T cell delivery of immune-stimulatory cytokines to enhance cancer immunotherapy

By

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Declaration

Except where explicitly stated, this thesis is the result of my own work. It includes nothing which is the outcome of work that was done in collaboration. The thesis has not been submitted in whole or part to any other university.

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Abstract

Adoptive cell therapy using TCR-engineered T cells is an exciting area of research and has emerged as a promising strategy for treating cancer patients. However, the effector function of TCR-engineered T cells can be tuned down by local mechanisms of tumour-associated immunosuppression. The potential of cytokines to reverse local immune suppression and enhance tumour immunity has been described in the past. The main aim of this project was to engineer T cell specificity as well as effector cytokine production as a strategy to enhance cancer immunotherapy. This was achieved by combining TCR gene transfer with genetic engineering to achieve IL-12 and IL-27 production in therapeutic T cells. *In vitro* validation data demonstrated not only an enhanced production of IL-12 and IL-27 by the engineered T cells but also an enhanced effector function upon antigen-specific stimulation. In order to circumvent previously described toxic side effects observed with systemic IL-12 delivery, a tet-regulated gene expression system was utilised to regulate cytokine production by engineered T cells *in vivo*. Adoptive transfer of TCR-redirected T cells expressing regulated IL-12 in B16F10 melanoma-bearing mice resulted in an enhanced accumulation of transferred $CD8^+$ T cells in the tumour and in a change of the innate immune cell composition in the tumour microenvironment. Importantly, regulated IL-12 delivery resulted in enhanced therapeutic efficacy of the transferred T cells without causing systemic toxicity. IL-27 delivery in engineered T cells also showed some effectiveness when combined with TCR gene therapy, although the therapeutic benefit of IL-27 was inferior to IL-12. The data in this study demonstrate the potency of additional genetic manipulation to tailor the TCR-redirected T cell effector function which can result in a substantial enhancement in their therapeutic efficacy, and thus, enhanced antitumor immune response.

This thesis is dedicated to my beloved parents and brothers …

It is also dedicated to people who have made contributions to its completion …

This achievement is also dedicated to my country Saudi Arabia …

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List of Abbreviations

APCs	Antigen presenting cells
AML	Acute myeloid leukemia
BFA	Brefeldin A
BFGF	Basic fibroblast growth factor
CAR	Chimeric antigen receptor
CCL22	CC-chemokine ligand 22
CT	Cancer-testis
CTLs	Cytotoxic T lymphocytes
CTLA-4	Cytotoxic T-lymphocyte antigen 4
CLF1	Cytokine-like factor 1
ConA	Concanavalin A
Cox-2	Cyclooxygenase-2
CRISPR	Clustered regularly interspaced short palindromic repeats
CXCL9	C-X-C motif chemokine 9
CXCL10	C-X-C motif chemokine 10
DCs	Dendritic cells
Dox	Doxycycline
EBV	Epstein-Barr virus
EBI3	Epstein-Barr virus-induced gene 3
FDA	Food and drug administration
Foxp3	Forkhead box P3
FSC	Forward scatter
GFP	Green fluorescence protein
GVHD	Graft-versus-host disease
HCV	Hepatitis C virus
HPV	Human papilloma virus
HLA	Human leukocyte antigen
hPGK	Human phosphoglycerate kinase
ICAM1	Intercellular adhesion molecule-1
ICOS	Inducible T cell co-stimulator
IDO	Indolamin 2,3-dioxygenase
IFNγ	Interferon-gamma

IFNγR	Interferon-gamma receptor
IgG	Immunoglobulin G
IgE	Immunoglobulin E
IL	Interleukin
iNOS	Nitric oxide synthase
IP-10	Interferon gamma (IFNγ)-induced protein 10
IRES	Internal ribosome entry site
JAK	Janus kinase
KO	Knock out
LAK	Lymphocyte activated killer
LFA1	Lymphocyte function-associated antigen-1
LMP2	Latent membrane protein 2
LMP7	Latent membrane protein 7
LPS	Lipopolysaccharide
LTR	Long terminal repeat
mAb	Monoclonal antibody
MART1	Melanoma antigen recognised by T cells 1
MCP-1	Monocyte/macrophage chemoattractant protein-1
MDSCs	Myeloid-derived suppressor cells
NFAT	Nuclear factor of activated T cells
MFI	Median fluorescent intensity
MHC	Major histocompatibility complex
MIG	Monokine-induced by interferon-gamma
MPSV	Myeloproliferative sarcoma virus
MQ	Macrophage
MTD	Maximum tolerable dose
NKSF	Natural killer cells (NKs)-stimulatory factor
NLRP3	NLR family pyrin domain containing 3
PD-1	Programmed cell death-1
PDAC	Pancreatic ductal adenocarcinoma
pDC	Plasmacytoid dendritic cell
PEG2	Prostaglandin
PRR	Pattern recognition receptor

RCC	Renal cell carcinoma
rIL-12	Recombinant interleukin-12
msc-IL27	Mouse single chain interleukin 27
siRNA	Short interfering ribonucleic acid
SOCS3	Suppressor of cytokine signalling 3
SSC	Side scatter
STAT	Signal transducer and activator of transcription
TAAs	Tumour-associated antigens
TALEN	Transcription activator-like effector nuclease
TAMs	Tumour-associated macrophages
TAP	Transporter associated with antigen presentation
TBI	Total body irradiation
TCR	T cell receptor
TCCR	T cell cytokine receptor
TGFβ	Transforming growth factor - beta
Th0	Naïve $CD4^+$ T cells
Th1	Type 1 helper T cells
TILs	Tumour infiltrating lymphocytes
Tim3	T cell immunoglobulin and mucin domain-3
TLR	Toll-like receptor
TNFα	Tumour necrosis factor-alpha
Tr1	Type-1 regulatory T cells
Treg	Regulatory T cell
TRP1	Tyrosinase-related protein-1
TRP2	Tyrosinase-related protein-2
TSAs	Tumour-specific antigens
TSA	Thricostatin-A
VEGF	Vascular endothelial growth factor
ZFNs	Zinc finger nuclease

Chapter 1
General Introduction

1.1 History of cancer immunosurveillance and immunoediting: does the immune system react against tumours?

The concept that the immune system can suppress tumour development was first postulated by Paul Ehrlich in 1909 [1]. However, this hypothesis could not be tested at that time due to the gap in knowledge about the structure and performance of the immune system. Five decades later, Burnet and Thomas reconsidered the concept of tumour immune surveillance, stating that the immune system physiological function is to recognise clones of transformed cells and eradicate them before they develop into tumours or destroy the developed tumours [1, 2]. When Stutman started to test this hypothesis utilizing immunodeficient nude mice compared to immunocopmetent wild type mice using induced and spontaneous cancer, no supportive data were obtained [3, 4]. Using improved mouse models, the immune surveillance concept has been supported by evidence generated from two different research groups. Mark Smyth's group showed that mice deficient in perforin, one of the key mediators of cell-induced cytotoxicity, were more susceptible to lymphoma compared to their immunocompetent mice counterpart [5]. The second piece of evidence that was provided by Robert Schreiber's group indicated that immunodeficient mice are more prone to spontaneous and carcinogen-induced cancer compared to the wild type mice [1, 6]. In addition, another finding showed that the immune system can reduce the tumour immunogenicity, due to immunological editing and tumour escape in immunocompetent mice [7]. These outcomes developed the wide concept of cancer immunoediting, which indicate the ability of the immune system to not only protect against tumours but to also select for tumour escape variants. The process of cancer immunoeditiong consists of three phases, which shape the antigenicity of tumours and include: elimination, equilibrium and escape phase (Figure 1.1) [8, 9]. In the elimination phase, both arms of the immune system, innate and adaptive immunity, work simultaneously to eliminate developing tumours resulting from the failure of intrinsic tumour suppressor mechanisms. If this process successfully rejected the tumour before they become clinically detectable, it will result in a tumour-free host. Alternatively, the outgrowth of rare cancer variants that escaped the elimination phase can enter a second phase of immunological control mainly by the adaptive immune response. This phase is called equilibrium phase in which tumour immunogenicity editing occurs; the tumour cells in this phase remain dormant for long time and could be for the host lifetime. However, immune selection pressure on dormant tumour cells, which are genetically unstable, can emerge as tumour escape variants (escape phase) that results in their

progressive growth to a clinically detectable disease. Tumour cells can use a number of escape mechanisms, which will be discussed later [8, 9]. Therefore, there is substantial evidence that supports the notion that the immune system can generate immune responses against tumours.

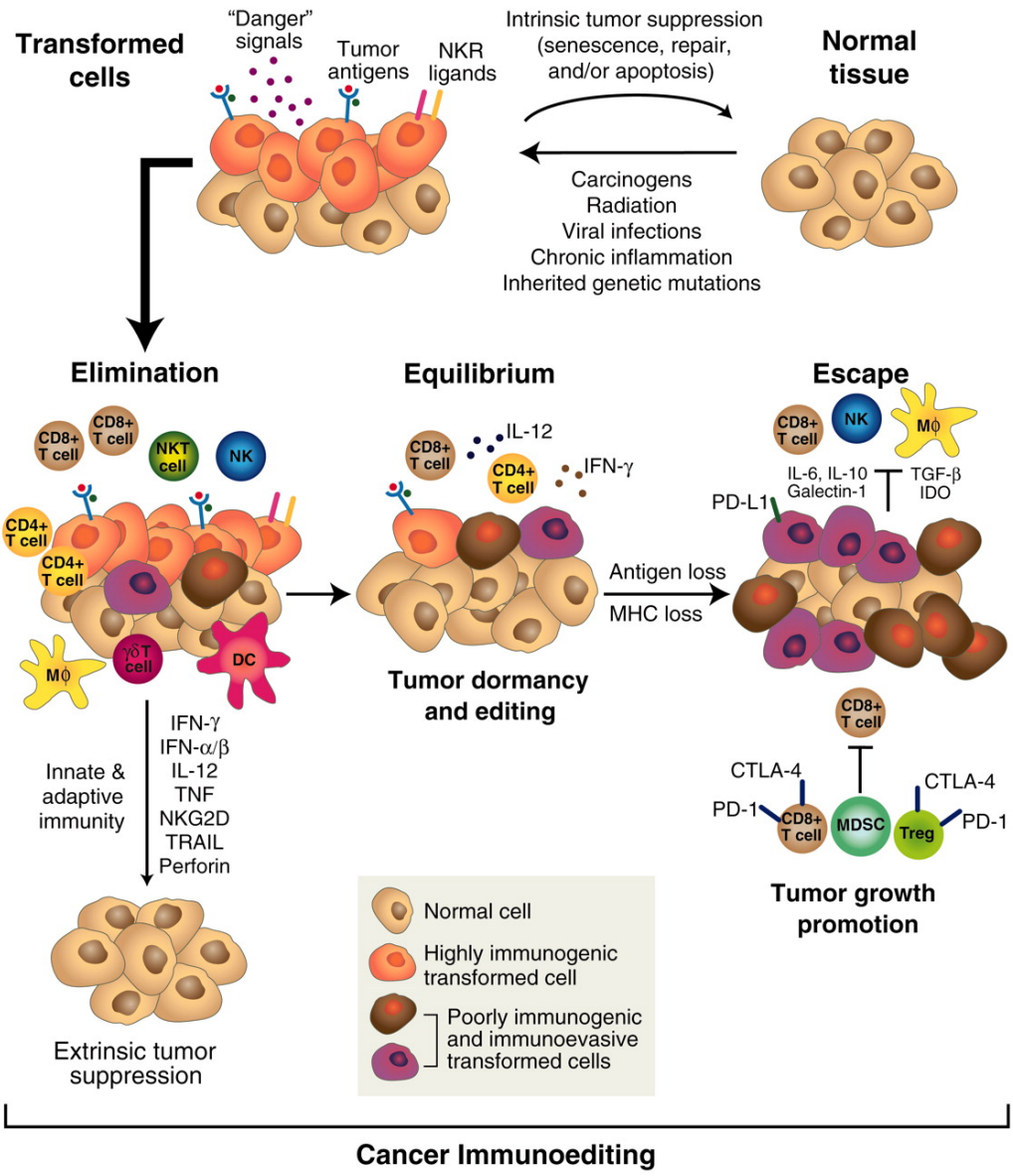


Figure 1.1 | The concept of cancer immunoediting. The process of cancer immunoediting emerges when intrinsic tumour suppression fails to protect the normal cells from being transformed by extrinsic tumour suppression mediators, such as carcinogen, radiation, viral infections etc. The transformed cells can then go through different phases of cancer immunoediting including: elimination, equilibrium and escape. In the elimination phase, the transformed cells can be removed by the innate and adaptive immune system. However, if a rare cancer variant is not eliminated, it may enter the next phase called equilibrium by which the adaptive immune mechanisms function to prevent the growth of the tumour cells. The tumour cells can remain dormant in this phase for long time, which could be for the host lifetime. Genetically unstable tumour cells can enter the escape phase as a result of immune selection pressure, resulting in tumour cells outgrowth to be clinically detectable (Adapted from Schuriber *et al.,* 2011 [8])

1.2 Antitumor immune responses

1.2.1 Tumour antigens

Originally, tumour antigens were divided into wide-ranging categories according to their patterns of expression including: tumour-specific antigens (TSAs) and tumour-associated antigens (TAAs). TSAs are antigens that are specifically expressed in tumour cells but no such expression can be found in normal cells. In theory, specificity to tumour cells would exclude any pre-existing immunological tolerance which usually found with normally expressed antigens, and therefore, immune responses directed toward TSAs will be unlikely to cause damage to normal tissues [10]. The second category, TAAs, are tumour antigens that are expressed on both tumour cells and normal cells. Compared to normal cells, TAAs are expressed at elevated levels on tumour cells, because they are mostly derived from abnormal or dysregulated expression of normal cellular genes. Although TAAs may not induce natural host immune response, they are considered as potential immunotherapeutic target or suitable as diagnostic markers for clinical use. Nowadays, tumour antigens are classified according to their molecular structure and antigenic source that can trigger immune T cell- or antibody-mediated responses [11]. Example of TSA involves products of mutated oncogenes and tumour suppressor genes with restricted expression in tumour cells only, such as RAS (mutant oncogene), Bcr/Abl fusion protein and p53 (mutant tumour suppressor gene) [12, 13]. Another example includes products of oncogenic viruses that are involved in the development of cancer cells from transformed infected cells, such as viral transforming gene products of Epstin-Barr virus (EBV) and human papilloma virus (HPV) which is associated with lymphomas and cervical cancers, respectively [14, 15] Virus-induced tumours are highly immunogenic due to the fact that viral antigens are foreign proteins. Apart from TSAs, the vast majority of the identified tumour antigens up to now are TAAs. One group of TAAs are unmutated cellular protein but they are abnormally expressed in tumour cells compared to normal cells such as HER2/Neu, which is associated with breast cancer and other carcinomas [11]. TAAs also include cancer-testis (CT) antigens which are proteins expressed in germ cells, placenta and were also found actively expressed in various cancers, but they are silent in most normal tissues [16, 17]. Examples of CT antigens are NY-ESO-1 and MAGE-1; the latter was the first human TAA identified from melanoma [8] [18-20]. Another group of TAAs are tissue-specific differentiation antigens, which are expressed in a specific cell lineage or a particular stage of cell differentiation. They are unmutated cellular proteins expressed

abnormally in tumour cells but are also expressed normally in the cells from which the tumour originated. A number of well-characterised melanoma antigens are related to melanocyte differentiation antigens, such as tyrosinase, tyrosinas-related protein (TRP1 and TRP2), gp 100 and MART-1/Melan A [21-23]. The CD20 molecule is also a differentiation antigen associated with B cell lymphomas and antibodies against this molecule (rituximab) have been used successfully as an immunotherapeutic treatment of non-Hodgkin's B cell lymphoma [24]

1.2.2 T cell-mediated immunity

In 1972, Freedman and colleagues were the first to provide evidence that T cells predominantly could kill tumour cells [25]. Almost 20 years later, studies in human tumours revealed that abundant infiltration of $CD8^+$ cytotoxic T cells (CTLs) in the tumours correlated with enhanced survival in cancer patients [13]. Subsequent studies reported similar beneficial outcome of CTLs infiltrating the tumours in many types of cancers, such as cutaneous melanoma, esophageal carcinoma, colorectal carcinoma, ovarian cancer and hepatocellular carcinoma [26-31]. Evidently, this seems to be a tumour-specific phenomena because circulating tumour antigen-specific CTLs showed no prognostic implication in patients with melanoma [32]. Moreover, a number of studies have demonstrated that adoptive transfer of tumour antigen-specific $CD8^+$ T cells can enhance tumour recognition and eradication in both mice and human [33-37].

CTLs mediate anti-tumour responses via different mechanisms including granules-mediated destruction (cell-mediated cytotoxicity) and cytokine release by CTLs [5, 38-41]. They do express cytotoxic effector molecules, such as granzymes and perforin, which are formed and released upon antigen-specific activation of CTLs, and consequently, can mediate tumour cell lysis in few minutes [42-44]. It seems to indicate that this is one of the important mechanism by which tumour cells are killed by CTLs, as tumour-killing activity has shown to be diminished in perforin-deficient mice [5, 38, 39, 45].

CTLs also express signature cytokines that are important in mediating anti-tumour responses such as interferon-gamma (IFNγ) and tumour necrosis factor-alpha (TNFα), which are produced upon antigen-specific activation. The essential role of IFNγ in tumour immunity has been established based on the results that tumour incidence increased in mice deficient in IFNγ, interferon-gamma receptor (IFNγR) or any component in the signalling cascade of IFNγ [40, 46, 47]. IFNγ was found to possess antiangiogenic

properties and play an important role in enhancing tumour regression [40, 48]. It has been suggested that IFNγ can directly improve tumour immunogenicity by binding to IFNγR on tumour cells, which in turn can upregulate their major histocompatibility complex (MHC) class I expression, the antigen presentation machinery, and increase their susceptibility to CTL-mediated killing. Another evidence showed that binding of IFNγ to its receptor on tumour cells can upregulate Fas (CD95) expression resulting in tumour cell lysis via interaction of their Fas with Fas ligand (Fas-L) on CTLs [7, 45, 49-52].
Importantly, for CTLs to be able to provoke an immune response against tumours, they must be primed and activated *in vivo* by recognising tumour antigens presented by host professional antigen-presenting cells (APCs), specifically dendritic cells (DCs). Although tumour cells are derived from nucleated cells, which are all expressing MHC class I, they usually lack the expression of costimulatory molecules or MHC Class II, and thereby, they will not be able to trigger T cell responses or prompt helper T cell-mediated $CD8^+$ T cell differentiation, respectively [11, 53]. Unlike tumour cells, DCs express co-stimulatory molecules, which together with their ability to present tumour antigen in the context of MHC class I, they can activate naïve $CD8^+$ T cells and promote their differentiation into effector CTLs; a process called cross-priming or cross-presentation [11, 54]. Tumour antigens ingested by DCs can also be processed and displayed by MHC class II molecules. Therefore, both $CD4^+$ T cells and $CD8^+$ T cells may recognise tumour antigens [53, 55].

The role of $CD4^+$ T cells in tumour immunity was thought initially to provide help for optimal priming of CTLs by secreting cytokines including: interleukin-2 (IL-2) and IFNγ, and by APCs licensing via CD40-CD40L interaction [56-62]. Yet, subsequent studies have shown that $CD4^+$ T cell contribution is more than just activating CTLs. $CD4^+$ T cells can also help memory $CD8^+$ T cell formation, at least in part, through a CD40-dependent process on $CD8^+$ T cells. For a functional $CD8^+$ memory T cells, Shedlock *et al* showed that provision of $CD4^+$ T cell help is required during the priming stage of $CD8^+$ T cells [63-66]. According to Marzo *et al*, adoptive co-transfer of tumour-specific $CD4^+$ T cells with $CD8^+$ T cells was not necessary for CTL generation, but rather, was needed for the maintenance of $CD8^+$ T cell numbers and for their tumour infiltration [67]. Furthermore, tumour-specific $CD4^+$ T cells can have direct anti-tumour activities by secreting cytokines such as IFNγ, which can increase expression of MHC class I molecules in tumour cells and increase their susceptibility to CTL-meditated lysis [68]. $CD4^+$ T cells can also

mediate cytotoxic activity and eradicate tumour in an MHC class II dependant fashion. This has been demonstrated in advance melanoma mouse model treated with a small number of naïve $CD4^+$ T cells following lymphodepletion [69]. $CD4^+$ T cell-mediated tumour rejection has also been demonstrated by transferring TRP1-specific $CD4^+$ T cells in lymphodepleted melanoma-bearing mice [70]. Similarly, the importance of $CD4^+$ T cell contribution in tumour immunity has also been demonstrated in human studies [71].

1.2.3 Tumour evasion of immune responses

Although there is supporting evidence that the immune system can mount an immune response against tumour, many tumour cells can develop mechanisms to evade anti-tumour immunity. These evasion mechanisms can be either intrinsic to the tumour cells or arbitrated by other immune cells.

- ***Escape through reduced antigenicity/immunogenicity.*** Tumour cells can be less immunogenic if they grow under the selection pressure of a normal immune system. This has been demonstrated by Robert Schreiber and colleagues, by which tumours established in immunocompetent mice will be able to grow when they are transplanted into a second immunocompetent mice [8]. In contrast, tumours established in immuno-deficient mice (lacking adaptive immune system) will not lose their immunogenicity and will be more frequently rejected following their transplantation into immunocompetent mice [6, 72]. Loss of immunogenicity can be developed by different mechanisms including: loss of immunogenic tumour antigens and/or down-regulation of MHC class I molecules [11, 73].

  Loss of tumour antigens or emergence of tumour escape variants is due to the genetic instability and high replication rate of tumour cells. If the antigens are not necessary for tumour growth or maintenance of the transformed phenotype, antigen-negative tumour cells will preferentially grow [8, 11].

  If tumour antigen loss is not the case, failure of tumour cell to present the antigen in the context of MHC molecules will render them invisible to immune recognition [74]. Tumour cells can down-regulate the antigen-presentation machinery to escape recognition by antigen-specific CTLs. This phenomenon has been found in nearly 20-60% of common solid tumours, such as melanoma, lung, renal and prostate cancer [75]. Down-modulation of MHC class I molecules can occur in several components of the (MHC class I) processing pathway including: the proteasome subunits (latent membrane protein-LMP2/7), the transporter associated with antigen presentation

(TAP), loss of MHC class I heavy chain, loss of β2-microglobulin and/or reduced transport of MHC I:peptide complex to the tumour cell surface [76]. Therefore, assessing the antigen presentation capabilities of tumour cells would be an effective way to address tumour immunogenicity when combined with analysing mutation frequencies. For example, mutational analyses of solid tumour studies indicated that there is a remarkable variability between immunogenic and non-immunogenic cancer. The former exhibited a higher rate of mutation such as melanoma, whereas the latter displayed a low mutation rate, such as pancreatic ductal adenocarcinoma (PDAC) [74]. In addition, MHC class I molecules down-regulation alone would not be considered as an effective evasion strategy by tumour cells, because NK cells can sense those cells expressing low levels of MHC class I molecules [77].

- ***Escape through suppression of immune responses.*** There are multiple inhibitory pathways that tumours can use to curb a potential anti-tumour immunity. There are well-defined molecules for their involvement in the T cell inhibitory pathways including programed cell death-1 (PD-1) and cytotoxic T-lymphocyte antigen 4 (CTLA-4). Secretion of IFNγ by tumour infiltrating lymphocytes (TILs) can induce upregulation of PD-L1, the immunoinhibotry molecule, by tumour cells leading to T cell inhibition [78]. PD-L1 is a ligand for the PD-1 receptor, which is usually expressed on T cells, and PD-L1/PD-1 interaction can inhibit T cell effector functions [79, 80]. In addition, inefficient activation of APCs in the tumour microenvironment may result in low levels of B7 (CD80/CD86) co-stimulation, which can result in the engagement of the high-affinity co-inhibitory receptor (CTLA-4) on T cells, rather than CD28, the co-stimulatory receptor [81]. Although CD28 is highly expressed on T cells, it has lower affinity to B7 molecules than CTLA-4 which is expressed in low abundance [82]. Therefore, the induction of one or both inhibitory pathways in T cells can diminish T cell activation upon antigen recognition.

