minimum of 30 (maximum 50) cells were assigned for tracking in an area that was 200μM from the centre of the image in all directions. Cells were manually tracked over the course of the time-lapse and path length (in pixels) derived by the software. Cells that migrated off the edge of the image were excluded from analysis. The average path length for the cells counted was then derived for each sample and the Mann Whitney test used to compare lenalidomide treated with untreated cells, P values <0.05 were taken as significant.

6.3.4 Cytotoxicity assays

The end product of both GvT and GvHD responses is the killing of target cells. In practice GvT is directed at allogeneic cells of the haematopoietic lineage, most commonly found in the bone marrow or lymph nodes. In order to assess the capacity of lenalidomide treated alloresponder cells to kill allogeneic haematopoietic targets a co-culture cytotoxicity assay was performed as described in Davies *et al.* [127].

On day 4 of the allogeneic co-culture a reserved aliquot of corresponding stimulator cells was thawed and cells were resuspended at 10^6cells/ml in supplemented media containing PHA-L at 3μg/ml and IL2 at 100IU/ml. Cells were then incubated at 37°C in humidified air with 5% CO_2 to derive PHA-blasts to act as allogeneic haematopoietic targets.

CFSE-stained alloresponder MACS-purified T cells or $CD8^+$ T cells and autologous control T cells/$CD8^+$ T cells were isolated from untreated or lenalidomide-treated allogeneic or autologous control co-cultures at day 9. Cells were counted and then resuspended in supplemented RPMI at $10x10^6$cells/ml.

PHA-blasts cells were counted, transferred to a sterile 15ml falcon tube and washed in serum-free RPMI. Cells were then centrifuged at 500g for 10 mins at RT to pellet and the supernatant removed completely with a pipette. PHA-blasts to be used as targets in cytotoxicity assays were then labelled with PKH-26 as per manufacturers protocol to allow their identification. Briefly, cells were resuspended in ethanolic diluent C provided and an equal volume of 2x dye solution (prepared by dilution of PKH 26 in diluent C) was added. Cells and dye were mixed by vigorous pipetting and incubated for 1 min. Staining was stopped by addition of a double volume of FBS and incubated for a further minute. Cells were then washed in supplemented media twice prior to resuspension in supplemented media at $1x10^6$cells/ml and transferred to a fresh sterile 15ml falcon tube.

Fifty thousand PKH-26 stained allogeneic and autologous target cells were then pipetted into designated wells in a u-bottom 96-well sterile culture plate. Serial dilutions of CFSE-labelled alloresponder T cells or $CD8^+$ T cells were then prepared to give ratios of 20:1, 10:1 and 2:1 effectors in 100μl of supplemented media. Effectors were then added to relevant allogeneic or autologous target cells in the 96-well plate. All samples were prepared in triplicate. A further

100µl of target cells was added to the negative control wells to ensure that the volume of media was the same in each well.

The plate was then incubated for 4 hours at 37°C in humidified air with 5% CO_2. Following incubation the plate was centrifuged at 500g for 5 mins at RT to pellet the cells, supernatant was discarded and the cells washed in FACS buffer. Cells were resuspended in annexin V binding buffer containing annexin V (Biolegend) and incubated in the dark at RT for 15 mins. Cells were then washed in annexin V binding buffer and transferred to 1.2ml microtubes and acquired on the BD Fortessa flow cytometer (Beckton Dickinson).

Analysis of co-staining patterns of PKH-26 and annexin V allowed identification of apoptotic target cells and comparison of % apoptosis in negative controls and those containing effectors in increasing ratios with targets. This method has been shown to be equivalent to the chromium^{51} release assay used to evaluate cytotoxicity, with the major advantage of avoiding exposure to radioactive isotopes (reviewed in Zaritskaya *et al.* [285]).

The average % apoptosis was calculated for each triplicate sample and the specific apoptosis caused by effector cell mediated cytotoxicity calculated using the formula:

% apoptosis of allogeneic targets when co-cultured with effector cells - % apoptosis of targets when cultured alone.

Wilcoxon matched pairs signed rank test was used to compared % target apoptosis in lenalidomide treated versus untreated samples and P value<0.05 taken as significant.

6.4 Results

6.4.1 Chemokine receptor expression changes during allogeneic co-culture appear to be dependent on the activation and proliferation status of the T cell.

Using multi-parameter flow cytometry I first examined the expression of the chemokine receptors CCR2, CXCR4, CCR5, CCR7, CCR9 and CCR10 on APB-derived T cells allostimulated in the presence of lenalidomide or vehicle control (DMSO). Baseline proportions of CCR-positive T cells were consistent with the published literature [378]. Expression of CCR on $CD4^+$ and $CD8^+$ T cells was assessed at baseline, day 7 and day 9 of allogeneic co-culture to determine how expression changed over time and with allostimulation.

To determine whether changes were due simply to the effect of *ex vivo* culture allostimulated T cells were compared to T cells from autologous co-culture (**Figure 6.2 & 6.3**). The proportion of CCR2 positive T cells remains static in allostimulated cells but dropped in T cells derived from autologous control co-cultures (**Figure 6.2, A**) and in contrast the proportion of CXCR4, CCR7 and CCR9 positive T cells decreases on allostimulation but remained static in cells from autologous co-cultures (**Figure 6.2, C, 6.3, A-B**). This indicated that the expression of CCR was modified by the degree to which the T cells are activated and stimulated rather than simply due to time spent in culture.

Both at baseline and on allostimulation the proportion of CCR7 positive cells was consistently higher in $CD4^+$ T cells than in $CD8^+$ T cells (**Figure 6.3, A**), while in contrast the proportion of CCR5 positive cells was consistently higher in $CD8^+$ T cells at baseline and after allostimulation (**Figure 6.2, B**).

Figure 6.2

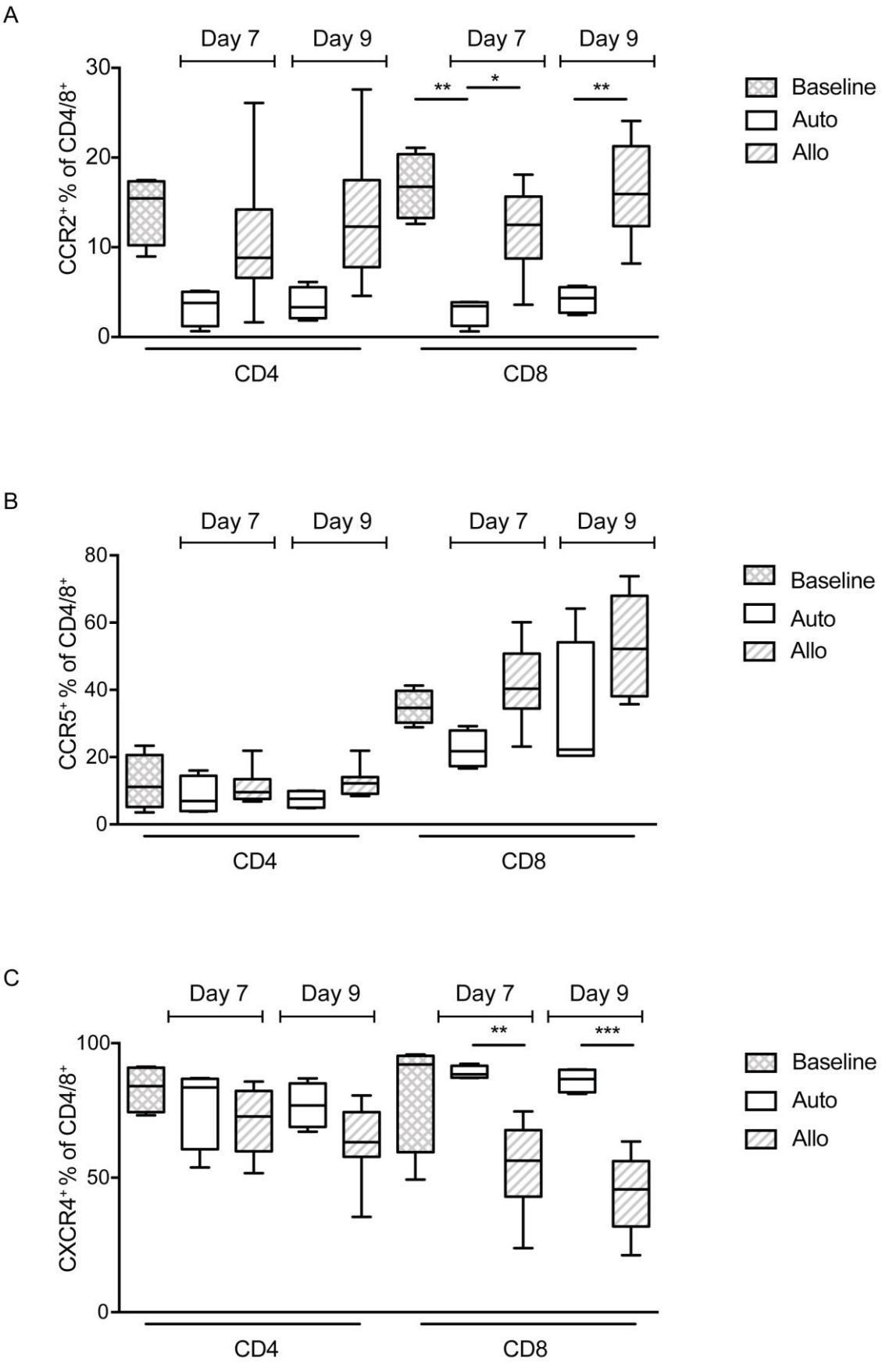

A
Day 7
Day 9
Day 7
Day 9
CCR2+ % of CD4/8+
30
20
10
0
**
*
**
CD4
CD8
Baseline
Auto
Allo
B
Day 7
Day 9
Day 7
Day 9
CCR5+ % of CD4/8+
80
60
40
20
0
CD4
CD8
Baseline
Auto
Allo
C
Day 7
Day 9
Day 7
Day 9
CXCR4+ % of CD4/8+
100
50
0
**
***
CD4
CD8
Baseline
Auto
Allo

Figure 6.2 Allostimulation alters frequencies of T cells expressing chemokine receptors

(A) Percentages of c-chemokine receptor 2 (CCR2) positive cells within $CD4^+$ and $CD8^+$ T cell subsets at baseline and after 7 and 9 days of allogeneic co-culture (Allo) or autologous control co-culture (Auto). Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 9 unique donor responder pairs. * = P<0.05, ** = P<0.01.

(B) Percentages of c-chemokine receptor 5 (CCR5) positive cells within $CD4^+$ and $CD8^+$ T cell subsets at baseline and after 7 and 9 days of allogeneic co-culture (Allo) or autologous control co-culture (Auto). Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 9 unique donor responder pairs.

(C) Percentages of cx-chemokine receptor 4 (CXCR4) positive cells within $CD4^+$ and $CD8^+$ T cell subsets at baseline and after 7 and 9 days of allogeneic co-culture (Allo) or autologous control co-culture (Auto). Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 9 unique donor responder pairs. ** = P<0.01, *** = P<0.001.

Figure 6.3

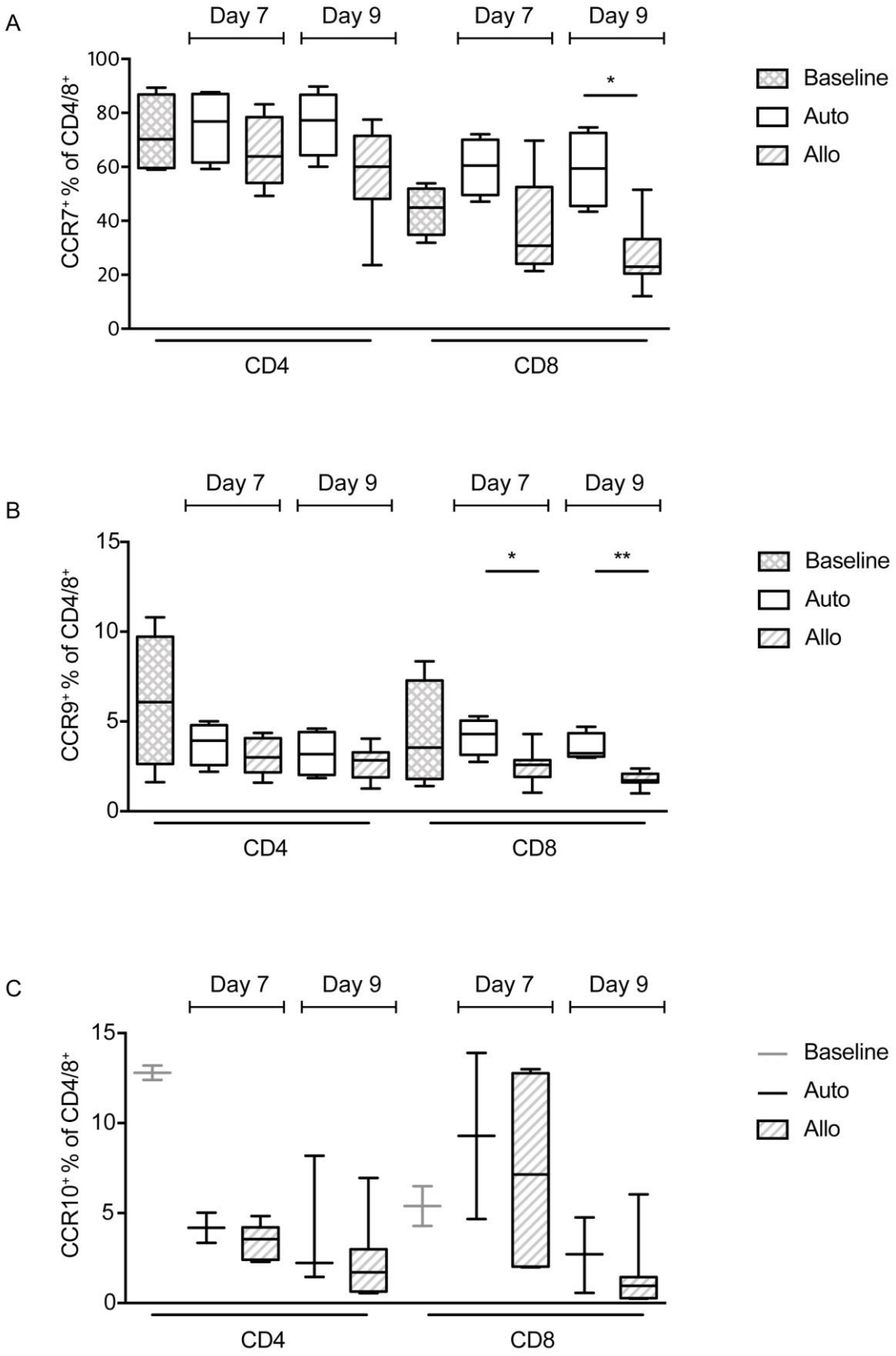

A
Day 7
Day 9
Day 7
Day 9
CCR7+ % of CD4/8+
100
80
60
40
20
0
*
CD4
CD8
Baseline
Auto
Allo
B
Day 7
Day 9
Day 7
Day 9
CCR9+ % of CD4/8+
15
10
5
0
*
**
CD4
CD8
Baseline
Auto
Allo
C
Day 7
Day 9
Day 7
Day 9
CCR10+ % of CD4/8+
15
10
5
0
CD4
CD8
Baseline
Auto
Allo

Figure 6.3 Allostimulation alters frequencies of T cells expressing chemokine receptors

(A) Percentages of c-chemokine receptor 7 (CCR7) positive cells within $CD4^+$ and $CD8^+$ T cell subsets at baseline and after 7 and 9 days of allogeneic co-culture (Allo) or autologous control co-culture (Auto). Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 9 unique donor responder pairs. * = $P<0.05$.

(B) Percentages of c-chemokine receptor 9 (CCR9) positive cells within $CD4^+$ and $CD8^+$ T cell subsets at baseline and after 7 and 9 days of allogeneic co-culture (Allo) or autologous control co-culture (Auto). Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 9 unique donor responder pairs. * = $P<0.05$, ** = $P<0.01$.

(C) Percentages of c-chemokine receptor 10 (CCR10) positive cells within $CD4^+$ and $CD8^+$ T cell subsets at baseline and after 7 and 9 days of allogeneic co-culture (Allo) or autologous control co-culture (Auto). Box (interquartile range, where there are 3 biological replicates) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 6 unique donor responder pairs.

6.4.2 Addition of lenalidomide to allogeneic co-culture causes additional changes in CCR2, CCR7 and CCR10 expression on alloproliferative CD8$^+$ T cells.

Taking into account that changes in the proportion of cells in a T cell population positive for a given CCR may reflect the proportion of those cells that have become activated and proliferative (as shown by changes in CCR expression on allostimulation vs autologous stimulation), and that lenalidomide causes a greater proportion of CD8$^+$ T cells to proliferate after allogeneic stimulus; to understand the effect of lenalidomide on CCR expression after allostimulation I next went on to concentrate on the expression of CCRs on alloproliferative CD4$^+$ and CD8$^+$ T cell subsets.

The greatest change in CCR expression after lenalidomide exposure was the significant up-regulation of CCR2 on both CD4$^+$ and CD8$^+$ alloproliferative T cells (23% (untreated) median compared to 34% (lenalidomide treated) and 20% to 36% respectively) (**Figure 6.4, A**). The proportion of CCR7$^+$ alloproliferative CD8$^+$ T cells was halved (4% (untreated) compared to median 2% (lenalidomide treated)) in keeping with my earlier finding that there is a decrease in naïve and CM (both CCR7$^+$ T cell subsets) cells after lenalidomide exposure (**Figure 6.5, A**).

The proportion of CCR10$^+$ cells was very small in CD8$^+$ alloproliferative cells. Exposure to lenalidomide halved the frequency of these cells from median 1% (untreated) to 0.4% median (lenalidomide treated) (**Figure 6.5, C**).

Figure 6.4

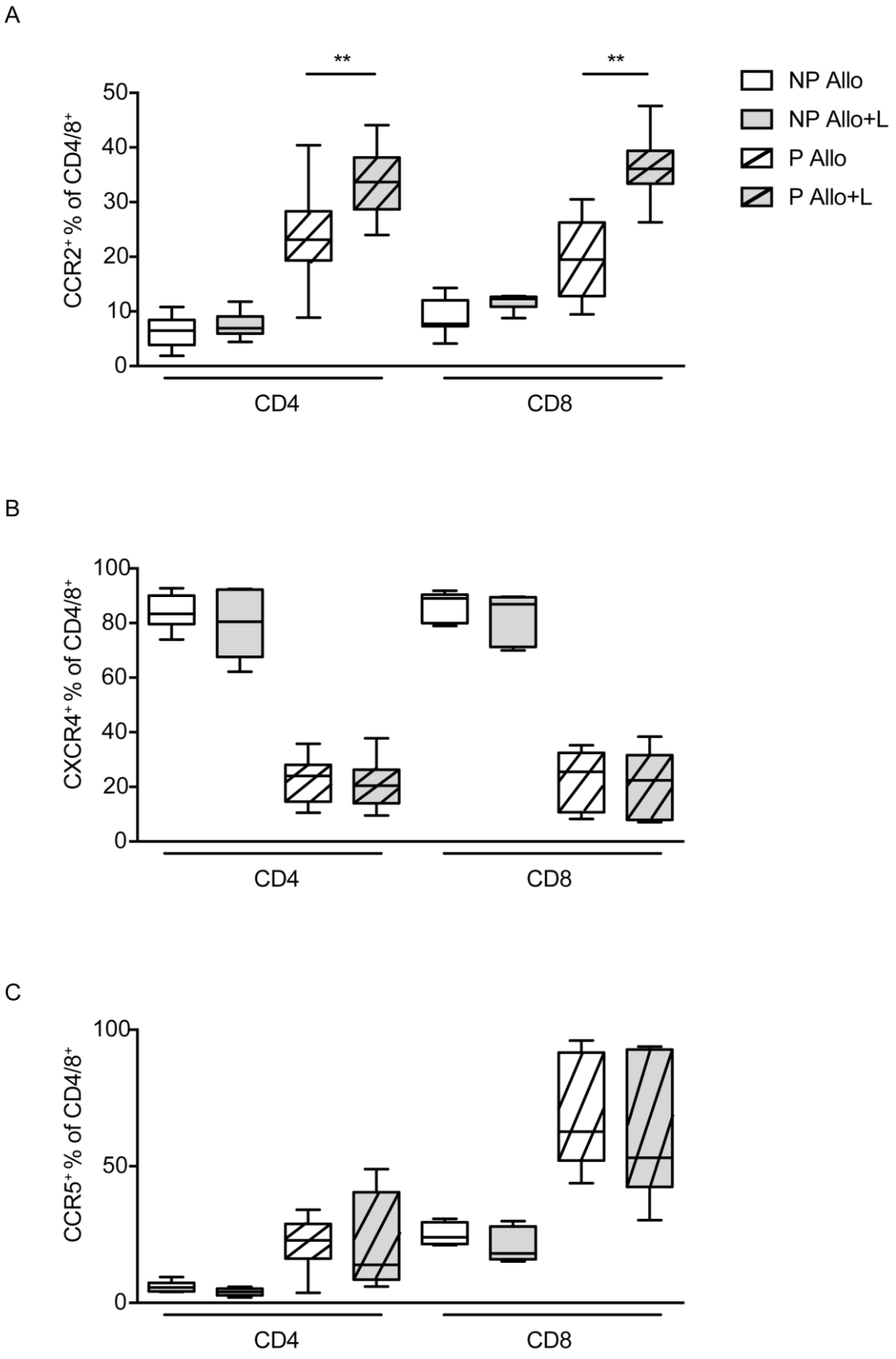

A
**
**
NP Allo
NP Allo+L
P Allo
P Allo+L
CCR2+ % of CD4/8+
50
40
30
20
10
0
CD4
CD8
B
CXCR4+ % of CD4/8+
100
80
60
40
20
0
CD4
CD8
C
CCR5+ % of CD4/8+
100
50
0
CD4
CD8

Figure 6.4 Allostimulation in the presence of lenalidomide leads to expansion of T cells expressing CCR2

(A) Percentages of c-chemokine receptor 2 (CCR2) positive cells within non-alloproliferative (NP) and alloproliferative (P) subsets of $CD4^+$ and $CD8^+$ T cells after 9 days of allogeneic co-culture in the absence (Allo) or presence of lenalidomde (Allo+L). Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 9 unique donor responder pairs. ** = $P<0.01$.

(B) Percentages of cx-chemokine receptor 4 (CXCR4) positive cells within non-alloproliferative (NP) and alloproliferative (P) subsets of $CD4^+$ and $CD8^+$ T cells after 9 days of allogeneic co-culture in the absence (Allo) or presence of lenalidomde (Allo+L). Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 9 unique donor responder pairs.

(C) Percentages of c-chemokine receptor 5 (CCR5) positive cells within non-alloproliferative (NP) and alloproliferative (P) subsets of $CD4^+$ and $CD8^+$ T cells after 9 days of allogeneic co-culture in the absence (Allo) or presence of lenalidomde (Allo+L). Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 9 unique donor responder pairs.