  In addition, it was also evident that there is a high number of tumour-infiltrating regulatory T cells (T_{regs}) in preclinical mouse models and cancer patients, which usually predict poor prognosis [83, 84]. Accumulation of T_{reg} cells in the tumour can occur in several ways, such as recruitment induced by CC-chemokine ligand 22 (CCL22), expansion and/or differentiation in the tumour microenvironment [62]. T_{reg} cells possess immunosuppressive functions that can dampen anti-tumour immunity

and facilitate tumour progression through different mechanisms in which both T cells and APCs can be targeted. T_{reg} cells can produce immunosuppressive cytokines such as IL-10 and TGFβ, which can suppress not only T cells but also APCs and NK cells in the tumour [84, 85]. They can also utilize cytolytic activities and induce apoptosis of effector CTLs and APCs through granzyme/perforin-mediated killing [86] .T_{reg} cells also have the ability to prompt B7-H4 expression on APCs including tumour associated macrophages (TAMs) and make them immunosuppressive [62, 87]. They can also cause metabolic disruption of T cells by competing for IL-2 consumption, which is an important factor for T cell growth and survival [88]. CTLA-4 is abundantly expressed by T_{reg} cells and, as described previously, it can compete with CD28 on T cells for binding to B7 molecules on APCs and, consequently, reduces T cell co-stimulation [89]. CTLA-4 expression on T_{reg} cells can also induce indoleamine 2,3-dioxygenase (IDO)-expression in DCs, which render them tolerogenic. IDO is an enzyme that catabolizes tryptophan that is required for T cell proliferation, and thereby, hindering T cell activation. IDO has also been found to be produced in the tumour microenvironment in response to IFNγ production by tumour-reactive T cells [90]. Of note, developing tumours can use more than one immune suppressive strategy to evade immune responses; for example, molecular profiling of melanoma revealed characteristic findings of three immunosuppressive strategies: i) PD-L1 upregulation, ii) $FOXP3^+$ T_{reg} infiltration and iii) IDO production [90].

Tumour itself can produce anti-inflammatory cytokines such as IL-10 and TGFβ that can mediate suppression of T cells, macrophages and DCs [11]. Previous studies in mouse tumour models and in human malignancies, such as melanoma, reported high levels of TGFβ, which can induce immune suppression [91-93]. For example, it has been evident that TGFβ is produced in advanced cancer, such as prostate carcinoma, and is associated with poor prognosis [94].

Furthermore, myeloid derived cells exhibiting suppressive phenotypes, such as TAMs and myeloid-derived suppressor cells (MDSCs), can also promote tumour progression by modulating the tumour microenvironment, which results in suppressing the anti-tumour immune responses. TAMs exhibit an M2-like phenotype (tumour promoter) which secrete immunosuppressive cytokines, such as IL10 that can affect T cell activation and impair their effector function. M2 macrophages can also produce proangiogenic chemokines, such as vascular endothelial growth factor (VEGF) and

monocyte/macrophage chemoattractant protein-1 (MCP-1) [62, 95]. TAMs can also produce CCL22, which is known for its influence on recruiting T_{reg} cells into the tumour microenvironment [96]. Intratumoral co-localization of both TAMs and T_{reg} cells has been observed in patients with melanoma [97]. It has also been reported a poor prognosis in patients with advanced melanoma infiltrated with TAMs [98].

The second immunosuppressive population, MDSCs, are a heterogeneous population of immature myeloid cells derived from the bone marrow and accumulate in the blood, secondary lymphoid tissues or tumours under the influence of many proinflammatory factors, such as IL-6 and VEGF [99]. MDSCs have been divided into two main subsets according to their origin: monocytic MDSCs (M-MDSCs) and granulocytic/polymorphonuclear MDSCs (G-MDSCs) [100]. MDSCs are present in any chronic inflammatory environment, not only tumours, and they possess pro-tumour properties by inhibiting anti-tumour immune responses via different mechanisms including: production of immunosuppressive cytokines (such as IL-10) that can abrogate activated APCs and inhibit their inflammatory functions, and production of peroxyntirite and IDO that hinders T cell proliferation and activation. MDSCs can also inhibit an effective T cell-mediated immunity in an indirect way through enhancing Treg cell development and shifting the differentiation of Th1 toward Th2 cells [11].

1.3 Adoptive cell therapy

As previously described, cumulative evidence indicated that the immune system can initiate immune responses against tumours [11]. However, in some instances, tumour immunity is impaired by natural mechanisms of central and peripheral immunological tolerance. Central tolerance results in clonal deletion of high affinity T cell receptor (TCR)-bearing thymocytes specific for self-antigens. T cells against TAAs have been found to be of low affinity/avidity as they are directed against self-antigens [101]. Although T cell recognition of tumour-specific antigens (also called neoantigens) have been reported, active suppression in the tumour microenvironment mediated by immune suppressive cells and anti-inflammatory cytokines often dominants and play a role in the impairment of tumour-reactive T cell effector functions [101-103]. It has been shown that tumour-specific T cells infiltrating the tumour are trapped in the local stromal cell network after they recognise their cognate antigens [104, 105].

Preclinical and clinical data have demonstrated that T cells play a key role in mediating cancer immunity. The concept of adoptive T cell therapy by which transferring *ex vivo* expanded T cells to treat an individual with cancer, has been established many years ago [106, 107]. This approach enables the isolation, activation and expansion of tumour-reactive T cells from resected tumour [107]. Early clinical trials, including the adoptive transfer of *ex vivo* expanded TILs, revealed minimal efficacy associated with poor engraftment and persistence of the transferred T cells [108]. However, lymphodepleting pre-conditioning regimen using myeloblative drugs, such as cyclophosphamide and fludarabine together with IL-2 enhanced T cell engraftment and functional activity, and demonstrated regression of metastatic melanoma in patients [108]. Unlike human, total body irradiation (TBI) in mice has been used as host conditioning prior to adoptive cell therapy. These different preconditioning strategies can potentially modulate the host immunologic environment, but can have different biological activities. For example, chemotherapy can facilitate the access of transferred T cells to solid tumours by altering the tumour vascular endothelium and inducing sensitization of tumor storma [109, 110]. TBI and/or chemotherapy can also impair the intestinal mucosal barrier integrity resulting in microbial translocation, which can serve as an immunological adjuvants by boosting the innate immune response [111]. Moreover, lympho-depleting pre-conditioning can greatly improve the therapeutic efficacy of transferred T cells in a number of ways, such as increasing the availability of homeostatic cytokines by reducing the competing endogenous population and decreasing the number of suppressive cells including T_{regs} and MDSCs [111] [112].

The success of TIL therapy has been limited to certain type of cancer including melanoma, cholangiocarcinoma and cervical cancer, while insufficient numbers of TILs with defined specificity were found in many malignancies [108, 113-115]. More recently, alternative approaches have been explored to overcome these limitations. The technologies of genetic engineering have provided a solution to redirect the specificity of a large number of T cells toward defined tumour antigens [107, 115]. There are two main approaches to genetically redirect T cell specificity; T cell receptor (TCR) and chimeric antigen receptor (CAR) gene therapy, using viral and non-viral gene delivery systems [115, 116]. TCRs can recognize antigens in the context of MHC or human leukocyte antigen (HLA) in mice and human, respectively [106]. Unlike TCR, CAR has an antibody-like specificity capable of activating T cells in an MHC/HLA-independent

fashion, which accounted for its elegant concept, that involves designing a signalling receptor composed of a variable region derived from antibody genes fused to intracellular TCR signalling domains (Figure 1.2). However, CARs can only recognize surface antigens [106]. CAR gene therapy will not be discussed in details here as it is beyond the scope of this project, but it can be found in earlier reviews [115-117].

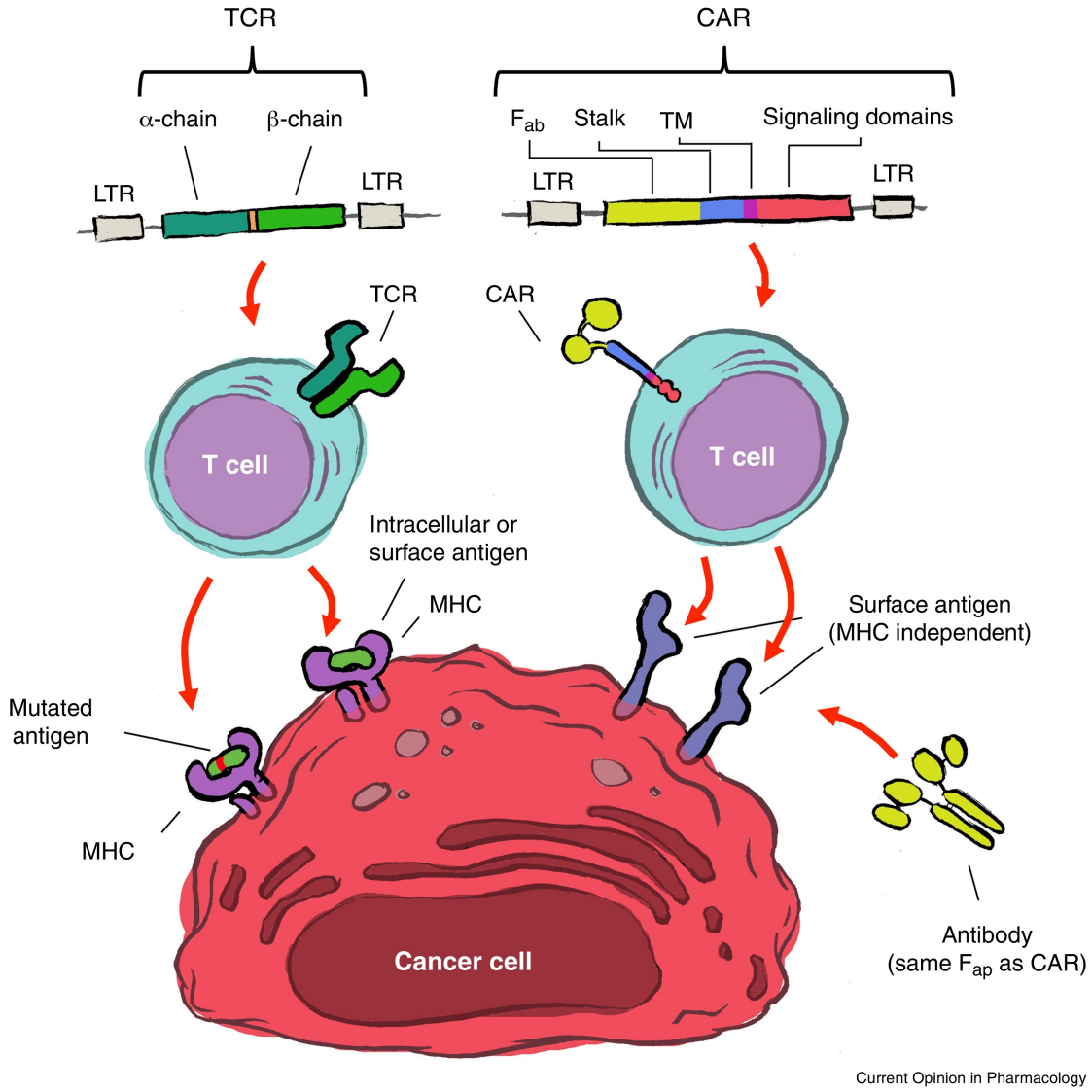


Figure 1.2 | Approaches to engineer T cell specificity. TCR and CAR gene therapy can be used to redirect the specificity of T cells toward a define tumour antigen. This can be achieved by transducing T cells with viral or non-viral vectors encoding the TCR or CAR genes. TCRs can recognise both surface and intracellular antigens in the context of MHC molecules, whereas CARs can recognise only surface antigens in an MHC-independent manner (Adapted from Zech *et al.*, 2015 [118]).

1.3.1 TCR gene therapy: general principles

T cells express TCRs which determine their antigen specificity. TCRs are heterodimers consist of two polypeptide chains α and β, which are linked together via a disulphide bond, and each receptor is unique to a single antigen-binding site [119]. TCRs can recognize a wide range of surface and intracellular tumour antigens, which are presented in the context of MHC or HLA in mice or human, respectively [106]. TCR gene therapy is an approach by which T cell specificity can be redirected through introducing a specific TCR, using viral vector encoding α and β TCR chains of defined antigen specificity, and this approach has been demonstrated in proof-of-concept studies [120-122]. These studies have also demonstrated that TCR gene transfer resulted in reconstitution of a functional cell surface TCR that, upon stimulation with the target antigen, can trigger antigen-specific CTLs producing pro-inflammatory cytokines. Moreover, a study by Kesseles *et al* has proposed the feasibility of the TCR gene transfer approach in generating specific immune responses against viruses and tumours *in vivo* by redirecting T cell specificity toward a virus or tumour antigen, respectively [123].

In order to generate a tumour-specific TCR, a proper tumour target needs to be identified, which can be achieved in several ways. One approach is to isolate tumour-specific T cells from cancer patients exhibiting good antitumor immunity (objective responses) and clone their TCRs into viral vectors, which can be used to transduce T cells derived from unrelated patients; this approach is called allogeneic TCR gene transfer [124, 125]. Another approach is to isolate TCRs from humanized mice (HLA transgenic mice) that have been immunised with tumour antigens of interest to generate T cells expressing tumour-specific TCRs against the immunised human antigens. These TCRs can then be isolated and cloned into viral vectors that can be used to genetically engineer autologous T cells derived from patients [126]. The feasibility of TCR gene therapy has been demonstrated in solid tumours and haematological malignancies [127-129]. The first clinical trial of TCR gene therapy was carried out in lymphodepleted melanoma patients, by adoptively transferring autologous T cells transduced with TCR recognising a melanoma differentiation antigen called MART-1 (melanoma antigen recognised by T cells 1) in the context of HLA-A2 [130].

1.3.2 Strategies to enhance TCR gene therapy

1.3.2.1 Optimising TCR sequences

In vitro engineering technologies have been employed to achieve an optimal TCR design that can enhance the introduced TCR expression considering a number of factors. Codon optimization of mRNA encoding TCR genes has been shown to enhance the levels of TCR expression [131]. In addition, introducing new TCRs can mispair with endogenous TCRs and increase the risk of generating TCRs with unknown specificity, which can give rise to autoreactive T cells against self-antigens. TCR mispairing has been shown to cause toxicity in the form of severe graft-versus-host disease (GVHD)-like pathology that was observed in experimental murine models [132]. To reduce the risk of mispairing with endogenous TCR chains and enhance the introduced TCR expression, several strategies have been developed. First strategy is to introduce a disulphide bond that links the constant domains of both α- and β-chains of TCR, which can result in preferential pairing of the introduced TCR [133, 134]. The second strategy is a complete or partial replacement of human constant region with murine-derived sequences (also refers as murinization), based on the observation that murinzed receptors showed enhanced assembly and stability of TCR-CD3 complex in human T cell compared to human TCR constant regions [135]. Another effective strategy to promote optimal expression of the introduced TCR and minimize mispairing is to knock-down endogenous TCR chains using gene editing tools, such as short interfering RNA (siRNA), Zinc finger nucleases, TALENs and most recently CRISPR technologies [115, 136, 137].

1.3.2.2 Engineering T cell effector functions

Initially, it was thought that increasing TCR affinity to a level similar to those of antibodies would enhance the TCR avidity, implying that T cells can be activated by low antigen density [115]. It is perhaps based on the fact that the majority of targeted tumour antigens in TCR gene therapy are TAAs which are also expressed by normal cells, and thereby, autologous T cells recognising these antigens would have lower affinity because high affinity clones have been removed by mechanisms of immunological tolerance [138]. Increasing TCR affinity was achieved by selecting TCR variants with higher binding affinity via site-directed mutagenesis targeting the TCR antigen-binding site [115]. However, it has been shown that increasing the TCR affinity can reduce T cell functional avidity [139]. Affinity maturation of a TCR beyond its natural affinity range

can affect the process of TCR serial triggering required to stimulate T cell effector function when the antigen concentration is low [140, 141]. In addition, there is a potential risk for the affinity-matured TCR to cross-react with irrelevant antigens resulted in so-called off-target toxicities [142].

An alternative strategy is to enhance the functional avidity of antigen-specific T cells by increasing the level of TCR expression on the T cell surface. Unlike TCR affinity maturation, enhancing T cell avidity is not associated with any modification in the TCR affinity or specificity, and therefore, it can reduce off-target cross-reactivity seen with enhanced TCR affinity. Recently, it has been demonstrated that co-transferring CD3 genes with TCR can increase the availability of CD3 molecules required for TCR/CD3 complexes to be fully assembled on the surface of T cells, resulting in improved functional avidity and better tumour protection *in vitro* and *in vivo*, respectively [143]. However, it has been suggested that additional CD3 can not only enhance the expression of the therapeutic TCR in gene engineered T cells but also can increase the risk of possible toxicity by enhancing the expression of endogenous and mispaired TCR [144].

Delivery of immune-stimulatory cytokines by T cells is another strategy that has been explored to enhance the engineered T cell functional activities, thus enhancing their antitumor immunity. One of the most extensively studied candidates is interleukin-12 (IL-12) that has shown a very promising result by its ability to eradicate tumour through different mechanisms; it will be discussed in more detail later in this chapter.

1.3.2.3 Engineering $CD4^+$ T cells, $CD8^+$ T cells, or both

Although early studies in cancer immunotherapy focused on tumour-specific $CD8^+$ T cells, there is growing evidence that $CD4^+$ T cells can play a role in tumour immunity and can efficiently eradicate tumour in preclinical models [106]. Recent clinical trial of adoptive cell therapy using autologous $CD4^+$ T cells specific for NY-ESO-1 resulted in complete response in a patient with metastatic melanoma [145]. In another clinical trial, adoptive transfer of $CD4^+$ T cells specific for a mutation in Erb-b2 interacting protein (ERBB2IP) present in cholangiocarcinoma led to a dramatic clinical response [113]. Moreover, co-transferring $CD4^+$ engineered T cells resulted in a great synergistic effect compared to transferring $CD8^+$ T cells only [146]. $CD4^+$ T cells can cooperate with antigen-specific $CD8^+$ T cells and enhance their effector function by facilitating effector cell recruitment into the tumour site, producing cytokines such as IL-2 and enhancing

clonal expansion [147, 148]. $CD4^+$ T cells are also capable to alter APC functions [149]. In addition to the $CD4^+$ T cell helper role, a more direct role in antitumor immunity has been reported [69, 70].

Due to the limited number of well-defined tumour antigens targeted by $CD4^+$ T cells, researchers have explored the possibility to generate Th responses by redirecting the specificity of $CD4^+$ T cells using MHC class I-restricted TCRs. This is based on the fact that most of the isolated high avidity TCRs against tumour antigens are MHC class I restricted, which require CD8 co-receptor for optimal function. Despite this, a number of TCRs have been found to function in $CD4^+$ T cells where no CD8 co-receptor is expressed, whereas co-introducing CD8α gene with the TCR into $CD4^+$ T cells was required for their specific binding to MHC Class I-peptide multimers [150-152]. Transferring MHC class I-restricted TCRs into $CD4^+$ T cells leads to their activation without the need of antigen-presentation by MHC class II, which is not commonly expressed by tumour cells [144]. One possible risk of redirecting $CD4^+$ T cells is developing such toxicity due to the wide-ranging expression of MHC class I in most tissues. However, anergy induction can impair the functional activities of $CD4^+$ T cells in the absence of costimulation, following interaction with cells or tissues that unable to provide costimulatory signals [144]. Importantly, the antitumor activities of $CD4^+$ T cells are largely dependent on their polarization [106, 153].

1.4 IL-12 family cytokines

IL-12 family cytokines is unique by which all cytokine members are heterodimers, and thereby, chain pairing is required to secret the biologically active form of the cytokine. To date, there are four main cytokine members including: IL-12, IL-23, IL-27 and IL-35. Each of these cytokines is composed of an α chain (p19, p28 or p35) and a β chain (p40 or Ebi3 (Epstein-Barr virus (EBV)-induced gene 3)); some subunits are shared among these cytokines. The P40 subunit can partner with p35 and p19, which gives rise to IL-12 and IL-23, respectively. The Ebi3 subunit can partner with p28 and p35, which gives rise to IL-27 and IL-35, respectively [154] (Figure 1.3). Despite sharing subuntis and structural similarities, these cytokines possess different biological activities. IL-12 and IL-23 are pro-inflmamatory cytokines, IL-27 is a bidirectional immunoregulatory cytokine and IL-35 is an anti-inflmmatory cytokine. These cytokines signal by binding to a heterodimeric receptor of two chains, which are also shared by multiple cytokines. IL-

12 signals by binding to IL-12Rβ1 and IL-12Rβ2, whereas IL-23 signalling occurs via IL-12Rβ1 and IL-23R. By contrast, IL-27 signalling occurs by binding to IL-27Rα and gp130, whereas IL-35 uses gp130 and 12Rβ2 [154]. This study focuses on modulating the effector function of T cells by overexpressing IL-12 and IL-27, therefore, these cytokines will be discussed in more detail below.

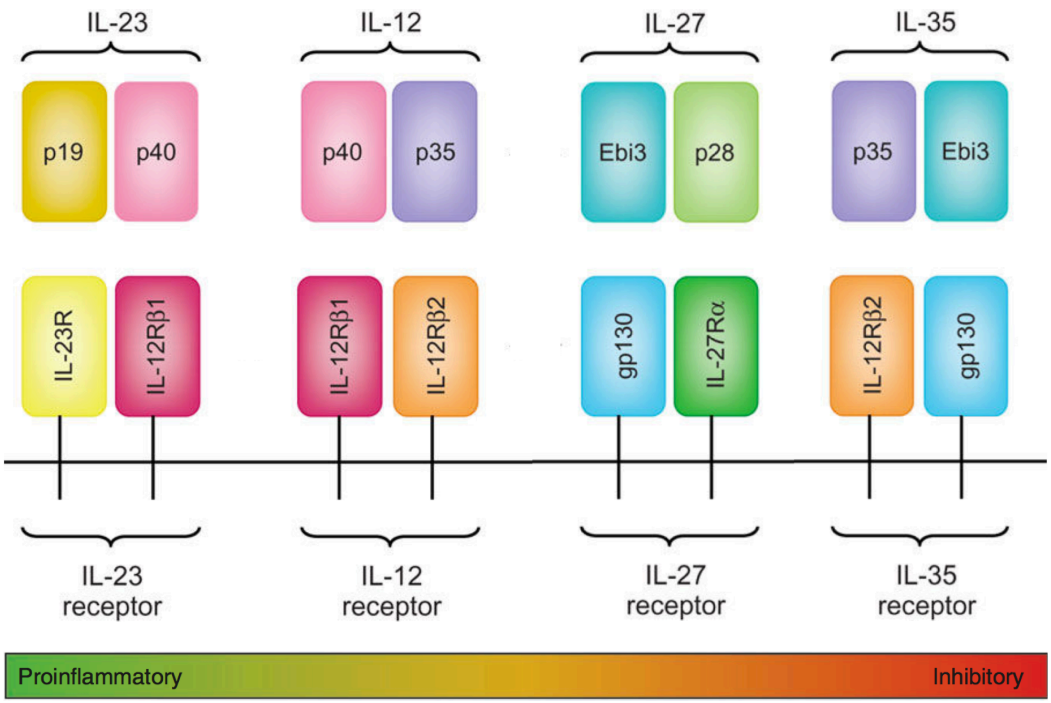


Figure 1.3 | IL-12 family cytokines. Members of the IL-12 family cytokines are shown together with their receptors including IL-23 and IL-12 (pro-inflammatory), IL-27 (bidirectional immunoregulatory) and IL-35 (anti-inflammatory). (Adapted and modified from Palmer, M.T. and Weaver, C.T. [155]).

1.4.1 Interleukin-12 (IL-12)

History and structure of IL-12/IL-12R complex

IL-12 was discovered in 1989 as a natural killer cells (NKs)-stimulatory factor (NKSF) for its ability to enhance cytotoxic function of NK cells [156]. IL-12 is a heterodimeric cytokine consisting of two covalently linked subunits by disulphide bridges: p35 and p40, that give rise to the biologically active protein (IL-12p70) [157]. The genes encoding p35 and p40 subunits are located on different chromosomes: chromosomes 6 and 11, and chromosomes 3 and 5 in mice and humans, respectively; therefore, the expression of IL-12 protein is independently regulated [157]. Co-expression of both subunits in the same cell leads to the production of the biologically active heterodimeric form of IL-12. The p35 subunit is expressed ubiquitously and constitutively at low levels by almost all type of cells, whereas p40 subunit is expressed only by phagocytic cells; therefore, IL-12 is produced mainly by antigen presenting cells (APCs) including DCs, monocytes and macrophages, and to a lesser extend B cells [158, 159]. In addition, the p40 subunit can form a homodimer (IL-12p40/p40), which can bind IL-12R and act as a competitive antagonist for IL12p70 in mice but not in human [158, 159]. IL-12 binds to its heterodimeric receptor (IL-12R), composed of IL-12Rβ1 and IL-12Rβ2 subunits expressed by T cells, NK cells and DCs. IL-12Rβ2 is important for signal transduction downstream of IL-12R complex; it is usually not expressed by naïve T cells but induced upon T cell activation via TCR signalling [160, 161]. Successful triggering of the IL-12R consequently activates the Janus Kinase (JAK)-signal transducer and activator of transcription (STAT) signalling pathway, mainly STAT4 [119, 156]. STAT4-deficient mice phenotypically resemble IL-12p40 deficient mice [162-164]. Two different types of stimuli can induce IL-12 production in its active form: priming and amplification signals. IL-12 can be produced in a T cell-independent manner via Toll-like receptors (TLRs) signalling and other patter recognition receptors (PRRs) expressed by cells of the innate immune system. These receptors are activated by microbial products, such as lipopolysaccharide (LPS), peptidoglycan and bacterial CpG DNA [11, 165]. IL-12 is also produced in a T cell-dependent manner through cell-to-cell interaction between CD40 on APCs and its ligand, CD40L ,on T cells. In addition, cytokine networks (such as; IFNγ or IL-15) can provide an amplification signals for IL-12 production.

IL-12 production: Biological activities and regulation

IL-12 is a pleiotropic cytokine, which is capable of linking both arms of the immune system: innate and adaptive immunity. Initially, IL-12 secretion induces IFNγ production, which is the most potent mediator of the IL-12-induced effects by NK cells, T cells and B cells. IFNγ upregulation is associated with IL-4 downregulation, which is known to mediate anti-inflammatory effects [166, 167]. IL-12 plays an important role in regulating adaptive immune responses. It enhances naïve $CD4^+$ T cell polarization toward type 1 helper T cells (Th1), which produces signature Th1 cytokines, such as IFNγ and TNFα, and enhances cell-mediated immunity [168, 169]. IL-12 enhances formation of cytotoxic $CD8^+$ T cells (CTLs) and lymphocyte activated killer (LAK) cells. It also boosts the cytolytic activities of CTLs and NK cells by inducing transcription of genes encoding molecules that are associated with cytotoxic granules, such as perforin and granzymes [162]. IL-12 has been found to activate B cells, either directly or indirectly, to produce immunoglobulin G (IgG) and suppress their production of IgE, through IL-12-polarized Th1 cells or by other IL-12-induced cytokines, such as IFNγ [170]. In addition, upon T cell activation through TCR engagement, IL-12 is one of the pro-inflammatory cytokines capable of providing signal 3 for optimal activation of T cells, which requires three signals include: peptide-MHC complex recognition by a specific TCR (signal 1), co-stimulation (signal 2) and pro-inflammatory cytokines (signal 3) [11, 171, 172].

IL-12 production is inhibited by negative regulatory mechanisms. One of the dominant inhibitors of IL-12 is IL-10, an anti-inflammatory cytokine, which prevents transcription of both genes encoding IL-12, p40 and p35 [162]. Studies in IL-10-deficient mice have shown lethal systemic inflammation in response to several pathogens [162, 173]. TGFβ is also another inhibitor of IL-12 and can decrease the stability of IL-12p40 mRNA [162].

Antitumor activities of IL-12

In 1993, IL-12 was reported to exhibit potent antitumor activity and thought to be mediated mainly through enhanced IFNγ production, which in turn can activate effector cells, such as NK cells and $CD8^+$ T cells [174, 175]. The importance of IL-12 signalling in tumour immunity was established by a number of observations in several preclinical studies [176-180]. The importance of endogenous IL-12 has been demonstrated in mice deficient in the components of IL-12: IL-12p35 or IL-12p40. An early formation of MCA-induced sarcomas has been observed in IL-12p40-deficient mice compared to wild

type mice [176], whereas mice deficient in p35 subunit were at greater risk of developing UV-induced tumours and were more susceptible to photocarcinogensis compared to wild type mice [179]. Moreover, IL-12 signalling through the IL-12R is also important as demonstrated by increased frequencies of spontaneous tumour development and enhanced progression of transplantable tumours in IL12Rβ2-deficient mice [178].

IL-12 has been shown to have a multidimensional effect in the tumour microenvironment involving both innate and adaptive immunity, which can directly affect tumour cells and/or can modulate tumour-infiltrating immune cells [181]. Therefore, IL-12-mediated antitumor effects may not always require tumour antigen recognition [182]. As described previously, IL-12 induces IFNγ expression by NK and T cells; IFNγ is a central player in IL-12-mediated antitumor immune responses. Induction of IFNγ by IL-12 can upregulate the antigen presentation machinery including MHC class I and II molecules on tumour cells, and thereby, enhances the presentation of tumour antigens. IL-12 can also upregulate other genes, such as IDO and iNOS (inducible nitric oxide synthase), which can slow down the tumour cell growth rate [183]. This was demonstrated in a murine model of spontaneous breast cancer and fibrosarcoma treated with systemic administration of IL-12 [183, 184].

In several murine tumour models treated with IL-12, increased intratumoral infiltration of effector cells has been reported, such as $CD8^+$ and $CD4^+$ T cells, NK cells and MQs [181, 183-185]. Depletion of T cells prior to IL-12 treatment abolished tumour immunity, indicating that lymphocyte infiltration is an important component of IL-12-mediated tumour protection [174, 175, 186]. In numerous models, enhancement of immune cell infiltration was associated with increased upregulation of lymphocyte adhesion molecules in tumours. This was demonstrated in mammary adenocarcinoma, a poorly immunogenic tumour, treated with IL-12 which showed an increased expression of vascular cell adhesion molecule-1 (VCAM-1) inside the tumour [184, 186]. Ogawa *et al* have reported that VCAM-1 induction increased lymphocyte migration to the tumour site, and thereby, enhanced T cell infiltration in IL-12-treated tumours [187]. Recently, it has been demonstrated in IL-12-treated mice bearing subcutaneous B16F10 melanoma that IL-12 stimulated a subset of lymphoid tissue-inducer (LTi) cells expressing NKp46, an NK cell-activating receptor, which was associated with the upregulation of adhesion molecules in the tumour microenvironment including: VCAM-1 and intercellular adhesion molecule 1

(ICAM-1) [188]. These studies demonstrate clearly the role of IL-12 in enhancing tumour accessibility to immune effector cells, which can promote tumour regression.

Apart from enhanced lymphocytic recruitment, IL-12 has the capacity to activate TILs and reprogram the immunosuppressive tumour microenvironment [185]. In this regard, a study by Kilinc *et al* has provided an insight into how IL-12 abolish intratumoral suppressor cells [185]. They have demonstrated that IL-12 activates pre-existing dysfunctional $CD8^+$ T cells with effector and memory phenotype and restores their effector function including IFNγ and granzyme B expression. This occurred simultaneously with a reduction of suppressor T_{reg} cells within the tumour; both IL-12-mediated effects were IFNγ-dependent. However, reactivation of T cells by IL-12 was short-lived, as they became apoptotic 4 days post treatment. This was followed by a wave of tumour infiltration with activated effector T cells on day 7 following IL-12 treatment [185]. Moreover, IL-12 can reverse anergic $CD4^+$ T cells in the tumour despite the existence of suppressive T_{reg} cells, allowing these $CD4^+$ T cells to expand, produce IFNγ and mediate tumour regression [189]. Another study showed that adoptive transfer of IL-12-transduced DCs in melanoma-bearing mice generated a pool of endogenous $CD8^+$ T cells with multiple specificity toward tumour stromal-associated antigens, resulting in tumour protection dependent on $CD8^+$ T cells [190]. In a murine lymphoma model, IL-12 has shown to suppress Treg expansion *in vitro* and *in vivo* in an IFNγ-dependent fashion as demonstrated by unaffected T_{reg} cells in IFNγR-deficient mice [191].

Beyond the effect of IL-12 on T cells, it can convert TAMs with a suppressive phenotype (known as M2) to an inflammatory phenotype (known as M1) in tumour-bearing mice treated with IL-12 microspheres [192, 193]. Tumours in mice treated with IL-12-engineered T cells displayed increased infiltration of activated MQs (M1) compared to control mice [194]. Furthermore, an elegant study by Kerkar *et al* indicated that IL-12-expressing $CD8^+$ T cells can destroy large vascularised tumours by reprogramming myeloid-derived cells in the tumour that exhibit a dysfunctional phenotype, partly through IFNγ, and by IL-12-mediated Fas induction [195, 196].

An important mechanism by which IL-12 modulates antitumor responses is by suppressing tumour angiogenesis, which has been described in 1995 [197]. IL-12 mediates its antiangiogenic activity through IFNγ production and downstream mediators including IFNγ-inducible protein 10 (IP-10, also known as CXCL10) and monokine induced by IFNγ (MIG, also known as CXCL9) [197-199]. Neutralising IFNγ or IP-10

can abrogate the IL-12-mediated angiogenesis inhibition. In addition, IL-12 has also been found to decrease pro-angiogenic mitogens on tumour cells, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (BFGF) [200, 201]. A study by Zhu *et al* demonstrated that blocking VEGF receptor (VEGFR) enhanced hepatocellular carcinoma metastasis by reducing host-derived IL-12p40 subunit [202]. Furthermore, subcutaneous implantation of B16 melanoma, C26 colon carcinoma and thricostatin A (TSA mammary adenocarcinoma) that were engineered to express IL-12 can lead to tumour elimination [161, 203, 204]. However, in some cancer type, such as C26 colon carcinoma, the amount of IL-12 secretion was important to mediate tumour inhibition and rejection.

IL-12 in the clinical setting

The IL-12 therapeutic effects have been extensively studied in a wide range of preclinical tumour models and provided a very promising platform. Utilization of recombinant IL-12 (rIL-12) monotherapy by systemic administration (intravenous, intraperitoneal and subcutaneous) or intratumoral administration have been explored in transplantable, spontaneous and induced tumour models [182, 205]. Combination therapy directed to improve tumour immunogenicity has been used to further enhance the IL-12 antitumor effects including radiation, chemotherapies, antitumor antibodies and peptide vaccines, which has been explored in different models, such as melanoma, lung and bladder carcinoma [206, 207]. Combining IL-12 with other cytokines, such as TNFα and IL-2 has also been investigated [208, 209]. Despite its positive effects, IL-12 systemic therapy in preclinical models was associated with adverse side effects, such as haematological toxicities, partly due to IL-12-induced IFNγ and TNFα [210, 211]. Another drawback of systemic IL-12 administration is the limited therapeutic index due to its short half-life as demonstrated following bolus administration [161]. Therefore, great efforts have been undertaken to develop approaches for therapeutic IL-12 delivery that overcome its associated toxicities and boost its therapeutic efficiency in the preclinical development. Gene therapy has been exploited to achieve local and sustainable production of IL-12 using several viral and non-viral vectors encoding IL-12, electroporation or cell transfer of tumour and immune cells that have been modified to overexpress IL-12; this have been described in a comprehensive review [212]. Nanoparticles and microspheres have also been exploited as vehicles to deliver IL-12 alone or in combination with other cytokines

[213-215]. Of note, it is clear that the efficacy of IL-12-mediated antitumor immunity is dose and context dependent [176, 182]. Despite the toxicity observed in preclinical models, translation of IL-12 into the clinic has not been hampered due to its potent antitumor activities. Recently, three clinical trials have started in different centres in USA including University of Pittsburgh (PA, USA), Genetic Institute (Cambridge, MA, USA) and Hoffmann La Roche (Nutley, NJ, USA) [216]. The first group initiated a small phase I trial of 4 patients with breast carcinoma or melanoma. They have received peritumoral injection of autologous fibroblasts engineered to secret IL-12 on a weekly basis; these cells showed enhanced lymphocytic infiltration and increased numbers of DCs. The other two groups started a larger pilot trial using rIL-12 administration, but the treatment regimen differed between the two trials in several respects. In phase I trial by Roche, only patients with renal cell carcinoma (RCC) were recruited and treated with subcutaneous injection of rIL-12 once or three times a week; in this study, no adverse effects have been reported. By contrast, phase I trial conducted at Genetic Institute aimed to determine the safety and tolerability of IL-12 in cancer therapy. This study was characterised by a daily consecutive injection of IL-12 via intravenous route with a maximum tolerable dose (MTD) of 500 ng/kg/day. However, upon initiating the phase II trial, the determined MTD led to an unexpected toxicity, and 15 out of 17 patients experienced severe adverse events in multiple organs including two deaths. Immediately, the US FDA decided to halt all phase II trials of IL-12 [217]. As a consequence, researchers have tried to investigate what could be the reason behind the observed differences in tolerability from phase I to phase II trials. They found that there was a difference in the dosing schedule in which a single dose of IL-12 was initially administered followed by consecutive doses in the phase I trial, which was not the case in the phase II trial that proceeded without the initial single dose [217]. A study by Lenoard *et al* revealed that the initial priming dose can protect against IL-12-induced toxicity by attenuating IFNγ effects following multiple doses of IL-12 [218]. This resulted in bringing back IL-12 in clinical trial, only in early phase (I) studies, which restarted again in various centers [219]. Since then, IL-12 antitumor activities have been assessed in different treatment regimens. Although pre-injection IL-12 as a priming dose resulted in a profound tolerable effect to subsequent doses, it seems that the therapeutic effect of IL-12 was not enhanced by this regimen [205]. Up to date, IL-12 has not yet been translated into clinics due to the toxicity-related issues and/or minimal efficacy which depends on the treatment regimen.

1.4.2 Interleukin-27 (IL-27)

History and structure of IL-27/IL-27R complex

In 2002, Pflanz and colleagues have described IL-27 as a cytokine associated with T cell activation [220]. Due to its structural similarities to IL-12, it has been classified as a member of IL-12 family; IL-27 is a heterodimeric cytokine composed of two subunits: EBI3 (also known as IL27β) and p28 (also known as IL-30) [220]. Unlike IL-12, both subunits of IL-27 are not disulphide-linked, and they are not always expressed together in the same cells [221]. EBI3 gene was recognized, in 1996, through its induction in B lymphocytes transformed by Epstein-Barr virus latent infection. It is related to IL-12p40, which is a secreted receptor lacking the membrane-binding motifs, and can form a heterodimer with p35 (IL-12α) subunit which gives rise to IL-35 (linked to the activities of T_{reg} cells) [222, 223]. The EB13 gene is localized on chromosome 19 and 17 in human and mouse, respectively [224]. The second subunit that forms IL-27, the p28, was not recognized until late 1990s through sequence-database searching [220]; p28 gene is localized on chromosome 16 and 7 in human and mouse, respectively [224]. Like EBI3, p28 subunit has an alternative partner, called cytokine-like factor-1 (CLF1), which can form another secreted complex, p28/CLF [225]. IL-27 is secreted by APCs, mainly by activated DCs and MQs [226].

The biological effects of IL-27 are mediated upon interaction with its receptor, IL-27R, which is a heterodimeric receptor composed of two chains: a ligand-binding chain called IL-27Rα (also known as WSX-1 or TCCR) and a signal-transducing chain called gp130; simultaneous expression of both chains is needed for IL-27 signal transduction [227]. The cytoplasmic domain of the WSX-1 chain contains a Box 1 motif that can bind to JAK proteins, and thereby, contributes to the WSX-1/gp130 heterodimeic signal transduction [228]. IL-27R is expressed by a variety of hematopoietic and non-hematopoietic cells. Although gp130 is widely expressed in many tissues, WSX-1 expression seems to be limited to the immune cells of hematopoietic origin, such as T cells, B cells and NK cells. WSX-1 expression has also been found in transformed and malignant cells [229, 230]. Therefore, IL-27-mediated effects depend on the type of cells by which IL-27R is expressed [226].

IL-27 is produced by APCs in response to TLR stimulation by microbial products, such as LPS, CpG-DNA, loxoribine and polyinosinic:polycytidylic acid. The TLR3 pathway is usually activated by viral infections, whereas TLR4 pathway is largely activated by

bacterial infections. Recently, it has been shown that apoptotic tumour cells can stimulate human DCs to produce IL-27 [231]. As a consequence of IL-27/IL-27R engagement, the JAK-STAT signalling pathway is activated, mainly STAT1 and STAT3, that mediates not only a pro-inflammatory effect but also mediates an anti-inflammatory effect [227].

Pro-inflammatory roles of IL-27

IL-27 production occurs during the early phase of an immune response, and consequently, controls the magnitude and quality of adaptive immune responses. Thus, IL-27 is important in linking innate and adaptive immune response [232]. Early reports revealed that APCs produce IL-27 at early stages of priming following antigen recognition that results in stimulating naïve $CD4^+$ T cells to clonally expand [220, 233, 234]. IL-27 promotes the differentiation of naïve $CD4^+$ T cells (Th0) into Th1 cells through the activation of STAT-1 and upregulation of T-bet expression, the Th1-specific transcription factor, and IL-12Rβ2 expression during the early stage of naïve $CD4^+$ T cell differentiation toward Th1 cell and prior to IL-12-mediated action [220, 233, 234]. IL-27 cooperates with IL-12 to mediate IFNγ production [220, 234]. However, IL-27 is not essential to stimulate IFNγ responses [235]. Alternatively, IL-27 can also induce differentiation of Th1 cells via a T-bet-independent mechanism; it induces Th1 cell differentiation in a STAT1-dependent manner by upregulating adhesion molecules which facilitates the interaction of intercellular adhesion molecule-1/lymphocyte function-associated antigen-1 (ICAM-1/LFA-1) [236]. Several studies have demonstrated the importance of WSX-1 receptor in mounting initial responses of Th1 cells utilizing WSX-1-knock-out (KO) mice; these mice exhibited a delay in the development of Th1 cells [234]. The impairment in Th1 cell development was correlated with a reduction in the levels of IFNγ expression compared to their wild-type counterparts upon stimulation with antigen or plate-bound anti-CD3 (polyclonal stimulation) [237, 238]. In a study by Yoshida *et al*, the reduction in IFNγ expression was seen in WSX-1 KO T cells exposed to a primary response *in vitro* to differentiate into Th1 cells but not in fully differentiated/activated WSX-1 KO Th1 cells receiving a secondary stimulation [238]. Moreover, WSX-1 KO mice were remarkably susceptible to intracellular infections by *Listeria monocytogenes* and *Leishmania major,* which was associated with a decrease in Th1 cells and an increase in Th2 cells [237, 238]. Yet, IFNγ production was not diminished in the later stages of infection in WSX-1 KO mice [238]. Together, these

studies imply that WSX-1 is crucial during the early phase of generating Th1 responses but expendable for their maintenance. Given the ability of IL-27 to revert the Th2 polarization, it can enhance the pre-existing response of antigen-specific Th1 cells [239, 240].

In the context of $CD8^+$ T cells, IL-27 can augment the expansion and generation of CTLs in infection and cancer [241]. IL-27 has been shown to stimulate the activation of STAT-1, -2, -3, -4 and -5, and consequently, upregulates T-bet, EOMES and IL-12Rβ2 which are concomitant with enhanced IFNγ expression. IL-27 enhances naïve $CD8^+$ T cell expansion; it also enhances the cytotoxic activity of mouse and human CTLs by promoting expression of granzyme B and perforin [241-243]. These IL-27-mediated effects on naïve $CD8^+$ T cells are induced in a T-bet-dependent and -independent fashion [241]. Matsui *et al* have shown that IL-27 exerts an adjuvant effect by improving the effectiveness of prime-boost immunization when an IL-27-endocing plasmid DNA is co-injected *in vivo*, resulting in enhanced the generation of hepatitis C (HCV)-specific $CD8^+$ T cell and production of IFNγ. They have also shown that pre-immunization with the IL-27-endocing plasmid DNA led to a marked increase in the number of IFNγ-expressing HCV-specific $CD8^+$ T cells [244].

Anti-inflammatory roles of IL-27

Although IL-27 was initially recognized as a pro-inflammatory cytokine, Villarino and colleagues were the first to propose the anti-inflammatory activity of IL-27 in the context of parasitic infection [220, 245]. They have demonstrated that WSX-1-deficient mice infected with *Toxoplasma gondii* were capable of establishing an acute inflammatory response and limiting parasite replication. Yet, these mice were unable to dampen down the adaptive immune response and developed a lethal inflammatory disease mediated by $CD4^+$ T cells, which was characterised by the maintenance of highly activated T cells, exaggerated production of IFNγ and IL-2, and increased T cell proliferation [245]. Thus, IL-27 possesses regulatory function by inhibiting cytokine production by T cells; this has also been shown in WSX-1-deficient mice infected with *Trypanosoma cruzi*. These mice showed increased mortality rates, prolonged elevated parasitemia and severe liver damage, which was associated with excessive Th1 and Th2 responses [246]. Studies by Pearl and colleagues have demonstrated that WSX-1-deficient mice infected with *Mycobacterium tuberculosis* exhibited reduced bacterial loads compared to control mice;

they have also acquired severe lung disease [247, 248]. Moreover, WSX-1-deficient mice showed an increased susceptibility to concanavalin A (conA)-induced hepatitis compared with their wild type counterparts, which correlated with high levels of IFNγ and IL-4 production by NKT cells [249]. Taken together, these studies indicate that the importance of IL-27 to inhibit effector T cell functions (e.g. proliferation and cytokine production) overcome its role in promoting Th1 cell responses in the presence of a strong stimuli, such as infections caused by bacteria or parasites [250]. In this context, although IL-27 has been shown to play an important role in early Th1 differentiation, some studies have found that IL-27 can negatively regulate activated Th1 responses in order to attenuate the inflammation intensity [251]. IL-27 has been shown to suppress the production of various cytokines by fully activated $CD4^+$ T cells *in vitro*, such as IFNγ, IL-2 and IL-4. The suppressive effect of IL-27 on fully activated $CD4^+$ T cells depends on the preferential activation of STAT3 pathway as demonstrated by impaired IL-27-mediated suppression in STAT3-deficient T cells [251]. The ability of IL-27 to suppress $CD4^+$ T cells to produce IL-2, an important mediator of T cell proliferation and survival, supports the finding that IL-27 could suppress the Th1 responses. This is in line with the observation that IL-2 is highly produced when there is a deficieny of the IL-27 receptor [250, 252]. IL-27-mediated suppression of IL-2 is correlated upregulation of suppressor of cytokine signaling 3 (SOCS3) [236].

Apart from the dual effects of IL-27 on Th1 response, the suppressive effect of IL-27 on Th2 response is well documented. IL-27 can inhibit $CD4^+$ T cell polarization toward a Th2 phenotype in a STAT1-dependent mechanism by restraining GATA3 expression, an important Th2-specific transcription factor [234, 251]. Moreover, IL-27-mediated suppression of Th2 response involves mechanisms distinct from generating Th1 cells [253]. Several studies have reported an excessive Th2 response in WSX-1 KO mice infected with gastrointestinal helminths, such as *Trichuris muris*, which displayed an enhanced resistance against these parasites [254-256]. The excessive Th2 responses indicated by elevated levels of Th2-type cytokines (including IL4, IL5 and IL-13) seems unlikely to be due to defective IFNγ production, which was confirmed by blocking Th1 responses in wild type mice *in vivo* [254]. This is consistent with the finding that IL-27 can directly inhibit Th2 responses in $CD4^+$ T cells independently from its ability to augment IFNγ production [250, 254]. Furthermore, through STAT1-mediated activation, IL-27 abrogates naïve $CD4^+$ T cell differentiation into Th17 cells by reducing the

expression of RORγt, the Th17-specific transcription factor, and thereby, suppresses IL-17 production and inhibits Th-17-related autoimmune diseases [240, 257, 258]. IL-27 has no effect on committed Th17 cells in mice, but it can be effective in suppressing IL-17 production in human naïve and memory T cells [259].