Figure 6.5

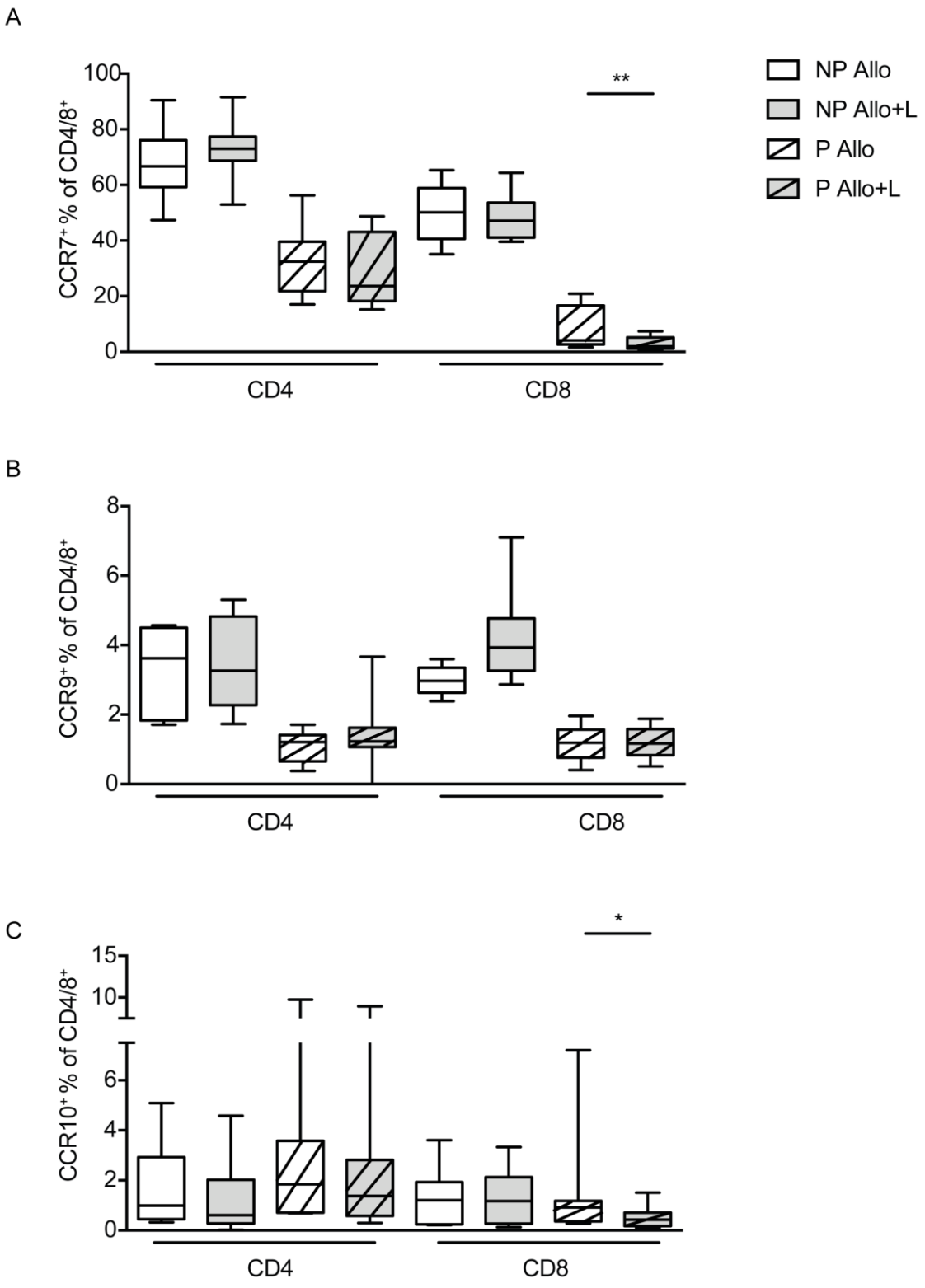

A
B
C
NP Allo
NP Allo+L
P Allo
P Allo+L
CCR7+ % of CD4/8+
CCR9+ % of CD4/8+
CCR10+ % of CD4/8+
CD4
CD8
**
*

Figure 6.5 Alloproliferation following lenalidomide exposure leads to decreased frequency of $CD8^+$ T cells expressing CCR7 and 10

(A) Percentages of c-chemokine receptor 7 (CCR7) positive cells within non-alloproliferative (NP) and alloproliferative (P) subsets of $CD4^+$ and $CD8^+$ T cells after 9 days of allogeneic co-culture in the absence (Allo) or presence of lenalidomde (Allo+L). Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 9 unique donor responder pairs. ** = $P<0.01$.

(B) Percentages of c-chemokine receptor 9 (CCR9) positive cells within non-alloproliferative (NP) and alloproliferative (P) subsets of $CD4^+$ and $CD8^+$ T cells after 9 days of allogeneic co-culture in the absence (Allo) or presence of lenalidomde (Allo+L). Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 9 unique donor responder pairs.

(C) Percentages of c-chemokine receptor 10 (CCR10) positive cells within non-alloproliferative (NP) and alloproliferative (P) subsets of $CD4^+$ and $CD8^+$ T cells after 9 days of allogeneic co-culture in the absence (Allo) or presence of lenalidomde (Allo+L). Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 6 unique donor responder pairs. * = $P<0.05$.

6.4.3 Addition of lenalidomide to allogeneic co-culture increases the frequency of alloproliferative $CD8^+$ T cells co-expressing CCR2 and CCR5.

I examined co-expression patterns of CCR and whether these were affected by lenalidomide exposure during allostimulation. The only CCR that were consistently co-expressed, and of which the co-expression was significantly altered by exposure to lenalidomide were CCR2 and CCR5. The proportion of $CCR2^+CCR5^+$ $CD4^+$ T cells was significantly higher in alloproliferative than non-alloproliferative cells, but the addition of lenalidomide did not increase the proportion of positive cells further. However, lenalidomide treatment significantly increased the proportion of $CD8^+$ alloproliferative T cells showed (median 16% (untreated) compared to 30% (lenalidomide treated)) co-expressing CCR2 and CCR5 (**Figure 6.6, B**).

Figure 6.6

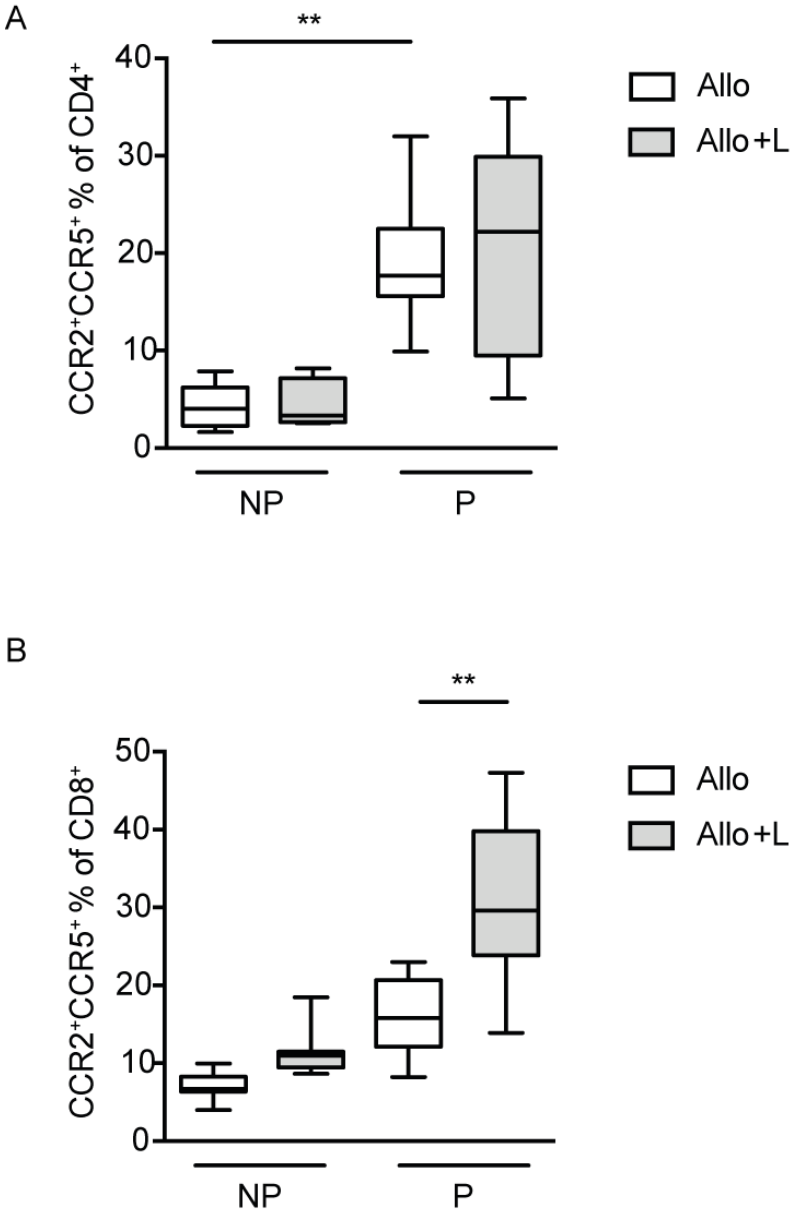

A
**
CCR2+CCR5+ % of CD4+
40
30
20
10
0
NP
P
Allo
Allo+L
B
**
CCR2+CCR5+ % of CD8+
50
40
30
20
10
0
NP
P
Allo
Allo+L

Figure 6.6 Allostimulation in the presence of lenalidomide leads to expansion of T cells co-expressing CCR2 and CCR5

(A) Percentages of $CD4^+$ T cells co-expressing c-chemokine receptor 2 (CCR2) and c-chemokine receptor 5 (CCR5) within non-alloproliferative (NP) and alloproliferative (P) subsets after 9 days of allogeneic co-culture in the absence (Allo) or presence of lenalidomide (Allo+L). Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 9 unique donor responder pairs. ** = $P<0.01$.

(B) Percentages of $CD8^+$ T cells co-expressing c-chemokine receptor 2 (CCR2) and c-chemokine receptor 5 (CCR5) within non-alloproliferative (NP) and alloproliferative (P) subsets after 9 days of allogeneic co-culture in the absence (Allo) or presence of lenalidomide (Allo+L). Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 9 unique donor responder pairs. ** = $P<0.01$.

6.4.4 Transwell migration of alloresponder PBMC to CCL2 is not significantly altered after allostimulation in the presence of lenalidomide.

Having examined CCR expression on T cells after allogeneic co-culture and determined that the greatest effect of lenalidomide treatment was to increase the proportion of $CCR2^+$ alloproliferative T cells, I next aimed to assess whether this change led to any functional change in the ability of allostimulated T cells to migrate in response to CCL2. CCL2 is an important chemokine in the pathogenesis of GvHD, directing T cells towards inflamed tissues where donor T cells encounter activated host-APC. As a comparison I also chose to examine migration to CXCL12 as a chemokine that mediates migration to an important site of GvT, the bone marrow.

Using a transwell system I first determined optimal concentrations of chemokine to achieve migration of PBMC. Migration of healthy steady state PBMC of approx 50% was achieved at CCL2 and CXCL12 concentrations of 100ng/ml, this concentration was used for all further experiments (**Figure 6.7, A).**

Next migration was determined for cells that had undergone 9 days of allostimulation in the presence or absence of lenalidomide. As expected there was a low level of basal migration (12%) through the transwell in the absence of any chemokine. Migration of both autologous responder and alloresponder PBMC in the presence of CCL2 and CXCL12 was increased over the basal level, however there was no significant difference in the migratory index of responder PBMC from lenalidomide treated co-cultures compared to untreated co-cultures (**Figure 6.7, B-C**).

Figure 6.7

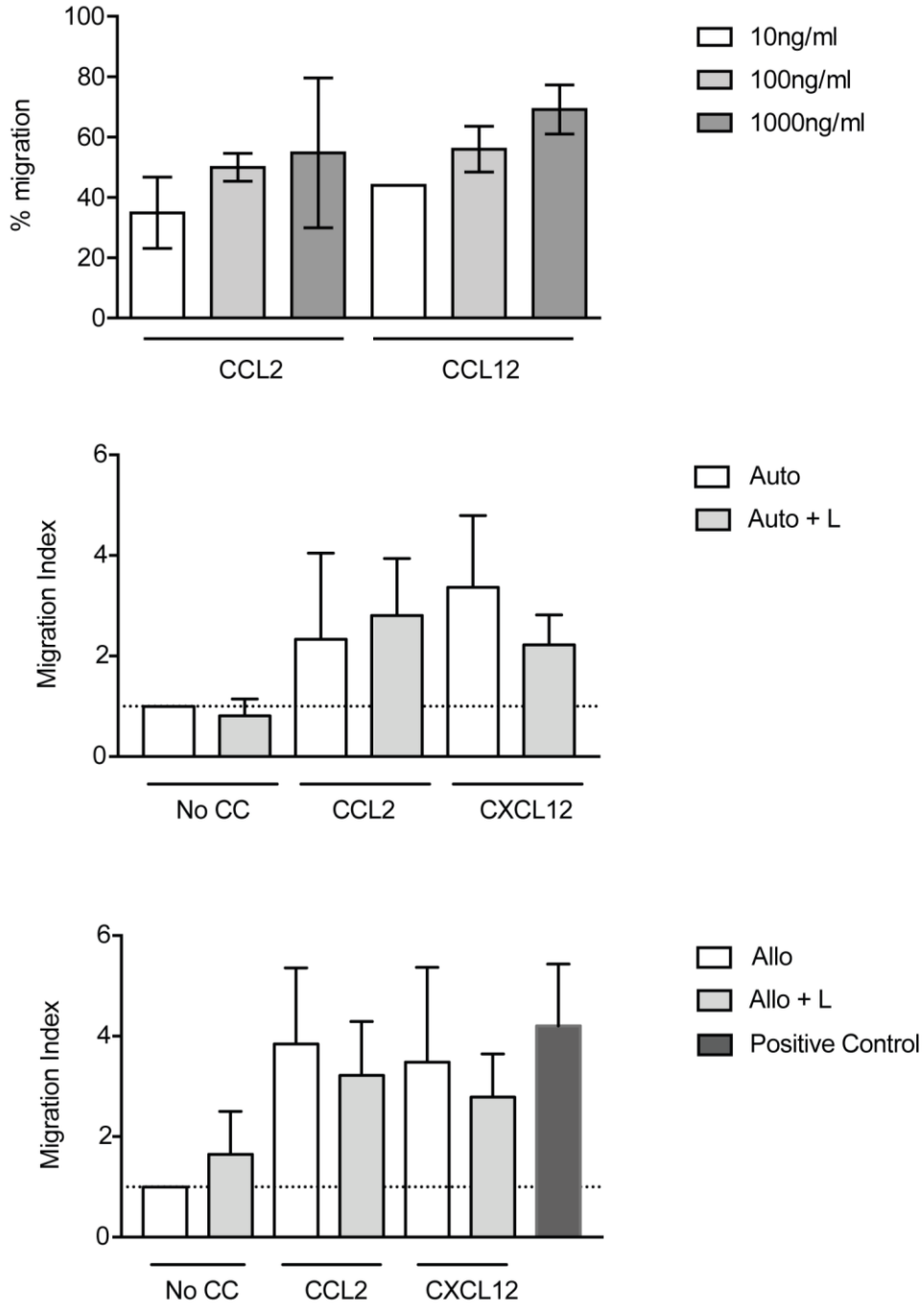

100
80
60
40
20
0
% migration
CCL2
CCL12
10ng/ml
100ng/ml
1000ng/ml
6
4
2
0
Migration Index
No CC
CCL2
CXCL12
Auto
Auto + L
6
4
2
0
Migration Index
No CC
CCL2
CXCL12
Allo
Allo + L
Positive Control

Figure 6.7 Allostimulation in the presence of lenalidomide does not significantly alter chemokine driven migration of alloresponder PBMC

(A) Mean (+/-SD) % transwell migration of 4 healthy donor PBMC in response to increasing concentrations of CCL2 or CXCL12.

(B) Mean (+/-SD) migration index (% migration in presence of chemokine/% migration in absence of chemokine) of autologous PBMC after 9 days of autologous co-culture in the absence (Auto) or presence (Auto+L) of lenalidomide. Results are shown for 3 unique autologous co-cultures. Dotted line represents migration of comparator group i.e. Untreated autologous responder cells in the absence of chemokine.

(C) Mean (+/-SD) migration index (% migration in presence of chemokine/% migration in absence of chemokine) of allogeneic responder PBMC after 9 days of allogeneic co-culture in the absence (Allo) or presence (Allo+L) of lenalidomide. Results are shown for 9 unique donor-responder pairs and 2 unique positive controls. Dotted line represents migration of comparator group i.e. Untreated allogeneic responder cells in the absence of chemokine. Positive control = CXCL12 2µg/ml stimulated freshly thawed PBMC.

Absence of an increase in migration to CCL2 in particular is perhaps surprising given that I have shown the proportion of $CCR2^+$ $CD4^+$ and $CCR2^+CD8^+$ T cells in this population to be increased after lenalidomide treatment. However as these experiments used bulk PBMC responders it is possible that lenalidomide may be negatively impacting on migration of other cell types, such as myeloid cells leading to an overall reduction in PBMC migration. In order to answer this question, cells that had migrated into the lower well of the transwell plate (migrated cells) were collected and interrogated by FACS. If migration of non-T cells were negatively impacted by lenalidomide they would be retained in the upper chamber and therefore the relative proportion of T cells in the lower chamber would be increased. There was no difference in the frequency of T cells, between the upper chamber and that in the lower chamber of the transwell plate in either treated or untreated co-cultures in any condition, indicating that there does not appear to be either a negative impact on non-T cells or a positive impact on T cell migration following lenalidomide treatment. (**Figure 6.8, A**).

Lenalidomide treatment leads to a proportional increase in activated T cells that are morphologically larger than their non-proliferative counterparts. These may have more difficulty transitioning through the pores of the transwell plate. These activated cells may also be more adherent and therefore are retained in the upper chamber? If this were the case then there would be proportionally fewer $CFSE^{dim}$ cells in the lower chamber than the upper chamber of the transwell plate. The frequency of $CFSE^{dim}$ cells was the same in both the upper and lower chambers of the transwell plate indicating that this is not the cause for the reduced migration of lenalidomide-treated cells (**Figure 6.8, B-D**).

Figure 6.8

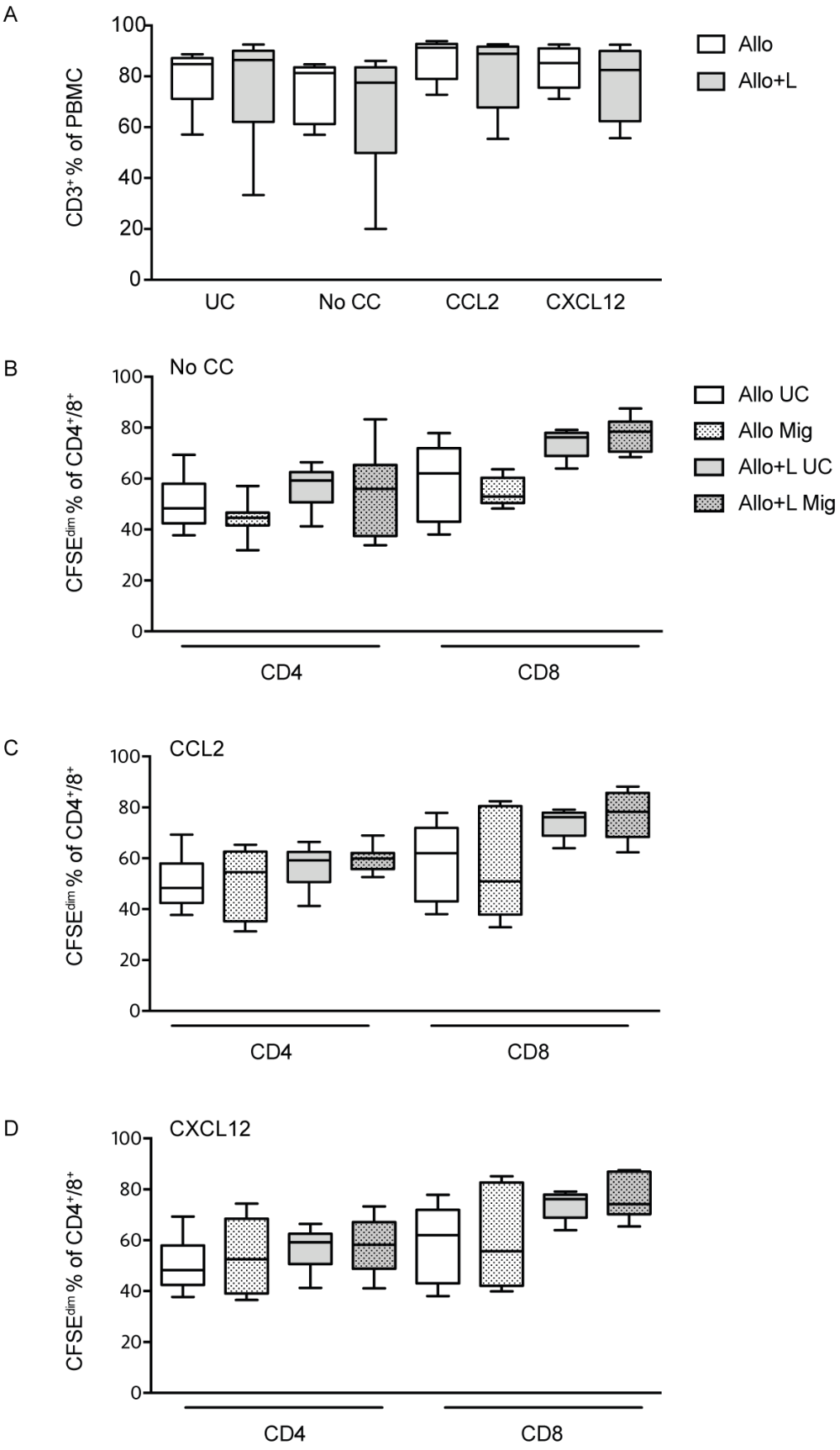

A
CD3+ % of PBMC
100
80
60
40
20
0
UC
No CC
CCL2
CXCL12
Allo
Allo+L
B
No CC
CFSEdim % of CD4+/8+
CD4
CD8
Allo UC
Allo Mig
Allo+L UC
Allo+L Mig
C
CCL2
D
CXCL12

Figure 6.8 Alloproliferative cells exposed to lenalidomide are able to migrate from the upper chamber to the lower chamber of the transwell plate

(A) Percentages of T cells within alloresponder PBMC after 9 days of allogeneic co-cultures in the absence (Allo) or presence of lenalidomide (Allo+L) in the upper chamber (UC) and lower chamber of the transwell plate in basal conditions (no chemokine (CC)) and stimulated by CCL2 or CXCL12. Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 9 unique donor responder pairs.

Percentages of alloproliferative ($CFSE^{dim}$) $CD4^+$ and $CD8^+$ T cells within alloresponder PBMC after 9 days of allogeneic co-cultures in the absence (Allo) or presence of lenalidomide (Allo+L) in the upper chamber (UC) and lower chamber (Mig) of the transwell plate in basal conditions (no chemokine (CC)) **(B)** and stimulated by CCL2 **(C)** or CXCL12 **(D)**. Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 9 unique donor responder pairs.

6.4.5 Allostimulation in the presence of lenalidomide alters expression of integrin VLA2

Integrins are a family of surface proteins that contribute to and control T cell migration [88]. I next examined whether allostimulation in the presence of absence of lenalidomide could impact on the expression of integrins on T cells in a way that could affect T cell migration. I first chose to examine the expression of very late antigen 2 (VLA2), following the observation that the gene expression of the alpha subunit of this integrin was in the top 10 significantly up/down-regulated genes in my gene expression data comparing treated alloproliferative cells compared to treated non-proliferative $CD8^+$ T cells (**Chapter 5**).

In both $CD4^+$ and $CD8^+$ T cells the proportion of cells expressing VLA2 was increased in alloproliferative cells compared to non-alloproliferative cells, but importantly allostimulation in the presence of lenalidomide led to a significantly increased frequency of $VLA2^+$ $CD4^+$ and $CD8^+$ T cells in alloproliferative subsets. There was a 2-fold increase in the proportion of $VLA2^+$ alloproliferative $CD4^+$cells and near 3-fold increase in the proportion of $VLA2^+$ alloproliferative $CD8^+$ cells (**Figure 6.9, C-D**).

I then assessed expression of lymphocyte function-associated antigen 1 (LFA1), as this is the major T cell integrin involved in T cell-endothelial and T cell-APC interactions. The proportion of $LFA1^+$ $CD4^+$ and $CD8^+$ T cells was high (median >80%) in both non-alloproliferative and alloproliferative cells following allostimulation. In the case of $CD4^+$ cells there was no change in the proportion of positive cells depending on proliferation status of the cells, whereas in the $CD8^+$ subset the proportion of $LFA1^+$ cells was significantly increased in alloproliferative cells, although lenalidomide exposure had no additional impact (**Figure 6.9, A-B**).