IL-27 triggers the expression of IL-10, by $CD4^+$ T cells; it induces IL-10 but not Foxp3 in IFNγ-expressing $CD4^+$ T cells, which is known as type-1 regulatory T cells (Tr1) that can regulate T cell function [260, 261]. The prominence of IL-27 in inducing IL-10 expression in T cells has been demonstrated in a variety of experimental models of infectious and autoimmune diseases [262]. The induction of IL-10-producing Tr1 cells by IL-27 occurs due to coordinated actions of c-Maf (transcription factor), costimulatory receptor ICOS (inducible T cell costimulator) and IL-21 [263]. In addition, $CD8^+$ memory T cells downregulate their gp130 receptor, rendering them unresponsive to IL-27 during the recall response, which results in their inability to produce IL-10 [264]. In addition, $CD4^+$ T cell primed by IL-27 have been demonstrated to upregulate programmed death ligand 1 (PD-L1) expression by a mechanism that is dependent on STAT1 pathway [265]. Adoptive transfer of IL-27-primed $CD4^+$ T cells *in vivo* suppressed Th17 cell development and diminished the severity of autoimmune encephalomyelitis [265]. Additionally, PD-L1 upregulation in response to IL-27 has also been reported in human monocyte-derived DCs and mouse pDCs in the liver, which was associated with poor antigen presentation capability [266, 267]. IL-27 can regulate T cell proliferation by inducing the immunosuppressive enzyme, IDO, in human monocyte/macrophage [268, 269].

Role of IL-27 in cancer

As stated above, IL-27 is an immunoregulatory cytokine and it been shown to play a dual role in tumour immunity, including both antitumor and protumor activities [270].

- ***Antitumor effects of IL-27***. In 2004, Hisada *et al* were the first to state that IL-27 exhibits potent antitumor activity in a mouse model of colon carcinoma C26 by a $CD8^+$ T cell-dependent mechanisms [271]. C26 cells were genetically engineered to express single chain IL-27 (scIL-27); challenging mice with these cells showed minimal tumour growth and enhanced survival with complete remission. A re-challenge experiment with the parental C26 revealed that these mice developed a

specific antitumor immune response, which was largely mediated by $CD8^+$ CTLs and increased IFNγ expression. The involvement of Th1 cells in the antitumor activity induced by IL-27 against C26 colon carcinoma was also reported, which was demonstrated by the necessity of Th1-related T-bet activation but not STAT4 [271]. In the same year, Salcedo *et al* showed that engineering TBJ neuroblastoma cells to secret IL-27 displayed a slow tumour growth rate compared to control tumours, and additional administration of IL-2 provided further enhancement which resulted in complete regression of metastatic neuroblastoma [272]. Protective immunity against TBJ neuroblastoma was mediated mainly by tumour-reactive $CD8^+$ T cells and enhanced IFNγ expression, accounting for the upregulation of MHC class I molecules on IL-27-expressing TBJ tumours compared to control tumours [272]. Since then, a growing body of evidence has confirmed that both endogenous and exogenous IL-27 can exert antitumor activities through different mechanisms in several preclinical tumour models, such as melanoma, head and neck carcinoma, colon carcinoma and lung cancer [270, 273-278]. In the context of endogenous IL-27, Natividad *et al* have demonstrated that endogenous IL-27 is required for the development of protective immunity against endogenous tumours, implying that IL-27 can play an essential role in tumour immune surveillance [270, 279].

In addition to the ability of IL-27 to improve tumour immunity by activating tumour-specific $CD8^+$ T cell, it can also enhance the activity of NK cells and the susceptibility of tumour cells to NK cell-mediated killing [253, 271, 272, 276, 280]. Modifying the poorly immunogenic B16F10 melanoma to overexpress scIL-27 resulted in a marked delay in tumour growth mediated via an IFNγ-independent mechanism including NK cells but not $CD8^+$ T cells [274]. Similar antitumor activities mediated by IL-27 on B16F10 were also maintained in IFNγ-deficient mice, suggesting that IFNγ is not essential for IL-27-mediated antitumor immune responses against B16F10 melanoma [273, 281].

IL-27 can directly act on B cells, resulting in enhanced proliferation and generation of a tumour-specific immunoglobulin (Ig) response, which together can provoke an antibody-dependent cellular cytotoxicity (ADCC) [226, 276]. Matsui *et al* have demonstrated that IL-27 mediates tumour suppression of NK-resistant head and neck squamous cell carcinoma via antibody-dependent cellular cytotoxicity mediated by NK cells following IL-27 induction of tumour-specific IgG antibody response [276].

In an elegant piece of research, it has been shown that sequential administration of DNA encoding IL-12 and IL-27 genes in a specific order including IL-12 followed by IL-27 (IL-12 → IL-27), but not IL-27 → IL-12, resulted in the induction of CTL responses and the eradication tumour cells [282]. This indicates that presence of IL-27 is not essential to improve priming of T cell responses against tumour, but rather enhances the survival of tumour-reactive T cells and program them toward a unique phenotype of stem cell-like effector cells [283]. In addition, IL-27 has been found to exert antiproliferative effects on melanoma cells co-expressing WSX-1 and gp130 and responding to IL-27 [275]. In this case, IL-27 can mediate the activation of STAT1 and STAT3, upregulation of MHC class I molecules and induction of cell growth arrest and apoptosis. Although various human melanoma cells express both subunits of IL-27R (WSX-1/gp130) render them susceptible to IL-27-mediated direct inhibition of tumour growth, mouse melanoma B16F10 cells lack WSX-1 expression, which make them unresponsive to IL-27 treatment. However, B16F10 transfected with WSX-1 responded to IL-27 treatment and showed tumour growth inhibition [275]. Apart from IL-27-mediated direct effect on tumours, IL-27 has been shown to exert antiangiogenic activities. Angiogenesis is an important process, which leads to tumour progression and metastasis [226]. IL-27 acts on surrounding endothelial cells and fibroblast to induce the expression of antiangiogenic CXC chemokines including IP-10 (also known as CXCL10) and monokine-induced by IFNγ (MIG, also known as CXCL9), which can recruit CXCR3-expressing effector cells, resulting in a tumour growth suppression [273, 281]. Unlike IL-12, the antiangiogenic effect of IL-27 is IFNγ-independent, but it is similar to those induced by IFNγ, which is attributed to the resemblance in using JAK-STAT signalling molecules, such as STAT1 [281]. IL-27 has also been shown to inhibit tumorigenesis in lung cancer cells by down-regulating the expression of cyclooxygenase-2 (COX-2) and prostaglandin E (PEG2) [277].

Furthermore, IL-27 has been shown to play a role in the regulation of T_{reg} cell responses; It suppresses the generation of $Foxp3^+$ T_{reg} cells [284]. It also inhibit the production of IL-2, an important survival factor for T_{reg} cells, and therefore, can affect T_{reg} cell homeostasis [285]. Signalling of IL-27 in T_{reg} cells promotes their differentiation into Th1-T_{reg} cells co-expressing IL-10 and IFNγ, which regulate Th1 responses [286]. These results indicate that IL-27-mediated antitumor activities include both T cell-dependent and -independent mechanisms.

- ***Protumour effects of IL-27.*** Apart from IL-27-mediated antitumor effects, emerging studies have shown that IL-27 could potentially induce protumor effects, which can suppress tumour-reactive T cells [232, 270]. IL-27 has been shown to inhibit T cell priming which is partly mediated through IL-27 induction of the immunoregulatory molecule CD39 in DCs. The upregulation of CD39 reduces the extracellular ATP concentration and downregulates nucleotide-dependent activation of the NLRP3 inflammasome that in turn can inhibit T cell responses [287]. Wang *et al* have demonstrated that IL-27 can down-modulate the antigen-presenting capabilities of DCs and their ability to promote Th1 responses by utilizing WSX-1-deficient DCs [288]. Given the ability of IL-27 to promote the generation of tumour-specific CTLs, its ability to suppress DCs is differentially regulated during development of tumour immunity [289]. Accordingly, it has been proposed that co-administration of wild type T cells with IL-27 signal-defective DCs can enhance therapeutic efficacy against tumours [289]. IL-27 can also induce the expression of programmed death ligand 1 (PD-L1) on T cells and DCs. Although it has been shown that PD-L1 induction on T cells render them tolerant, another report showed that PD-L1 expression is required on tumour-reactive $CD8^+$ T cells to enhance their survival [265, 290]. Human monocyte-derived DCs exposed to IL-27 displayed an immunosuppressive phenotype and low capacity to stimulate T cells in a PD-L1-dependent fashion [266].

  Another mechanism by which IL-27 can mediate immunosuppression is the ability to stimulate IL-10 production by both mouse and human Tr1 cells, which can act as a negative feedback loop for limiting inflammation [291, 292]. The role of IL-10 in tumour immunity is controversial. In one hand, IL-10 can exert an immunosuppressive effect on DC and MQ subsets by reducing their expression of MHC class II molecules and suppressing IL-12 production in activated DCs and MQs [226]. On the other hand, IL-10 has been found to induce activation and expansion of intratumoral antigen-specific CTLs, enhance their cytotoxicity and IFNγ production; engineering tumour cells to overexpress IL-10 showed tumour protection in mice [293, 294]. Apart from IL-10, IL-27 can also induce the expression of T cell immunoglobulin and mucin domain-3 (Tim3), an inhibitory receptor associated with T cell exhaustion; on mouse tumour-infiltrating T cells [295].

  Although that human tumour cells co-expressing IL-27R subunits are prone to IL-27-mediated direct inhibition of cell proliferation, this is not the case with adult acute

myeloid leukaemia (AML) cells, which showed an enhanced survival and resistant to chemotherapeutic drugs mediated by IL-27 [296].

IL-27 translation to the clinic: incomplete puzzle

Cumulative studies in preclinical tumour models revealed that the antitumor activities of IL-27 dominate over its protumor activities. However, IL-27-mediated effect is largely dependent on tumour cell properties [281]. Although IL-27 antitumor effects have been acknowledged over a decade *in vitro* and *in vivo*, no IL-27-based treatment in the context of immunotherapy has been developed and translated into the clinic. In the context of gene therapy to treat cancer, delivery of IL-27 as a single agent has shown encouraging results in an animal model of metastatic prostate cancer [297]. The advantages of IL-27 in enhancing the survival of effector T cells and diminishing T_{reg} population give IL-27 an opportunity to explore its contribution in cancer immunotherapy [279, 283]. Given its ability to induce similar antitumor activity of IL-12 without inducing lethal systemic toxicity, it maybe worth considering IL-27 as a potential candidate. However, due to the immune suppressive effect of IL-27, the potential of IL-27 in the context of adoptive cell therapy remains to be determined.

1.5 General aims

TCR gene therapy is an alternative approach to generate a therapeutic cellular product of redirected T cells against specific tumour antigens. The feasibility of TCR gene therapy has been demonstrated in recent clinical trials. However, the limited success of this approach in some type of cancers has been attributed to a number of factors that can potentially influence the therapeutic efficacy of adoptively transferred TCR-engineered T cells, such as engraftment and persistence in the host, long-term maintenance of effector function and overcoming immune suppression in the tumour microenvironment.

Here, I propose to combine TCR gene therapy with genetic engineering to achieve IL-12 and IL-27 expression in TCR-redirected T cells. Manipulating cytokine production in TCR-redirected T cells can modulate their effector functions in addition to their re-directed specificity as a strategy to enhance therapeutic efficacy of adoptive T cells therapy.

The antitumor effects of IL-12 have been well documented. Despite its potent antitumor activity, its promising effectiveness is offset by severe systemic toxicity that renders its safety profile unacceptable. Unlike IL-12, IL-27 is not associated with severe adverse effects. However, there is a clear knowledge gap regarding the dual roles of IL-27 in tumour immunity. Therefore, there is a need to further decipher the utilization of IL-27 in adoptive cell therapy (Figure 1.3).

In this project, I aimed to

- Develop a strategy to safely deliver IL-12 in engineered T cells.
- Explore the role of IL-27 in the context of T cell-based therapy.
- Explore the role of regulated IL-12 and IL-27 delivery in tumour immunity.

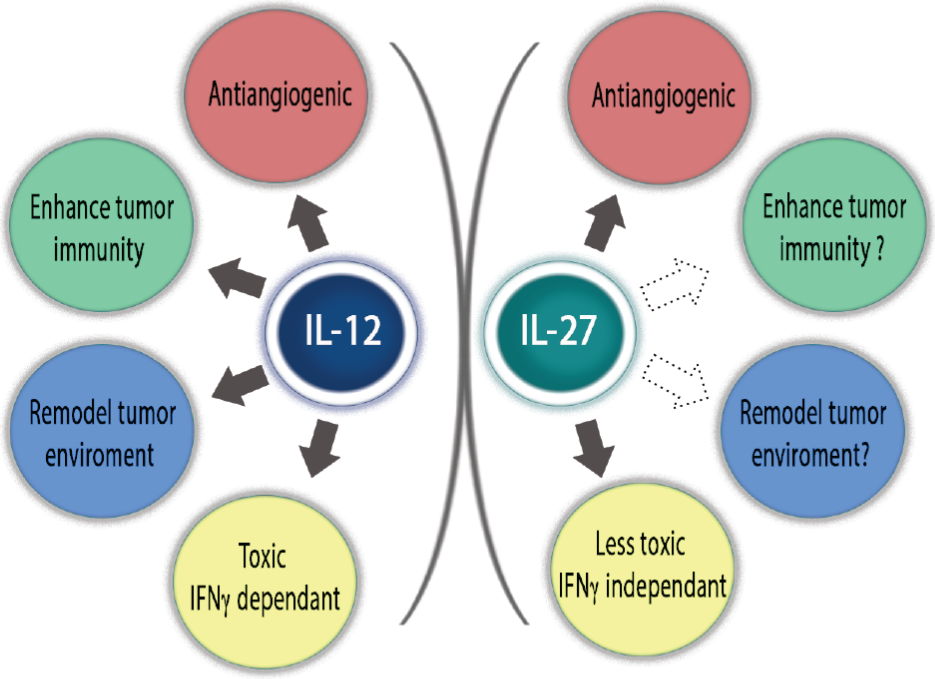


Figure 1.4 | Project background.

Chapter 2
Materials and Methods

2.1 Molecular Cloning

2.1.1 Generation of constitutive retroviral vectors

Two constitutive retroviral vectors were generated including pMP71-mscIL12-IRES-GFP and pMP71-mscIL27-IRES-GFP. MscIL12 region of p40 and p35 subunits linked by $(Gly_4Ser)_3$ flexible linker were amplified using Phusion High-Fidelity DNA Polymerases set (for high performance PCR) (ThermoFisher Scientific, F-530L) from pSIN-$(NFAT)_6$-mscIL12 vector (a kind gift from Dr.Gavin Bendle, Kite Pharma EU B.V., Amsterdam) using set of primers I (table 1) where NotI and SalI restriction site were introduced. The following PCR program was used: denaturation step at 98 °C for 30 seconds, 30 cycles of denaturation step at 98 °C for 10 seconds, annealing step at 72℃ for 50 seconds, extension step for at 72 °C for 50 seconds (1 min/ Kb of plasmid length) and a final extension step at 72 °C for 10 minutes. Then, the PCR product was transferred into pMP71 retroviral vector through NotI at the 5' end, and SalI at the 3' end, by which mscIL12 is linked to GFP gene via IRES sequence (mscIL12-IRES-GFP).

The codon optimized mscIL-27 was synthesized by GeneArt® (Regensburg, Germany) by which p28 and EBi3 regions are linked by the flexible linker, in a pMA-RQ vector. The mscIL27 was first amplified by high fidelity PCR using set of primers II (table 1) and the same PCR program as described above to introduce the restriction sites. The PCR product was then transferred into pMP71 retroviral vector via NotI at the 5' end, and SalI at the 3' end, by which mscIL27 is linked to GFP gene via IRES sequence (mscIL27-IRES-GFP) (See also Chapter 3, *section 3.3.1.1*).

2.1.2 Generation of tet-regulated retroviral vectors

mscIL12 was amplified by high fidelity PCR from pSIN-$(NFAT)_6$-mscIL12 vector using set of primers III (Table 1) where AclI restriction site followed by a Kozak sequence was introduced at the 5' end and P2A sequence at the 3' end respectively. eGFP sequence was amplified from pSERS-FU.P2A-eGFP vector by high fidelity PCR using set of primers IV (Table 1). Both PCR products, mscIL12 and eGFP regions, were then fused together by Phusion® High-Fidelity PCR master mix with HF buffer (New England Biolabs). Then, the mscIL12-Fu.P2A-eGFP fused product was transferred into tet-regulated retroviral vector through AccI, which is a ClaI-compatible site in the tet-regulated retroviral vector backbone, at the 5' end and BsrGI at the 3' end.

To construct a tet-regulated mscIL27 retroviral vector, mscIL27 has already been synthesized by GeneArt in the following order mscIL27-Fu.P2A-eGFP with ClaI at the 5' end and BsrGI at the 3' end. This was then transferred into the tet-regulated retroviral vector via ClaI and BsrGI restriction at the 5' end and at the 3' end, respectively (See also Chapter 3, *section 3.3.1.2*).

#	Direction	Sequence
I	FWD	5' ATT GAG CGG CCG CCA ACA TGG GTC CT 3'
	REV	5' GCA TGA GTC GAC TCA GGC GGA GCT CA 3'
II	FWD	5'ATA TGC GGC CGC CAC CAT GAG CAA GC 3'
	REV	5'ATA TGT CGA CTT ATG CTG TCC CAG GCG 3'
III	FWD	5' ATA TAA CGT TGC CAA CAT GGG TCC TCA 3'
	REV	5' CTC TTG GCT CTG GCG GAG CTC AGA TA 3'
IV	FWD	5' AGA GCC AAG AGA GGC GCC ACC A 3'
	REV	5' AAT TGG ACT AAT CCG GAG CGG CCG 3'

Table | Primers used in the PCR assays for molecular assembly of vectors designed in this study

2.1.3 Restriction digest and ligation

Digestion mix was set up as following: 1µl of the DNA (1µg/µl), 1µl of each restriction enzyme (NEB), 1µl 10X NEB buffer[1], 1µl BSA (if required), and nuclease free water (Qiagen 129114) was added to a final volume of 10µl. Digestion mix were allowed to react for 1-2 hours in a 37°C incubator, and then were separated by running the digested products in gel loading solution in 1:5 dilution (Sigma G2526) on a 1% agrose gel containing Ethidium Bromide, along with 8µl of Hyper Ladder 1 (Bioline 33025) in an electrophoresis chamber. Gel was then visualized under UV light using Ultrospec 1100 pro (Amersham Biosciences). The appropriate bands corresponding to expected sizes of DNA fragments were then excised from the gel and the desired DNA was gel extracted

[1] The right NEB buffer was chosen according to its compatibility with both restriction enzymes in the digestion mixture. It can be found online on NEB website (https://www.neb.com/tools-and-resources/usage-guidelines/nebuffer-performance-chart-with-restriction-enzymes).

using QIAquick Gel Extraction Kit (Qiagen 28704) following the manufacturers' instructions; DNA yield was eluted in 50µl of nuclease-free H_2O.

To ligate the insert into the vector backbone, a Quick Ligation Kit (NBE) (M2200S) was used. A specific molar ratio of insert:vector was identified based on the extracted band intensity. The ligation reaction was set up in a total volume of 20µl as following: insert and vector backbone (according to the identified ratio), 10µl 2X Quick Ligase Reaction Buffer, 6µl nuclease-free H_2O and 1µl Quick T4 DNA Ligase, and incubated for 10-15 minutes at room temperature. Ligation product was then kept on ice or at 4°C and proceeded with bacterial transformation.

2.1.4 Bacterial transformation

Ligated plasmids were transformed using High Efficiency 5-alpha competent E.coli (DH5α) (NEB C2987I) following the manufacturer's protocol. 50µl of the DH5α was added to an Eppendorf tube (placed on ice) and 2µl of the ligated product was added to the bacteria. Transformation mix was kept on ice for 30 minutes, and a heat shock step was performed at 42°C for 30 seconds (on a heat plate). Immediately, the tube was placed on ice for 2 minutes and 300µl of super optimal broth (S.O.C) medium (provided in the kit) was added, and was then incubated at 37°C for 1hr with vigorous shaking (~225rpm). Following incubation, the transformation mix was spread onto ampicillin-LB agar plates and incubated at 37°C overnight. The following day, colonies were picked up and inoculated in 5ml of ampicillin-LB broth medium (0.1mg/ml ampicillin, Sigma-Aldrich) in a 5ml universal tube. The tube was then incubated at 37°C with a vigorous shaking overnight (12-16hrs) or for 8hrs in order to do MiniPrep or MaxiPrep, respectively. For the MiniPrep, plasmid DNA was extracted from bacteria using QiaPrep Spin MiniPrep kit (Qiagen 27106) following the manufacturer's protocol. For the MaxiPrep, following the 8hrs of incubation, bacterial suspension was further diluted in 1:500 – 1:1000 in 100ml of ampicillin-LB broth medium and incubated at 37°C with a vigorous shaking overnight. On the following day, QIAfilter Plasmid Midi or Maxi kit (Qiagen 12243 or 12163) was used to perform bacterial DNA extraction following the manufacturer's protocol.

In the case of a subcloning transformation, which usually carried out following site-directed mutagenesis, subcloning efficiency DH5α bacteria (Invitrogen 18265-017) was used.

2.1.5 Site-directed mutagenesis

This method was used to modify the tet-regulated vector backbone and introduce ClaI restriction site according to the manufacturer's instructions in QuickChange II XL Site-Directed Mutagenesis kit (Agilent). The following mutagenesis PCR program was used: denaturation step at 95 °C for 1 minute, 18 cycles of denaturation step at 95 °C for 30 seconds, annealing step at 60 °C for 30 seconds and extension step for at 68 °C for 7.30 minutes (1 min/ Kb of plasmid length) and a final extension step at 68 °C for 7 minutes. PCR product was digested by DpnI (NEB) at 37 °C for 1 hour and then transformed into XL10-Gold ultracompetent cells provided with the kit. QiaPrep Spin MiniPrep kit (Qiagen 27106) was used to isolate DNA, and a digestion reaction was performed using BsrGI and ClaI restriction enzymes (NEB) to identify the right DNA. After identifying the right DNA, a subcloning transformation was performed using subcloning efficiency DH5α bacteria (Invitrogen 18265-017) followed by bacterial DNA extraction using QiaPrep Spin MaxiPrep kit (Qiagen 12243).

All acquired plasmid DNAs were confirmed by re-digestion with appropraite restriction ezymes and sequencing (Eurofins MWG Operon). The DNA concentration was quantified by a Nanodrop spectrophotometer.

2.2 *In vitro* cell culture

2.2.1 Cell lines and culture conditions

***Packaging cell line*:** Phoenix ecotropic cells (Phoenix-Eco), an adherent packaging cell line (Nolan Laboratory, Stanford, CS, USA), were maintained in Iscove's Modified Dulbecco medium (IMDM) (Lonza BE12-726F) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Sigma, 011M3395), 1% of 200mM L-glutamine (GIBCO 25030) and 1% of 100U/ml Penicillin/streptomycin (GIBCO 15070). Cells were split every 2-3 days when they are ~80% confluent.

Tumour cell lines: BW cells (BW5147) (H-2^k) is a murine lymphoma cell line derived from AKR/J mice spontaneously develop thymoma. EL4 is a murine lymphoma cell line (H-2^b) derived from induced lymphoma by 9,10-dimethyl-1,2-benzanthracene in C57BL mice. EL4 was transfected to stably express influenza derived nucleoprotein (NP) peptide which is presented in the context of H-$2D^b$, a kind gift from Dr. B. Stockinger (National Institute for Medical Research, London). EL4-NP transfected cells carry antibiotic

resistance gene to Geneticin as a selection marker (G418 disulfate salt solution 50 mg/ml, Sigma-A1720). EL4-NP Luciferase-positive cells, a kind gift from Dr. M. Pule (UCL, London), were generated by transfecting EL4-NP cells with plasmid encoding a red-shifted luciferase. All these cells were split every other day to maintain 0.1-1x10^6 cells/ml density. B16F10 melanoma cell line (H-2^b) is an adherent cell line derived from the skin of C57BL/6J mice developed melanoma, a kind gift from Dr. S. Quezada (UCL Cancer Institute, London). B16F10 cells usually express no or low levels of MHCI (K^b, D^b) and MHC class II (I-A^b) molecules (Waltraud, 1998). All tumour cell lines were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Lonza BE12-167F) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Sigma, 011M3395), 1% of 200mM L-glutamine (GIBCO 25030) and 1% of 100U/ml Penicillin/streptomycin (GIBCO 15070) (referred to as tumour medium "TM"). B16F10 cells were split every 2-3 days when they are ~80% confluent.

All adherent cell lines were treated with 3-5ml of 0.05% Trypsin/EDTA (GIBCO 25300) for up to 5 minutes to facilitate their detachment for subculturing. If cells are not detaching easily, they were placed at 37°C to enable their dispersal.

Primary T cells: Murine T cells were maintained in RPMI 1640 medium (Lonza BE12-167F) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Labtech, 40507), 1% of 200mM L-glutamine (GIBCO 25030), 1% of 100U/ml Penicillin/streptomycin (GIBCO 15070) and 50μM final concentration of 2-β-Mercaptoethanol (referred to as T cell medium "TCM").

All cells were cultured at 37°C in a humidified atmosphere of 5% CO_2. All tissue culture work was performed in a class II hood (Biohit Biological Safety Cabinet).

2.2.2 Cell separation

Selection of $CD3^+$ T cells was performed using Pan T cell isolation kit (Miltenyi Biotec, Cat. no. 130-095-130) on a MidiMACS separator in combination with LS columns (Miltenyi Biotec 130-042-401), following manufacturer's protocol. This routinely yielded >95% $CD3^+$ T cell purity as determined by flow cytometry. Bulk splenocytes were first harvested from wild type B6 mice, ACK lysed (Gibco™, A10492-01) and counted prior to cell selection.

2.2.3 Cell count and viability assessment

Using a haemocytometer, cells were counted under a light microscope. Cells harvested from different tissues were lysed with ACK lysing buffer (Gibco™, A10492-01). Cell viability was then assessed by dye exclusion test using 0.1% trypan blue (Sigma-Aldrich-T8154) in PBS; It was mixed with cell suspension in 1:1 ratio. Trypan blue selectively penetrates dead cell membrane and stains them blue, whereas intact membrane of viable cells excludes the dye and remains uncoloured.

2.2.4 Storage of cells

To store cells, a freezing medium (1ml/vial) of 10% dimethyl sulphoxide (DMSO) (Sigma, UK) and 90% supplemented medium was prepared. Packaging cells (Phenoix eco) were frozen in cold 10% DMSO and 90% IMDM. All tumour cell lines were frozen in ice-cold 10% DMSO and 90% RPMI. Cells were spun down at 494g for 5 minutes and resuspended in cold freezing medium. Cell vials were frozen at -80°C overnight and transferred to liquid nitrogen storage on the following day.

To grow up cells from a frozen stock, cells were thawed at 37°C in a water bath and then added drop-wise to a universal tube containing 5ml of warmed medium. Cells were then spun and resuspended with the appropriate amount of medium.

2.3 Retroviral transduction

2.3.1 Transfection and production of retroviral particles

The Phoenix-Eco packaging cells were used to produce viral supernatants for transduction of mouse T cells. On day0, which is 24hrs before transfection, $1.5x10^6$ packaging cells were plated out on 60.1 cm^2 Poly-L-lysine coated tissue culture plates in 8ml of supplemented IMDM medium (TPP 93100). Plated cells were kept overnight at 37°C in a humidified atmosphere of 5% CO_2, allowing them to adhere to the plate and grow. The following day (day1), 3 hours before transfection, the IMDM medium was replaced with 5ml fresh medium. For transfection, FuGENE® HD transfection reagent (Promega E2312) was used. Transfection mixture was prepared into two steps; in a 1.5ml Eppendorf tube, 25µl of Fugene was added to 750µl of Opti-mem medium (GIBCO 31985). The mixture was incubated for 5min in the hood before adding to a second tube containing the DNA mix: 2.6µg of vector DNA, 1.5µg of pCL-eco DNA and sterile distilled H_2O up to a total volume of 50µl. Upon mixing both tubes of DNA and Opti-

mem-Fugene, the mixture was then incubated for 15-30 minutes at room temperature inside the hood. The mixture was then applied drop-wise to PhEco cells that had been plated the day before (day0). 24hrs later (day2), medium was replaced with 5.5ml supplemented RPMI medium and cells were incubated for another 24hrs. On day 3 (48 hours following Phoenix-Eco transfection), virus-containing supernatants were harvested and spun for 5 minutes at 459g to remove any residual Phoenix-Eco cells. The viral supernatants can be used immediately for transduction or stored as aliquots at -80℃.

2.3.2 Retroviral transduction of BW cell line

On day 0, the day of transduction, BW cells were counted and $5x10^4$ viable cells were re-suspended in 100µl of neat viral supernatant; then, it was transferred into 96 well tissue culture-treated plate (round bottom) (TPP 92697). The plate was then spun at 712g, for 90min at 32°C with no break. After centrifugation, cells were transferred into T25 tissue culture flask (TPP 90026) containing 10ml of TM medium and incubated under standard tissue culture conditions for at least 3 days before they were analysed.

2.3.3 Retroviral transduction of primary mouse T cells

To transduce primary T cells, splenocytes were harvested from WT B6 mice lysed with ACK lysing buffer (Gibco™, A10492-01) and activated as bulk cells or preceded with CD3 T cell enrichment step (as described in section 2.2). On the same day, primary T cells were then activated using CD3/CD28 antibody coated beads (Dynabeads-Mouse T cell activator, Gibco-11453D). 25µl of the dynabeads were used to activate $1x10^6$ CD3-enriched T cells or $2x10^6$ bulk splenocytes. The magnatic beads were washed with sterile PBS (in 1:1 ratio) and placed on a magnet (DynaMag™-15), and the washed beads were collected. The beads were then used to activate the cells which were seeded at $1\text{-}1.5x10^6$ cells/ml in TCM medium containing 100U/ml of IL-2 (Chiron). Cells were incubated in standard tissue culture conditions for at least 24hrs before transduction. The following day (day1), activated cells were counted and $1.5x10^6$ cell were re-suspended in 500µl neat viral supernatant for single transduction and 500µl of each viral supernatant (1ml in total) for double transduction, and were then transferred into pre-treated non tissue culture 24 well plate[2]. This was followed by spinning the plate at 712g, for 90minutes at 32°C, and the plate were then incubated in standard tissue culture conditions. On day2, beads were removed from transduced cells; the cells were then re-suspended in 2ml fresh TCM containing 20U/ml IL-2 (Roche) and incubated in standard tissue culture conditions. On day4 (72hrs after transduction), the cells were stained to assess transduction efficiency by flow cytometry.

2.4 Flow cytometry: reagents and staining protocols

2.4.1 Cell surface staining

To stain for cell surface markers, cells were harvested and washed in FACS staining buffer (1% hi-FCS in PBS) and pelleted. The cells were resuspended in 50-100µl of FACS buffer containing the desired fluorescent-labelled monoclonal antibodies (according to appropriate dilution that has been previously determined), and they were incubated at 4°C in the dark for 15-20 minutes. The cells were then washed twice in FACS buffer and resuspended in 300µl of FACS buffer for analysis. Cells that were harvested from different tissues following an *in vivo* experiment were blocked first by

[2] The plate was pre-treated by coating with retronectin (Takahara-bio-Otsu, Japan) for 3-4hrs at room temperature or overnight at 4°C; then, after removing the retronectin, 2% bovine serum albumin (BSA) in PBS was added as blocking agent for 30minutes and washed twice (x2) with sterile PBS.

50µl FACS buffer containing Fc block (anti-mouse CD16/32) for 15 minutes at 4°C in the dark to prevent non-specific binding and washed in FACS buffer before proceeding with cell surface staining step.

Prior to FACS analysis, 1µl of propidium iodide (PI) (BD-Parmingen 51-6621E) was added to the cell suspension (300µl) allowing the discrimination of live cells from dead ones. PI cannot penetrate live cells, and thus all PI^- cells would be included in the analysis. All samples were acquired using BD™ LSRII or BD LSRFortessa™ flow cytometer (BD Bioscience, USA). Staining procedures were performed using 96 well tissue culture-treated plates, round bottom (TPP-92027), or in some cases, BD Falcon™ 5ml round-bottom tubes (BD, 352052) were used. All flow cytometry data were analysed using FlowJo software (Tree Star, Ashland, OR, USA).

2.4.2 Intracellular cytokine staining (ICS)

ICS staining protocol can be used alone to assess cytokine release or in combination with cell surface staining protocol. To stain for cell surface markers, ICS protocol was carried out following the cell surface staining as described in previous section. To determine intracellular cytokines, cells were treated with 10µg/ml Brefeldin A (BFA) (Sigma-B7651) to block cytokine secretion for 2hrs or 4hrs in the case of BW cells or primary T cells, respectively. Cells were then washed in FACS buffer and permeabilised in 50-100µl Fixation/Permeabilization™ solution (Fix/Perm) provided with the BD Cytofix/Cytoperm™ kit (BD-554714) that was used for ICS. The cells were then incubated at 4°C in the dark for 20 minutes and washed with 150-200µl BD Perm/Wash™ buffer (also provided in the kit). The cells were then stained with 50µl of Perm/Wash™ buffer containing the desired fluorescent-labelled monoclonal antibodies (in an appropriate dilution) and incubated at 4°C in the dark for 30 minutes. After incubation, the cells were washed twice, once with 200µl of Perm/Wash™ buffer followed by a second wash with 200µl of FACS buffer. The cells were then resuspended in 300µl FACS buffer for analysis.

2.4.3 Intracellular Ki-67 staining

Ki-67 staining was carried out using eBioscience™ Foxp3/Transcription Factor Staining Buffer Set (eBioscience 00-5523-00), which has been validated by the company for staining with antibodies agains transcription factors and nuclear proteins. Cells were first

surface stained as described in section 2.4.1. To perform Ki-67 ICS, the cells were washed once with FACS buffer before proceeding with the cell permeabilization step. The cells were then resuspended in 200µl of a freshly prepared Fix/Perm working solution, by mixing 1 part of Fix/Perm concentrate with 3 parts of Fix/Perm diluent. The cell suspension was kept in the dark at 4°C for at least 30 minutes (up to 18 hours for mouse samples). The cells were then pelleted by centrifugation at 577g for 5 minutes and washed with 1X of permeabilization buffer that is supplied as 10X with the kit, and therefore, the 10X permeabilization buffer was diluted 1:10 in dH_2O. The cell pellets were resuspended in 50µl of permeabilization buffer containing the appropriate amount of Ki-67-conjugated antibody and were then incubated in the dark at 4°C for at least 30 minutes. After incubation, the cells were washed twice, once with 200µl of permeabilization buffer followed by a second wash with 200µl of FACS buffer. The cells were then resuspended in 300µl of FACS buffer and analysed by flow cytometer machine.

2.4.4 Fixable viability dye staining

Identifying viable cells can be achieved by adding PI as mentioned previously. However, PI cannot be used with fixed cells, and therefore, it was only used with cell surface staining. Alternatively, a Fixable Viability Dye (FVD) was used when ICS needs to be conducted.

Prior to ICS, cells were washed twice in 150-200µl protein/serum-free PBS (also azide-free). The cells were then resuspended in 100µl of FVD diluted in PBS and were incubated in the dark at 4°C for 15 minutes. Following incubation, cells were washed twice, once with PBS and once with FACS buffer and proceeded to the ICS protocol for cytokine or Ki-67 staining (section 2.4.2 & 2.4.3).

2.4.5 Annexin V vs Fixable Viability Dye staining

Annexin V staining with Fixable Viability Dye were used to identify early apoptotic cells (FVD^-/Annexin V^+) and late apoptotic cells (FVD^+/Annexin V^+). Annexin V staining was performed following cell surface and FDA staining steps using eBioscience™ Annexin V Apoptosis Detection Kit PerCP-eFluor™ 710 (eBioscience, 88-8008-72). The cells were washed in 200µl of 1X binding buffer that was supplied as 10X in the kit, and therefore, the 10X binding buffer was diluted 1:10 in dH_2O. The cell pellets were resuspended in 100µl of binding buffer containing the appropriate amount of Annexin V-conjugated antibody and were incubated in the dark at room temperature for 10-15 minutes. Following incubation, cells were washed with 1X binding buffer and followed by the ICS protocol (section 2.4.2).

2.4.6 List of antibodies used for flow cytometry

Antibody	Fluorochrome	Clone	Company/ Catalogue no.
Annexin V	PerCP-eFluor™ 710	-	eBioscience 88-8008-72
B220	BV786	RA3-6B2	BD 563894
CD3ε	BV711	145-2C11	BD 563123
CD3ε	BV605	145-2C11	BD 563004
CD3ε	BUV395	145-2C11	BD 563565
CD4	APC-H7	GK1.5	BD 580181
CD8a	V450	53-6.7	BD 560469
CD11b	V450	M1/70	eBioscience 48-0112
CD11c	APC	HL3	BD 550261
CD19	PerCP-Cy5.5	ID3	eBioscience 45-0193
CD45.1	BV650	A20	BD 563754
CD62L	Alexa 700	Mel-14	BD 560517
CD107a	BV786	1D4B	BD 564349
CD127	eFluor660	A7R34	eBioscience 50-1271-80
CD279 (PD-1)	BV605	J43	BD 563059
F4/80	APC-eFluor® 780	BM8	eBioscience 47-4801
Fas (CD95)	PE-Cy7	Jo2	BD 557653

FC block	None	93	eBioscience 14-0161-86
Foxp3	Alexa Fluor® 700	FJK-16s	eBioscience 56-5773
Gr-1	Biotin	RB6-8C5	553124
H-2D^b (MHCI)	FITC	KH95	BD 553573
H-2K^b (MHCI)	FITC	AF6-88.5	BD 553569
Human CD34 (Q8)	Biotin	QBEND/10	AbDSerotec-MCA547B
I-A^b (MHCII)	PE	AF6-120.1	BD 553552
IFNγ	APC	XMG1.2	BD 554413
IL-4	PE	11B11	Pharmigen 554435
IL-10	APC	JES5-16E3	Pharmigen 554468
IL-10	BV650	JES5-16E3	BD 564083
IL-12 (p40/p70)	APC	C15.6	Pharmigen 554480
IL-27 (p28)	APC	MM27-7BI	Biolegend 516906
IL-27 (p28)	PE	MM27-7BI	Biolegend 516908
IL-17A	PE-CF594	TC11-18H10	BD 562542
Ki-67	eFluor660	SolA15	eBioscience 50-5698
NK1.1	BV605	PK136	BD 563220
Streptavidin	APC	-	BD 554067
Streptavidin	PE	-	BD 554061
Streptavidin	V500	-	BD 561419
Streptavidin	BUV737	-	BD 564293
Thy1.1 (CD90.1)	PE-Cy7	H1S51	eBioscience 25-0900-82
TNFα	PE	MP6-XT22	BD 554419
Vβ3	PE	KJ25	BD 553209
Vβ11	Biotin	RR3-15	BD 553196
Vβ11	PE	RR3-15	BD 553198

2.5 *In vitro* functional assay

2.5.1 Cytokine release assay

Engineered T cells were stimulated and assessed 4-5 days following transduction. According to transduction efficiency and cell viability, transduced T cells were co-cultured with tumour cells either EL4 or EL4-NP (in a responder-to-target ratio of 1:2),

and incubated in 1ml or 200µl culture medium (TCM) in 24 well tissue culture (TC) plates (TPP Z707791) or 96 well round-bottom TC-culture plates (TPP 92097), respectively. T cells were also stimulated (nonspecifically) with PMA (50ng/ml) and Ionomycin (1µg/ml) as a positive control for intracellular cytokine staining. Stimulated T cells were treated with BFA (10µg/ml) at the beginning of the stimulation and were incubated for 4-5hrs under standard tissue culture conditions. Following the incubation period, cells were washed and stained as described previously for FACS analysis.

2.5.2 Enzyme-linked immunosorbent assay (ELISA)

Cytokine secreted in cell culture supernatant (IL-12) was quantified by ELISA using the BD OptEIA™ kit (BD-555256) following the manufacturer's protocol. A 96 well ELISA plate (BD Falcon-353279) was coated with 100µl/well of capture antibody diluted 1:250 (provided in the kit) in coating buffer and kept at 4°C overnight. The following day, the coating buffer/antibody was aspirated and the plate was washed 3 times with 300µl/well of wash buffer (0.05% Tween20/PBS). The plate was then blocked with 200µl/well assay diluent (BD Pharmingen™ Assay Diluent-555213) for 1hr at room temperature. The plate was then washed 3 times with wash buffer, and 100 µl/well of pre-diluted cell culture supernatants or standards were added. The plate was sealed and incubated for 2hrs at room temperature. Standards were prepared by reconstituting the lyophilised recombinant mouse IL-12 (rIL-12p70) (provided in the kit) in 1 ml of assay diluent to obtain the highest standard concentration (4000 pg/ml). Serial dilution was performed within the plate to yield a concentration range from 4000 pg/ml to 62.5 pg/ml in duplicate. Following incubation, the plate was washed 5 times with wash buffer and 100 µl/well of working detector which compose of detection antibody (biotinylated anti-mouse IL-12 monoconal antibody) and Sav-HRP enzyme reagent (streptavidin-horseradish peroxidase conjugate). The plate was sealed and incubated for 1hr at room temperature. This was followed by 7 times washing step. 100 µl/well of substrate solution (TMB substrate reagent set from BD-555214) was added, and the plate was then incubated in the dark at room temperature for 30 minutes. Finally, 50 µl/well of stop solution (BD-51-2608KZ) was added and the plate was read at 450nm with λ correction 570nm within 30 minutes of stopping the enzymatic reaction using a microplate reader.

2.6 *In vivo* experiments

2.6.1 Mice

All C57BL/6 female mice used as recipients or tissue donors were bred in-house in the animal facility (at University College London (UCL)-based at the Royal Free Hospital) within individually ventilated cages (IVCs) or purchased from Charles River animal facility. All animal procedures were performed according to the United Kingdom Home office regulations.

2.6.2 Adoptive transfer and tumour challenge

Before starting tumour challenge experiments, all mice were weighed, ear tagged and shaved on their right flank.

For EL4-NP tumour challenge experiments, on day 0, Thy1.2 C57BL/6 female recipient mice were irradiated with 4Gy total body irradiation (TBI), and 3-4hrs later, they were injected subcutaneously (s.c.) with 100µl/mouse of $1x10^6$ EL4-NP tumour cells resuspended in sterile PBS (Gibco™, cat no. 20012019). Tumour was measured overtime at different time intervals using a digital caliper; tumour size was calculated using the following formula (a x b x π^3 /4), where a is the horizontal diameter and b is the vertical diameter of the tumour. On day 5, mice received intravenous (i.v.) injection of $0.5x10^6$ transduced T cells. Mice were sacrificed when they lost >20% of their initial body weight or reached a lethal tumour burden (tumour size exceeded 15mm) according to the regulations of the UK home office. Where it is stated, mice were sacrificed at specific time points.

For B16F10 tumour challenge experiments, on day 0, Thy1.2 C57BL/6 female recipient mice were implanted with s.c. injection of $0.5x10^6$ B16F10 tumour cells, and tumour measurements were taken as described above. On day 10 post tumour injection, mice were sublethally irradiated with 4Gy TBI and received i.v. injection of $2x10^6$ transduced T cells.

[3] π= 3.14159

2.6.3 Isolation of tumour infiltrating lymphocytes

A standard protocol from the research group of Prof. Sergio Quezada (UCL Cancer Institute, London, UK) for extracting tumour infiltrating cells was followed. B16 tumours were harvested from sacrificed mice, transferred to a clean bijoux tube and weighed. The tumours were then chopped into small pieces and incubated in 1ml/tumour sample of dissociation solution, which contains of RPMI medium (924µl) supplemented with 66µl of 5mg/ml Liberas[4] (Roche-05401020001) and 10µl of 20mg/ml DNase I[5] (Roche-10104159001). The tubes containing the tumour and dissociation solution were place in 37°C water bath for 30 minutes and mixed every 10 minutes. Following incubation, the tubes were immediately transferred onto ice and tumour samples were further processed using a solution of 5mM EDTA in PBS. Tumour suspension were then passed/mashed through a 70µm cell strainer (BD Falcon, 352340) and centrifuged at 459g for 5 minutes. Cell pellets were resuspended in 3ml of supplemented RPMI medium and layered over 3ml of histopaque, a lymphocyte separation medium (Sigma, 10771), and spun at 815g for 20 minutes at room temperature with no break. From each sample, the interphase was collected, washed and resuspended in 1ml media. FACS staining was carried out as previously described and FACS analysis was performed using FlowJo software.

2.6.4 Re-isolation of tumour cells

Harvested tumours were treated as described in previous section. After collecting, washing and resuspending the interphase, suspensions were spun and resuspended in freezing medium and stored in liquid nitrogen (section 2.2.4) to assess all re-isolated tumours at the same time. To evaluate tumour re-isolates, frozen tumour samples were thawed and cultured under the same conditions as the parental B16F10 tumour cells (section 2.2.1).

[4] One vial of liberase was reconstituted in 1ml of RPMI to obtain a concentration of 5mg/ml. It can be aliquoted and stored at -80°C

[5] One vial of DNase was resuspended in 5ml of dH_2O to obtain a working concentration of 20mg/ml. It can be aliquoted and stored at -80°C

2.6.5 *In vivo* bioluminescence imaging

To image EL4-NP Luciferase^{+} tumour, mice were injected intraperitoneally (i.p.) with 150μg/g D-Luciferin firefly (Biosynth). 10 minutes later, mice were anaesthetized and bioluminescence signals were obtained by Xenogen IVIS-100 (Caliper Life Sciences).

2.7 Statistical analysis

All statistical analysis was performed using GraphPad Prism version 6.0 software (GraphPad Software, USA). Two-tailed Mann Whitney test, Wilcoxon matched-pairs signed rank or Log-rank (Mantel Cox) were utilized to calculate *P values.*

Chapter 3

Manipulation of cytokine production: *in vitro* validation

3.1 Introduction

T cell engineering is a fast growing field for treating cancer. One approach is the use of TCR gene therapy to induce anti-tumour immune response. This approach is based on redirecting T cell specificity by introducing antigen-specific TCR using viral gene transfer. However, the effector function of the TCR-modulated T cells can be impaired in the suppressive tumour microenvironment [298]. In an attempt to overcome the suppressive effect and enhance the efficacy of T cell therapy, engineered T cells have been supplied with immune stimulatory molecules. Owing to the pleiotropic effect of cytokines, a number of cytokines have been explored as an adjuvant that can enhance anti-tumour immunity and overcome immune suppressive mechanisms that are operational in the tumour microenvironment. IL-12 holds promise in cancer treatment due to its ability to modulate the anti-tumour immune response of both arms of the immune system; innate and adaptive immune cells [165]. As described previously, IL-12 is a heterodimeric cytokine consisting of two covalently linked subunits; p35 and p40, which give rise to the biologically active protein p70, produced mainly by APCs. It binds to its receptor, which is composed of IL-12β1 and IL-12β2 subunits expressed by T cells and natural killer (NK) cells [119, 156]. IL-12 plays an important role in T cell-mediated immunity. It stimulates IFNγ production by T cells and NK cells, and it has the ability to enhance the cytolytic activity of NK cells and $CD8^+$ T cells. IL-12 also contributes to $CD4^+$ T cell-driven differentiation toward Th1 cells and reduces IL-4-mediated immune suppression by other immune cells [162, 299]. Early studies of IL-12 reported that it exhibited an anti-tumour activity that was thought to be mainly mediated through enhanced IFNγ production [174, 175]. It has also been shown that adoptive transfer of antigen specific-$CD8^+$ T cells equipped with IL-12 in tumour bearing mice revealed improved functional activity of the transferred T cells and enhanced overall survival [300]. Despite IL-12 showing encouraging results in pre-clinical animal models, clinical application of IL-12 has been hindered by its severe systemic toxicity resulting from IFNγ overproduction [182, 186, 218, 281, 301]. Taking this into consideration, another approach has been designed to control IL-12 expression by using an NFAT-responsive promoter which drives IL-12 expression upon TCR engagement [302]. A recent clinical trial reported cytokine-mediated toxicity from treating patients with T cells engineered to express IL-12 regulated by the NFAT promoter [303]. To date, IL-12 therapy has not yet been successfully translated into clinic despite its promising results in pre-clinical models.

So further refinement needs to be considered such as exploiting another controlled-release system. On the other hand, IL-27, a member of IL-12 family, is an immune regulatory cytokine, but it is less toxic than IL-12. IL-27 is composed of two subunits, EBI3 and p28 (known as IL-30), and it is produced mainly by APCs [220]. The biological effects of IL-27 are mediated by its interaction with IL-27R complex including WSX-1/TCCR and gp130, and it is expressed by a variety of hematopoietic and non-hematopoietic cells [226]; and thus, it can affect various types of cells. Accumulating evidence suggests that IL-27 can play a role in anti-tumour immunity by promoting Th1 and cytotoxic T lymphocyte function. It was shown to enhance the expression of T-bet and Eomes transcription factors in naïve human $CD8^+$ T cell stimulated with anti-CD3 antibodies. In these conditions, IL-27 also increased the expression of cytotoxic effector molecules such as perforin and granzyme B [243]. It has also been found to promote the differentiation of naïve $CD4^+$ T cells into Th1 cells and inhibit the polarization of Th2 cells [233, 271, 304]. Through STAT1-mediated activation, IL-27 inhibits the expression of Th17 transcription factors and thereby abrogates naïve $CD4^+$ T cell differentiation into Th17 cells. Given the ability of IL-27 to revert the development of a polarized Th2-type immune response, it can enhance pre-existing responses of antigen-specific Th1 cells [239, 240]. Apart from the pro-inflammatory effects of IL-27, it can also act as a negative regulator in preventing excessive inflammation that can result in severe organ damage or subsequent autoimmune disease. The anti-inflammatory response of IL-27 is mediated by suppressing IL-2 production by $CD4^+$ T cells, inhibiting Th17 development and inducing IL-10 production by $CD4^+$ and $CD8^+$ T cells [250, 305].