Figure 6.9

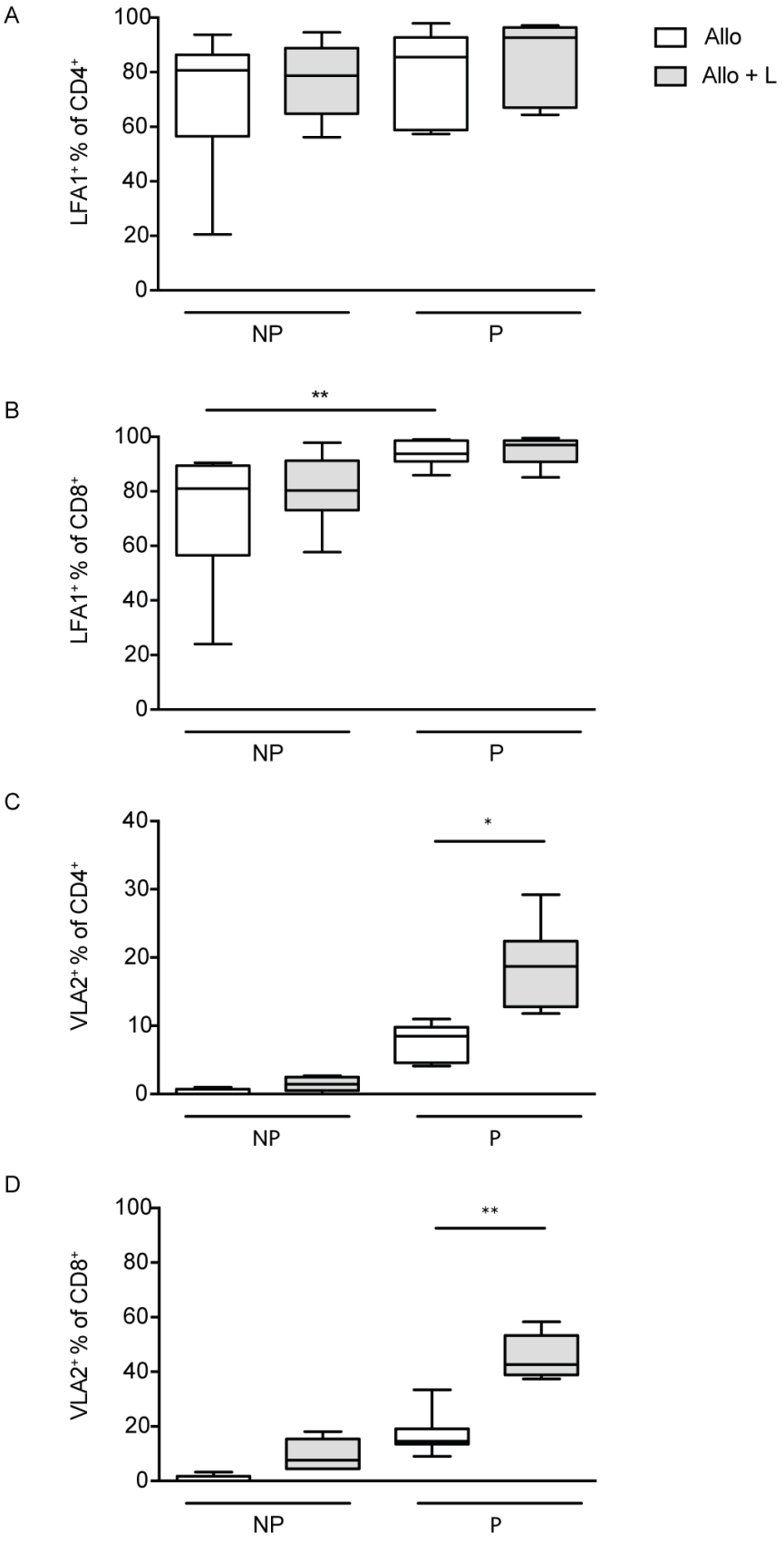

A
B
C
D
Allo
Allo + L
LFA1+ % of CD4+
LFA1+ % of CD8+
VLA2+ % of CD4+
VLA2+ % of CD8+
NP
P
**
*
**

Figure 6.9 Allostimulation in the presence of lenalidomide leads to increased frequency of alloproliferative T cells expressing VLA2

(A) Percentages of leukocyte functional antigen 1 (LFA1) positive $CD4^+$ T cells in non-alloproliferative (NP) and alloproliferative (P) subsets after 9 days of allogeneic co-culture in the absence (Allo) or presence of lenalidomide (Allo+L). Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 9 unique donor-responder pairs.

(B) Percentages of leukocyte functional antigen 1 (LFA1) positive $CD8^+$ T cells in non-alloproliferative (NP) and alloproliferative (P) subsets after 9 days of allogeneic co-culture in the absence (Allo) or presence of lenalidomide (Allo+L). Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 9 unique donor-responder pairs. ** P <0.01.

(C) Percentages of very late activation antigen (VLA2) positive $CD4^+$ T cells in non-alloproliferative (NP) and alloproliferative (P) subsets after 9 days of allogeneic co-cuture in the absence (Allo) or presence of lenalidomide (Allo+L). Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 9 unique donor-responder pairs. * P = <0.05.

(D) Percentages of very late activation antigen (VLA2) positive $CD8^+$ T cells in non-alloproliferative (NP) and alloproliferative (P) subsets after 9 days of allogeneic co-cuture in the absence (Allo) or presence of lenalidomide (Allo+L). Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 9 unique donor-responder pairs. ** = P<0.01.

Other work from our group has shown that lenalidomide treatment was able to confer greater activity to LFA1 on T cells from CLL patients by inducing a conformational-change to a more active 'extended' form. I therefore finally assessed the expression of extended-LFA1 on cells allostimulated in the presence or absence of lenalidomide. Surprisingly I found that the proportion of $CD4^+$ T cells expressing extended-LFA1 was very low (mean less than 0.5%) after allostimulation in the presence or absence of lenalidomide. The proportion was higher in $CD8^+$ T cells, but was still a fraction of the number of cells that stained positive for the presence of the integrin (**Figure 6.10, A-B**). Also unexpected was the finding that the proportion of T cells staining positively for extended-LFA1 appeared to be reduced in cells allostimulated in the presence of lenalidomide compared to controls. Additionally on a per-cell basis lenalidomide treatment appeared to reduce extended-LFA1 expression (measured by median fluorescence intensity, **Figure 6.10, C**). Further samples are required to confirm these findings as the number of unique donor-responder pairs examined was limited (n=3).

Figure 6.10

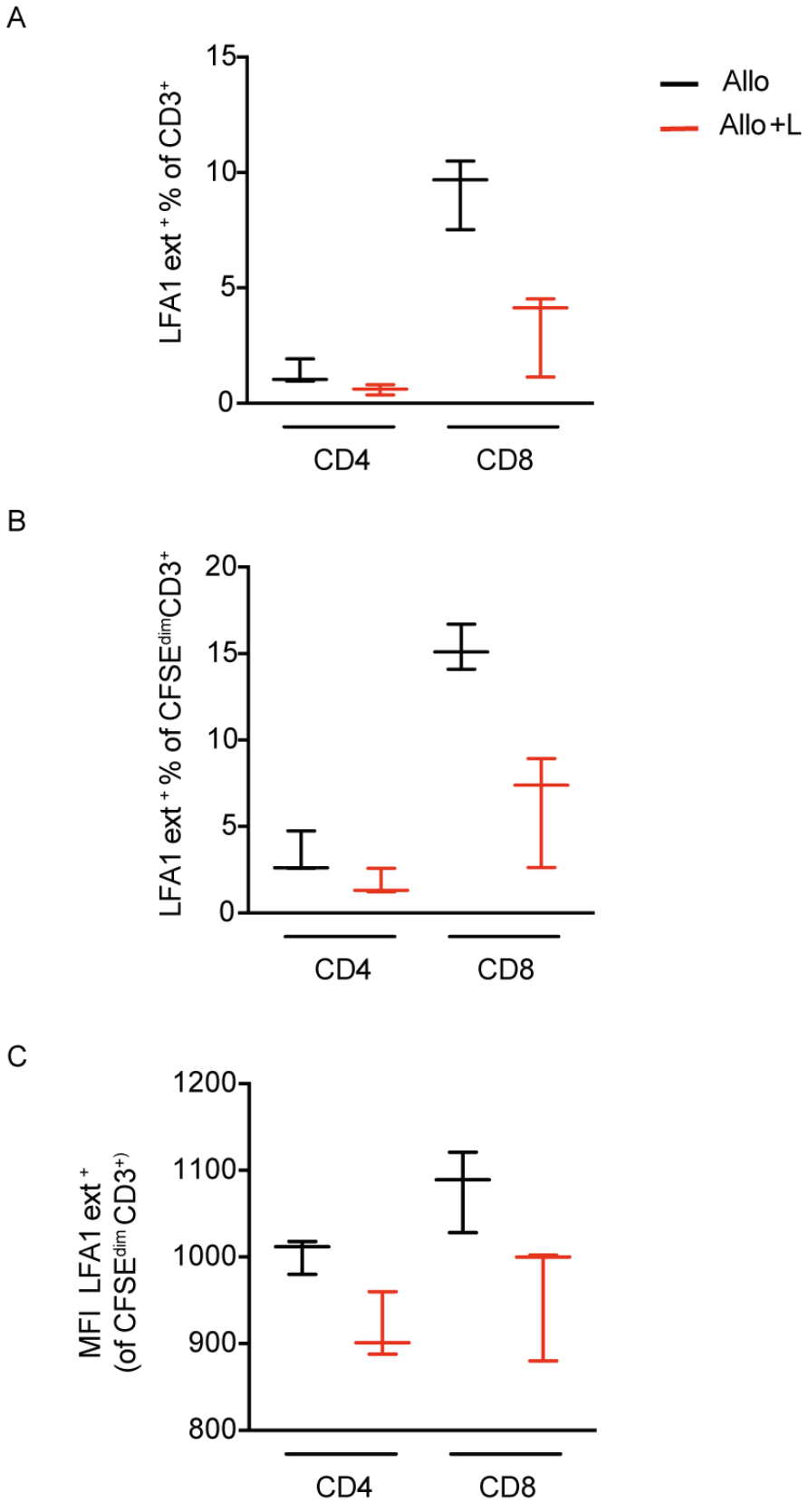

A
15
10
5
0
LFA1 ext + % of CD3+
Allo
Allo +L
CD4
CD8
B
20
15
10
5
0
LFA1 ext + % of CFSEdimCD3+
CD4
CD8
C
1200
1100
1000
900
800
MFI LFA1 ext +
(of CFSEdim CD3+)
CD4
CD8

Figure 6.10 Allostimulation in the presence of lenalidomide leads to decreased expression of extended LFA1 on alloesponder T cells

(A) Percentages of $CD4^+$ and $CD8^+$ T cells expressing extended LFA1 after 9 days of allogeneic co-culture in the absence (Allo) or presence of lenalidomide (Allo+L). Line and whisker (maximum and minimum) plots are shown. Horizontal lines represent medians. Results are shown for 3 unique donor responder pairs.

(B) Percentages of alloproliferative $CD4^+$ and $CD8^+$ T cells expressing extended LFA1 after 9 days of allogeneic co-culture in the absence (Allo) or presence of lenalidomide (Allo+L). Line and whisker (maximum and minimum) plots are shown. Horizontal lines represent medians. Results are shown for 3 unique donor responder pairs.

(C) Expression (median fluorescence intensity) of extended LFA1 on alloproliferative $CD4^+$ and $CD8^+$ T cells after 9 days of allogeneic co-culture in the absence (Allo) or presence of lenalidomide (Allo+L). Line and whisker (maximum and minimum) plots are shown. Horizontal lines represent medians. Results are shown for 3 unique donor responder pairs.

6.4.6 Chemokinesis of alloresponder T cells is not significantly altered after allostimulation in the presence of lenalidomide.

It has been shown that lenalidomide is capable of 'repairing' migratory defects in T cells induced by contact with CLL cells [379]. In experiments using time-lapse microscopy investigators were able to demonstrate that healthy T cells co-cultured with CLL cells had reduced motility compared to cells co-cultured with healthy B cells, and that lenalidomide treatment could restore motility to normal levels. The experiment used slides coated in ICAM1 and media containing CXCL12. Although this system doesn't assess directional migration (as in the transwell assay), the addition of ICAM1 has the advantage of assessing two aspects of T cell migration, integrin-dependent as well as chemokine stimulated movement.

For these experiments T cells were purified from allogeneic co-cultures at day 9 prior to incubation on ICAM1-coated plates and acquisition of images. I could detect no significant difference in the chemokinesis of lenalidomide-treated T cells (compared to untreated), in response to ICAM1 alone, in the presence of CCL2 or in the presence of CXCL12 (**Figure 6.11, A-C)**. It is uncertain as to whether a change in the amount of extended-LFA1 present on lenalidomide treated cells had impacted on the results of these experiments.

Figure 6.11

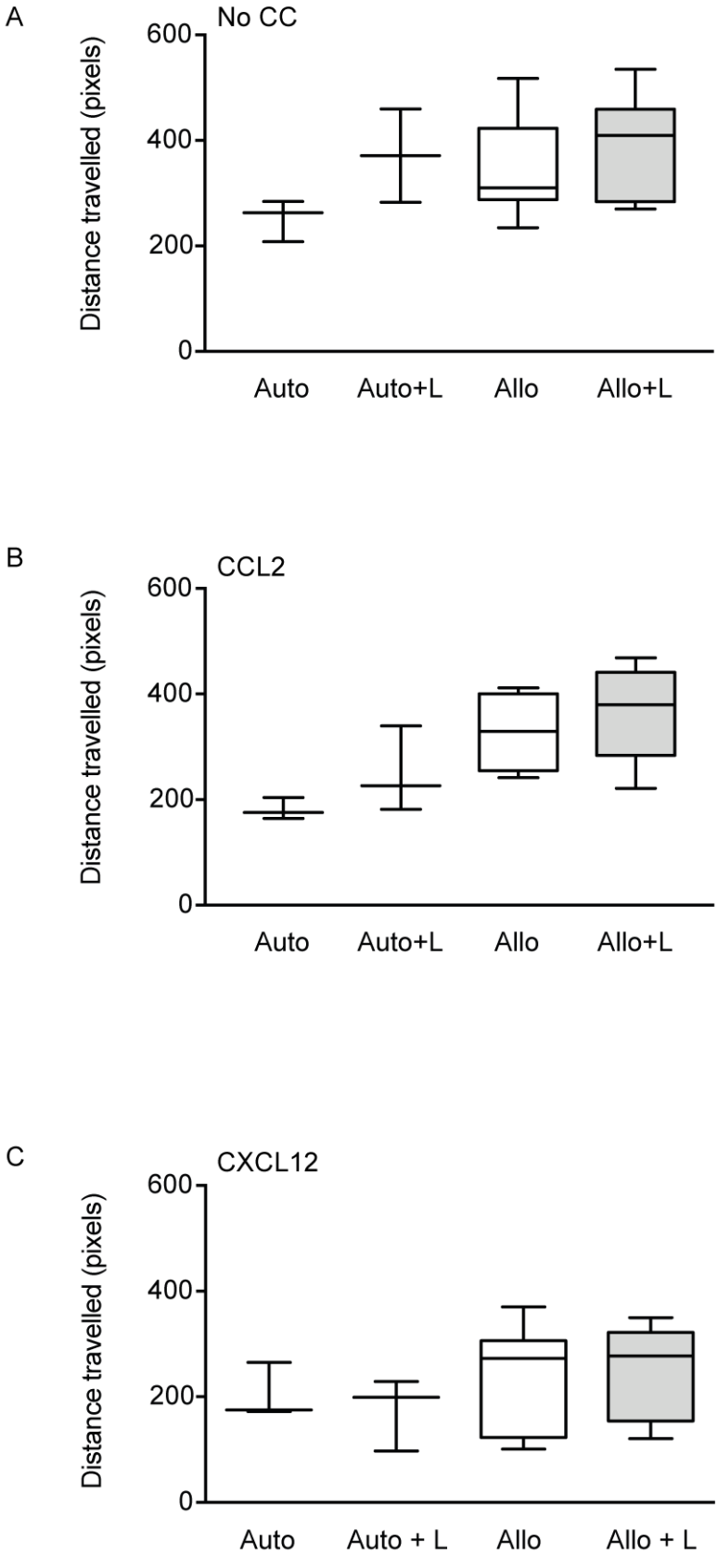

A
No CC
Distance travelled (pixels)
600
400
200
0
Auto
Auto+L
Allo
Allo+L
B
CCL2
Distance travelled (pixels)
600
400
200
0
Auto
Auto+L
Allo
Allo+L
C
CXCL12
Distance travelled (pixels)
600
400
200
0
Auto
Auto + L
Allo
Allo + L

Figure 6.11 Allostimulation in the presence of lenalidomide does not affect chemokine driven migration in response to ICAM1

(A) Distance travelled by alloresponder T cells on intercellular adhesion molecule 1 (ICAM1) after 9 days of autologous or allogeneic co-culture in the absence (Auto/Allo) or presence of lenalidomide (Auto+L/Allo+L). Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 9 unique donor-responder pairs and their corresponding 3 autologous controls.
(B) Distance travelled by alloresponder T cells on intercellular adhesion molecule 1 (ICAM1) in the presence of CCL2 after 9 days of autologous or allogeneic co-culture in the absence (Auto/Allo) or presence of lenalidomide (Auto+L/Allo+L). Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 9 unique donor-responder pairs and their corresponding 3 autologous controls.
(C) Distance travelled by alloresponder T cells on intercellular adhesion molecule 1 (ICAM1) in the presence of CXCL12 after 9 days of autologous or allogeneic co-culture in the absence (Auto/Allo) or presence of lenalidomide (Auto+L/Allo+L). Box (interquartile range) and whisker (maximum and minimum) plots are shown. Horizontal lines are medians. Results are shown for 9 unique donor-responder pairs and their corresponding 3 autologous controls.

6.4.7 Lenalidomide treated CD8$^+$ alloresponder T cells demonstrate increased cytotoxicity against allogeneic haematopoietic targets

Having found no evidence that lenalidomide treatment impacted on alloresponder T cell migratory capacity in a way that might differentially direct T cells to sites of GvHD or GvT, I next went on to assess a functional parameter that could provide further insight into the capacity of lenalidomide-treated cells to exert GvT effects. PBMC from donors used as stimulators in allogeneic co-culture were incubated with PHA-L and IL2 to generate PHA-blasts for use as haematopoietic targets in a flow based cytotoxicity assay. The up-regulation of markers indicating 'blast' formation, CD25, CD45RO, CD69 and HLA-DR [380] was seen after 5 days of culture (**Figure 6.12, A**).

I first examined whether T cells from allogeneic co-cultures treated with lenalidomide induced greater target cell apoptosis than those from untreated co-cultures. The % target cell apoptosis was calculated as:

% apoptosis of allogeneic target cells when co-cultured with effector cells - % apoptosis of target cells when cultured alone.

I observed a decrease in % apoptosis of autologous target cells when co-cultured with increasing numbers of effectors from lenalidomide treated and untreated autologous co-cultures (**Figure 6.12, B**). This would indicate that the cells were in some way protected from apoptosis, this may be due to the lenalidomide or possibly simply due to the targets being protected when cultured together with greater numbers of cells.

This may also explain why there was also a decrease in allogeneic target cell apoptosis when co-cultured with increasing numbers of effectors from untreated allogeneic co-cultures, whereas one would expect an increase in apoptosis. In contrast where effectors were from lenalidomide treated co-cultures a gradual increase in % target apoptosis was seen as the ratio of effectors to targets increased, although this did not reach statistical significance it suggested that there was likely to be an increase in cytotoxicity exerted by lenalidomide treated T cells.

I therefore went on to purify CD8$^+$ alloresponder T cells from allogeneic co-cultures for use as effector cells. I chose to use CD8$^+$ T cells alone as this is the cellular subset that I would expect

to exert greater cytotoxic effects against haematopoietic targets and secondly as this is the subset of cells in which I observed an increased in the frequency of CD107a expression and greater polyfunctional cytokine capacity. Therefore by removing the $CD4^+$ T cells it should be possible to more easily resolve any change in target cell apoptosis. Indeed when purified $CD8^+$ T cells from lenalidomide treated and untreated co-cultures were used as effectors a gradual increase in cytotoxicity was observed as the ratio of effector to target cells increased. Importantly $CD8^+$ T cells from lenalidomide treated co-cultures demonstrated a greater degree of target cell killing compared to cells from untreated co-cultures at a 10:1 and 20:1 ratio of effectors to targets (**Figure 6.12, C**).

Figure 6.12

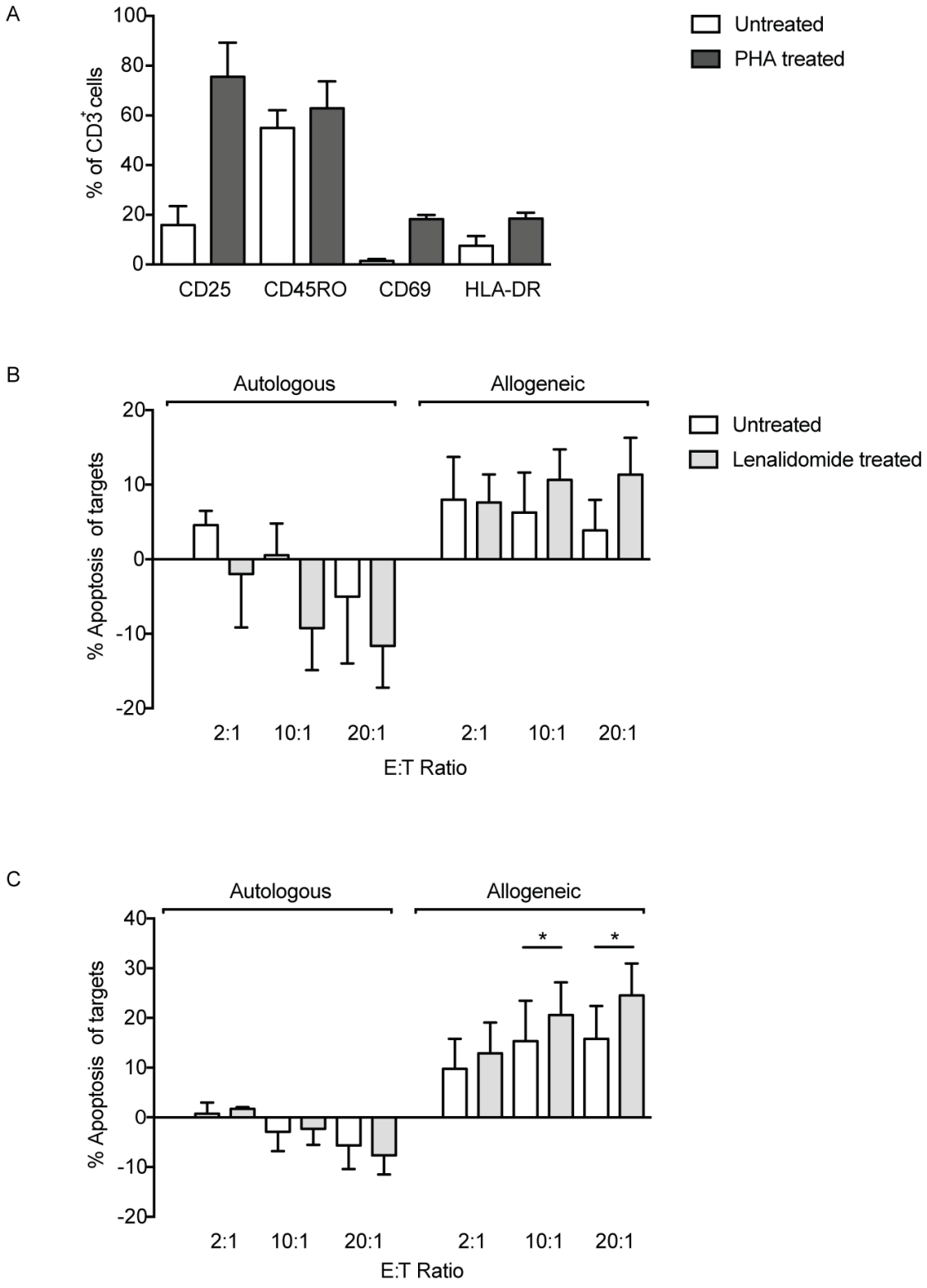

A
100
80
60
40
20
0
% of CD3+ cells
CD25
CD45RO
CD69
HLA-DR
Untreated
PHA treated
B
Autologous
Allogeneic
20
10
0
-10
-20
% Apoptosis of targets
2:1
10:1
20:1
2:1
10:1
20:1
E:T Ratio
Untreated
Lenalidomide treated
C
Autologous
Allogeneic
40
30
20
10
0
-10
-20
% Apoptosis of targets
*
*
2:1
10:1
20:1
2:1
10:1
20:1
E:T Ratio

Figure 6.12 Alloresponder $CD8^+$ T cells exposed to lenalidomide exert increased cytotoxicity against allogeneic haematopoietic targets

(A) Treatment of healthy PBMC with interleukin 2 (IL2) and phytohaemaglutinin (PHA) leads to increased expression of CD25, CD45RO, CD69 and HLA-DR after 5 days in line with published literature. Mean (+/- SEM) are shown for 4 unique healthy donors.

(B) Mean (+/- SD) percentage apoptosis of autologous and allogeneic target cells (T) in co-culture with increasing ratios of effector T cells (E) from lenalidomide treated or untreated autologous and allogeneic co-cultures at day 9. Results are shown for 9 unique donor-responder pairs.

(C) Mean (+/- SD) percentage apoptosis of autologous and allogeneic target cells (T) in co-culture with increasing ratios of effector $CD8^+$ T cells (E) from lenalidomide treated or untreated autologous and allogeneic co-cultures at day 9. Results are shown for 9 unique donor-responder pairs. * = $P<0.05$.

6.5 Discussion

The separation of GvT and GvHD by modulation of organ specific migration could have many advantages, not least that other aspects of T cell function important for GvT could be preserved. As with other aspects of the alloresponse, it was unknown whether the use of lenalidomide after AHSCT impacts on the migratory capacity of T cells. This could be anticipated in view of changes in cytoskeletal and migratory proteins observed in autologous T cell responses [186,209,379].