3.2 Aims

Here, I sought to construct retroviral vectors constitutively expressing IL-12 and IL-27 or under the regulation of doxycycline (Dox). Also, I have sought to demonstrate the functional activities of these modulated T cells *in vitro* in an antigen-specific manner mediated via an introduced TCR. In this chapter, I have tested the following hypothesis:

- Gene transfer can be used to achieve constitutive IL-12 and IL-27 expression in T cells.
- Regulation of IL-12 and IL-27 production in T cells can be achieved by Tet-controlled gene expression.
- Co-transduction of IL-12 or IL-27 can modulate the effector function of TCR-redirected T cells in vitro.

3.3 Results

3.3.1 Vector Design

Two types of retrovirus-based vectors encoding my gene of interest were used, which are mouse single chain IL-12 (mscIL-12) and mouse single chain IL-27 (mscIL-27). The mscIL-12 and mscIL-27 resulted from fusing p40 and p35, or EBI3 and p28 subunits with a flexible linker, respectively. The purpose of the flexible linker is to ensure both subunits of IL-12 and IL-27 are linked together allowing the production of the biologically active form of these cytokines.

3.3.1.1 Constructing retroviral vectors constitutively expressing IL-12 and IL-27

A validated pMP71 retroviral vector that is capable of producing high levels of constitutive transgene expression in primary T cells was selected [306]. As described previously (materials & methods), the pMP71 vector backbone was modified to encode either mscIL-12 or mscIL-27 linked to green fluorescent protein (GFP) as a reporter via an internal ribosome entry site sequence (IRES). In pMP71 vector, msIL-12 or msIL-27 are constitutively expressed by a myeloproliferative sarcoma virus (MPSV) long terminal repeat (LTR) promoter.

To generate *pMP71-mscIL12-IRES-GFP* vector, mscIL12 region of p40 and p35 subunits linked by (Gly4Ser)3 flexible linker was amplified from pSIN-(NFAT)6 -mscIL12 vector and inserted into the retroviral pMP71 backbone as illustrated in details in figure 3.1.

To generate *pMP71-mscIL27-IRES-GFP* vector, mscIL27 region of EBI3 and p28 subunits linked by (Gly4Ser)3 flexible linker was amplified from 13AAXDVP vector synthesized by GeneArt and inserted into the retroviral pMP71 backbone as illustrated in details in figure 3.2.

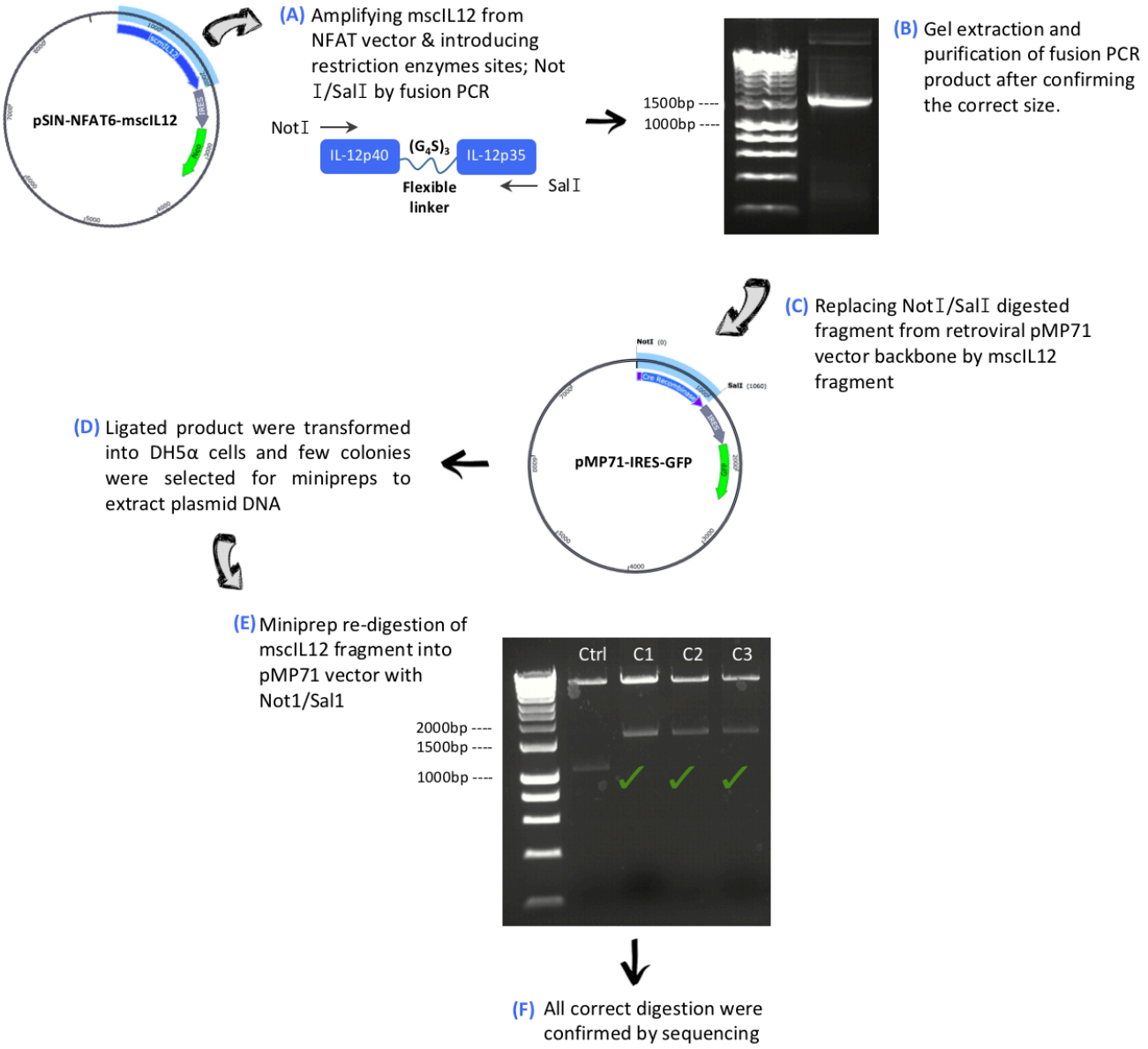


Figure 3.1 | Cloning mscIL-12 into pMP71 vector. (A) mscIL12 fragment of p40 and p35 subunits linked by (Gly4Ser)3 flexible linker (IL12p40-(G_4S)3-IL12p35) was amplified by high fidelity PCR from pSIN-(NFAT)6-mscIL12 vector using set of primers tagged with NotI/SalI restriction sites to be introduced. **(B)** Fusion PCR product was run on 1% agarose gel to detect and quantify the size of the product NotI-mscIL-12-SalI (1643bp), which was then gel extracted and purified to be inserted into a retroviral pMP71 backbone. **(C)** The purified and digested mscIL-12 fragment was then replaced the NotI/SalI digested fragment from the retroviral pMP71 vector backbone by a ligation step. **(D)** Ligated product was transformed into DH5α cells and few colonies were selected for minipreps to extract plasmid DNA. **(E)** Acquired plasmid DNA was re-digested with NotI/SalI restriction enzymes and all correct digestion products were confirmed by sequencing.

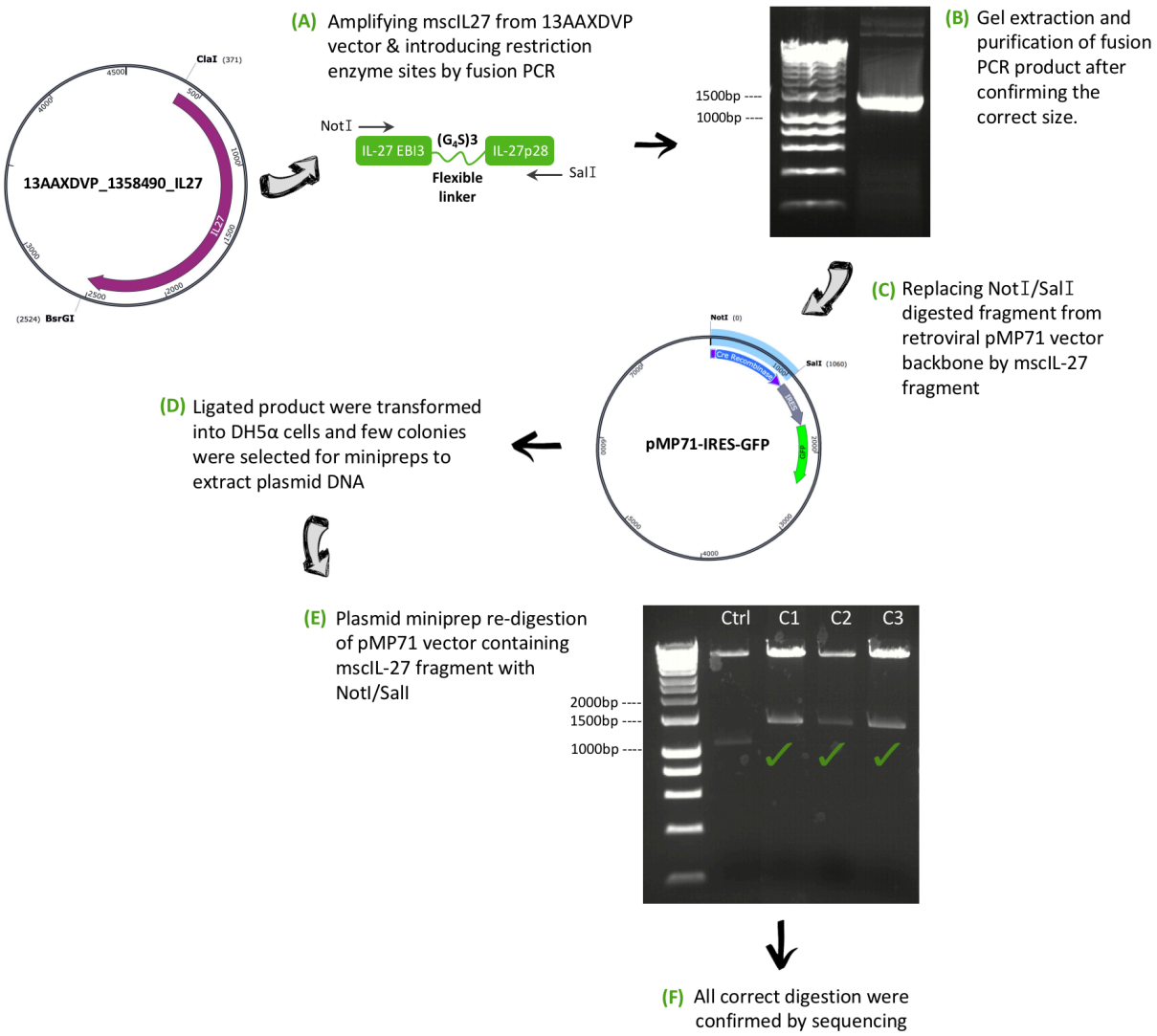


Figure 3.2 | Cloning mscIL-27 into pMP71 vector. (A) mscIL-27 fragment of EBI3 p40-like subunit and p35 subunit linked by (Gly4Ser)3 flexible linker (IL27 EBI3-(G_4S)3-IL27p28) was amplified by high fidelity PCR from 13AAXDVP vector made by GeneArt using set of primers tagged with NotI and SalI restriction sites to be introduced. **(B)** Fusion PCR product was run on 1% agarose gel to detect and quantify the size of the product NotI-mscIL27-SalI (1365bp), which was then gel extracted and purified to be inserted into a retroviral pMP71 backbone. **(C)** The purified mscIL-27 fragment was then replaced the NotI/SalI digested fragment from the retroviral pMP71 vector backbone by a ligation step. **(D)** Ligated product was transformed into DH5α cells and few colonies were selected for minipreps to extract plasmid DNA. **(E)** Acquired plasmid DNA was re-digested with NotI/SalI and all correct digestion products were confirmed by sequencing.

3.3.1.2 Constructing retroviral vectors inducibly expressing IL-12 and IL-27

A pSERS retroviral vector, all-in-one Tet-On inducible system, was developed to regulate the expression of IL-12 (iIL12) and IL-27 (iIL27). It was selected as it has been demonstrated to give a high level of regulated gene expression mediated by tetracycline or its derivative doxycycline [307]. The pSERS retroviral vector is composed of two main elements to express a tet-regulated transgene including: the reverse tet-responsive transactivator and the regulated expression cassette. The reverse tet-responsive transactivator (rtTA2-M2) was fused to a Q8 tag via 2A sequence, which is a fusion protein served as a transduction marker. The Q8 tag is a fusion protein composed of a 42 amino acid-long CD8α stalk "8" and a minimal epitope of truncated human CD34 (16 amino acid-long), which can be stained with anti-human CD34 monoclonal antibody called QBEnd10 "Q" [308]. This fusion protein (Q8-2A-rtTA2-M2) is constitutively expressed by the human phosphoglycerate kinase (hPGK) promoter. The second element, which is the regulated expression cassette, contains the gene of interest linked to GFP via 2A sequences, which is under the control of tet-responsive promoter (containing a repeat of tet-operator fused to a minimal promoter) and is off in the absence of tetracycline or its derivatives doxycycline. Upon tetracycline/doxycycline administration, the transactivator binds to the tet-responsive promoter and induces the expression of the gene of interest and GFP. Thus, in this context, GFP serves as an induction marker, allowing us to investigate the regulatory activities of this vector.

Initially, the vector backbone was modified to introduce ClaI restriction site by site-directed mutagenesis as illustrated in figure 3.3. Following that, the vector was modified to encode our gene of interest, either mscIL-12 or mscIL-27 linked to GFP gene via 2A sequence (Figure 3.4) (Figure 3.5).

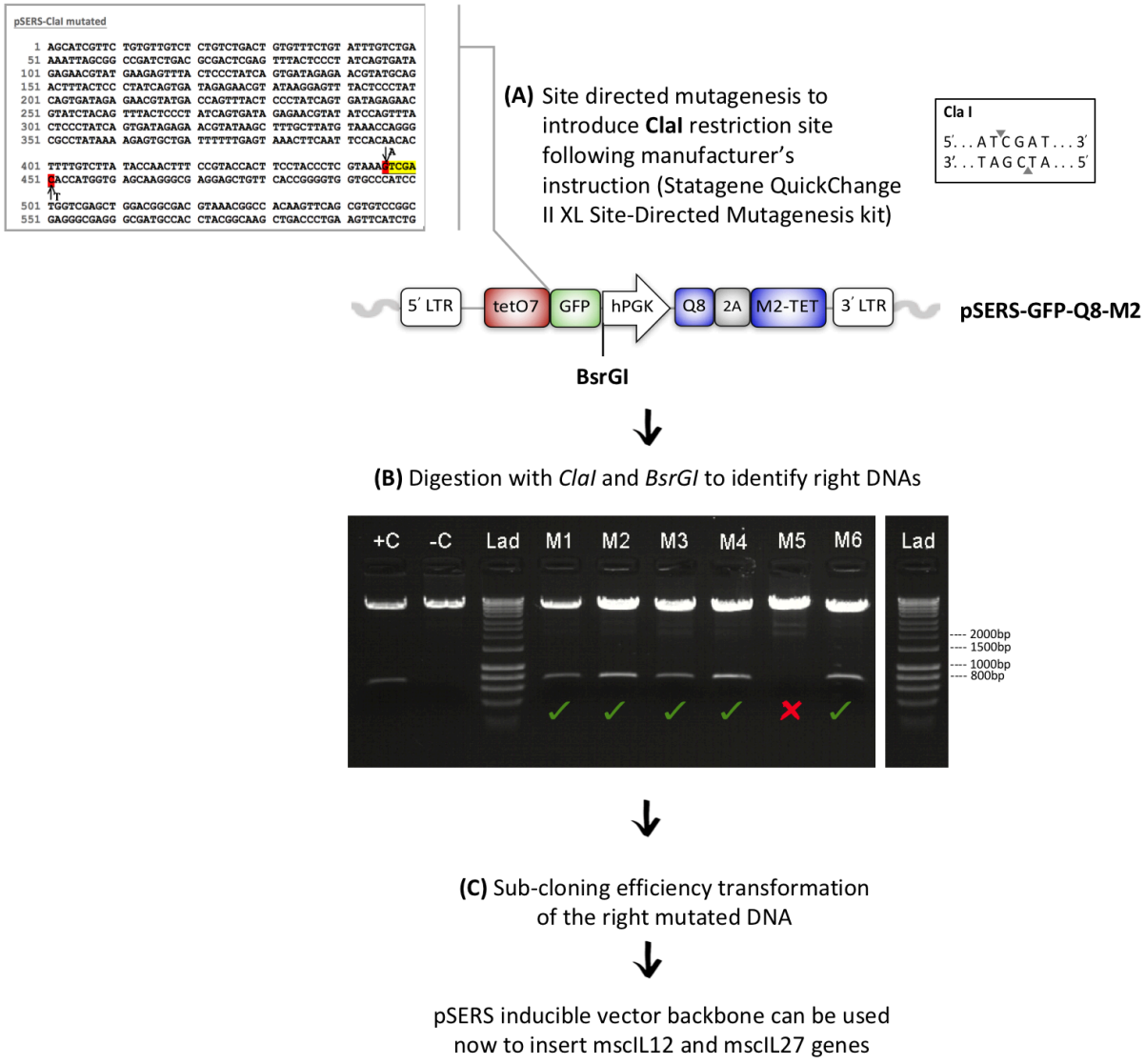


Figure 3.3 | Directed mutagenesis to introduce ClaI site into pSERS inducible vector. **(A)** Vector control (iGFP) was used as a template to introduce ClaI restriction site by inserting a mutation at position 446 and 451 to exchange Guanine 'G' with Adenine 'A', and Cytosine 'C' with Thymine 'T', respectively, using Quick change II XL site-directed mutagenesis kit and specifically designed primers. Colonies from the transformed new product into the chemically competent cells were digested with ClaI and BsrGI restriction enzymes to check the new product containing the introduced mutation. **(B)** Indicative agrose gel of the digested products identified the right mutated DNA products. **(C)** Sub-cloning the mutated DNA by transformation into DH5α cells for high efficiency transformation. Purify and extract DNA including pSERS inducible vector incorporates ClaI site in its backbone.

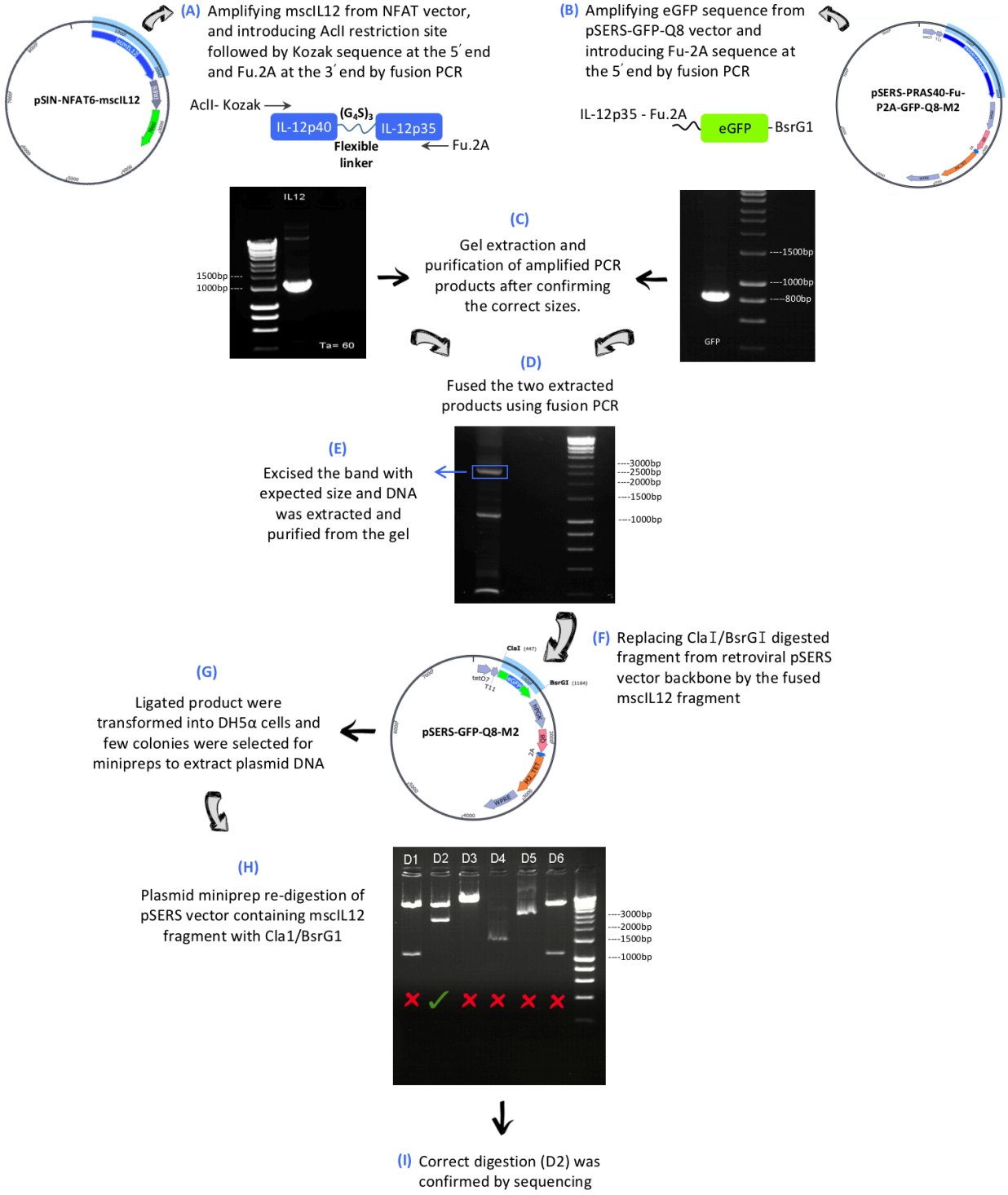

pSIN-NFAT6-mscIL12
(A) Amplifying mscIL12 from NFAT vector, and introducing AclI restriction site followed by Kozak sequence at the 5′ end and Fu.2A at the 3′ end by fusion PCR
AclI- Kozak
IL-12p40
(G4S)3
IL-12p35
Flexible linker
Fu.2A
(B) Amplifying eGFP sequence from pSERS-GFP-Q8 vector and introducing Fu-2A sequence at the 5′ end by fusion PCR
IL-12p35 - Fu.2A
eGFP
BsrG1
pSERS-PRAS40-Fu-P2A-GFP-Q8-M2
IL12
1500bp
1000bp
Ta= 60
(C)
Gel extraction and purification of amplified PCR products after confirming the correct sizes.
----1500bp
----1000bp
-----800bp
GFP
(D)
Fused the two extracted products using fusion PCR
(E)
Excised the band with expected size and DNA was extracted and purified from the gel
----3000bp
----2500bp
----2000bp
----1500bp
----1000bp
(F) Replacing ClaI/BsrGI digested fragment from retroviral pSERS vector backbone by the fused mscIL12 fragment
pSERS-GFP-Q8-M2
(G)
Ligated product were transformed into DH5α cells and few colonies were selected for minipreps to extract plasmid DNA
(H)
Plasmid miniprep re-digestion of pSERS vector containing mscIL12 fragment with Cla1/BsrG1
D1 D2 D3 D4 D5 D6
----3000bp
----2000bp
----1500bp
----1000bp
(I) Correct digestion (D2) was confirmed by sequencing

Figure 3.4 | Cloning mscIL-12 into pSERS vector. A diagram illustrates the steps used in cloning mscIL-12-2A-GFP into pSERS vector. **(A)** mscIL12 fragment of p40 and p35 subunits linked by (Gly4Ser)3 flexible linker (IL12p40-(G_4S)3-IL12p35) was amplified by high fidelity PCR from pSIN-(NFAT)6-mscIL12 vector using set of primers including forward (FW) primer tagged with AclI restriction site followed by kozak sequence to be introduced at the 5' end, and reverse (RV) primer tagged with P2A sequence to be introduced at the 3' end of the mscIL-12 fragment to have a PCR product size of 1656bp **(B)** eGFP sequence containing BsrGI restriction site at the 3' end was amplified by high fidelity PCR from pSERS-GFP-Q8 vector using set of primers to have a PCR product size of 818bp when it runs on 1% agarose gel. The FW primer was tagged with a short sequence of (IL12p35-Fu.2A). **(C)** Both PCR products obtained from step A & B were run on a 1% agarose gel. After confirming the right size of both expected PCR products, bands were extracted from the gel and purified. **(D)** A second fusion PCR reaction was set up to fuse both PCR products obtained from the primary PCR amplification step using set of primers (the FW primer used in step A and RV primer used in step B). **(E)** The product of the second PCR reaction was run on a 1% agarose gel to detect the fusion product (2414bp), which were then extracted and purified from the gel. **(F)** The purified fusion fragment digested with ClaI/BsrGI was then replaced the ClaI/BsrGI digested fragment from the retroviral pSERS vector backbone by a ligation step using T4 quick DNA ligase. **(G)** Ligated product was transformed into DH5α cells and few colonies were selected for minipreps to extract plasmid DNA. **(H)** Acquired plasmid DNA was re-digested with ClaI/BsrGI and **(I)** the correct digestion product was confirmed by sequencing.

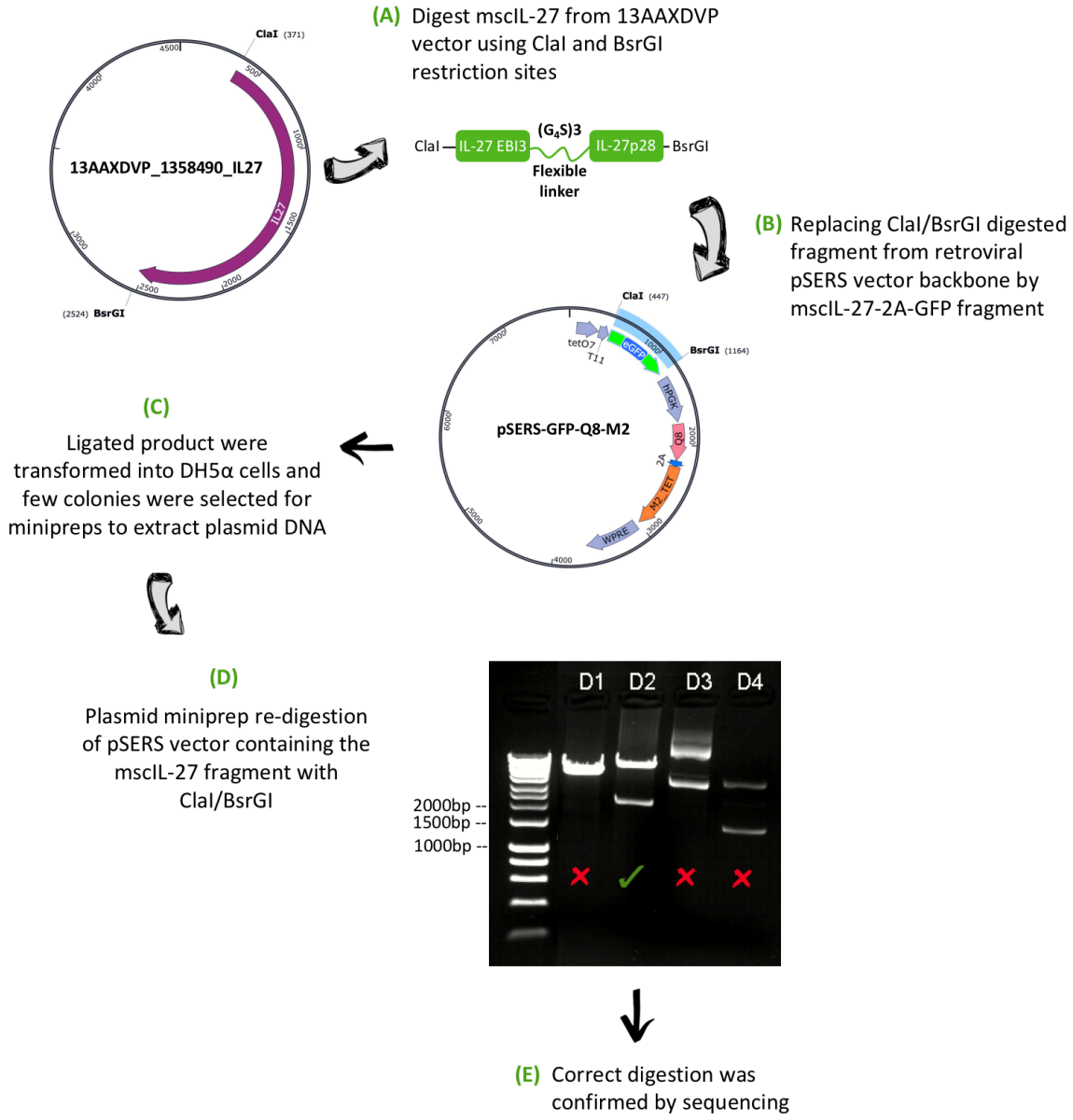


Figure 3.5 | Cloning mscIL-27 into pSERS vector. A diagram illustrates the steps used in cloning mscIL-27-2A-GFP into pSERS vector. **(A)** mscIL-27-2A-GFP fragment was digested from 13AAXDVP vector (synthesized by GeneArt) using ClaI/BsrGI. **(B)** The digested mscIL-27-2A-GFP fragment was then replaced the ClaI/BsrGI digested fragment from the retroviral pSERS vector backbone by a ligation step using T4 quick DNA ligase. **(C)** Ligated product was transformed into DH5α cells and few colonies were selected for minipreps to extract plasmid DNA. **(D)** Acquired plasmid DNA was re-digested with ClaI/BsrGI and **(E)** all correct digestion products were confirmed by sequencing.

3.3.2 Genetic engineering enhances IL-12 and IL-27 production by primary T cells

Firstly, we set out to assess the ability of engineered T cells to express elevated levels of IL-12 and IL-27 following retroviral transduction with the constructs encoding msIL-12 or msIL-27. A color-coded system has been applied in creating chapter's figures to make it easy to follow including: black (mock-Td T cells), blue (IL-12-Td T cells), green (IL-27-Td T cells) and orange (TCR-Td T cells).

Initial validation experiments were performed in the BW cell line as they are easily transduced, and the expression of IL-12 and IL-27 was assessed. BW cells were transduced with pMP71-IL-12 ($T_{cIL\text{-}12}$), pMP71-IL-27 ($T_{cIL\text{-}27}$) or mock-transduced (T_{mock}). 3days post-transduction, BW cells were treated with brefeldin A (BFA) for 2hrs to block cytokine secretion. Intracellular cytokine expression of IL-12 and IL-27 was detected by flow cytometry together with GFP expression as a transduction marker (Figure 3.6). In the case of BW cells that were transduced with the tet-regulated vectors, an overnight induction with Dox (1µg/ml) was set up followed by the assessment of transduction and induction efficiency via Q8 staining and GFP expression, respectively (Figure 3.7).

After the successful validation of retroviral constructs in BW cell line, the constructs were tested in primary T cells. Splenocytes from wild type C57BL/6 mice were harvested, enriched for CD3 and activated with CD3/CD28 beads (as described in materials and methods). 24 hours following activation, T cells were transduced with pMP71-IL12, pMP71-IL27 or mock-transduced, and intracellular expression of constitutive IL-12 and IL-27 were examined by flow cytometry 3 days post-transduction. This demonstrated that transduced T cells expressed high levels of IL-12 and IL-27 compared to mock-transduced cells (figure 3.8 A). In the case of primary T cells transduced with the tet-regulated vectors, assessment of intracellular IL-12 and IL-27 expressions along with GFP expression (the induction marker) showed a well-regulated expression of cytokines and GFP in Dox-treated cells compared to cells that did not receive Dox (Figure 3.8 B).

3.3.3 Transduction of T cells with IL-12 and IL-27 does not alter the CD4/CD8 ratio

Given that IL-12 and IL-27 are potent immunomodulatory cytokines, we asked whether transducing T cells with these cytokines would alter the CD4/CD8 ratio. Splenocytes from C57BL/6 mice were activated with CD3/CD28 beads and transduced with IL-12, IL-27 or mock-transduced after 24hrs of activation. 3 days post-transduction, transduced T cells were stained with anti-CD4 and anti-CD8 antibodies. This demonstrated that neither IL-12 nor IL-27 has a profound effect on altering CD4/CD8 ratio upon transduction of T cells (Figure 3.9).

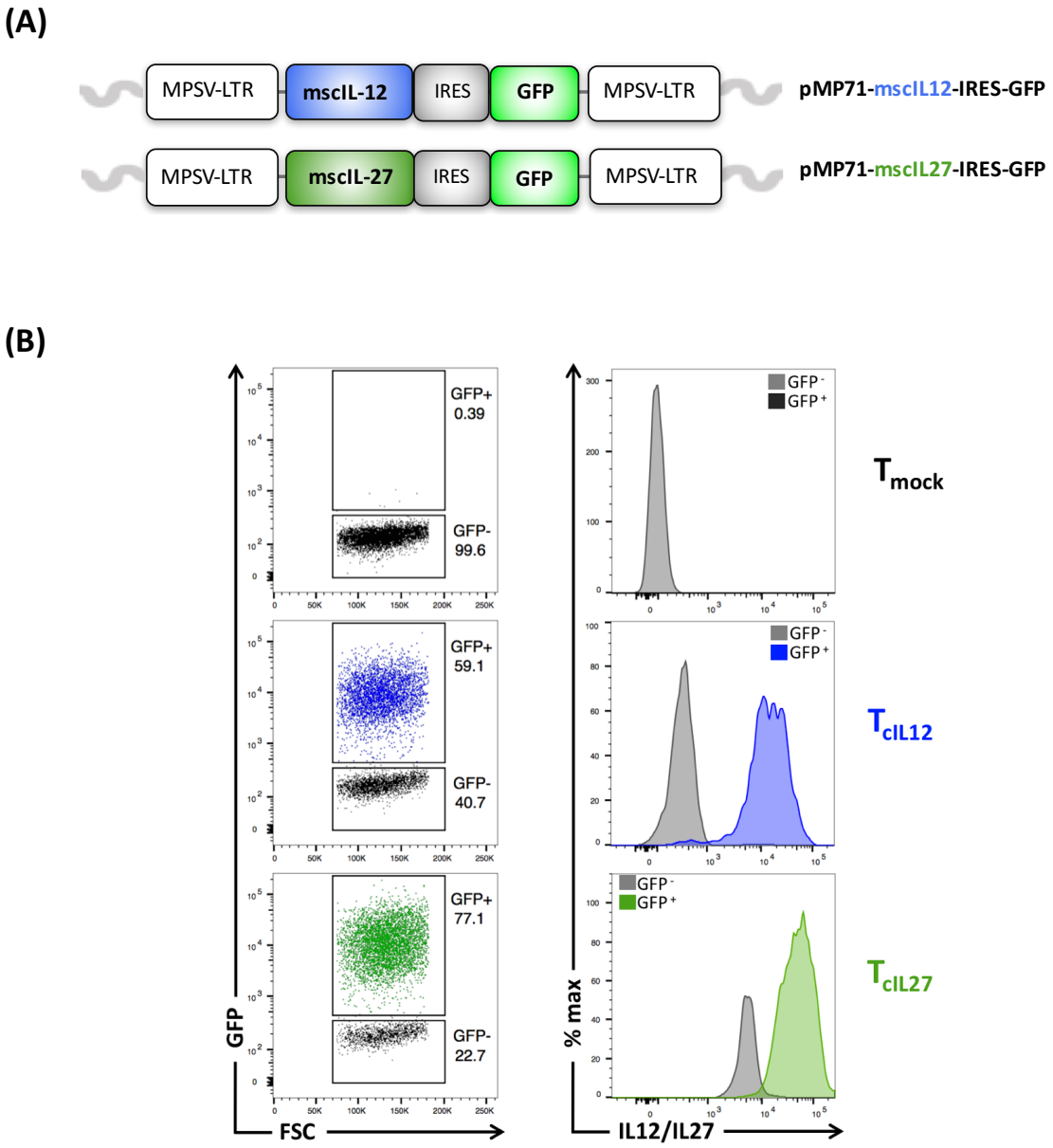
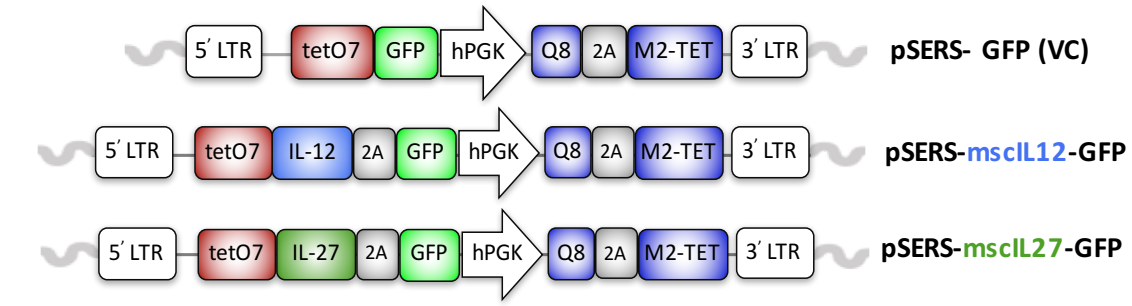


Figure 3.6 | *In vitro* validation of IL-12 and IL-27 constitutive expression in BW cell line. (A) Schematic diagram representing the molecular structure of the constitutive pMP71 retroviral vector encoding mouse single chain IL-12 or IL-27 linked to green fluorescent protein (GFP) reporter gene via an internal ribosome entry site (IRES). **(B)** BW cells were transduced with pMP71-IL12, pMP71-IL27 retrovirus or mock-transduced. 3 days later, BW cells were treated with BFA (10µg/ml) to block cytokine secretion for 2hrs and were stained intracellularly with anti-IL-12 or anti-IL-27 antibodies. Representative plots showing GFP expression marks the transduction efficiency ***(left)*** and IL-12 or IL-27 expression within GFP^+ (transduced) compared to GFP^- (un-transduced) population ***(right)***

(A)


5′ LTR
tetO7
GFP
hPGK
Q8
2A
M2-TET
3′ LTR
pSERS- GFP (VC)
5′ LTR
tetO7
IL-12
2A
GFP
hPGK
Q8
2A
M2-TET
3′ LTR
pSERS-mscIL12-GFP
5′ LTR
tetO7
IL-27
2A
GFP
hPGK
Q8
2A
M2-TET
3′ LTR
pSERS-mscIL27-GFP

(B)

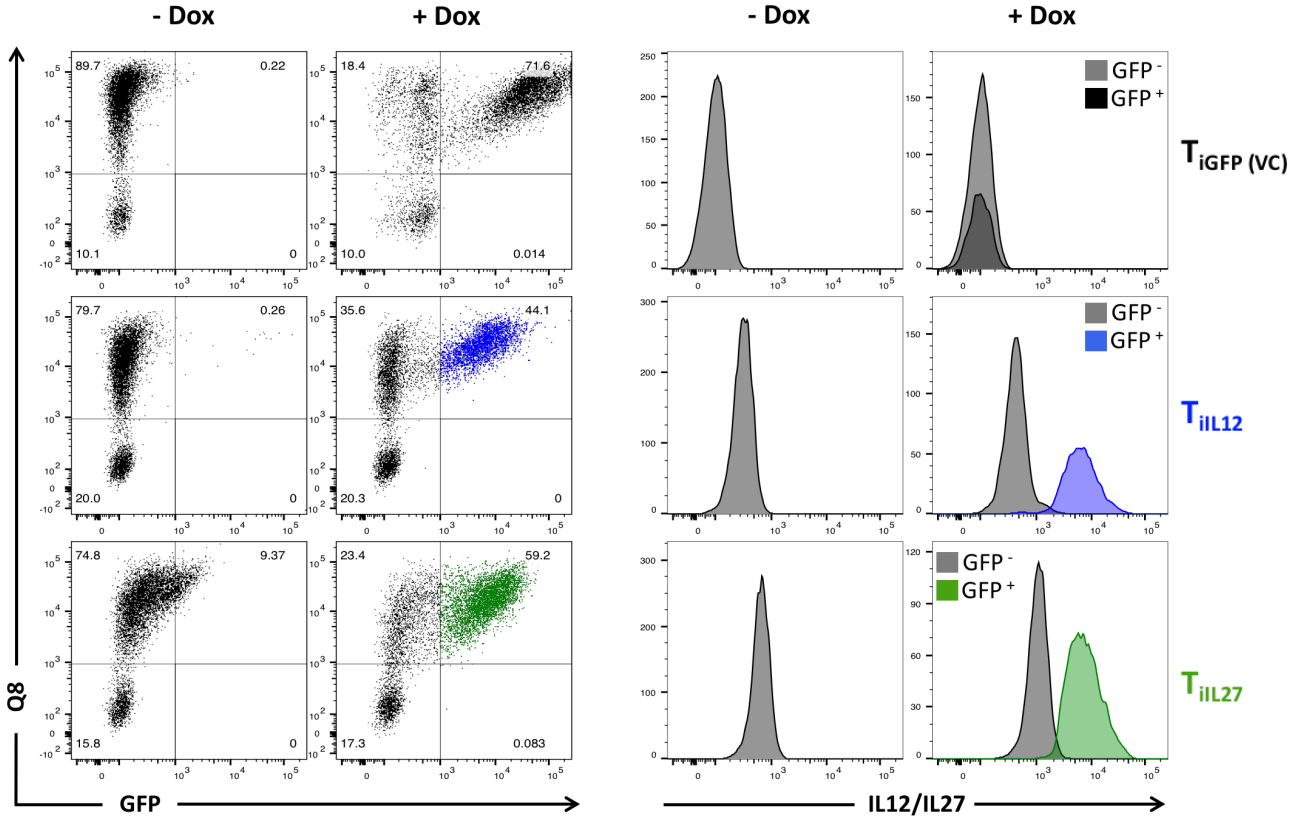
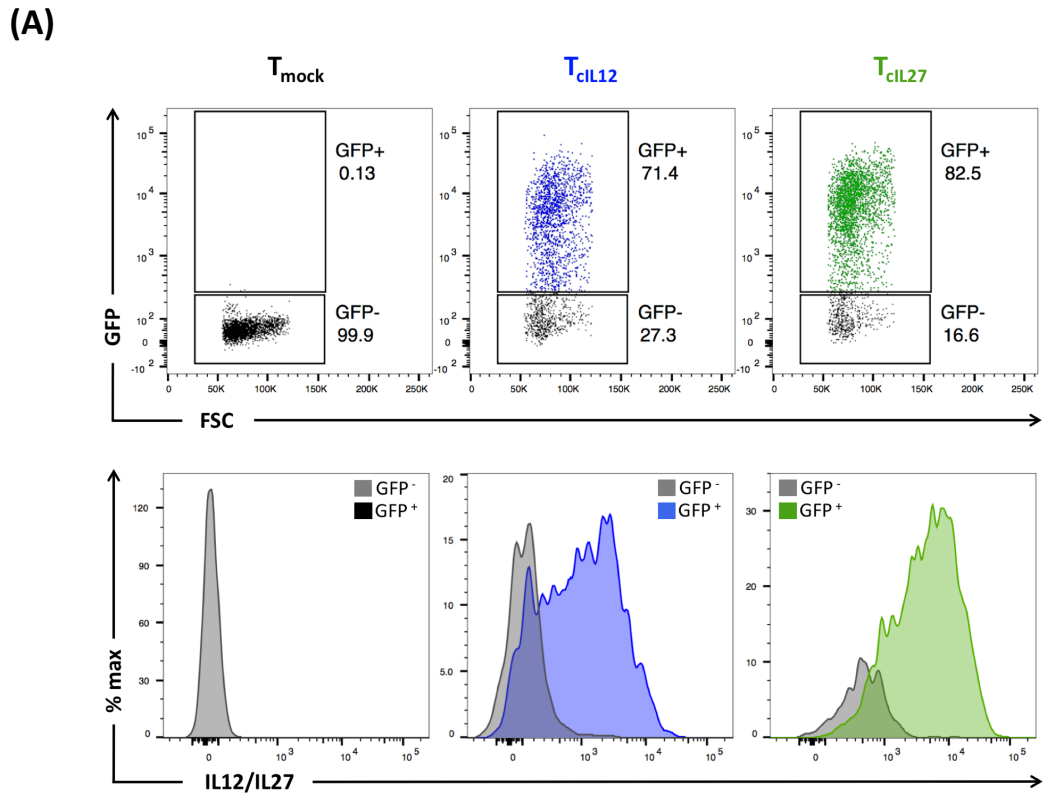

- Dox
+ Dox
- Dox
+ Dox
89.7
0.22
10.1
0
18.4
71.6
10.0
0.014
79.7
0.26
20.0
0
35.6
44.1
20.3
0
74.8
9.37
15.8
0
23.4
59.2
17.3
0.083
Q8
GFP
GFP -
GFP +
T iGFP (VC)
T iIL12
T iIL27
IL12/IL27

Figure 3.7 | *In vitro* validation of IL-12 and IL-27 expression under the regulation of tet-inducible promoter in BW cell line. (A) Schematic diagram representing the molecular structure of the pSERS retroviral-based tet regulated vector encoding mscIL-12 or mscIL-27 linked to a GFP reporter gene via 2A sequences, or encoding GFP only which served as vector control. hPGK promoter constitutively expresses the reverse tet-responsive transactivator. The regulated expression cassette containing mscIL-12 or mscIL-27 is under the control of tet-responsive promoter, which consists of repeats of tet operator sequences fused to a minimal promoter. Tet-responsive promoter is 'On' in the presence of tetracycline/doxycycline. **(B)** BW cells were transduced with pSERS-IL12, pSERS-IL27 or vector control (pSERS-GFP). 3 days post-transduction, transduced BW cells were treated with Dox (1μg/ml) or left untreated overnight. The following day, cytokine expression was blocked for 2hrs by culturing the cells in BFA-containing medium. The cells were then stained with anti-Q8 (htCD34) to assess the transduction efficiency and with GFP to assess the efficiency of induction following Dox administration. They were also stained for intracellular IL-12 and IL-27. Representative plots showing Q8 and GFP staining profile of transduced BW cells in the presence and absence of Dox ***(left)*** and IL-12 or IL-27 expression within GFP^+ (induced) compared to GFP^- (non-induced) population ***(right).***


(A)
T mock
T cIL12
T cIL27
GFP+ 0.13
GFP- 99.9
GFP+ 71.4
GFP- 27.3
GFP+ 82.5
GFP- 16.6
GFP
FSC
GFP -
GFP +
% max
IL12/IL27

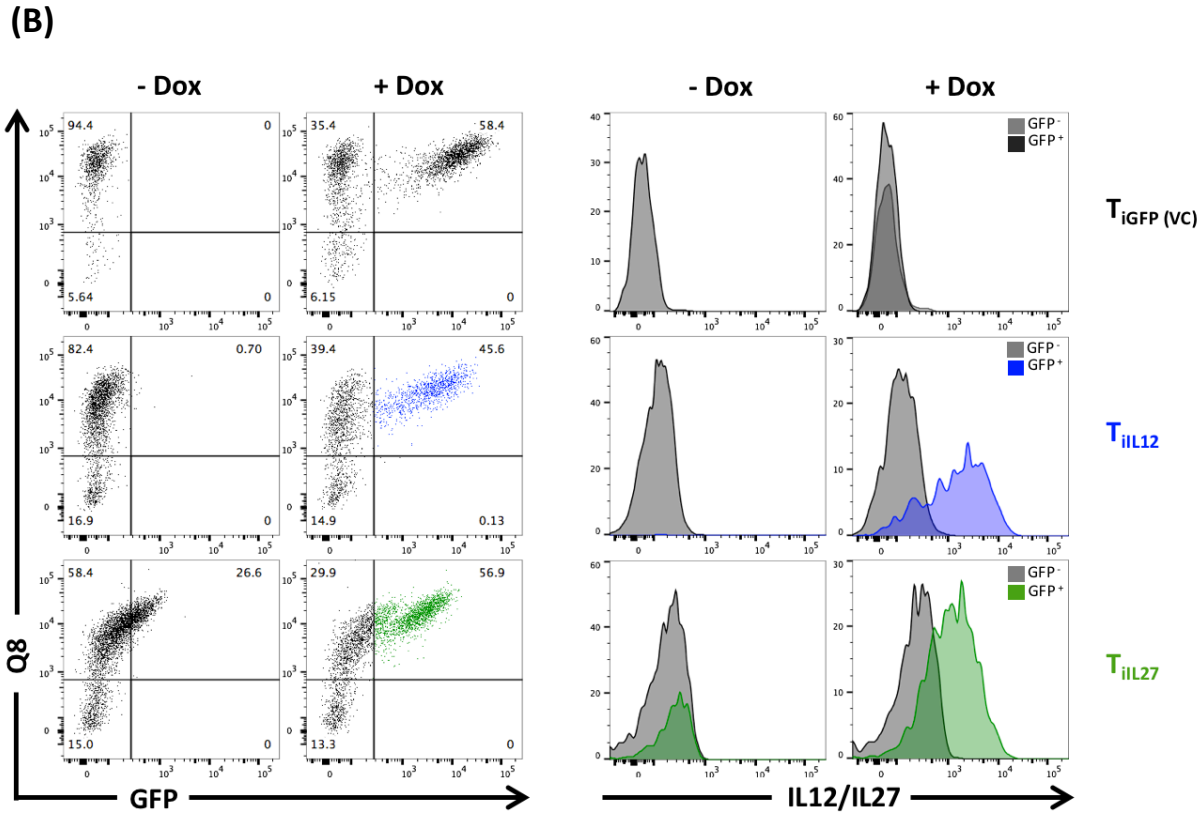

(B)
- Dox
+ Dox
- Dox
+ Dox
94.4
0
5.64
0
35.4
58.4
6.15
0
82.4
0.70
16.9
0
39.4
45.6
14.9
0.13
58.4
26.6
15.0
0
29.9
56.9
13.3
0
Q8
GFP
GFP -
GFP +
IL12/IL27
T iGFP (VC)
T iIL12
T iIL27

Figure 3.8 | *In vitro* validation of constructs encoding IL-12 or IL-27 in primary T cells. Splenocytes from wild type C56BL/6 mice were activated with CD3/CD28 beads and transduced with constitutive (pMP71) or inducible (pSERS) retrovirus constructs. 3 days later, T cells were treated with BFA for 4hrs to block cytokine secretion. **(A)** Representative plots showing transduction efficiency of T cells transduced with constitutive vectors (pMP71-IL12, pMP71-IL27, or mock-transduced) assessed by GFP expression ***(left)*** and intracellular expression of IL-12 or IL-27 within GFP^+ (transduced) cells compared to GFP^- (un-transduced) cells ***(right).*** **(B)** T cells transduced with inducible vectors including pSERS-IL12, pSERS-IL27, or pSERS-GFP (VC) were treated with Dox (1μg/ml) overnight or left untreated. Representative plots showing Q8 and GFP expression in transduced T cells which correlates with transduction efficiency and the level of induction, respectively in the presence and absence of Dox ***(left)*** and IL-12 or IL-27 expression within GFP^+ (induced) compared to GFP^- (non-induced) population ***(right).*** Data shown represents at least 3 independent experiments.