The experiments detailed in this chapter demonstrate that although lenalidomide-treatment alters frequencies of alloproliferative T cells expressing particular CCR, it does not appear to do this in a pattern that would be expected to confer organ-specific migratory capacity. Lenalidomide treatment also did not significantly impact functional assays of T cell migration to chemokines demonstrated to be important for directing donor T cells to sites of GvHD (CCL2) or to sites of GvT (CXCL12). This would suggest that potentiation of migration is not a contributor to the increased GvHD observed following clinical use of lenalidomide after AHSCT. The findings may also explain why the GvHD observed does not appear to target any one specific organ system.

Additionally these experiments also demonstrate that a statistically significant increase in the frequency of a subset of CCR positive cells does not necessarily equate to a change in the functional chemotaxis of a mixed population of cells. The transwell assays performed could be repeated with either purified T cells or purified $CD8^+$ T cells to increase the possibility of detecting a change in the subset specific migratory capacity of these cells. However given that there was no difference observed in the time-lapse microscopy assays, in which isolated T cells were used I am not sure that this would provide additional useful information.

The functional capacity of cells to respond to CCL2 was the focus of the functional experiments, as there was a significant increase in the frequency of CCR2 positive cells. However it is possible that while the frequency of cells expressing other CCR (i.e. CCR9) doesn't change this does not necessarily equate to no difference in function. It is possible that the responses of these cells to chemokine could be either enhanced or repressed by factors other than the degree of receptor expression. Therefore it might still be informative to assess functional migratory responses to chemokines such as CCR9 and CCR10 that direct migration specifically to the gut and skin, important sites of acute GvHD.

The co-culture system used did not include addition of cytokines, chemokines or other pro-inflammatory stimuli, with the aim of maximising the ability to resolve changes in the alloresponse due to lenalidomide treatment from changes in T cell responses to external stimuli. These have already been assessed in other settings (LPS, SEE, PMA, Ionomycin, IL2). However *in vivo* the levels of these cytokines and chemokines will impact on CCR expression and migratory responses. Therefore, an assessment of CCR expression changes or migration in the presence of LPS may provide additional information on lenalidomide treated alloresponses in the pro-inflammatory environment that occurs following AHSCT.

The marked expansion of a $CCR2^+CCR5^+$ population of T cells (without a corresponding increase in motility) is interesting. CCR2 and CCR5 have been described as markers of activation and effector differentiation as well as having roles in survival of T cells in addition to their roles as chemokine receptors. Individually CCR2 has been implicated in preventing apoptosis of activated $CD8^+$ T cells during the normal contraction phase of the immune response to viruses, leading to a prolonged effector response and greater proliferation of responder $CD8^+$ T cells [381], which is in agreement with changes observed in the lenalidomide treated alloresponse. CCR5 has been shown to be preferentially expressed on Th1 cells versus Th2 and therefore may play a role in differentiation towards type 1 responses [382], however the expression of this chemokine alone on alloresponder cells was not altered by lenalidomide exposure. The co-expression of CCR2 and CCR5 is also associated with differentiation of $CD4^+$ cells from naïve through to CM and EM phenotype and with acquisition of enhanced cytokine secretion and rapid recall responses to antigens [383]. The same has not been demonstrated for $CD8^+$ T cells but the experimental findings support this role in this subset. Therefore while lenalidomide modulation of CCR in the alloresponse may not impact on migration of cells, this is further evidence that lenalidomide enhances effector differentiation of alloresponder T cells.

The enhanced alloresponder $CD8^+$ effector phenotype I observed after lenalidomide-treatment of allogeneic co-cultures might be expected to result in enhanced target-cell apoptosis. In the post-transplant setting this may affect either tumour or healthy cells. My data supports an enhanced GvT effect, demonstrating an increased cytotoxicity of $CD8^+$ alloresponder T cells against allogeneic haematopoietic targets. However I have not examined the effect on non-haematopoietic targets. It may be possible to perform similar experiments using peripheral blood derived endothelial cells or skin biopsy derived fibroblasts as target cells as in Nonn *et al.* [107]. An alternative and validated approach would be to perform skin-explant models [384,385], to

allow histological evaluation of the effect of donor cells allostimulated in the presence or absence of lenalidomide on recipient tissue that is a common target of aGvHD. The limitation of this approach is that it uses only the skin as a target of GvHD, whereas aGvHD can affect multiple tissues that also include the gut and liver.

It is currently not possible to assess GvHD and GvT alloresponses simultaneously in an *in vitro* model. For this reason the greater proportion of transplant research is conducted using murine models. While helpful, the results obtained from these murine systems are often limited by the fact that they are either measuring: murine immune responses, which are different from human ones or xeno-responses when human cells are transplanted into immunodeficient mice. They are also often restricted to measuring responses of selected $CD4^+$ or $CD8^+$ T cell subsets.

An advantage of murine models is the ability to examine GvT and GvHD in one model system. However in order to simultaneously measure GvHD and GvT both tumour and responder T cells must be engrafted into the mouse, often leading to additional complexity as these tumours are often heterotopic (e.g. subcutaneous) and therefore may be inaccessible to T cells that have migratory programmes for other tissues (reviewed in Schroeder *et al.* [386]). With the development of new murine models such as tumour-bearing Nod-SCID-gamma (NSG) mice transgenic for human MHC class I and II, these models may provide better insight into alloresponses [387].

Although the *in vitro* experiments presented have limitations, they are conducted using human cells and with minimum manipulation of the alloresponse. Additional experiments to further explore the effect of lenalidomide on allostimulated T cell migration in the presence or absence of pro-inflammatory stimuli may reveal differences in organ specific migratory capacity that would be clinically relevant.

Importantly I have shown that cytotoxicity against haematopoietic targets, representing tumours of haemato-lymphoid origin is enhanced following allostimulation in the presence of lenalidomide. This supports the use of lenalidomide to enhance GvT effects after AHSCT, not restricted to myeloma alone. However an assessment of cytotoxicity against non-haematopoietic targets is still an important area requiring investigation.

Chapter 7 - Final discussion and future work

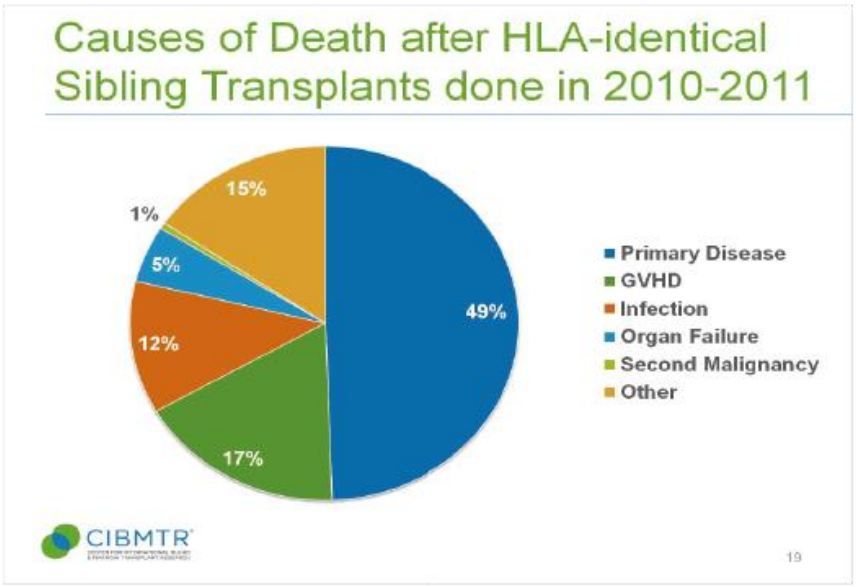
7.1 Final discussion

AHSCT is an effective treatment for many patients with haematological malignancy and curative in approximately 50% of patients overall (Center for International Blood and Marrow Transplant Research, https://www.**cibmtr**.org/). Relapse of primary disease is now the major cause of death following AHSCT, followed by GvHD (**Figure 7.1**). Given the toxicity to the patient and expense to the healthcare budget of AHSCT, strategies to reduce the relapse rates are urgently needed.

Cellular therapies such as DLI have been effective for some types of relapsing malignancy, particularly CML, but have much lower success rates in acute leukaemia and myelodysplastic syndrome [26,388]. DLI are also associated with significant risk of acute and chronic GvHD. The success rates may be increased by strategies to improve the efficacy of DLI, either by selection of specific subsets of cells (T, Treg or NK), selection of tumour antigen specific T cells or post-transplant vaccination prior to DLI.

The availability of cells however remains a major limitation of DLI. It is usually possible to return to a sibling or related donor for further collection of cells but re-accessing an unrelated donor is more challenging and in the case of UCB units, impossible. The recent advent of CAR T cells bearing a modified TCR construct of high affinity and avidity that appears to bypass HLA-dependent allorecognition may broaden the options for DLI. In case series reported to date CAR T cells derived from AHSCT donors have not been observed to cause acute GvHD [389]. It is possible that in the future third-party CAR T cells could be used as an 'off the shelf' treatment for patients where DLI are otherwise unavailable. However adoptive cellular therapies are, and are likely to remain expensive and time-consuming and with each level of manipulation required to prepare the cells prior to transfer the cost and technical difficulty rises.

Figure 7.1



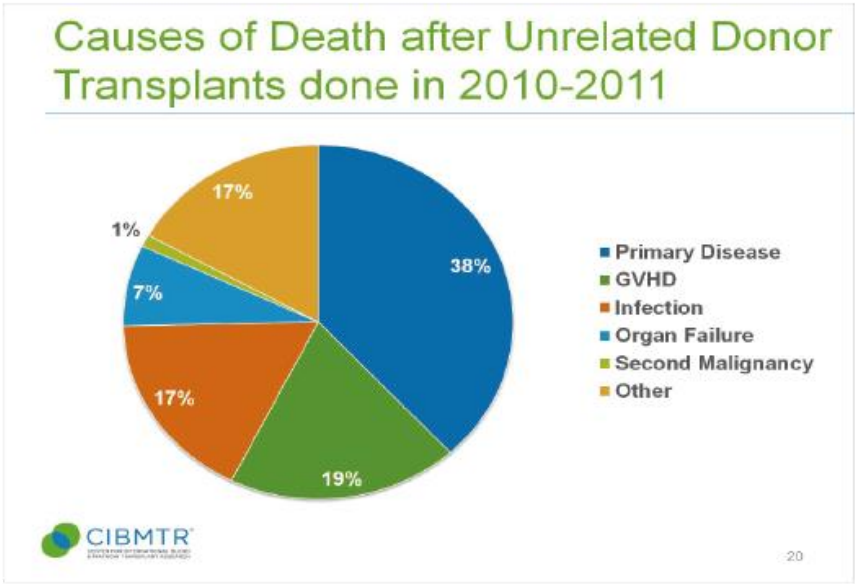


Figure 7.1 Causes of death following AHSCT

Reproduced from CIBMTR data registry summary slides.

Pharmacological methods of boosting GvT alone or in combination with cellular therapies are an attractive option. Hypomethylating agents such as the DNA-methy-transferase inhibitor azacytidine have direct anti-tumour effects in AML and MDS and have additional immunomodulatory effects. Azacytidine has been shown to cause up-regulation of tumour-associated antigens and HLA on leukaemia cells that may augment tumour recognition by donor T cells [390-392]. However the effect on T cells may also be immunosuppressive, with reductions in $CD8^+$ T cell cytotoxicity against AML blasts, increases in Treg and suppression of Th1 responses [392,393].

Trials of azacytidine as maintenance or relapse therapy, and in combination with DLI have shown favourable toxicity in terms of GvHD and good response rates [394,395], although durability of responses may be an issue, with one trial of single agent azacytidine for treatment of MRD reporting an 80% response rate but 65% eventual relapse [396].

IMiDs such as thalidomide and lenalidomide have been used following AHSCT, with the aim of capitalising on both their direct anti-tumour and T cell immunomodulatory effects. However pre-clinical and *in vitro* data detailing the changes caused by lenalidomide on the T cell alloresponse has to date been lacking.

In this study I have shown that exposure to clinically relevant concentrations of the widely used IMiD lenalidomide has contrasting effects on net alloresponses of T cells derived from two cell sources commonly used in AHSCT, APB and UCB. Lenalidomide has a common effect, potentiating alloresponses of cytotoxic $CD8^+$ T cells from both APB and UCB. Importantly however, lenalidomide decreases alloproliferation and expands $CD4^+$ Treg frequencies of $CD4^+$ T cells from UCB but not APB, resulting in a net reduction in proliferative UCB T cell alloresponses.

The observed changes in alloresponses of APB derived T cells are in keeping with, and help to explain the clinical observation of GvHD following lenalidomide treatment after AHSCT. I observe a general increase in effector and pro-inflammatory cytokine alloresponses by $CD8^+$ T cells that are likely to mediate MHC Class I directed cytotoxicity that can affect healthy recipient tissue, resulting in GvHD as well as exert a GvT effect. This occurs alongside a decrease in counter-regulatory $CD4^+$ Treg.

Importantly however, while lenalidomide increases alloproliferation and effector differentiation of UCB $CD8^+$ T cells it also decreases alloproliferation of $CD4^+$ T cells from UCB and expands $CD4^+$ Treg frequencies. This results in a net reduction in overall alloproliferative UCB T cell alloresponses. The findings, which suggest that lenalidomide could be used to provide direct anti-tumour activity after allogeneic UCB transplant with less potential for induction of harmful GvHD have important implications for the future use of IMiDs in the post-transplant setting.

Despite the clinical experience and data presented in this thesis, there still remains the question of whether lenalidomide can contribute significantly to GvT effects and therefore mitigate the accompanying risk of GvHD. The clinical trials have not been designed to detect differences in relapse related outcomes, although response rates were promising. There are also intriguing case reports where induction of remission was attributed to lenalidomide after AHSCT [262,266]. Functional data presented in this thesis demonstrates that lenalidomide exposure during allostimulation results in enhanced $CD8^+$ T cell cytotoxicity using both the surrogate marker CD107a and cell mediated cytotoxicity against haematopoietic target cells that would support enhanced GvT after lenalidomide exposure.

While I believe that lenalidomide could be used to boost GvT effects mediated by donor T cells, and also possibly NK cells, I have thus far found no evidence that the drug intrinsically has any differential effects that could be expected to selectively promote GvT over GvHD. It is more likely, given the data presented in this thesis that it is the inherent sensitivity of the cells exposed to lenalidomide that results in differential effects on net alloresponses that could influence clinical outcomes.

If a case is to be made for the use of lenalidomide after AHSCT to boost GvT, even with the concurrent risk of GvHD, then the next question is: what is the best scenario in which to use lenalidomide? In which type of transplant or type of patient? When after transplant? And finally at what dose?

The data regarding the alloresponses of UCB-derived T cells suggests that lenalidomide may be used 'more safely' after UCBT due to expansions of tolerogenic Treg. An alternative transplant scenario where lenalidomide may be beneficial is following haploidentical-AHSCT. In this scenario where rigorous T cell depletion is required, lenalidomide could be utilised to boost NK

cell alloresponses, with a reduced risk of T cell mediated GvHD. However further investigation of NK cell alloresponses would be required to support this.

In conventional AHSCT using APB-derived T cells, lenalidomide may have a place in the treatment of patients at high risk of relapse. In this scenario the clinical experience would suggest that lenalidomide used more than 6 months after transplant is less likely to result in induction of severe aGvHD. However in high-risk patients this may well be too late as relapse is likely to be early after AHSCT. As I have shown a significant impact of a single dose of lenalidomide on T cell alloresponses, one option therefore may be to use lenalidomide at an earlier time-point, but to reduce the frequency of lenalidomide dosing. A short course of lenalidomide administered soon after donor T cell engraftment could potentiate priming of donor T cells and enhance GvT alloresponses.

A more conservative approach may be to consider lenalidomide as an adjunct to DLI for patients who have already failed to respond to one DLI. These patients have experienced a failure of alloreactivity and would be at relatively low risk of GvHD and very high risk of death from relapse. Therefore the addition of lenalidomide before and after a second DLI may potentiate donor T cell alloresponses and convert the recipient from non-responder to responder status.

Importantly phenotypic characterisation of lenalidomide enhanced alloresponder T cells in this thesis provides a potential tool for immunomonitoring of lenalidomide treatment following AHSCT. A panel of surface markers including CD8, VLA2, CCR2 and CCR5 could identify expanded populations of effector $CD8^+$ T cells responsive to lenalidomide and facilitate identification of response to treatment as well as decisions to withdraw treatment if required. As flow cytometry forms an integral part of the diagnostic and monitoring pathway for AHSCT recipients, this would be a feasible addition to existing management regimes.

An alternative approach to treatment of transplant recipients directly with lenalidomide may be to use lenalidomide to augment *ex vivo* expansion of donor T cells for adoptive transfer. For example lenalidomide could augment expansion of $CD8^+$ tumour antigen specific T cells [140], with potentially enhanced polyfunctional cytokine and cytotoxic capacity. Alternatively lenalidomide could also be used to augment existing strategies to expand Treg from UCB for prevention or treatment of GvHD [397].

The mechanism of action underlying the altered T cell alloresponse following lenalidomide exposure is at least in part due to ikaros depletion via enhanced cereblon activity. This is true of both APB and UCB-derived T cells. However as there appears to be no difference in either cereblon expression or ikaros expression in UCB compared to APB this cannot explain the differential effect of the drug on these two cell sources.

In addition gene expression profiling of alloproliferative $CD8^+$ T cells from APB revealed that lenalidomide treatment led to a significant change in the gene expression of these cells that cannot all be attributed to the actions of ikaros. Up or down regulation of a number of genes involved in T cell activation and proliferation as well as genes involved in pathways that govern T cell metabolic switches were found. This indicates that either lenalidomide acts via additional non-cereblon dependent pathways, or that cereblon has additional non-ikaros substrates that can alter alloresponses. Therefore it is interesting that a group from the Mayo clinic have very recently published the discovery of another cereblon binding protein, Argonaut 2 (Ago2) [398].

Ago2 plays a critical role in micro-RNA (miRNA) maturation, stability and function, forming part of the miRNA induced silencing complex (miRISC) [399] (**Figure 7.2**). The authors have only presented data for the anti-Ago2 activity of lenalidomide in myeloma cell lines, however it is likely this mechanism also occurs in T cells. As a single miRNA can post-transcriptionally regulate a number of mRNAs, a decrease in the miRISC would lead to a profound change in the miRNA and mRNA milieu inside a cell. Therefore in any cell type a decrease in the miRISC is likely to result in pleiotropic effects on cell function, as occurs on lenalidomide treatment of T cells.

In support of this, miRNA have been demonstrated to play important roles in the differentiation of $CD8^+$ T cells following antigenic stimulation, with increased and decreased expression of miRNA such as miR-21 and miR-16 corresponding with naïve, effector and memory phenotypes [400]. The overall level of miRNA was also found to correlate with the 'activity' of the cells, with the highest levels found in quiescent naïve and memory cells and the lowest in actively dividing and functionally competent effectors.

Figure 7.2

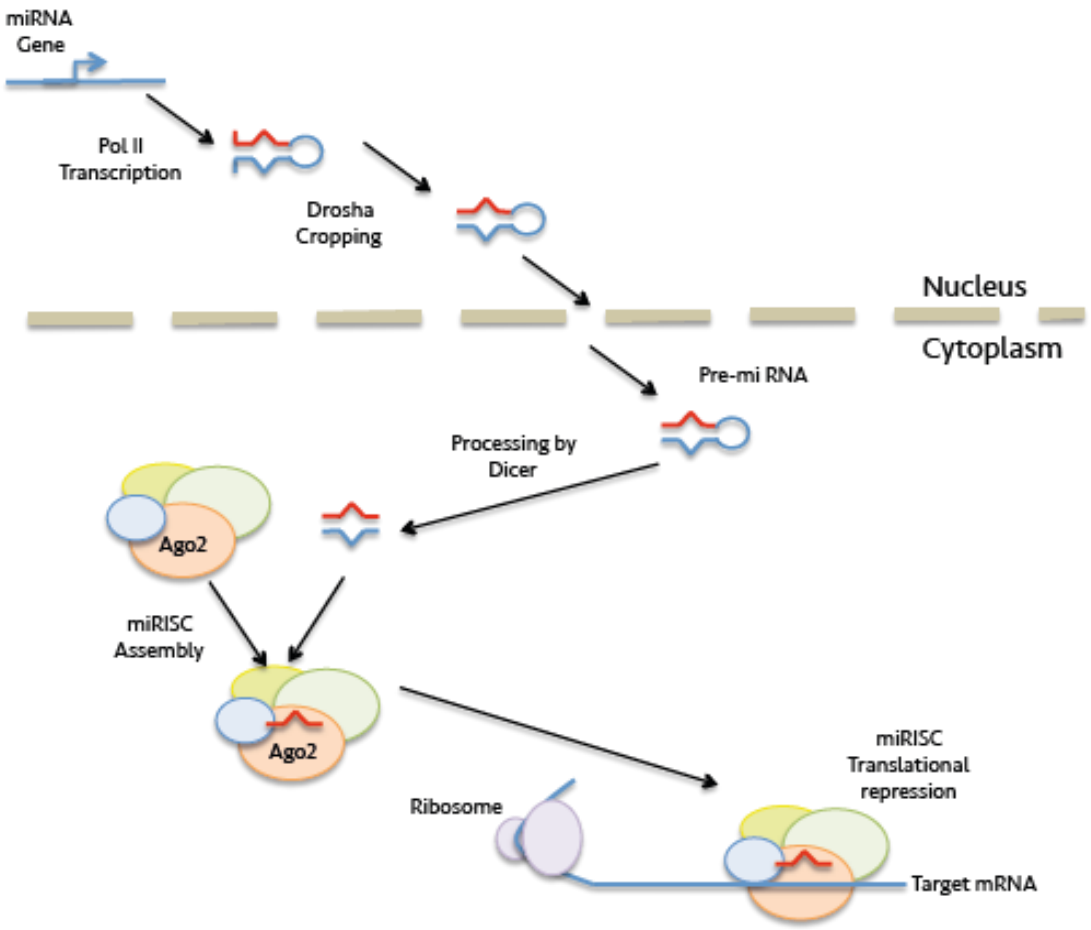


Figure 7.2 Processing and action of miRNA

Schematic representing transcription of microRNA (miRNA) from miRNA gene by Pol II and processing to mature miRNA by Drosha and Dicer enzymes, prior to incorporation of single strand miRNA into the micro RNA induced silencing complex (miRISC) containing argonaute 2 (Ago2).

The miRISC then exerts post-transcriptional repression of gene expression by preventing translation of complementary mRNA by the ribosome.

Adapted from Rana 2007 [403]

Interestingly work by Bronevetsky *et al.* has shown that activation induces the post-transcriptional down-regulation of Ago2 in $CD4^+$ T cells, by increased ubiquitination and proteasomal degradation [401], which would fit with a cereblon-dependent mechanism. This group also found that naïve T cells with reduced Ago2 differentiated more readily into cytokine producing cells. Finally work by Marcais *et al.* showed that in a model where peripheral T cells lack *Dicer*, and therefore are unable to form miRNA, $CD4^+$ T cells were able to become activated in the absence of co-stimulation, and at lower levels of TCR stimulation than wild-type T cells [402].

The commonalities seen between cells with reduced levels of Ago2 and the changes I have observed in my experiments with lenalidomide suggests that lenalidomide induced degradation of Ago2 via cereblon may be another contributor to the modification of the alloresponse seen with lenalidomide. Importantly there are known to be differences in miRNA expression between UCB and APB T cells [404,405], therefore this may explain the differential effect of lenalidomide on UCB. miRNA are also known to be critically important to the post-transcriptional regulation of FOXP3 in UCB Treg [406,407].

It is important to note that lenalidomide is only one of a family of IMiD drugs derived from thalidomide, including pomalidomide, CC-11006 and CC-122 (all Celgene). These may also have a potential role to play in manipulation of the human alloresponse. However it is important to note that none of these drugs have yet been used in this setting, and while one might anticipate similar actions existing data has highlighted that both potency and specific changes in immune parameters may be different [174,202,408-410]. This is evident from the findings that pomalidomide is active in patients who are or have become refractory to lenalidomide therapy, and in the different toxicities of the two drugs [411,412]. Therefore *in vitro* characterisation of the effects of these newer IMiDs on human alloresponses will help to evaluate the possible advantages or disadvantages of their use compared to lenalidomide following AHSCT.

In summary, lenalidomide augments human alloresponses *in vitro* and *in vivo* by increasing the proliferation of allospecific $CD8^+$ T cells with an enhanced capacity to produce pro-inflammatory cytokines and to kill their cellular targets. In addition, in APB lenalidomide treatment leads to an increase in the $CD4^+$ Teff to Treg cell ratio due to a selective reduction in $CD4^+$ Treg, thereby increasing $CD4^+$ help to $CD8^+$ T cells and reducing inhibitory effects of Treg on alloresponses.

Importantly in UCB lenalidomide exposure during allostimulation has a differential effect on $CD4^+$ alloresponses, leading to expansion of Treg cells that may provide protection from GvHD.

While the exact molecular mechanisms underlying the effect of lenalidomide on the alloresponse remain to be fully understood, they are at least in part due to cereblon-dependent ikaros degradation and require the presence of the drug during antigen-priming. However it appears from the gene expression data that additional pathways are involved in the effect of lenalidomide on $CD8^+$ T cells. In light of the recent finding of another cereblon substrate Ago2, it is possible that some of these changes are due to actions via Ago2 on the miRNA environment in T cells but they may also be due to effects on substrates of lenalidomide-cereblon not yet identified.

These findings can help to inform the optimum clinical use of lenalidomide following AHSCT and also provide additional data that can be used to design monitoring strategies and *ex vivo* strategies for cellular manipulation as well as new studies to further understand the complex mechanisms underlying the effect of this drug.

7.2 Future work

1. Extended evaluation of the effect of lenalidomide on different graft sources for AHSCT

- To further confirm that the effect of lenalidomide on HLA-matched samples is similar to that observed in HLA-mismatched.
- To examine the effect on haploidentical alloresponses.
- To confirm the suppressive capacity of expanded Treg populations from UCB.

2. Investigation of the mechanism underlying the differential actions of lenalidomide on UCB $CD4^+$ T cells.

- Gene expression profiling of lenalidomide treated UCB derived $CD4^+$ T cells versus APB derived.
- Investigation of expression levels of Ago2 in APB and UCB T cells to assess for differing levels of expression.
- Pathways and candidate immunoregulatory targets from gene expression data such as Myc pathway and SOCS2 should be validated by examining protein level expression in $CD8^+$ T cells allostimulated in the presence or absence of lenalidomide.

3. Further functional validation of lenalidomide-related effects on T cell alloresponses.

- Assessment of UCB derived $CD8^+$ T cells cytotoxicity against allogeneic haematopoietic targets.
- Assessment of tumour antigen specific T cell responses of lenalidomide treated alloresponders using Wilms Tumour antigen as a model haematopoietic tumour-associated antigen.
- Assessment of collagen dependent migratory capacity of lenalidomide treated cells, in view of increased expression of VLA2.

4. Investigation of GvHD effects of lenalidomide

- Skin-explant model of GvHD using responder cells allostimulated in the presence or absence of lenalidomide.

References

1. Horowitz MM, Gale RP, Sondel PM, et al. Graft-versus-leukemia reactions after bone marrow transplantation. *Blood* 1990; **75**(3): 555-62.
2. Naujokat C, Berges C, Fuchs D, Sadeghi M, Opelz G, Daniel V. Antithymocyte globulins suppress dendritic cell function by multiple mechanisms. *Transplantation* 2007; **83**(4): 485-97.
3. Gyurkocza B, Sandmaier BM. Conditioning regimens for hematopoietic cell transplantation: one size does not fit all. *Blood* 2014; **124**(3): 344-53.
4. Krenger W, Blazar BR, Holländer GA. Thymic T-cell development in allogeneic stem cell transplantation. *Blood* 2011; **117**(25): 6768-76.
5. Erlich HA, Opelz G, Hansen J. HLA DNA typing and transplantation. *Immunity* 2001; **14**(4): 347-56.
6. Nowak J. Role of HLA in hematopoietic SCT. *Bone Marrow Transplant* 2008; **42 Suppl 2**: S71-6.
7. Anasetti C, Aversa F, Brunstein CG. Back to the future: mismatched unrelated donor, haploidentical related donor, or unrelated umbilical cord blood transplantation? *Biol Blood Marrow Transplant* 2012; **18**(1 Suppl): S161-5.
8. Holtick U, Albrecht M, Chemnitz JM, et al. Bone marrow versus peripheral blood allogeneic haematopoietic stem cell transplantation for haematological malignancies in adults. *Cochrane Database Syst Rev* 2014; **4**: Cd010189.
9. Kolb HJ. Graft-versus-leukemia effects of transplantation and donor lymphocytes. *Blood* 2008; **112**(12): 4371-83.
10. Arai S, Vogelsang GB. Management of graft-versus-host disease. *Blood Rev* 2000; **14**(4): 190-204.
11. Storb R, Epstein RB, Ragde H, Bryant J, Thomas ED. Marrow engraftment by allogeneic leukocytes in lethally irradiated dogs. *Blood* 1967; **30**(6): 805-11.
12. Storb R, Kolb HJ, Graham TC, Kolb H, Weiden PL, Thomas ED. Treatment of established graft-versus-host disease in dogs by antithymocyte serum or prednisone. *Blood* 1973; **42**(4): 601-9.
13. Kolb H. A receptor for 'self' on lymphocytes. *Immunology* 1977; **33**(6): 859-63.
14. Rodt H, Kolb HJ, Netzel B, et al. GVHD suppression by incubation of bone marrow grafts with anti-T-cell globulin: effect in the canine model and application to clinical bone marrow transplantation. *Transplant Proc* 1979; **11**(1): 962-6.
15. Rodt H, Kolb HJ, Netzel B, et al. Effect of anti-T-cell globulin on GVHD in leukemic patients treated with BMT. *Transplant Proc* 1981; **13**(1 Pt 1): 257-61.
16. Blazar BR, Murphy WJ, Abedi M. Advances in graft-versus-host disease biology and therapy. *Nat Rev Immunol* 2012; **12**(6): 443-58.
17. Afzali B, Lechler RI, Hernandez-Fuentes MP. Allorecognition and the alloresponse: clinical implications. *Tissue Antigens* 2007; **69**(6): 545-56.
18. AK A, AH L, S. P. Cellular and molecular immunology: Elsevier Health Sciences; 2014.
19. Jagasia MH, Greinix HT, Arora M, et al. National Institutes of Health Consensus Development Project on Criteria for Clinical Trials in Chronic Graft-versus-Host Disease: I. The 2014 Diagnosis and Staging Working Group report. *Biol Blood Marrow Transplant* 2015; **21**(3): 389-401.e1.
20. Atkinson K, Farrelly H, Cooley M, O'Flaherty E, Downs K, Biggs J. Human marrow T cell dose correlates with severity of subsequent acute graft-versus-host disease. *Bone Marrow Transplant* 1987; **2**(1): 51-7.
21. Maraninchi D, Gluckman E, Blaise D, et al. Impact of T-cell depletion on outcome of allogeneic bone-marrow transplantation for standard-risk leukaemias. *Lancet* 1987; **2**(8552): 175-8.

22. Atkinson K, Biggs J, Dodds A, et al. High incidence of early leukemic relapse in patients given cyclosporin and T cell depleted HLA-identical sibling marrow transplants for acute leukemia in first remission. *Aust N Z J Med* 1988; **18**(4): 587-93.
23. Apperley JF, Mauro FR, Goldman JM, et al. Bone marrow transplantation for chronic myeloid leukaemia in first chronic phase: importance of a graft-versus-leukaemia effect. *Br J Haematol* 1988; **69**(2): 239-45.
24. Kolb HJ, Mittermuller J, Clemm C, et al. Donor leukocyte transfusions for treatment of recurrent chronic myelogenous leukemia in marrow transplant patients. *Blood* 1990; **76**(12): 2462-5.
25. Drobyski WR, Keever CA, Roth MS, et al. Salvage immunotherapy using donor leukocyte infusions as treatment for relapsed chronic myelogenous leukemia after allogeneic bone marrow transplantation: efficacy and toxicity of a defined T-cell dose. *Blood* 1993; **82**(8): 2310-8.
26. Marks DI, Lush R, Cavenagh J, et al. The toxicity and efficacy of donor lymphocyte infusions given after reduced-intensity conditioning allogeneic stem cell transplantation. *Blood* 2002; **100**(9): 3108-14.
27. Mackinnon S, Papadopoulos EB, Carabasi MH, et al. Adoptive immunotherapy evaluating escalating doses of donor leukocytes for relapse of chronic myeloid leukemia after bone marrow transplantation: separation of graft-versus-leukemia responses from graft-versus-host disease. *Blood* 1995; **86**(4): 1261-8.
28. Kircher B, Wolf M, Stevanovic S, et al. Hematopoietic lineage-restricted minor histocompatibility antigen HA-1 in graft-versus-leukemia activity after donor lymphocyte infusion. *J Immunother* 2004; **27**(2): 156-60.
29. Orsini E, Bellucci R, Alyea EP, et al. Expansion of tumor-specific CD8+ T cell clones in patients with relapsed myeloma after donor lymphocyte infusion. *Cancer Res* 2003; **63**(10): 2561-8.
30. Gill S, Olson JA, Negrin RS. Natural killer cells in allogeneic transplantation: effect on engraftment, graft- versus-tumor, and graft-versus-host responses. *Biol Blood Marrow Transplant* 2009; **15**(7): 765-76.
31. Della Chiesa M, Marcenaro E, Sivori S, Carlomagno S, Pesce S, Moretta A. Human NK cell response to pathogens. *Semin Immunol* 2014; **26**(2): 152-60.
32. Ruggeri L, Capanni M, Casucci M, et al. Role of natural killer cell alloreactivity in HLA-mismatched hematopoietic stem cell transplantation. *Blood* 1999; **94**(1): 333-9.
33. Ruggeri L, Mancusi A, Burchielli E, Aversa F, Martelli MF, Velardi A. Natural killer cell alloreactivity in allogeneic hematopoietic transplantation. *Curr Opin Oncol* 2007; **19**(2): 142-7.
34. Klingemann HG. Cellular therapy of cancer with natural killer cells-where do we stand? *Cytotherapy* 2013; **15**(10): 1185-94.
35. Billingham RE. The biology of graft-versus-host reactions. *Harvey Lect* 1966; **62**: 21-78.
36. Ferrara JL, Levine JE, Reddy P, Holler E. Graft-versus-host disease. *Lancet* 2009; **373**(9674): 1550-61.
37. Jamil MO, Mineishi S. State-of-the-art acute and chronic GVHD treatment. *Int J Hematol* 2015; **101**(5): 452-66.
38. Cao TM, Wong RM, Sheehan K, et al. CD34, CD4, and CD8 cell doses do not influence engraftment, graft-versus-host disease, or survival following myeloablative human leukocyte antigen-identical peripheral blood allografting for hematologic malignancies. *Exp Hematol* 2005; **33**(3): 279-85.
39. Hartmann N, Leithauser F, Albers C, et al. In vitro-established alloantigen-specific CD8+ CTLs mediate graft-versus-tumor activity in the absence of graft-versus-host disease. *Leukemia* 2011; **25**(5): 848-55.
40. Suzuki Y, Adachi Y, Minamino K, et al. A new strategy for treatment of malignant tumor: intra-bone marrow-bone marrow transplantation plus CD4- donor lymphocyte infusion. *Stem Cells* 2005; **23**(3): 365-70.

41. Du W, Leigh ND, Bian G, et al. Granzyme B-Mediated Activation-Induced Death of CD4+ T Cells Inhibits Murine Acute Graft-versus-Host Disease. *J Immunol* 2015; **195**(9): 4514-23.
42. Fricke S, Hilger N, Fricke C, et al. Prevention of graft-versus-host-disease with preserved graft-versus-leukemia-effect by ex vivo and in vivo modulation of CD4(+) T-cells. *Cell Mol Life Sci* 2014; **71**(11): 2135-48.
43. Atkinson K, Cooley M, Farrelly H, O'Flaherty E, Ashby M, Biggs J. CD4+ T cells appear capable of initiating graft-versus-host disease across non-major histocompatibility complex (MHC) barriers in man. *Bone Marrow Transplant* 1987; **2**(1): 79-84.
44. Champlin R, Ho W, Gajewski J, et al. Selective depletion of CD8+ T lymphocytes for prevention of graft-versus-host disease after allogeneic bone marrow transplantation. *Blood* 1990; **76**(2): 418-23.
45. Nimer SD, Giorgi J, Gajewski JL, et al. Selective depletion of CD8+ cells for prevention of graft-versus-host disease after bone marrow transplantation. A randomized controlled trial. *Transplantation* 1994; **57**(1): 82-7.
46. Giralt S, Hester J, Huh Y, et al. CD8-depleted donor lymphocyte infusion as treatment for relapsed chronic myelogenous leukemia after allogeneic bone marrow transplantation. *Blood* 1995; **86**(11): 4337-43.
47. Chakraverty R, Eom HS, Sachs J, et al. Host MHC class II+ antigen-presenting cells and CD4 cells are required for CD8-mediated graft-versus-leukemia responses following delayed donor leukocyte infusions. *Blood* 2006; **108**(6): 2106-13.
48. Zeis M, Uharek L, Hartung G, Glass B, Steinmann J, Schmitz N. Graft-vs-leukemia activity and graft-vs-host disease induced by allogeneic Th1- and Th2-type CD4+ T cells in mice. *Hematol J* 2001; **2**(2): 136-44.
49. Lu Y, Sakamaki S, Kuroda H, et al. Prevention of lethal acute graft-versus-host disease in mice by oral administration of T helper 1 inhibitor, TAK-603. *Blood* 2001; **97**(4): 1123-30.
50. Fowler DH, Odom J, Steinberg SM, et al. Phase I clinical trial of costimulated, IL-4 polarized donor CD4+ T cells as augmentation of allogeneic hematopoietic cell transplantation. *Biol Blood Marrow Transplant* 2006; **12**(11): 1150-60.
51. Liu J, Anderson BE, Robert ME, et al. Selective T-cell subset ablation demonstrates a role for T1 and T2 cells in ongoing acute graft-versus-host disease: a model system for the reversal of disease. *Blood* 2001; **98**(12): 3367-75.
52. Farber DL. Biochemical signaling pathways for memory T cell recall. *Semin Immunol* 2009; **21**(2): 84-91.
53. Anderson BE, McNiff J, Yan J, et al. Memory CD4+ T cells do not induce graft-versus-host disease. *J Clin Invest* 2003; **112**(1): 101-8.
54. Macedo C, Orkis EA, Popescu I, et al. Contribution of naive and memory T-cell populations to the human alloimmune response. *Am J Transplant* 2009; **9**(9): 2057-66.
55. Distler E, Bloetz A, Albrecht J, et al. Alloreactive and leukemia-reactive T cells are preferentially derived from naive precursors in healthy donors: implications for immunotherapy with memory T cells. *Haematologica* 2011; **96**(7): 1024-32.
56. Touzot F, Neven B, Dal-Cortivo L, et al. CD45RA depletion in HLA-mismatched allogeneic hematopoietic stem cell transplantation for primary combined immunodeficiency: A preliminary study. *J Allergy Clin Immunol* 2015; **135**(5): 1303-9.e1-3.
57. Rezvani K, Mielke S, Ahmadzadeh M, et al. High donor FOXP3-positive regulatory T-cell (Treg) content is associated with a low risk of GVHD following HLA-matched allogeneic SCT. *Blood* 2006; **108**(4): 1291-7.
58. Mielke S, Rezvani K, Savani BN, et al. Reconstitution of FOXP3+ regulatory T cells (Tregs) after CD25-depleted allotransplantation in elderly patients and association with acute graft-versus-host disease. *Blood* 2007; **110**(5): 1689-97.

59. Wolf D, Wolf AM, Fong D, et al. Regulatory T-cells in the graft and the risk of acute graft-versus-host disease after allogeneic stem cell transplantation. *Transplantation* 2007; **83**(8): 1107-13.
60. Fujioka T, Tamaki H, Ikegame K, et al. Frequency of CD4(+)FOXP3(+) regulatory T-cells at early stages after HLA-mismatched allogeneic hematopoietic SCT predicts the incidence of acute GVHD. *Bone Marrow Transplant* 2013; **48**(6): 859-64.
61. Hicheri Y, Bouchekioua A, Hamel Y, et al. Donor regulatory T cells identified by FoxP3 expression but also by the membranous CD4+CD127low/neg phenotype influence graft-versus-tumor effect after donor lymphocyte infusion. *J Immunother* 2008; **31**(9): 806-11.
62. Edinger M, Hoffmann P, Ermann J, et al. CD4+CD25+ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. *Nat Med* 2003; **9**(9): 1144-50.
63. Kambayashi T, Laufer TM. Atypical MHC class II-expressing antigen-presenting cells: can anything replace a dendritic cell? *Nat Rev Immunol* 2014; **14**(11): 719-30.
64. Toubai T, Mathewson N, Reddy P. The role of dendritic cells in graft-versus-tumor effect. *Front Immunol* 2014; **5**: 66.
65. Merad M, Hoffmann P, Ranheim E, et al. Depletion of host Langerhans cells before transplantation of donor alloreactive T cells prevents skin graft-versus-host disease. *Nat Med* 2004; **10**(5): 510-7.
66. Reddy P, Maeda Y, Liu C, Krijanovski OI, Korngold R, Ferrara JL. A crucial role for antigen-presenting cells and alloantigen expression in graft-versus-leukemia responses. *Nat Med* 2005; **11**(11): 1244-9.
67. Horváth R, Budinský V, Kayserová J, et al. Kinetics of dendritic cells reconstitution and costimulatory molecules expression after myeloablative allogeneic haematopoetic stem cell transplantation: implications for the development of acute graft-versus host disease. *Clin Immunol* 2009; **131**(1): 60-9.
68. Middleton PG, Taylor PR, Jackson G, Proctor SJ, Dickinson AM. Cytokine gene polymorphisms associating with severe acute graft-versus-host disease in HLA-identical sibling transplants. *Blood* 1998; **92**(10): 3943-8.
69. Holler E, Kolb HJ, Möller A, et al. Increased serum levels of tumor necrosis factor alpha precede major complications of bone marrow transplantation. *Blood* 1990; **75**(4): 1011-6.
70. Levine JE, Paczesny S, Mineishi S, et al. Etanercept plus methylprednisolone as initial therapy for acute graft-versus-host disease. *Blood* 2008; **111**(4): 2470-5.
71. Ferrara JL, Cooke KR, Pan L, Krenger W. The immunopathophysiology of acute graft-versus-host-disease. *Stem Cells* 1996; **14**(5): 473-89.
72. Burman AC, Banovic T, Kuns RD, et al. IFNgamma differentially controls the development of idiopathic pneumonia syndrome and GVHD of the gastrointestinal tract. *Blood* 2007; **110**(3): 1064-72.
73. Wang H, Yang YG. The complex and central role of interferon-γ in graft-versus-host disease and graft-versus-tumor activity. *Immunol Rev* 2014; **258**(1): 30-44.
74. Dinarello CA. Biologic basis for interleukin-1 in disease. *Blood* 1996; **87**(6): 2095-147.
75. Antin JH, Weisdorf D, Neuberg D, et al. Interleukin-1 blockade does not prevent acute graft-versus-host disease: results of a randomized, double-blind, placebo-controlled trial of interleukin-1 receptor antagonist in allogeneic bone marrow transplantation. *Blood* 2002; **100**(10): 3479-82.
76. Tawara I, Koyama M, Liu C, et al. Interleukin-6 modulates graft-versus-host responses after experimental allogeneic bone marrow transplantation. *Clin Cancer Res* 2011; **17**(1): 77-88.
77. Symington FW, Symington BE, Liu PY, Viguet H, Santhanam U, Sehgal PB. The relationship of serum IL-6 levels to acute graft-versus-host disease and hepatorenal disease after human bone marrow transplantation. *Transplantation* 1992; **54**(3): 457-62.

78. Roddy JV, Haverkos BM, McBride A, et al. Tocilizumab for steroid refractory acute graft-versus-host disease. *Leuk Lymphoma* 2016; **57**(1): 81-5.
79. Vignali DA, Kuchroo VK. IL-12 family cytokines: immunological playmakers. *Nat Immunol* 2012; **13**(8): 722-8.
80. Reddy V, Winer AG, Eksioglu E, Meier-Kriesche HU, Schold JD, Wingard JR. Interleukin 12 is associated with reduced relapse without increased incidence of graft-versus-host disease after allogeneic hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant* 2005; **11**(12): 1014-21.
81. Sykes M, Pearson DA, Taylor PA, Szot GL, Goldman SJ, Blazar BR. Dose and timing of interleukin (IL)-12 and timing and type of total-body irradiation: effects on graft-vs.-host disease inhibition and toxicity of exogenous IL-12 in murine bone marrow transplant recipients. *Biol Blood Marrow Transplant* 1999; **5**(5): 277-84.
82. Leonard JP, Sherman ML, Fisher GL, et al. Effects of single-dose interleukin-12 exposure on interleukin-12-associated toxicity and interferon-gamma production. *Blood* 1997; **90**(7): 2541-8.
83. Tanaka J, Imamura M, Kasai M, et al. Cytokine gene expression in the mixed lymphocyte culture in allogenic bone marrow transplants as a predictive method for transplantation-related complications. *Br J Haematol* 1994; **87**(2): 415-8.
84. Schmidt-Hieber M, Fietz T, Knauf W, et al. Efficacy of the interleukin-2 receptor antagonist basiliximab in steroid-refractory acute graft-versus-host disease. *Br J Haematol* 2005; **130**(4): 568-74.
85. Zorn E, Mohseni M, Kim H, et al. Combined CD4+ donor lymphocyte infusion and low-dose recombinant IL-2 expand FOXP3+ regulatory T cells following allogeneic hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant* 2009; **15**(3): 382-8.
86. Groom JR. Moving to the suburbs: T-cell positioning within lymph nodes during activation and memory. *Immunol Cell Biol* 2015; **93**(4): 330-6.
87. Sackstein R. A revision of Billingham's tenets: the central role of lymphocyte migration in acute graft-versus-host disease. *Biol Blood Marrow Transplant* 2006; **12**(1 Suppl 1): 2-8.
88. Marelli-Berg FM, Cannella L, Dazzi F, Mirenda V. The highway code of T cell trafficking. *J Pathol* 2008; **214**(2): 179-89.
89. Kunkel EJ, Campbell JJ, Haraldsen G, et al. Lymphocyte CC chemokine receptor 9 and epithelial thymus-expressed chemokine (TECK) expression distinguish the small intestinal immune compartment: Epithelial expression of tissue-specific chemokines as an organizing principle in regional immunity. *J Exp Med* 2000; **192**(5): 761-8.
90. Anderson BE, Taylor PA, McNiff JM, et al. Effects of donor T-cell trafficking and priming site on graft-versus-host disease induction by naive and memory phenotype CD4 T cells. *Blood* 2008; **111**(10): 5242-51.
91. Masopust D, Schenkel JM. The integration of T cell migration, differentiation and function. *Nat Rev Immunol* 2013; **13**(5): 309-20.
92. New JY, Li B, Koh WP, et al. T cell infiltration and chemokine expression: relevance to the disease localization in murine graft-versus-host disease. *Bone Marrow Transplant* 2002; **29**(12): 979-86.
93. Terwey TH, Kim TD, Kochman AA, et al. CCR2 is required for CD8-induced graft-versus-host disease. *Blood* 2005; **106**(9): 3322-30.
94. Bogunia-Kubik K, Duda D, Suchnicki K, Lange A. CCR5 deletion mutation and its association with the risk of developing acute graft-versus-host disease after allogeneic hematopoietic stem cell transplantation. *Haematologica* 2006; **91**(12): 1628-34.
95. Wysocki CA, Burkett SB, Panoskaltsis-Mortari A, et al. Differential roles for CCR5 expression on donor T cells during graft-versus-host disease based on pretransplant conditioning. *J Immunol* 2004; **173**(2): 845-54.
96. Reshef R, Luger SM, Hexner EO, et al. Blockade of lymphocyte chemotaxis in visceral graft-versus-host disease. *N Engl J Med* 2012; **367**(2): 135-45.

97. Chen X, Dodge J, Komorowski R, Drobyski WR. A critical role for the retinoic acid signaling pathway in the pathophysiology of gastrointestinal graft-versus-host disease. *Blood* 2013; **121**(19): 3970-80.
98. Aoyama K, Saha A, Tolar J, et al. Inhibiting retinoic acid signaling ameliorates graft-versus-host disease by modifying T-cell differentiation and intestinal migration. *Blood* 2013; **122**(12): 2125-34.
99. Keshav S, Vaňásek T, Niv Y, et al. A randomized controlled trial of the efficacy and safety of CCX282-B, an orally-administered blocker of chemokine receptor CCR9, for patients with Crohn's disease. *PLoS One* 2013; **8**(3): e60094.
100. Reiss Y, Proudfoot AE, Power CA, Campbell JJ, Butcher EC. CC chemokine receptor (CCR)4 and the CCR10 ligand cutaneous T cell-attracting chemokine (CTACK) in lymphocyte trafficking to inflamed skin. *J Exp Med* 2001; **194**(10): 1541-7.
101. Faaij CM, Lankester AC, Spierings E, et al. A possible role for CCL27/CTACK-CCR10 interaction in recruiting CD4 T cells to skin in human graft-versus-host disease. *Br J Haematol* 2006; **133**(5): 538-49.
102. Bach FH. Donor-recipient pairing for transplantation: present limitations and future objectives. *Rev Eur Etud Clin Biol* 1970; **15**(2): 141-3.
103. Kekre N, Kim HT, Thanarajasingam G, et al. Efficacy of immune suppression tapering in treating relapse after reduced intensity allogeneic stem cell transplantation. *Haematologica* 2015; **100**(9): 1222-7.
104. Gatza E, Choi SW. Approaches for the prevention of graft-versus-host disease following hematopoietic cell transplantation. *Int J Hematol Oncol* 2015; **4**(3): 113-26.
105. Abdul Wahid SF, Ismail NA, Mohd-Idris MR, et al. Comparison of reduced-intensity and myeloablative conditioning regimens for allogeneic hematopoietic stem cell transplantation in patients with acute myeloid leukemia and acute lymphoblastic leukemia: a meta-analysis. *Stem Cells Dev* 2014; **23**(21): 2535-52.
106. van Dijk JM, TerBrugge KG, Willinsky RA, Wallace MC. Selective disconnection of cortical venous reflux as treatment for cranial dural arteriovenous fistulas. *J Neurosurg* 2004; **101**(1): 31-5.
107. Nonn M, Herr W, Khan S, et al. Selective depletion of alloreactive T lymphocytes using patient-derived nonhematopoietic stimulator cells in allograft engineering. *Transplantation* 2008; **86**(10): 1427-35.
108. Montagna D, Yvon E, Calcaterra V, et al. Depletion of alloreactive T cells by a specific anti-interleukin-2 receptor p55 chain immunotoxin does not impair in vitro antileukemia and antiviral activity. *Blood* 1999; **93**(10): 3550-7.
109. André-Schmutz I, Le Deist F, Hacein-Bey-Abina S, et al. Immune reconstitution without graft-versus-host disease after haemopoietic stem-cell transplantation: a phase 1/2 study. *Lancet* 2002; **360**(9327): 130-7.
110. Kuniyasu Y, Takahashi T, Itoh M, Shimizu J, Toda G, Sakaguchi S. Naturally anergic and suppressive CD25(+)CD4(+) T cells as a functionally and phenotypically distinct immunoregulatory T cell subpopulation. *Int Immunol* 2000; **12**(8): 1145-55.
111. Hartwig UF, Nonn M, Khan S, Meyer RG, Huber C, Herr W. Depletion of alloreactive T cells via CD69: implications on antiviral, antileukemic and immunoregulatory T lymphocytes. *Bone Marrow Transplant* 2006; **37**(3): 297-305.
112. Davies JK, Koh MB, Lowdell MW. Antiviral immunity and T-regulatory cell function are retained after selective alloreactive T-cell depletion in both the HLA-identical and HLA-mismatched settings. *Biol Blood Marrow Transplant* 2004; **10**(4): 259-68.
113. Al-Homsi AS, Roy TS, Cole K, Feng Y, Duffner U. Post-Transplant High-Dose Cyclophosphamide for the Prevention of Graft-versus-Host Disease: A Review. *Biol Blood Marrow Transplant* 2014.
114. Goldsmith SR, Slade M, DiPersio JF, et al. Cytomegalovirus viremia, disease, and impact on relapse in T-cell replete peripheral blood haploidentical hematopoietic cell transplantation with post-transplant cyclophosphamide. *Haematologica* 2016.
115. Berger M, Lanino E, Cesaro S, et al. Feasibility and Outcome of Haploidentical Hematopoietic Stem Cell Transplantation with Post-Transplant High-Dose

Cyclophosphamide for Children and Adolescents with Hematologic Malignancies: An AIEOP-GITMO Retrospective Multicenter Study. *Biol Blood Marrow Transplant* 2016; **22**(5): 902-9.
116. Bacigalupo A, Dominietto A, Ghiso A, et al. Unmanipulated haploidentical bone marrow transplantation and post-transplant cyclophosphamide for hematologic malignanices following a myeloablative conditioning: an update. *Bone Marrow Transplant* 2015; **50 Suppl 2**: S37-9.
117. Holtick U, Chemnitz JM, Shimabukuro-Vornhagen A, et al. OCTET-CY: a phase II study to investigate the efficacy of post-transplant cyclophosphamide as sole graft-versus-host prophylaxis after allogeneic peripheral blood stem cell transplantation. *Eur J Haematol* 2016; **96**(1): 27-35.
118. Kanakry CG, O'Donnell PV, Furlong T, et al. Multi-institutional study of post-transplantation cyclophosphamide as single-agent graft-versus-host disease prophylaxis after allogeneic bone marrow transplantation using myeloablative busulfan and fludarabine conditioning. *J Clin Oncol* 2014; **32**(31): 3497-505.
119. Bonini C, Ferrari G, Verzeletti S, et al. HSV-TK gene transfer into donor lymphocytes for control of allogeneic graft-versus-leukemia. *Science* 1997; **276**(5319): 1719-24.
120. Ciceri F, Bonini C, Marktel S, et al. Antitumor effects of HSV-TK-engineered donor lymphocytes after allogeneic stem-cell transplantation. *Blood* 2007; **109**(11): 4698-707.
121. Ciceri F, Bonini C, Stanghellini MT, et al. Infusion of suicide-gene-engineered donor lymphocytes after family haploidentical haemopoietic stem-cell transplantation for leukaemia (the TK007 trial): a non-randomised phase I-II study. *Lancet Oncol* 2009; **10**(5): 489-500.
122. Tuthill M, Chen F, Paston S, De La Peña H, Rusakiewicz S, Madrigal A. The prevention and treatment of cytomegalovirus infection in haematopoietic stem cell transplantation. *Cancer Immunol Immunother* 2009; **58**(9): 1481-8.
123. Mailly L, Leboeuf C, Tiberghien P, Baumert T, Robinet E. Genetically engineered T-cells expressing a ganciclovir-sensitive HSV-tk suicide gene for the prevention of GvHD. *Curr Opin Investig Drugs* 2010; **11**(5): 559-70.
124. Zhou X, Dotti G, Krance RA, et al. Inducible caspase-9 suicide gene controls adverse effects from alloreplete T cells after haploidentical stem cell transplantation. *Blood* 2015; **125**(26): 4103-13.
125. Zhou X, Di Stasi A, Tey SK, et al. Long-term outcome after haploidentical stem cell transplant and infusion of T cells expressing the inducible caspase 9 safety transgene. *Blood* 2014; **123**(25): 3895-905.
126. June CH, Bluestone JA, Nadler LM, Thompson CB. The B7 and CD28 receptor families. *Immunol Today* 1994; **15**(7): 321-31.
127. Davies JK, Yuk D, Nadler LM, Guinan EC. Induction of alloanergy in human donor T cells without loss of pathogen or tumor immunity. *Transplantation* 2008; **86**(6): 854-64.
128. Davies JK, Gribben JG, Brennan LL, Yuk D, Nadler LM, Guinan EC. Outcome of alloanergized haploidentical bone marrow transplantation after ex vivo costimulatory blockade: results of 2 phase 1 studies. *Blood* 2008; **112**(6): 2232-41.
129. Roncarolo MG, Battaglia M. Regulatory T-cell immunotherapy for tolerance to self antigens and alloantigens in humans. *Nat Rev Immunol* 2007; **7**(8): 585-98.
130. Hoffmann P, Ermann J, Edinger M, Fathman CG, Strober S. Donor-type CD4(+)CD25(+) regulatory T cells suppress lethal acute graft-versus-host disease after allogeneic bone marrow transplantation. *J Exp Med* 2002; **196**(3): 389-99.
131. Matthews K, Lim Z, Afzali B, et al. Imbalance of effector and regulatory CD4 T cells is associated with graft-versus-host disease after hematopoietic stem cell transplantation using a reduced intensity conditioning regimen and alemtuzumab. *Haematologica* 2009; **94**(7): 956-66.

132. Brunstein CG, Miller JS, Cao Q, et al. Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics. *Blood* 2011; **117**(3): 1061-70.
133. Di Ianni M, Falzetti F, Carotti A, et al. Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. *Blood* 2011; **117**(14): 3921-8.
134. Koreth J, Kim HT, Jones KT, et al. Efficacy, durability, and response predictors of low-dose interleukin-2 therapy for chronic graft-versus-host disease. *Blood* 2016; **128**(1): 130-7.
135. Prentice HG, Blacklock HA, Janossy G, et al. Depletion of T lymphocytes in donor marrow prevents significant graft-versus-host disease in matched allogeneic leukaemic marrow transplant recipients. *Lancet* 1984; **1**(8375): 472-6.
136. Herve P, Flesch M, Cahn JY, et al. Removal of marrow T cells with OKT3-OKT11 monoclonal antibodies and complement to prevent acute graft-versus-host disease. A pilot study in ten patients. *Transplantation* 1985; **39**(2): 138-43.
137. Lang P, Schumm M, Taylor G, et al. Clinical scale isolation of highly purified peripheral CD34+progenitors for autologous and allogeneic transplantation in children. *Bone Marrow Transplant* 1999; **24**(6): 583-9.
138. Apperley JF, Jones L, Hale G, et al. Bone marrow transplantation for patients with chronic myeloid leukaemia: T-cell depletion with Campath-1 reduces the incidence of graft-versus-host disease but may increase the risk of leukaemic relapse. *Bone Marrow Transplant* 1986; **1**(1): 53-66.
139. Pirsch JD, Maki DG. Infectious complications in adults with bone marrow transplantation and T-cell depletion of donor marrow. Increased susceptibility to fungal infections. *Ann Intern Med* 1986; **104**(5): 619-31.
140. Falkenburg JH, Wafelman AR, Joosten P, et al. Complete remission of accelerated phase chronic myeloid leukemia by treatment with leukemia-reactive cytotoxic T lymphocytes. *Blood* 1999; **94**(4): 1201-8.
141. Maus MV, Grupp SA, Porter DL, June CH. Antibody-modified T cells: CARs take the front seat for hematologic malignancies. *Blood* 2014; **123**(17): 2625-35.
142. Morgan RA, Dudley ME, Wunderlich JR, et al. Cancer regression in patients after transfer of genetically engineered lymphocytes. *Science* 2006; **314**(5796): 126-9.
143. Ochi T, Fujiwara H, Okamoto S, et al. Novel adoptive T-cell immunotherapy using a WT1-specific TCR vector encoding silencers for endogenous TCRs shows marked antileukemia reactivity and safety. *Blood* 2011; **118**(6): 1495-503.
144. Anderson LD, Savary CA, Mullen CA. Immunization of allogeneic bone marrow transplant recipients with tumor cell vaccines enhances graft-versus-tumor activity without exacerbating graft-versus-host disease. *Blood* 2000; **95**(7): 2426-33.
145. Teshima T, Liu C, Lowler KP, Dranoff G, Ferrara JL. Donor leukocyte infusion from immunized donors increases tumor vaccine efficacy after allogeneic bone marrow transplantation. *Cancer Res* 2002; **62**(3): 796-800.
146. Anderson LD, Mori S, Mann S, Savary CA, Mullen CA. Pretransplant tumor antigen-specific immunization of allogeneic bone marrow transplant donors enhances graft-versus-tumor activity without exacerbation of graft-versus-host disease. *Cancer Res* 2000; **60**(20): 5797-802.
147. Hashii Y, Sato-Miyashita E, Matsumura R, et al. WT1 peptide vaccination following allogeneic stem cell transplantation in pediatric leukemic patients with high risk for relapse: successful maintenance of durable remission. *Leukemia* 2012; **26**(3): 530-2.
148. Kitawaki T, Kadowaki N, Kondo T, et al. Potential of dendritic-cell immunotherapy for relapse after allogeneic hematopoietic stem cell transplantation, shown by WT1 peptide- and keyhole-limpet-hemocyanin-pulsed, donor-derived dendritic-cell vaccine for acute myeloid leukemia. *Am J Hematol* 2008; **83**(4): 315-7.
149. Billiau AD, Fevery S, Rutgeerts O, Landuyt W, Waer M. Crucial role of timing of donor lymphocyte infusion in generating dissociated graft-versus-host and graft-versus-

leukemia responses in mice receiving allogeneic bone marrow transplants. *Blood* 2002; **100**(5): 1894-902.
150. Tomblyn M, Lazarus HM. Donor lymphocyte infusions: the long and winding road: how should it be traveled? *Bone Marrow Transplant* 2008; **42**(9): 569-79.
151. Porter DL, Levine BL, Bunin N, et al. A phase 1 trial of donor lymphocyte infusions expanded and activated ex vivo via CD3/CD28 costimulation. *Blood* 2006; **107**(4): 1325-31.
152. Ottinger HD, Beelen DW, Scheulen B, Schaefer UW, Grosse-Wilde H. Improved immune reconstitution after allotransplantation of peripheral blood stem cells instead of bone marrow. *Blood* 1996; **88**(7): 2775-9.
153. Rezvani K, Rouce RH. The Application of Natural Killer Cell Immunotherapy for the Treatment of Cancer. *Front Immunol* 2015; **6**: 578.
154. Choi I, Yoon SR, Park SY, et al. Donor-derived natural killer cells infused after human leukocyte antigen-haploidentical hematopoietic cell transplantation: a dose-escalation study. *Biol Blood Marrow Transplant* 2014; **20**(5): 696-704.
155. Ruggeri L, Capanni M, Urbani E, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science* 2002; **295**(5562): 2097-100.
156. Meinhardt K, Kroeger I, Bauer R, et al. Identification and characterization of the specific murine NK cell subset supporting graft-versus-leukemia- and reducing graft-versus-host-effects. *Oncoimmunology* 2015; **4**(1): e981483.
157. Olson JA, Leveson-Gower DB, Gill S, Baker J, Beilhack A, Negrin RS. NK cells mediate reduction of GVHD by inhibiting activated, alloreactive T cells while retaining GVT effects. *Blood* 2010; **115**(21): 4293-301.
158. Foley B, Felices M, Cichocki F, Cooley S, Verneris MR, Miller JS. The biology of NK cells and their receptors affects clinical outcomes after hematopoietic cell transplantation (HCT). *Immunol Rev* 2014; **258**(1): 45-63.
159. Aversa F, Terenzi A, Tabilio A, et al. Full haplotype-mismatched hematopoietic stem-cell transplantation: a phase II study in patients with acute leukemia at high risk of relapse. *J Clin Oncol* 2005; **23**(15): 3447-54.
160. Mancusi A, Ruggeri L, Urbani E, et al. Haploidentical hematopoietic transplantation from KIR ligand-mismatched donors with activating KIRs reduces nonrelapse mortality. *Blood* 2015; **125**(20): 3173-82.
161. Scquizzato E, Zambello R, Teramo A, et al. KIR/HLA-I mismatching and risk of relapse in paediatric patients undergoing non-haploidentical allogeneic haematopoietic stem cell transplantation. *Pediatr Transplant* 2011; **15**(2): 198-204.
162. Beelen DW, Ottinger HD, Ferencik S, et al. Genotypic inhibitory killer immunoglobulin-like receptor ligand incompatibility enhances the long-term antileukemic effect of unmodified allogeneic hematopoietic stem cell transplantation in patients with myeloid leukemias. *Blood* 2005; **105**(6): 2594-600.
163. Stern M, Passweg JR, Meyer-Monard S, et al. Pre-emptive immunotherapy with purified natural killer cells after haploidentical SCT: a prospective phase II study in two centers. *Bone Marrow Transplant* 2013; **48**(3): 433-8.
164. Shah NN, Baird K, Delbrook CP, et al. Acute GVHD in patients receiving IL-15/4-1BBL activated NK cells following T-cell-depleted stem cell transplantation. *Blood* 2015; **125**(5): 784-92.
165. Pérez-Martínez A, Fernández L, Valentín J, et al. A phase I/II trial of interleukin-15--stimulated natural killer cell infusion after haplo-identical stem cell transplantation for pediatric refractory solid tumors. *Cytotherapy* 2015; **17**(11): 1594-603.
166. Bastos-Oreiro M, Anguita J, Martínez-Laperche C, et al. Inhibitory killer cell immunoglobulin-like receptor (iKIR) mismatches improve survival after T-cell-repleted haploidentical transplantation. *Eur J Haematol* 2016; **96**(5): 483-91.
167. Clausen J, Wolf D, Petzer AL, et al. Impact of natural killer cell dose and donor killer-cell immunoglobulin-like receptor (KIR) genotype on outcome following human

leucocyte antigen-identical haematopoietic stem cell transplantation. *Clin Exp Immunol* 2007; **148**(3): 520-8.
168. Giebel S, Nowak I, Dziaczkowska J, et al. Activating killer immunoglobulin-like receptor incompatibilities enhance graft-versus-host disease and affect survival after allogeneic hematopoietic stem cell transplantation. *Eur J Haematol* 2009; **83**(4): 343-56.
169. Ghadially H, Ohana M, Elboim M, et al. NK cell receptor NKp46 regulates graft-versus-host disease. *Cell Rep* 2014; **7**(6): 1809-14.
170. Kong Y, Zhang J, Claxton DF, et al. PD-1(hi)TIM-3(+) T cells associate with and predict leukemia relapse in AML patients post allogeneic stem cell transplantation. *Blood Cancer J* 2015; **5**: e330.
171. Bachireddy P, Hainz U, Rooney M, et al. Reversal of in situ T-cell exhaustion during effective human antileukemia responses to donor lymphocyte infusion. *Blood* 2014; **123**(9): 1412-21.
172. Davids MS, Kim HT, Bachireddy P, et al. Ipilimumab for Patients with Relapse after Allogeneic Transplantation. *N Engl J Med* 2016; **375**(2): 143-53.
173. Yared JA, Hardy N, Singh Z, et al. Major clinical response to nivolumab in relapsed/refractory Hodgkin lymphoma after allogeneic stem cell transplantation. *Bone Marrow Transplant* 2016; **51**(6): 850-2.
174. Corral LG, Haslett PA, Muller GW, et al. Differential cytokine modulation and T cell activation by two distinct classes of thalidomide analogues that are potent inhibitors of TNF-alpha. *J Immunol* 1999; **163**(1): 380-6.
175. Muller GW, Corral LG, Shire MG, et al. Structural modifications of thalidomide produce analogs with enhanced tumor necrosis factor inhibitory activity. *J Med Chem* 1996; **39**(17): 3238-40.
176. Chen N, Wen L, Lau H, Surapaneni S, Kumar G. Pharmacokinetics, metabolism and excretion of [(14)C]-lenalidomide following oral administration in healthy male subjects. *Cancer Chemother Pharmacol* 2012; **69**(3): 789-97.
177. Richardson PG, Schlossman RL, Weller E, et al. Immunomodulatory drug CC-5013 overcomes drug resistance and is well tolerated in patients with relapsed multiple myeloma. *Blood* 2002; **100**(9): 3063-7.
178. Tong Z, Yerramilli U, Surapaneni S, Kumar G. The interactions of lenalidomide with human uptake and efflux transporters and UDP-glucuronosyltransferase 1A1: lack of potential for drug-drug interactions. *Cancer Chemother Pharmacol* 2014; **73**(4): 869-74.
179. Chen N, Lau H, Kong L, et al. Pharmacokinetics of lenalidomide in subjects with various degrees of renal impairment and in subjects on hemodialysis. *J Clin Pharmacol* 2007; **47**(12): 1466-75.
180. Singhal S, Mehta J, Desikan R, et al. Antitumor activity of thalidomide in refractory multiple myeloma. *N Engl J Med* 1999; **341**(21): 1565-71.
181. Hideshima T, Chauhan D, Shima Y, et al. Thalidomide and its analogs overcome drug resistance of human multiple myeloma cells to conventional therapy. *Blood* 2000; **96**(9): 2943-50.
182. Bonkowski J, Vermeulen LC, Kolesar JM. The clinical utility of lenalidomide in multiple myeloma and myelodysplastic syndromes. *J Oncol Pharm Pract* 2010; **16**(4): 223-32.
183. Arumainathan A, Kalakonda N, Pettitt AR. Lenalidomide can be highly effective in chronic lymphocytic leukaemia despite T-cell depletion and deletion of chromosome 17p. *Eur J Haematol* 2011; **87**(4): 372-5.
184. Shanafelt TD, Ramsay AG, Zent CS, et al. Long-term repair of T-cell synapse activity in a phase II trial of chemoimmunotherapy followed by lenalidomide consolidation in previously untreated chronic lymphocytic leukemia (CLL). *Blood* 2013; **121**(20): 4137-41.
185. Ramsay AG, Gribben JG. Immune dysfunction in chronic lymphocytic leukemia T cells and lenalidomide as an immunomodulatory drug. *Haematologica* 2009; **94**(9): 1198-202.

186. Ramsay AG, Clear AJ, Kelly G, et al. Follicular lymphoma cells induce T-cell immunologic synapse dysfunction that can be repaired with lenalidomide: implications for the tumor microenvironment and immunotherapy. *Blood* 2009; **114**(21): 4713-20.
187. Witzig TE, Vose JM, Zinzani PL, et al. An international phase II trial of single-agent lenalidomide for relapsed or refractory aggressive B-cell non-Hodgkin's lymphoma. *Ann Oncol* 2011; **22**(7): 1622-7.
188. Tilly H, Morschhauser F, Salles G, et al. Phase 1b study of lenalidomide in combination with rituximab-CHOP (R2-CHOP) in patients with B-cell lymphoma. *Leukemia* 2013; **27**(1): 252-5.
189. Zhang L, Qian Z, Cai Z, et al. Synergistic antitumor effects of lenalidomide and rituximab on mantle cell lymphoma in vitro and in vivo. *Am J Hematol* 2009; **84**(9): 553-9.
190. Dawar R, Hernandez-Ilizaliturri F. The emerging role of lenalidomide in the management of mantle cell lymphoma (MCL). *Best Pract Res Clin Haematol* 2012; **25**(2): 185-90.
191. Morschhauser F, Fitoussi O, Haioun C, et al. A phase 2, multicentre, single-arm, open-label study to evaluate the safety and efficacy of single-agent lenalidomide (Revlimid(R)) in subjects with relapsed or refractory peripheral T-cell non-Hodgkin lymphoma: The EXPECT trial. *Eur J Cancer* 2013.
192. Beckers MM, Huls G. Therapy refractory angioimmunoblastic T-cell lymphoma in complete remission with lenalidomide. *Eur J Haematol* 2013; **90**(2): 162-3.
193. Bartlett JB, Michael A, Clarke IA, et al. Phase I study to determine the safety, tolerability and immunostimulatory activity of thalidomide analogue CC-5013 in patients with metastatic malignant melanoma and other advanced cancers. *Br J Cancer* 2004; **90**(5): 955-61.
194. Glaspy J, Atkins MB, Richards JM, et al. Results of a multicenter, randomized, double-blind, dose-evaluating phase 2/3 study of lenalidomide in the treatment of metastatic malignant melanoma. *Cancer* 2009; **115**(22): 5228-36.
195. Keizman D, Zahurak M, Sinibaldi V, et al. Lenalidomide in nonmetastatic biochemically relapsed prostate cancer: results of a phase I/II double-blinded, randomized study. *Clin Cancer Res* 2010; **16**(21): 5269-76.
196. Mathew P, Tannir N, Tu SM, Carter CM, Bekele NB, Pagliaro L. A modular Phase I study of lenalidomide and paclitaxel in metastatic castration-resistant prostate cancer following prior taxane therapy. *Cancer Chemother Pharmacol* 2010; **65**(4): 811-5.
197. Lopez-Girona A, Heintel D, Zhang LH, et al. Lenalidomide downregulates the cell survival factor, interferon regulatory factor-4, providing a potential mechanistic link for predicting response. *Br J Haematol* 2011; **154**(3): 325-36.
198. Kotla V, Goel S, Nischal S, et al. Mechanism of action of lenalidomide in hematological malignancies. *J Hematol Oncol* 2009; **2**: 36.
199. Geitz H, Handt S, Zwingenberger K. Thalidomide selectively modulates the density of cell surface molecules involved in the adhesion cascade. *Immunopharmacology* 1996; **31**(2-3): 213-21.
200. Verhelle D, Corral LG, Wong K, et al. Lenalidomide and CC-4047 inhibit the proliferation of malignant B cells while expanding normal CD34+ progenitor cells. *Cancer Res* 2007; **67**(2): 746-55.
201. Sheskin J. The treatment of lepra reaction in lepromatous leprosy. Fifteen years' experience with thalidomide. *Int J Dermatol* 1980; **19**(6): 318-22.
202. Marriott JB, Clarke IA, Dredge K, Muller G, Stirling D, Dalgleish AG. Thalidomide and its analogues have distinct and opposing effects on TNF-alpha and TNFR2 during co-stimulation of both CD4(+) and CD8(+) T cells. *Clin Exp Immunol* 2002; **130**(1): 75-84.
203. Schafer PH, Gandhi AK, Loveland MA, et al. Enhancement of cytokine production and AP-1 transcriptional activity in T cells by thalidomide-related immunomodulatory drugs. *J Pharmacol Exp Ther* 2003; **305**(3): 1222-32.

204. LeBlanc R, Hideshima T, Catley LP, et al. Immunomodulatory drug costimulates T cells via the B7-CD28 pathway. *Blood* 2004; **103**(5): 1787-90.
205. Payvandi F, Wu L, Naziruddin SD, et al. Immunomodulatory drugs (IMiDs) increase the production of IL-2 from stimulated T cells by increasing PKC-theta activation and enhancing the DNA-binding activity of AP-1 but not NF-kappaB, OCT-1, or NF-AT. *J Interferon Cytokine Res* 2005; **25**(10): 604-16.
206. Huang MC, Greig NH, Luo W, et al. Preferential enhancement of older human T cell cytokine generation, chemotaxis, proliferation and survival by lenalidomide. *Clin Immunol* 2011; **138**(2): 201-11.
207. De Keersmaecker B, Allard SD, Lacor P, Schots R, Thielemans K, Aerts JL. Expansion of polyfunctional HIV-specific T cells upon stimulation with mRNA electroporated dendritic cells in the presence of immunomodulatory drugs. *J Virol* 2012; **86**(17): 9351-60.
208. Luptakova K, Rosenblatt J, Glotzbecker B, et al. Lenalidomide enhances anti-myeloma cellular immunity. *Cancer Immunol Immunother* 2013; **62**(1): 39-49.
209. Ramsay AG, Johnson AJ, Lee AM, et al. Chronic lymphocytic leukemia T cells show impaired immunological synapse formation that can be reversed with an immunomodulating drug. *J Clin Invest* 2008; **118**(7): 2427-37.
210. Lim H, Kane L, Schwartz JB, et al. Lenalidomide enhancement of human T cell functions in human immunodeficiency virus (HIV)-infected and HIV-negative CD4 T lymphocytopenic patients. *Clin Exp Immunol* 2012; **169**(2): 182-9.
211. Ramsay AG, Clear AJ, Fatah R, Gribben JG. Multiple inhibitory ligands induce impaired T-cell immunologic synapse function in chronic lymphocytic leukemia that can be blocked with lenalidomide: establishing a reversible immune evasion mechanism in human cancer. *Blood* 2012; **120**(7): 1412-21.
212. Lee BN, Gao H, Cohen EN, et al. Treatment with lenalidomide modulates T-cell immunophenotype and cytokine production in patients with chronic lymphocytic leukemia. *Cancer* 2011; **117**(17): 3999-4008.
213. Chung DJ, Pronschinske KB, Shyer JA, et al. T-cell Exhaustion in Multiple Myeloma Relapse after Autotransplant: Optimal Timing of Immunotherapy. *Cancer Immunol Res* 2016; **4**(1): 61-71.
214. Busch A, Zeh D, Janzen V, et al. Treatment with lenalidomide induces immunoactivating and counter-regulatory immunosuppressive changes in myeloma patients. *Clin Exp Immunol* 2014.
215. Gandhi AK, Shi T, Li M, et al. Immunomodulatory effects in a phase II study of lenalidomide combined with cetuximab in refractory KRAS-mutant metastatic colorectal cancer patients. *PLoS One* 2013; **8**(11): e80437.
216. Idler I, Giannopoulos K, Zenz T, et al. Lenalidomide treatment of chronic lymphocytic leukaemia patients reduces regulatory T cells and induces Th17 T helper cells. *Br J Haematol* 2010; **148**(6): 948-50.
217. Harada T, Ozaki S, Oda A, et al. Association of Th1 and Th2 cytokines with transient inflammatory reaction during lenalidomide plus dexamethasone therapy in multiple myeloma. *Int J Hematol* 2013; **97**(6): 743-8.
218. Galustian C, Meyer B, Labarthe MC, et al. The anti-cancer agents lenalidomide and pomalidomide inhibit the proliferation and function of T regulatory cells. *Cancer Immunol Immunother* 2009; **58**(7): 1033-45.
219. Grygorowicz MA, Borycka IS, Nowak E, et al. Lenalidomide potentiates CD4(+)CD25(+)Treg-related suppression of lymphoma B-cell proliferation. *Clin Exp Med* 2016.
220. Govindaraj C, Madondo M, Kong YY, Tan P, Wei A, Plebanski M. Lenalidomide-based maintenance therapy reduces TNF Receptor 2 on CD4 T cells and enhances immune effector function in acute myeloid leukemia patients. *Am J Hematol* 2014.
221. Muthu Raja KR, Kovarova L, Hajek R. Induction by lenalidomide and dexamethasone combination increases regulatory cells of patients with previously untreated multiple myeloma. *Leuk Lymphoma* 2012; **53**(7): 1406-8.

222. Haslett PA, Corral LG, Albert M, Kaplan G. Thalidomide costimulates primary human T lymphocytes, preferentially inducing proliferation, cytokine production, and cytotoxic responses in the CD8+ subset. *J Exp Med* 1998; **187**(11): 1885-92.
223. Haslett PA, Hanekom WA, Muller G, Kaplan G. Thalidomide and a thalidomide analogue drug costimulate virus-specific CD8+ T cells in vitro. *J Infect Dis* 2003; **187**(6): 946-55.
224. Neuber B, Herth I, Tolliver C, et al. Lenalidomide enhances antigen-specific activity and decreases CD45RA expression of T cells from patients with multiple myeloma. *J Immunol* 2011; **187**(2): 1047-56.
225. McDaniel JM, Pinilla-Ibarz J, Epling-Burnette PK. Molecular action of lenalidomide in lymphocytes and hematologic malignancies. *Adv Hematol* 2012; **2012**: 513702.
226. Noonan K, Rudraraju L, Ferguson A, et al. Lenalidomide-induced immunomodulation in multiple myeloma: impact on vaccines and antitumor responses. *Clin Cancer Res* 2012; **18**(5): 1426-34.
227. Henry JY, Labarthe MC, Meyer B, Dasgupta P, Dalgleish AG, Galustian C. Enhanced cross-priming of naive CD8(+) T cells by dendritic cells treated by the IMiDs((R)) immunomodulatory compounds lenalidomide and pomalidomide. *Immunology* 2013; **139**(3): 377-85.
228. Sakamaki I, Kwak LW, Cha SC, et al. Lenalidomide enhances the protective effect of a therapeutic vaccine and reverses immune suppression in mice bearing established lymphomas. *Leukemia* 2013.
229. Davies FE, Raje N, Hideshima T, et al. Thalidomide and immunomodulatory derivatives augment natural killer cell cytotoxicity in multiple myeloma. *Blood* 2001; **98**(1): 210-6.
230. Hayashi T, Hideshima T, Akiyama M, et al. Molecular mechanisms whereby immunomodulatory drugs activate natural killer cells: clinical application. *Br J Haematol* 2005; **128**(2): 192-203.
231. Hernandez-Ilizaliturri FJ, Reddy N, Holkova B, Ottman E, Czuczman MS. Immunomodulatory drug CC-5013 or CC-4047 and rituximab enhance antitumor activity in a severe combined immunodeficient mouse lymphoma model. *Clin Cancer Res* 2005; **11**(16): 5984-92.
232. Dauguet N, Fournie JJ, Poupot R, Poupot M. Lenalidomide down regulates the production of interferon-gamma and the expression of inhibitory cytotoxic receptors of human Natural Killer cells. *Cell Immunol* 2010; **264**(2): 163-70.
233. Wu L, Parton A, Lu L, Adams M, Schafer P, Bartlett JB. Lenalidomide enhances antibody-dependent cellular cytotoxicity of solid tumor cells in vitro: influence of host immune and tumor markers. *Cancer Immunol Immunother* 2011; **60**(1): 61-73.
234. Reddy N, Hernandez-Ilizaliturri FJ, Deeb G, et al. Immunomodulatory drugs stimulate natural killer-cell function, alter cytokine production by dendritic cells, and inhibit angiogenesis enhancing the anti-tumour activity of rituximab in vivo. *Br J Haematol* 2008; **140**(1): 36-45.
235. Wu L, Adams M, Carter T, et al. lenalidomide enhances natural killer cell and monocyte-mediated antibody-dependent cellular cytotoxicity of rituximab-treated CD20+ tumor cells. *Clin Cancer Res* 2008; **14**(14): 4650-7.
236. Chang DH, Liu N, Klimek V, et al. Enhancement of ligand-dependent activation of human natural killer T cells by lenalidomide: therapeutic implications. *Blood* 2006; **108**(2): 618-21.
237. Song W, van der Vliet HJ, Tai YT, et al. Generation of antitumor invariant natural killer T cell lines in multiple myeloma and promotion of their functions via lenalidomide: a strategy for immunotherapy. *Clin Cancer Res* 2008; **14**(21): 6955-62.
238. Schulz A, Durr C, Zenz T, et al. Lenalidomide reduces survival of chronic lymphocytic leukemia cells in primary cocultures by altering the myeloid microenvironment. *Blood* 2013; **121**(13): 2503-11.

239. Acebes-Huerta A, Huergo-Zapico L, Gonzalez-Rodriguez AP, et al. Lenalidomide induces immunomodulation in chronic lymphocytic leukemia and enhances antitumor immune responses mediated by NK and CD4 T cells. *Biomed Res Int* 2014; **2014**: 265840.
240. Chanan-Khan AA, Chitta K, Ersing N, et al. Biological effects and clinical significance of lenalidomide-induced tumour flare reaction in patients with chronic lymphocytic leukaemia: in vivo evidence of immune activation and antitumour response. *Br J Haematol* 2011; **155**(4): 457-67.
241. Ito T, Ando H, Suzuki T, et al. Identification of a primary target of thalidomide teratogenicity. *Science* 2010; **327**(5971): 1345-50.
242. Zhu YX, Braggio E, Shi CX, et al. Cereblon expression is required for the antimyeloma activity of lenalidomide and pomalidomide. *Blood* 2011; **118**(18): 4771-9.
243. Lopez-Girona A, Mendy D, Ito T, et al. Cereblon is a direct protein target for immunomodulatory and antiproliferative activities of lenalidomide and pomalidomide. *Leukemia* 2012; **26**(11): 2326-35.
244. Bedewy AM, El-Maghraby SM. Do baseline Cereblon gene expression and IL6 receptor expression determine the response to thalidomide-dexamethazone treatment in Multiple myeloma patients? *Eur J Haematol* 2013.
245. Broyl A, Kuiper R, van Duin M, et al. High cereblon expression is associated with better survival in patients with newly diagnosed multiple myeloma treated with thalidomide maintenance. *Blood* 2013; **121**(4): 624-7.
246. Egan JB, Kortuem KM, Kurdoglu A, et al. Extramedullary myeloma whole genome sequencing reveals novel mutations in Cereblon, proteasome subunit G2 and the glucocorticoid receptor in multi drug resistant disease. *Br J Haematol* 2013; **161**(5): 748-51.
247. Lu G, Middleton RE, Sun H, et al. The myeloma drug lenalidomide promotes the cereblon-dependent destruction of Ikaros proteins. *Science* 2014; **343**(6168): 305-9.
248. Gandhi AK, Kang J, Havens CG, et al. Immunomodulatory agents lenalidomide and pomalidomide co-stimulate T cells by inducing degradation of T cell repressors Ikaros and Aiolos via modulation of the E3 ubiquitin ligase complex CRL4. *Br J Haematol* 2013.
249. Zhu YX, Braggio E, Shi CX, et al. Identification of cereblon-binding proteins and relationship with response and survival after IMiDs in multiple myeloma. *Blood* 2014; **124**(4): 536-45.
250. Wang JH, Nichogiannopoulou A, Wu L, et al. Selective defects in the development of the fetal and adult lymphoid system in mice with an Ikaros null mutation. *Immunity* 1996; **5**(6): 537-49.
251. Harker N, Naito T, Cortes M, et al. The CD8alpha gene locus is regulated by the Ikaros family of proteins. *Mol Cell* 2002; **10**(6): 1403-15.
252. Avitahl N, Winandy S, Friedrich C, Jones B, Ge Y, Georgopoulos K. Ikaros sets thresholds for T cell activation and regulates chromosome propagation. *Immunity* 1999; **10**(3): 333-43.
253. Bandyopadhyay S, Dure M, Paroder M, Soto-Nieves N, Puga I, Macian F. Interleukin 2 gene transcription is regulated by Ikaros-induced changes in histone acetylation in anergic T cells. *Blood* 2007; **109**(7): 2878-86.
254. Thomas RM, Chunder N, Chen C, Umetsu SE, Winandy S, Wells AD. Ikaros enforces the costimulatory requirement for IL2 gene expression and is required for anergy induction in CD4+ T lymphocytes. *J Immunol* 2007; **179**(11): 7305-15.
255. Quirion MR, Gregory GD, Umetsu SE, Winandy S, Brown MA. Cutting edge: Ikaros is a regulator of Th2 cell differentiation. *J Immunol* 2009; **182**(2): 741-5.
256. Kneppers E, van der Holt B, Kersten MJ, et al. Lenalidomide maintenance after nonmyeloablative allogeneic stem cell transplantation in multiple myeloma is not feasible: results of the HOVON 76 Trial. *Blood* 2011; **118**(9): 2413-9.
257. Sockel K, Bornhaeuser M, Mischak-Weissinger E, et al. Lenalidomide maintenance after allogeneic HSCT seems to trigger acute graft-versus-host disease in

patients with high-risk myelodysplastic syndromes or acute myeloid leukemia and del(5q): results of the LENAMAINT trial. *Haematologica* 2012; **97**(9): e34-5.
258. Coman T, Bachy E, Michallet M, et al. Lenalidomide as salvage treatment for multiple myeloma relapsing after allogeneic hematopoietic stem cell transplantation: a report from the French Society of Bone Marrow and Cellular Therapy. *Haematologica* 2013; **98**(5): 776-83.
259. Lioznov M, El-Cheikh J, Jr., Hoffmann F, et al. Lenalidomide as salvage therapy after allo-SCT for multiple myeloma is effective and leads to an increase of activated NK (NKp44(+)) and T (HLA-DR(+)) cells. *Bone Marrow Transplant* 2010; **45**(2): 349-53.
260. Wolschke C, Stubig T, Hegenbart U, et al. Postallograft lenalidomide induces strong NK cell-mediated antimyeloma activity and risk for T cell-mediated GvHD: Results from a phase I/II dose-finding study. *Exp Hematol* 2013; **41**(2): 134-42 e3.
261. Blum W, Klisovic RB, Becker H, et al. Dose escalation of lenalidomide in relapsed or refractory acute leukemias. *J Clin Oncol* 2010; **28**(33): 4919-25.
262. Ades L, Eclache V, Gardin C, et al. Efficacy of lenalidomide in an sAML patient with del 5q relapsing after reduced-intensity allogeneic stem cell transplantation performed in complete remission. *Bone Marrow Transplant* 2010; **45**(4): 791-2.
263. Ford CD, Asch J, Konopa K, Petersen FB. CR with lenalidomide in del(5)(q13q33) AML relapsing after allogeneic hematopoietic SCT. *Bone Marrow Transplant* 2010; **45**(2): 403-4.
264. Olivieri A, Attolico I, Cimminiello M, Discepoli G, Cifarelli RA. Lenalidomide can induce graft versus leukemia effect in primary plasma cell leukemia: a case report. *Leuk Res* 2009; **33**(11): e191-3.
265. Mehta RS, Di Stasi A, Hosing C, et al. Lenalidomide-Induced Graft-Vs.-Leukemia Effect in a Patient With Chronic Lymphocytic Leukemia Who Relapsed After Allogeneic Stem Cell Transplant. *Clin Lymphoma Myeloma Leuk* 2013.
266. Mandac I, Kolonic SO. Lenalidomide induced good clinical response in a patient with multiple relapsed and refractory Hodgkin's lymphoma. *J Hematol Oncol* 2010; **3**: 20.
267. Alsina M, Becker PS, Zhong X, et al. Lenalidomide Maintenance for High Risk Multiple Myeloma after Allogeneic Hematopoietic Cell Transplantation. *Biol Blood Marrow Transplant* 2014.
268. Kroger N, Badbaran A, Lioznov M, et al. Post-transplant immunotherapy with donor-lymphocyte infusion and novel agents to upgrade partial into complete and molecular remission in allografted patients with multiple myeloma. *Exp Hematol* 2009; **37**(7): 791-8.
269. Kroger N, Zabelina T, Klyuchnikov E, et al. Toxicity-reduced, myeloablative allograft followed by lenalidomide maintenance as salvage therapy for refractory/relapsed myeloma patients. *Bone Marrow Transplant* 2013; **48**(3): 403-7.
270. Bensinger WI, Green DJ, Burwick N, Becker PS. A prospective study of lenalidomide monotherapy for relapse after Allo-SCT for multiple myeloma. *Bone Marrow Transplant* 2014.
271. El-Cheikh J, Crocchiolo R, Furst S, et al. Lenalidomide plus donor-lymphocytes infusion after allogeneic stem-cell transplantation with reduced-intensity conditioning in patients with high-risk multiple myeloma. *Exp Hematol* 2012; **40**(7): 521-7.
272. Minnema MC, van der Veer MS, Aarts T, Emmelot M, Mutis T, Lokhorst HM. Lenalidomide alone or in combination with dexamethasone is highly effective in patients with relapsed multiple myeloma following allogeneic stem cell transplantation and increases the frequency of CD4+Foxp3+ T cells. *Leukemia* 2009; **23**(3): 605-7.
273. Davies JK, Taussig D, Oakervee H, et al. Long-term survival with low toxicity after allogeneic transplantation for acute myeloid leukaemia and myelodysplasia using non-myeloablative conditioning without T cell depletion. *Br J Haematol* 2013; **162**(4): 525-9.
274. Iqbal M, Wani TA, Khalil NY, Darwish IA. Development and validation of ultra-performance liquid chromatographic method with tandem mass spectrometry for determination of lenalidomide in rabbit and human plasma. *Chem Cent J* 2013; **7**(1): 7.

275. Fulcher D, Wong S. Carboxyfluorescein succinimidyl ester-based proliferative assays for assessment of T cell function in the diagnostic laboratory. *Immunol Cell Biol* 1999; **77**(6): 559-64.
276. Hasbold J, Gett AV, Rush JS, et al. Quantitative analysis of lymphocyte differentiation and proliferation in vitro using carboxyfluorescein diacetate succinimidyl ester. *Immunol Cell Biol* 1999; **77**(6): 516-22.
277. Marin L, Minguela A, Torio A, et al. Flow cytometric quantification of apoptosis and proliferation in mixed lymphocyte culture. *Cytometry A* 2003; **51**(2): 107-18.
278. Quah BJ, Parish CR. The use of carboxyfluorescein diacetate succinimidyl ester (CFSE) to monitor lymphocyte proliferation. *J Vis Exp* 2010; (44).
279. Scheinberg P, Price DA, Ambrozak DR, Barrett AJ, Douek DC. Alloreactive T cell clonotype recruitment in a mixed lymphocyte reaction: implications for graft engineering. *Exp Hematol* 2006; **34**(6): 788-95.
280. Bishara A, Brautbar C, Nagler A, et al. Prediction by a modified mixed leukocyte reaction assay of graft-versus-host disease and graft rejection after allogeneic bone marrow transplantation. *Transplantation* 1994; **57**(10): 1474-9.
281. Bishara A, Brautbar C, Cohen I, Nagler A. Positivity in a modified mixed leukocyte reaction test correlates with molecular HLA-C disparity in prediction of unrelated bone marrow transplantation outcome. *Hum Immunol* 1999; **60**(9): 833-9.
282. Tanaka J, Imamura M, Kasai M, Sakurada K. Transplantation-related complications predicted by cytokine gene expression in the mixed lymphocyte culture in allogeneic bone marrow transplants. *Leuk Lymphoma* 1995; **19**(1-2): 27-32.
283. Mehrotra A, Leventhal J, Purroy C, Cravedi P. Monitoring T cell alloreactivity. *Transplant Rev (Orlando)* 2015; **29**(2): 53-9.
284. Bendall SC, Nolan GP, Roederer M, Chattopadhyay PK. A deep profiler's guide to cytometry. *Trends Immunol* 2012; **33**(7): 323-32.
285. Zaritskaya L, Shurin MR, Sayers TJ, Malyguine AM. New flow cytometric assays for monitoring cell-mediated cytotoxicity. *Expert Rev Vaccines* 2010; **9**(6): 601-16.
286. Freer G, Rindi L. Intracellular cytokine detection by fluorescence-activated flow cytometry: basic principles and recent advances. *Methods* 2013; **61**(1): 30-8.
287. Allan AL, Keeney M. Circulating tumor cell analysis: technical and statistical considerations for application to the clinic. *J Oncol* 2010; **2010**: 426218.
288. Chen JC, Chang ML, Muench MO. A kinetic study of the murine mixed lymphocyte reaction by 5,6-carboxyfluorescein diacetate succinimidyl ester labeling. *J Immunol Methods* 2003; **279**(1-2): 123-33.
289. Lyons AB. Analysing cell division in vivo and in vitro using flow cytometric measurement of CFSE dye dilution. *J Immunol Methods* 2000; **243**(1-2): 147-54.
290. Martins SL, St John LS, Champlin RE, et al. Functional assessment and specific depletion of alloreactive human T cells using flow cytometry. *Blood* 2004; **104**(12): 3429-36.
291. Hsieh MH, Patterson AE, Korngold R. T-cell subsets mediate graft-versus-myeloid leukemia responses via different cytotoxic mechanisms. *Biol Blood Marrow Transplant* 2000; **6**(3): 231-40.
292. Davis MM, Bjorkman PJ. T-cell antigen receptor genes and T-cell recognition. *Nature* 1988; **334**(6181): 395-402.
293. Rowen L, Koop BF, Hood L. The complete 685-kilobase DNA sequence of the human beta T cell receptor locus. *Science* 1996; **272**(5269): 1755-62.
294. Wei S, Charmley P, Robinson MA, Concannon P. The extent of the human germline T-cell receptor V beta gene segment repertoire. *Immunogenetics* 1994; **40**(1): 27-36.
295. Callan MF, Steven N, Krausa P, et al. Large clonal expansions of CD8+ T cells in acute infectious mononucleosis. *Nat Med* 1996; **2**(8): 906-11.
296. Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 1999; **401**(6754): 708-12.

297. Taniguchi T, Minami Y. The IL-2/IL-2 receptor system: a current overview. *Cell* 1993; **73**(1): 5-8.
298. Kaech SM, Tan JT, Wherry EJ, Konieczny BT, Surh CD, Ahmed R. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. *Nat Immunol* 2003; **4**(12): 1191-8.
299. Casazza JP, Betts MR, Price DA, et al. Acquisition of direct antiviral effector functions by CMV-specific CD4+ T lymphocytes with cellular maturation. *J Exp Med* 2006; **203**(13): 2865-77.
300. Stuller KA, Cush SS, Flaño E. Persistent gamma-herpesvirus infection induces a CD4 T cell response containing functionally distinct effector populations. *J Immunol* 2010; **184**(7): 3850-6.
301. Betts MR, Nason MC, West SM, et al. HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. *Blood* 2006; **107**(12): 4781-9.
302. Wimmers F, Aarntzen EH, Duiveman-deBoer T, et al. Long-lasting multifunctional CD8(+) T cell responses in end-stage melanoma patients can be induced by dendritic cell vaccination. *Oncoimmunology* 2016; **5**(1): e1067745.
303. Cao T, Soto A, Zhou W, et al. Ex vivo expanded human CD4+CD25+Foxp3+ regulatory T cells prevent lethal xenogenic graft versus host disease (GVHD). *Cell Immunol* 2009; **258**(1): 65-71.
304. Björkström NK, Ljunggren HG, Michaëlsson J. Emerging insights into natural killer cells in human peripheral tissues. *Nat Rev Immunol* 2016; **16**(5): 310-20.
305. Romagnani C, Juelke K, Falco M, et al. CD56brightCD16- killer Ig-like receptor-NK cells display longer telomeres and acquire features of CD56dim NK cells upon activation. *J Immunol* 2007; **178**(8): 4947-55.
306. Wu Z, Frascaroli G, Bayer C, Schmal T, Mertens T. Interleukin-2 from Adaptive T Cells Enhances Natural Killer Cell Activity against Human Cytomegalovirus-Infected Macrophages. *J Virol* 2015; **89**(12): 6435-41.
307. Fehniger TA, Carson WE, Caligiuri MA. Costimulation of human natural killer cells is required for interferon gamma production. *Transplant Proc* 1999; **31**(3): 1476-8.
308. Klangsinsirikul P, Russell NH. Peripheral blood stem cell harvests from G-CSF-stimulated donors contain a skewed Th2 CD4 phenotype and a predominance of type 2 dendritic cells. *Exp Hematol* 2002; **30**(5): 495-501.
309. Tayebi H, Kuttler F, Saas P, et al. Effect of granulocyte colony-stimulating factor mobilization on phenotypical and functional properties of immune cells. *Exp Hematol* 2001; **29**(4): 458-70.
310. Rutella S, Pierelli L, Bonanno G, et al. Role for granulocyte colony-stimulating factor in the generation of human T regulatory type 1 cells. *Blood* 2002; **100**(7): 2562-71.
311. Toh HC, Sun L, Soe Y, et al. G-CSF induces a potentially tolerant gene and immunophenotype profile in T cells in vivo. *Clin Immunol* 2009; **132**(1): 83-92.
312. Leitner GC, Faschingbauer M, Wenda S, Weigel G, Fischer G. Administration of recombinant human granulocyte-colony-stimulating factor does not induce long-lasting detectable epigenetic alterations in healthy donors. *Transfusion* 2014; **54**(12): 3121-6.
313. St John LS, Wan L, He H, et al. PR1-specific cytotoxic T lymphocytes are relatively frequent in umbilical cord blood and can be effectively expanded to target myeloid leukemia. *Cytotherapy* 2016; **18**(8): 995-1001.
314. Hiwarkar P, Qasim W, Ricciardelli I, et al. Cord blood T cells mediate enhanced antitumor effects compared with adult peripheral blood T cells. *Blood* 2015; **126**(26): 2882-91.
315. Torelli GF, Maggio R, Peragine N, et al. Functional analysis and gene expression profile of umbilical cord blood regulatory T cells. *Ann Hematol* 2012; **91**(2): 155-61.
316. Szabolcs P, Park KD, Reese M, Marti L, Broadwater G, Kurtzberg J. Coexistent naïve phenotype and higher cycling rate of cord blood T cells as compared to adult peripheral blood. *Exp Hematol* 2003; **31**(8): 708-14.

317. Sol MA, Thomsen M, Durand M, et al. Flow cytometric characterization of proliferating natural killer lymphocytes from bone marrow donors in the mixed lymphocyte reaction. *Cytometry* 1998; **33**(1): 67-75.
318. Beatty PG, Mori M, Milford E. Impact of racial genetic polymorphism on the probability of finding an HLA-matched donor. *Transplantation* 1995; **60**(8): 778-83.
319. Henslee-Downey PJ, Abhyankar SH, Parrish RS, et al. Use of partially mismatched related donors extends access to allogeneic marrow transplant. *Blood* 1997; **89**(10): 3864-72.
320. Rocha V, Wagner JE, Sobocinski KA, et al. Graft-versus-host disease in children who have received a cord-blood or bone marrow transplant from an HLA-identical sibling. Eurocord and International Bone Marrow Transplant Registry Working Committee on Alternative Donor and Stem Cell Sources. *N Engl J Med* 2000; **342**(25): 1846-54.
321. Eapen M, Rocha V, Sanz G, et al. Effect of graft source on unrelated donor haemopoietic stem-cell transplantation in adults with acute leukaemia: a retrospective analysis. *Lancet Oncol* 2010; **11**(7): 653-60.
322. Chevallier P, Robillard N, Illiaquer M, et al. Characterization of various blood and graft sources: a prospective series. *Transfusion* 2013; **53**(9): 2020-6.
323. D'Arena G, Musto P, Cascavilla N, et al. Flow cytometric characterization of human umbilical cord blood lymphocytes: immunophenotypic features. *Haematologica* 1998; **83**(3): 197-203.
324. Risdon G, Gaddy J, Stehman FB, Broxmeyer HE. Proliferative and cytotoxic responses of human cord blood T lymphocytes following allogeneic stimulation. *Cell Immunol* 1994; **154**(1): 14-24.
325. Matthews NC, Wadhwa M, Bird C, Borras FE, Navarrete CV. Sustained expression of CD154 (CD40L) and proinflammatory cytokine production by alloantigen-stimulated umbilical cord blood T cells. J Immunol. United States; 2000: 6206-12.
326. Chalmers IM, Janossy G, Contreras M, Navarrete C. Intracellular cytokine profile of cord and adult blood lymphocytes. *Blood* 1998; **92**(1): 11-8.
327. Lee YS, Kim TS, Kim DK. T lymphocytes derived from human cord blood provide effective antitumor immunotherapy against a human tumor. *BMC Cancer* 2011; **11**: 225.
328. Brown JA, Boussiotis VA. Umbilical cord blood transplantation: basic biology and clinical challenges to immune reconstitution. *Clin Immunol* 2008; **127**(3): 286-97.
329. Lucchini G, Perales MA, Veys P. Immune reconstitution after cord blood transplantation: peculiarities, clinical implications and management strategies. *Cytotherapy* 2015; **17**(6): 711-22.
330. Godfrey WR, Spoden DJ, Ge YG, et al. Cord blood CD4+CD25+-derived T regulatory cell lines express FoxP3 protein and manifest potent suppressor function. *Blood* 2005; **105**(2): 750-8.
331. Lin SJ, Lu CH, Yan DC, Lee PT, Hsiao HS, Kuo ML. Expansion of regulatory T cells from umbilical cord blood and adult peripheral blood CD4(+)CD25 (+) T cells. *Immunol Res* 2014; **60**(1): 105-11.
332. Hoffmann P, Eder R, Boeld TJ, et al. Only the CD45RA+ subpopulation of CD4+CD25high T cells gives rise to homogeneous regulatory T-cell lines upon in vitro expansion. *Blood* 2006; **108**(13): 4260-7.
333. Luevano M, Daryouzeh M, Alnabhan R, et al. The unique profile of cord blood natural killer cells balances incomplete maturation and effective killing function upon activation. *Hum Immunol* 2012; **73**(3): 248-57.
334. Gandhi AK, Kang J, Havens CG, et al. Immunomodulatory agents lenalidomide and pomalidomide co-stimulate T cells by inducing degradation of T cell repressors Ikaros and Aiolos via modulation of the E3 ubiquitin ligase complex CRL4(CRBN.). *Br J Haematol* 2014; **164**(6): 811-21.
335. Fleige S, Pfaffl MW. RNA integrity and the effect on the real-time qRT-PCR performance. *Mol Aspects Med* 2006; **27**(2-3): 126-39.
336. Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* 2004; **3**: Article3.

337. Benjamini Y, Drai D, Elmer G, Kafkafi N, Golani I. Controlling the false discovery rate in behavior genetics research. *Behav Brain Res* 2001; **125**(1-2): 279-84.
338. Knosp CA, Carroll HP, Elliott J, et al. SOCS2 regulates T helper type 2 differentiation and the generation of type 2 allergic responses. *J Exp Med* 2011; **208**(7): 1523-31.
339. Palmer DC, Restifo NP. Suppressors of Cytokine Signaling (SOCS) in T cell differentiation, maturation, and function. *Trends Immunol* 2009; **30**(12): 592-602.
340. Tannahill GM, Elliott J, Barry AC, Hibbert L, Cacalano NA, Johnston JA. SOCS2 can enhance interleukin-2 (IL-2) and IL-3 signaling by accelerating SOCS3 degradation. *Mol Cell Biol* 2005; **25**(20): 9115-26.
341. Telang S, Clem BF, Klarer AC, et al. Small molecule inhibition of 6-phosphofructo-2-kinase suppresses t cell activation. *J Transl Med* 2012; **10**: 95.
342. Pollizzi KN, Powell JD. Integrating canonical and metabolic signalling programmes in the regulation of T cell responses. *Nat Rev Immunol* 2014; **14**(7): 435-46.
343. Nguyen XH, Lang PA, Lang KS, et al. Toso regulates the balance between apoptotic and nonapoptotic death receptor signaling by facilitating RIP1 ubiquitination. *Blood* 2011; **118**(3): 598-608.
344. Murakami Y, Narayanan S, Su S, et al. Toso, a functional IgM receptor, is regulated by IL-2 in T and NK cells. *J Immunol* 2012; **189**(2): 587-97.
345. Liu F, Rehmani I, Esaki S, et al. Pirin is an iron-dependent redox regulator of NF-kappaB. *Proc Natl Acad Sci U S A* 2013; **110**(24): 9722-7.
346. Owusu-Ansah E, Banerjee U. Reactive oxygen species prime Drosophila haematopoietic progenitors for differentiation. *Nature* 2009; **461**(7263): 537-41.
347. Yang Z, Shen Y, Oishi H, et al. Restoring oxidant signaling suppresses proarthritogenic T cell effector functions in rheumatoid arthritis. *Sci Transl Med* 2016; **8**(331): 331ra38.
348. Kiaii S, Clear AJ, Ramsay AG, et al. Follicular lymphoma cells induce changes in T-cell gene expression and function: potential impact on survival and risk of transformation. *J Clin Oncol* 2013; **31**(21): 2654-61.
349. Keever CA. Characterization of cord blood lymphocyte subpopulations. *J Hematother* 1993; **2**(2): 203-6.
350. Gorelik L, Flavell RA. Transforming growth factor-beta in T-cell biology. *Nat Rev Immunol* 2002; **2**(1): 46-53.
351. Hough KP, Chisolm DA, Weinmann AS. Transcriptional regulation of T cell metabolism. *Mol Immunol* 2015; **68**(2 Pt C): 520-6.
352. Chou C, Pinto AK, Curtis JD, et al. c-Myc-induced transcription factor AP4 is required for host protection mediated by CD8+ T cells. *Nat Immunol* 2014; **15**(9): 884-93.
353. Karmaus PW, Chi H. c-Myc and AP4: a relay team for metabolic reprogramming of CD8+ T cells. *Nat Immunol* 2014; **15**(9): 828-9.
354. Finlay DK, Rosenzweig E, Sinclair LV, et al. PDK1 regulation of mTOR and hypoxia-inducible factor 1 integrate metabolism and migration of CD8+ T cells. *J Exp Med* 2012; **209**(13): 2441-53.
355. Heintel D, Rocci A, Ludwig H, et al. High expression of cereblon (CRBN) is associated with improved clinical response in patients with multiple myeloma treated with lenalidomide and dexamethasone. *Br J Haematol* 2013; **161**(5): 695-700.
356. Bjorklund CC, Ma W, Wang ZQ, et al. Evidence of a role for activation of Wnt/beta-catenin signaling in the resistance of plasma cells to lenalidomide. *J Biol Chem* 2011; **286**(13): 11009-20.
357. Maciolek JA, Pasternak JA, Wilson HL. Metabolism of activated T lymphocytes. *Curr Opin Immunol* 2014; **27**: 60-74.
358. Jeffrey K. Davies DCT, Heather Oakervee,Andrew J. Davies, Samir G. Agrawal, John G. Gribben,T. Andrew Lister, and Jamie D. Cavenagh. Long-Term Follow-Up After Reduced-Intensity Conditioning Allogeneic Transplantation for Acute Myeloid

Leukemia/Myelodysplastic Syndrome: Late CNS Relapses Despite Graft-Versus-Host Disease. *Journal of Clinical Oncology* 2006.
359. Fanning SL, Zilberberg J, Stein J, et al. Unraveling graft-versus-host disease and graft-versus-leukemia responses using TCR Vbeta spectratype analysis in a murine bone marrow transplantation model. *J Immunol* 2013; **190**(1): 447-57.
360. Childs R, Chernoff A, Contentin N, et al. Regression of metastatic renal-cell carcinoma after nonmyeloablative allogeneic peripheral-blood stem-cell transplantation. *N Engl J Med* 2000; **343**(11): 750-8.
361. Khouri IF, Saliba RM, Giralt SA, et al. Nonablative allogeneic hematopoietic transplantation as adoptive immunotherapy for indolent lymphoma: low incidence of toxicity, acute graft-versus-host disease, and treatment-related mortality. *Blood* 2001; **98**(13): 3595-9.
362. Bleakley M, Riddell SR. Exploiting T cells specific for human minor histocompatibility antigens for therapy of leukemia. *Immunol Cell Biol* 2011; **89**(3): 396-407.
363. Casucci M, Perna SK, Falcone L, et al. Graft-versus-leukemia effect of HLA-haploidentical central-memory T-cells expanded with leukemic APCs and modified with a suicide gene. *Mol Ther* 2013; **21**(2): 466-75.
364. Rosinski SL, Stone B, Graves SS, et al. Development of a Minor Histocompatibility Antigen Vaccine Regimen in the Canine Model of Hematopoietic Cell Transplantation. *Transplantation* 2015; **99**(10): 2083-94.
365. Yuan J, Ren HY, Shi YJ, Liu W. In Vitro Immunological Effects of Blocking CCR5 on T Cells. *Inflammation* 2015; **38**(2): 902-10.
366. Hayashida JN, Nakamura S, Toyoshima T, et al. Possible involvement of cytokines, chemokines and chemokine receptors in the initiation and progression of chronic GVHD. *Bone Marrow Transplant* 2013; **48**(1): 115-23.
367. Jaksch M, Remberger M, Mattsson J. Increased gene expression of chemokine receptors is correlated with acute graft-versus-host disease after allogeneic stem cell transplantation. *Biol Blood Marrow Transplant* 2005; **11**(4): 280-7.
368. Luster AD. Chemokines--chemotactic cytokines that mediate inflammation. *N Engl J Med* 1998; **338**(7): 436-45.
369. Zlotnik A, Yoshie O. The chemokine superfamily revisited. *Immunity* 2012; **36**(5): 705-16.
370. Bogunia-Kubik K, Mizia S, Gronkowska A, et al. CCR5 gene polymorphism affects the risk of GvHD after haematopoietic stem cell transplantation from an unrelated donor. *Br J Haematol* 2015.
371. Rao AR, Quinones MP, Garavito E, et al. CC chemokine receptor 2 expression in donor cells serves an essential role in graft-versus-host-disease. *J Immunol* 2003; **171**(9): 4875-85.
372. Loeffler J, Ok M, Morton OC, Mezger M, Einsele H. Genetic polymorphisms in the cytokine and chemokine system: their possible importance in allogeneic stem cell transplantation. *Curr Top Microbiol Immunol* 2010; **341**: 83-96.
373. Palmer LA, Sale GE, Balogun JI, et al. Chemokine receptor CCR5 mediates alloimmune responses in graft-versus-host disease. *Biol Blood Marrow Transplant* 2010; **16**(3): 311-9.
374. Coghill JM, Carlson MJ, Panoskaltsis-Mortari A, et al. Separation of graft-versus-host disease from graft-versus-leukemia responses by targeting CC-chemokine receptor 7 on donor T cells. *Blood* 2010; **115**(23): 4914-22.
375. Chewning JH, Zhang W, Randolph DA, Swindle CS, Schoeb TR, Weaver CT. Allogeneic Th1 cells home to host bone marrow and spleen and mediate IFNgamma-dependent aplasia. *Biol Blood Marrow Transplant* 2013; **19**(6): 876-87.
376. BOYDEN S. The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leucocytes. *J Exp Med* 1962; **115**: 453-66.
377. Kramer N, Walzl A, Unger C, et al. In vitro cell migration and invasion assays. *Mutat Res* 2013; **752**(1): 10-24.

378. Lim HW, Broxmeyer HE, Kim CH. Regulation of Trafficking Receptor Expression in Human Forkhead Box P3+ Regulatory T Cells. *The Journal of Immunology* 2006; **177**(2): 840-51.
379. Ramsay AG, Evans R, Kiaii S, Svensson L, Hogg N, Gribben JG. Chronic lymphocytic leukemia cells induce defective LFA-1-directed T-cell motility by altering Rho GTPase signaling that is reversible with lenalidomide. *Blood* 2013; **121**(14): 2704-14.
380. Salgado FJ, Lojo J, Fernandez-Alonso CM, Vinuela JE, Cordero OJ, Nogueira M. Interleukin-dependent modulation of HLA-DR expression on CD4 and CD8 activated T cells. *Immunol Cell Biol* 2002; **80**(2): 138-47.
381. Diaz-Guerra E, Vernal R, del Prete MJ, Silva A, Garcia-Sanz JA. CCL2 inhibits the apoptosis program induced by growth factor deprivation, rescuing functional T cells. *J Immunol* 2007; **179**(11): 7352-7.
382. Kim CH, Rott L, Kunkel EJ, et al. Rules of chemokine receptor association with T cell polarization in vivo. *J Clin Invest* 2001; **108**(9): 1331-9.
383. Zhang HH, Song K, Rabin RL, et al. CCR2 identifies a stable population of human effector memory CD4+ T cells equipped for rapid recall response. *J Immunol* 2010; **185**(11): 6646-63.
384. Sviland L, Dickinson AM. A human skin explant model for predicting graft-versus-host disease following bone marrow transplantation. *J Clin Pathol* 1999; **52**(12): 910-3.
385. Wang XN, Collin M, Sviland L, et al. Skin explant model of human graft-versus-host disease: prediction of clinical outcome and correlation with biological risk factors. *Biol Blood Marrow Transplant* 2006; **12**(2): 152-9.
386. Schroeder MA, DiPersio JF. Mouse models of graft-versus-host disease: advances and limitations. *Dis Model Mech* 2011; **4**(3): 318-33.
387. Najima Y, Tomizawa-Murasawa M, Saito Y, et al. Induction of WT1-specific human CD8+ T cells from human HSCs in HLA class I Tg NOD/SCID/IL2rgKO mice. *Blood* 2016; **127**(6): 722-34.
388. Kolb HJ, Schattenberg A, Goldman JM, et al. Graft-versus-leukemia effect of donor lymphocyte transfusions in marrow grafted patients. *Blood* 1995; **86**(5): 2041-50.
389. Brudno JN, Somerville RP, Shi V, et al. Allogeneic T Cells That Express an Anti-CD19 Chimeric Antigen Receptor Induce Remissions of B-Cell Malignancies That Progress After Allogeneic Hematopoietic Stem-Cell Transplantation Without Causing Graft-Versus-Host Disease. *J Clin Oncol* 2016; **34**(10): 1112-21.
390. Hambach L, Ling KW, Pool J, et al. Hypomethylating drugs convert HA-1-negative solid tumors into targets for stem cell-based immunotherapy. *Blood* 2009; **113**(12): 2715-22.
391. Almstedt M, Blagitko-Dorfs N, Duque-Afonso J, et al. The DNA demethylating agent 5-aza-2'-deoxycytidine induces expression of NY-ESO-1 and other cancer/testis antigens in myeloid leukemia cells. *Leuk Res* 2010; **34**(7): 899-905.
392. Goodyear OC, Dennis M, Jilani NY, et al. Azacitidine augments expansion of regulatory T cells after allogeneic stem cell transplantation in patients with acute myeloid leukemia (AML). *Blood* 2012; **119**(14): 3361-9.
393. Stübig T, Badbaran A, Luetkens T, et al. 5-azacytidine promotes an inhibitory T-cell phenotype and impairs immune mediated antileukemic activity. *Mediators Inflamm* 2014; **2014**: 418292.
394. Czibere A, Bruns I, Kröger N, et al. 5-Azacytidine for the treatment of patients with acute myeloid leukemia or myelodysplastic syndrome who relapse after allo-SCT: a retrospective analysis. *Bone Marrow Transplant* 2010; **45**(5): 872-6.
395. de Lima M, Giralt S, Thall PF, et al. Maintenance therapy with low-dose azacitidine after allogeneic hematopoietic stem cell transplantation for recurrent acute myelogenous leukemia or myelodysplastic syndrome: a dose and schedule finding study. *Cancer* 2010; **116**(23): 5420-31.
396. Platzbecker U, Wermke M, Radke J, et al. Azacitidine for treatment of imminent relapse in MDS or AML patients after allogeneic HSCT: results of the RELAZA trial. *Leukemia* 2012; **26**(3): 381-9.

397. Brunstein CG, Miller JS, McKenna DH, et al. Umbilical cord blood-derived T regulatory cells to prevent GVHD: kinetics, toxicity profile and clinical effect. *Blood* 2015.
398. Xu Q, Hou YX, Langlais P, et al. Expression of the cereblon binding protein argonaute 2 plays an important role for multiple myeloma cell growth and survival. *BMC Cancer* 2016; **16**(1): 297.
399. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004; **116**(2): 281-97.
400. Wu H, Neilson JR, Kumar P, et al. miRNA profiling of naive, effector and memory CD8 T cells. *PLoS One* 2007; **2**(10): e1020.
401. Bronevetsky Y, Villarino AV, Eisley CJ, et al. T cell activation induces proteasomal degradation of Argonaute and rapid remodeling of the microRNA repertoire. *J Exp Med* 2013; **210**(2): 417-32.
402. Marcais A, Blevins R, Graumann J, et al. microRNA-mediated regulation of mTOR complex components facilitates discrimination between activation and anergy in CD4 T cells. *J Exp Med* 2014; **211**(11): 2281-95.
403. Rana TM. Illuminating the silence: understanding the structure and function of small RNAs. *Nat Rev Mol Cell Biol* 2007; **8**(1): 23-36.
404. Merkerova M, Vasikova A, Belickova M, Bruchova H. MicroRNA expression profiles in umbilical cord blood cell lineages. *Stem Cells Dev* 2010; **19**(1): 17-26.
405. Weitzel RP, Lesniewski ML, Haviernik P, et al. microRNA 184 regulates expression of NFAT1 in umbilical cord blood CD4+ T cells. *Blood* 2009; **113**(26): 6648-57.
406. Rouas R, Fayyad-Kazan H, El Zein N, et al. Human natural Treg microRNA signature: role of microRNA-31 and microRNA-21 in FOXP3 expression. *Eur J Immunol* 2009; **39**(6): 1608-18.
407. Liu X, Robinson SN, Setoyama T, et al. FOXP3 is a direct target of miR15a/16 in umbilical cord blood regulatory T cells. *Bone Marrow Transplant* 2014; **49**(6): 793-9.
408. Knight R. IMiDs: a novel class of immunomodulators. *Semin Oncol* 2005; **32**(4 Suppl 5): S24-30.
409. Mitsiades CS, Chen-Kiang S. Immunomodulation as a therapeutic strategy in the treatment of multiple myeloma. *Crit Rev Oncol Hematol* 2013; **88 Suppl 1**: S5-S13.
410. Quach H, Ritchie D, Stewart AK, et al. Mechanism of action of immunomodulatory drugs (IMiDS) in multiple myeloma. *Leukemia* 2010; **24**(1): 22-32.
411. Mark TM, Coleman M, Niesvizky R. Preclinical and clinical results with pomalidomide in the treatment of relapsed/refractory multiple myeloma. *Leuk Res* 2014; **38**(5): 517-24.
412. Shortt J, Hsu AK, Johnstone RW. Thalidomide-analogue biology: immunological, molecular and epigenetic targets in cancer therapy. *Oncogene* 2013.

Appendix A – Reagents

Table 1A Monoclonal antibodies used for flow cytometry

Target	Fluorochrome	Clone	Manufacturer
CD3	APC	HIT3a	Biolegend
CD3	APC Cy7	SK7	Biolegend
CD3	PerCP	OKT3	Biolegend
CD4	AlexaFluor 400	RPA-T4	Biolegend
CD8	PE	HIT8a	Biolegend
CD8	Brilliant Violet 605	RPA-T4	Biolegend
CD16	PerCP	3G8	Biolegend
CD25	Brilliant Violet 421	BC96	Biolegend
CD45RA	PeCy7	HI100	Biolegend
CD56	PE	MEM-188	Biolegend
CD69	PerCP	FN-50	Beckton Dickinson
CD127	PECy5	A109D5	Biolegend
CCR2	APC	K0362C	Biolegend
CCR5	PECy7	J418F1	Biolegend
CXCR4	Brilliant Violet 605	12G5	Biolegend
CCR7	APC	G043H7	Biolegend
CCR9	PECy7	L053E8	Biolegend
CCR10	PE	1B5	Beckton Dickinson
Ikaros	PE	R32-1149	Beckton Dickinson
FOXP3	PE	206D	Biolegend
IFNγ	Brilliant Violet 421	4S.B3	Biolegend
TNF	PerCP Cy 5.5	MAb11	Biolegend

CD107a	PECy7	H4A3	Biolegend
IL2	PECy7	MQI-17H12	Biolegend
HLA-DR	APC	L243	Miltenyi

Table 2A Probe/Primers used for qRT-PCR

Gene of interest	Probe/Primer
Pirin	TaqMan® Primer/Probe set Hs01125822_m1 (Life technologies)
SOCS2	TaqMan® Primer/Probe set Hs00919620_m1 (Life technologies)
PFKFB4	TaqMan® Primer/Probe set Hs00190096_m1 (Life technologies)
PMCH	TaqMan® Primer/Probe set Hs01041242_g1 (Life technologies)
FAIM3	TaqMan® Primer/Probe set Hs00193770_m1 (Life technologies)
Cereblon	TaqMan® Primer/Probe set Hs00372271_m1 (Life technologies)
18s	TaqMan® Primer/Probe set Hs99999901_s1 (Life technologies)
GAPDH	TaqMan® Primer/Probe set Hs03929097_g1 (Life technologies)