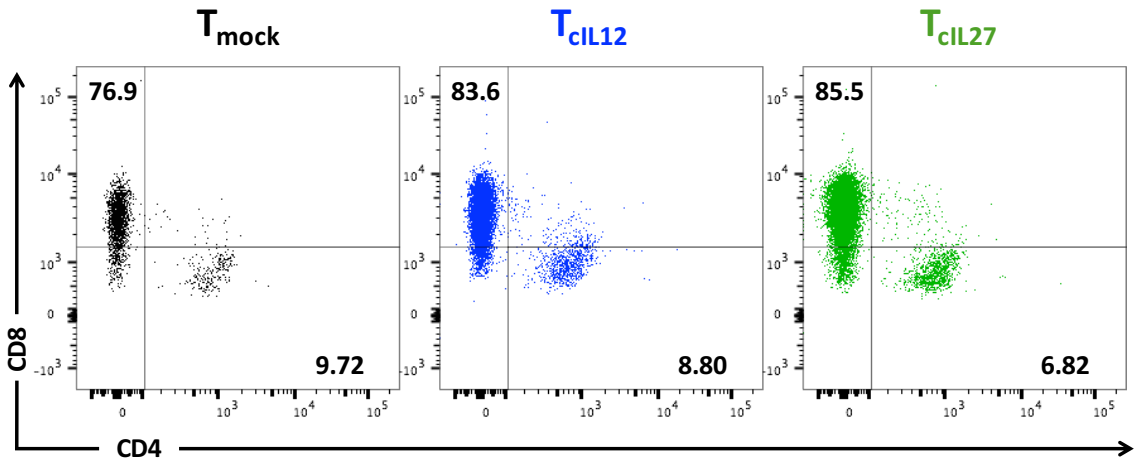


Figure 3.9 | CD4/CD8 ratio upon T cell transduction with IL-12 and IL-27. Bulk splenocytes from WT mice were activated with CD3/CD28 beads and transduced with pMP71-IL12, pMP71-IL-27 or mock-transduced. Representative plots depicting CD4 and CD8 staining profile of the transduced T cells 3 days post-transduction.

3.3.4 IL-12 expression but not IL-27 by engineered T cells results in a substantial increase in IFNγ production upon antigen-specific stimulation

Having shown the ability of engineered T cells to express elevated levels of IL-12 and IL-27, we next explored the functional activity of these manipulated T cells in vitro. We hypothesized that the functional activity of TCR-redirected T cells can potentially be changed by IL-12 and IL-27 expression. To test this hypothesis, *in vitro* stimulation assay was carried out using EL4 thymoma cells and EL4-NP, which is a modified version of EL4 expressing influenza NP peptide that can be recognised by the F5 TCR in the context of MHC class I (H-2D^b). Initially, splenocytes from normal C57BL/6 mice were retrovirally transduced with the influenza specific-F5 TCR (T_{TCR}), F5-TCR and IL-12 ($T_{TCR+cIL12}$), F5-TCR and IL-27 ($T_{TCR+cIL27}$) or mock-transduced T cells (T_{mock}). 3 days post-transduction, transduced cells were stimulated for 4 hours with EL4 and EL4-NP to achieve non-specific and antigen-specific stimulation, respectively. Intracellular expression of IFNγ and TNFα as Th1 signature cytokines, IL10 and IL4 as Th2 signature cytokines, and IL17A as Th17 signature cytokine were examined by flow cytometry (Figure 3.10 A). This demonstrated that IFNγ and TNFα production increased by T_{TCR} cells in response to antigen-specific stimulation with EL4-NP tumour cells with 24.9% and 58.7% responding T cells, respectively. The frequency of TNFα expressing cells remained largely unchanged by TCR-transduced cells secreting IL-12 ($T_{TCR+cIL12}$) (55.6%) or IL-27 ($T_{TCR+cIL27}$) (60.2%). In contrast, IL-12 had a substantial effect on the production of antigen-specific IFNγ with 66.3% compared to 24.9% and 24.6% produced by T_{TCR} and $T_{TCR+cIL27}$ cells, respectively. As expected, the majority of cells (>95%) producing IFNγ and TNFα were CD8$^+$ T cells because, as mentioned previously, F5 TCR is class I restricted. In addition, IL-12 expression not only increased the frequency of IFNγ$^+$ cells but also produced higher amounts of IFNγ (higher MFI) by up to seven-fold increase compared to T_{TCR} cells, whereas IL-27 expression did not.

Moreover, low levels of IFNγ production in the absence of antigen-specific stimulation, with EL4 tumour cells, was observed in $T_{TCR+cIL12}$ cells, but the level of expression per cell (MFI) was much lower compared to the level of IFNγ produced following antigen-specific stimulation (Figure 3.10 B).

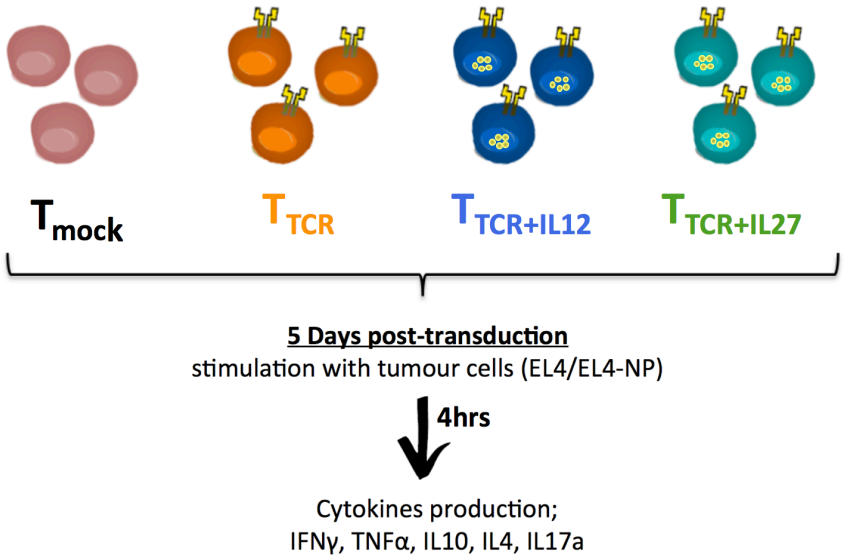
3.3.5 IL-12 expression in TCR-transduced cells enhances IL-10 production upon antigen specific stimulation

Intracellular cytokine staining profile of IL-10 and IL-4 in T cells transduced with TCR, TCR+IL12 or TCR+IL27 revealed that there was no detectable expression of IL-4 and IL-10 in response to antigen-specific stimulation, except in one case. Interestingly, antigen-specific production of IL-10 and, to a lesser extend, IL4 was observed by $T_{TCR+cIL12}$ cells in $CD8^+$ T cell population after stimulation with EL4-NP tumour cells (Figure 3.11). Together, these data indicated that although TCR+IL-12 engineered T cells produced large amounts of IFNγ and TNFα, they also produced IL-10 in response to antigen stimulation.

3.3.6 IL-12 induces antigen-specific IL-10 and IFNγ production from the same T cells

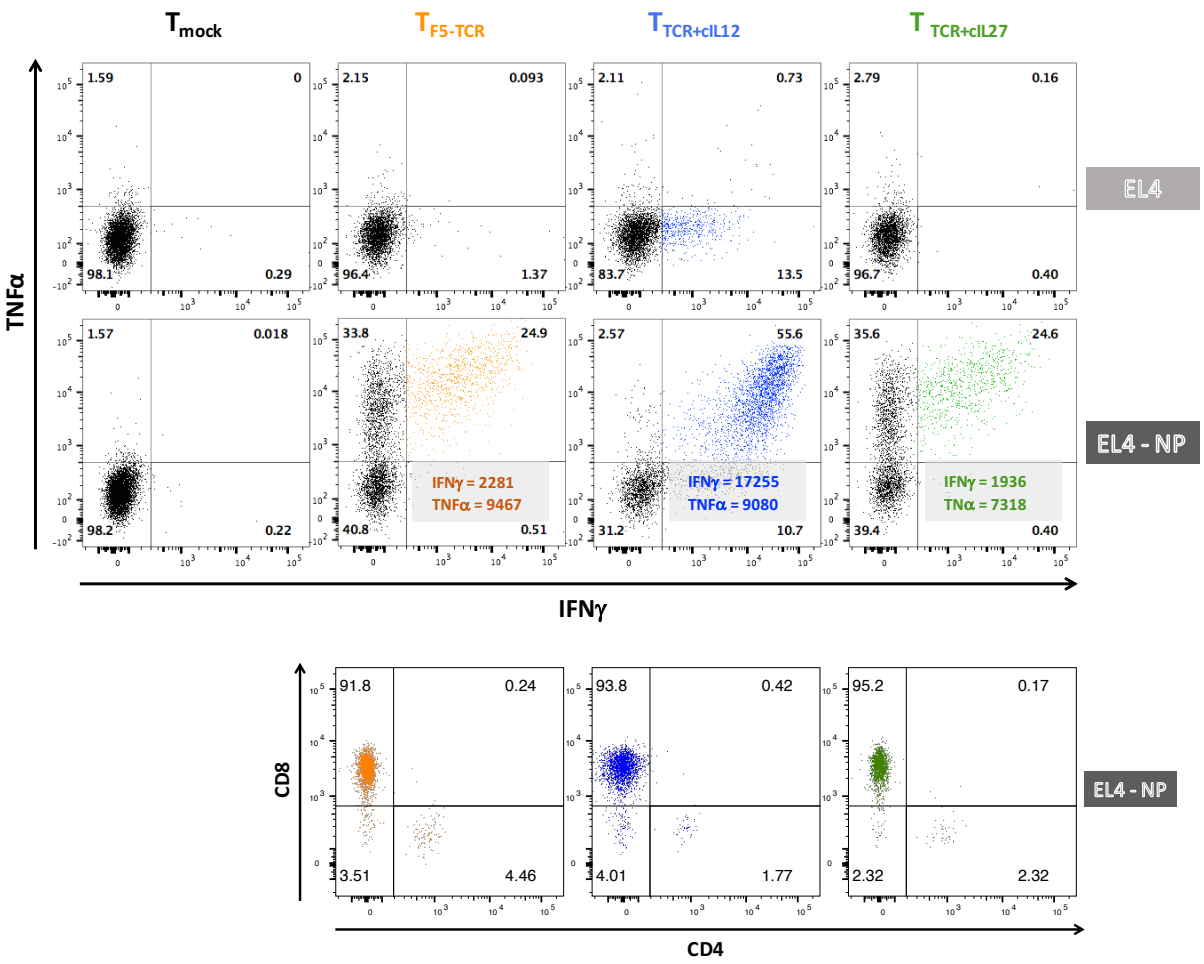
The ability of IL-12 to induce IL-10 production by Th1 cells has been previously reported as a negative regulator of IL-12-mediated immune response. According to Saraiva and colleagues, stimulation of T cells in the presence of IL-12 can generate IFNγ/IL-10 double producer T cell clones [309-313]. Having shown that IL-12-induced antigen-specific production of IL-10, I assessed whether these cells were also co-expressing Th1-like cytokines including IFNγ and TNFα. Interestingly, IL-10 was found to be produced by high IFNγ-expressing cells in response to antigen-specific stimulation in the presence of IL-12. IL-10 and IFNγ co-producing cells were also producing TNFα, and this was in the $CD8^+$ T cell population (Figure 3.12). It is possible that IL-10 produced by the highly activated antigen-specific T cells may serve as a negative feedback mechanism.

(A)


T mock
T TCR
T TCR+IL12
T TCR+IL27
5 Days post-transduction
stimulation with tumour cells (EL4/EL4-NP)
4hrs
Cytokines production;
IFNγ, TNFα, IL10, IL4, IL17a

(B)

(B.a)


T mock
T F5-TCR
T TCR+cIL12
T TCR+cIL27
TNFα
IFNγ
EL4
EL4 - NP
1.59
0
98.1
0.29
2.15
0.093
96.4
1.37
2.11
0.73
83.7
13.5
2.79
0.16
96.7
0.40
1.57
0.018
98.2
0.22
33.8
24.9
40.8
0.51
IFNγ = 2281
TNFα = 9467
2.57
55.6
31.2
10.7
IFNγ = 17255
TNFα = 9080
35.6
24.6
39.4
0.40
IFNγ = 1936
TNα = 7318
CD8
CD4
EL4 - NP
91.8
0.24
3.51
4.46
93.8
0.42
4.01
1.77
95.2
0.17
2.32
2.32

(B.b)

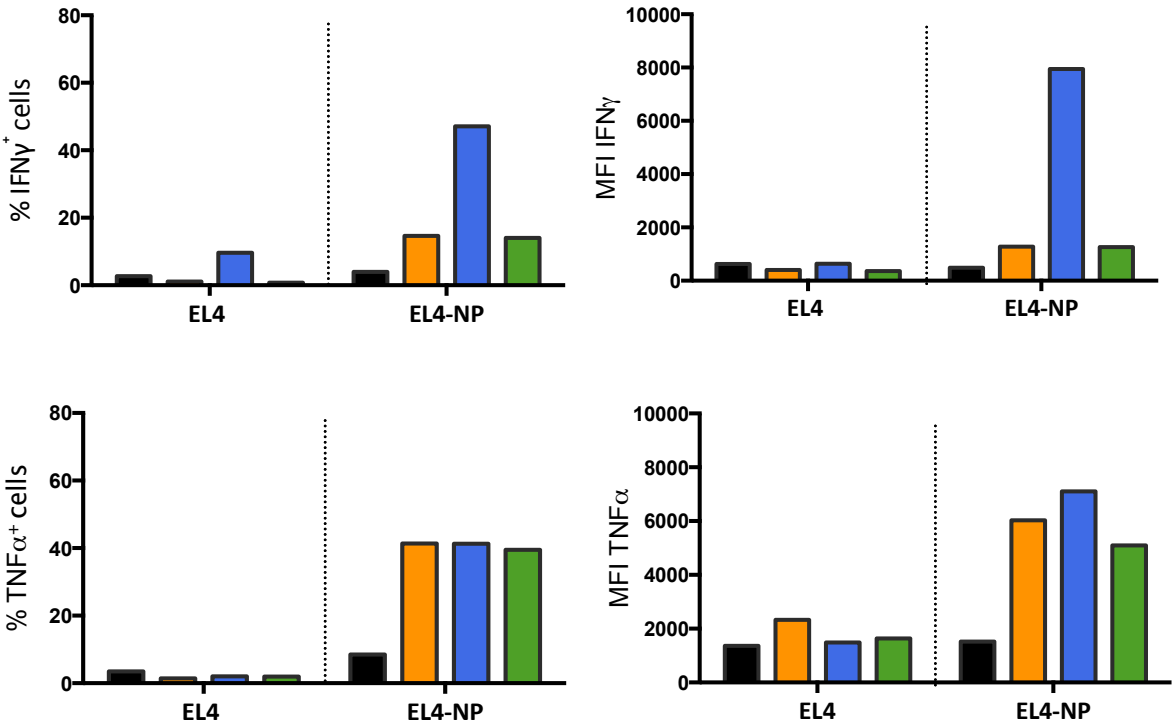


Figure 3.10 | *In vitro* effector function: the effects of IL-12 and IL-27 expression on the levels of IFNγ an TNFα production upon antigen-specific stimulation. (A) Experimental design. Splenocytes from wild type C57BL/6 mice were activated with CD3/28 beads. 24hrs following activation, cells were transduced with influenza-specific F5 TCR (T_{TCR}), F5 TCR and IL-12 ($T_{TCR+cIL12}$), F5 TCR and IL-27 ($T_{TCR+cIL27}$) or mock-transduced (T_{mock}). 4-5 days post-transduction, transduced T cells were stimulated with tumour cells expressing the cognate antigen (EL4-NP) or with EL4 to achieve antigen-specific or non-specific stimulation, respectively. Stimulated cells were stained and assessed for cytokine expression including: IFNγ, TNFα, IL-4, IL-10, IL17α. **(B)** Representative flow cytometry plots depicting similar TNFα expression, but an increased expression of IFNγ by $T_{TCR+IL12}$ cells in response to antigen-specific stimulation with EL4-NP tumour cells. Dot plots show live-gated TCR-expressing cells ($CD19^+$). IFNγ and TNFα production was mainly produced by $CD8^+$ transduced T cells *(a)*. Summary data showing the frequency and mean fluorescence intensity (MFI) of IFNγ and TNFα expression. Data pooled from 3 independent experiments *(b)*.

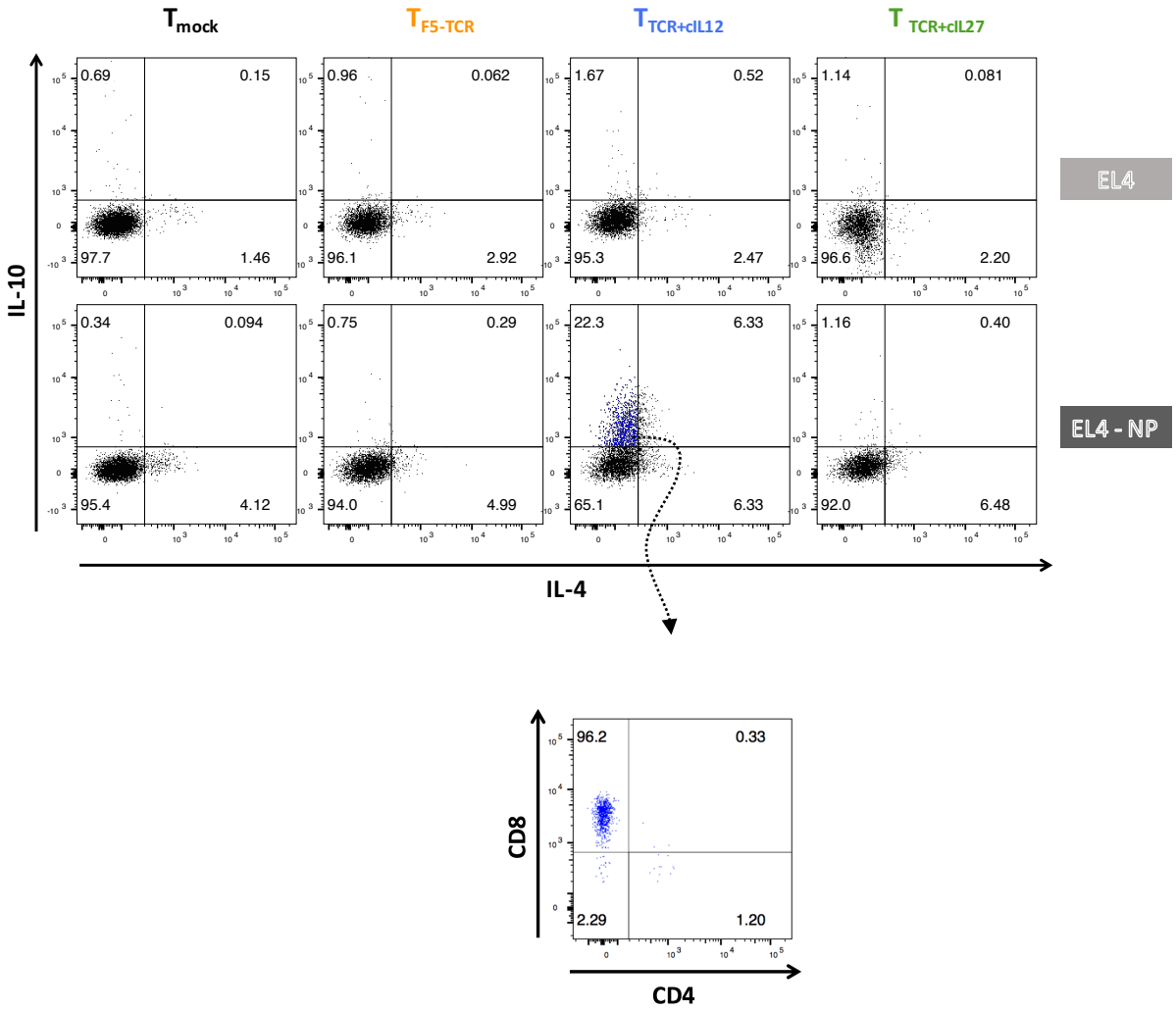


Figure 3.11 | Expression profile of IL-4 and IL-10 in TCR-redirected T cells secreting IL-12 and IL-27 following *in vitro* stimulation. Bulk cells harvested from wild type C57BL/6 mice were activated with CD3/CD28 beads and transduced with influenza-specific F5 TCR (T_{TCR}), F5 TCR and IL-12 ($T_{TCR+cIL12}$), F5 TCR and IL-27 ($T_{TCR+cIL27}$) or mock-transduced (T_{mock}). 4-5 days later, transduced cells were stimulated with tumour cells expressing cognate antigen (EL4-NP) or with EL4. Stimulated cells were stained and assessed for cytokine production including: IFNγ, TNFα, IL-4, IL-10, IL17A. Representative flow cytometry plots of intracellular IL-4 and IL-10 expression showed no IL-4 production by transduced T cells, but IL-12-induced IL-10 production (to leser extend, IL-4) in response to antigen-specific stimulation with EL4-NP. Dot plots show live-gated TCR-expressing cells ($CD19^+$). The majority of IL-10-expressing cells were $CD8^+$ T cells as shown by the CD4/CD8 staining profile. Data shown represents 3 independent experiments.

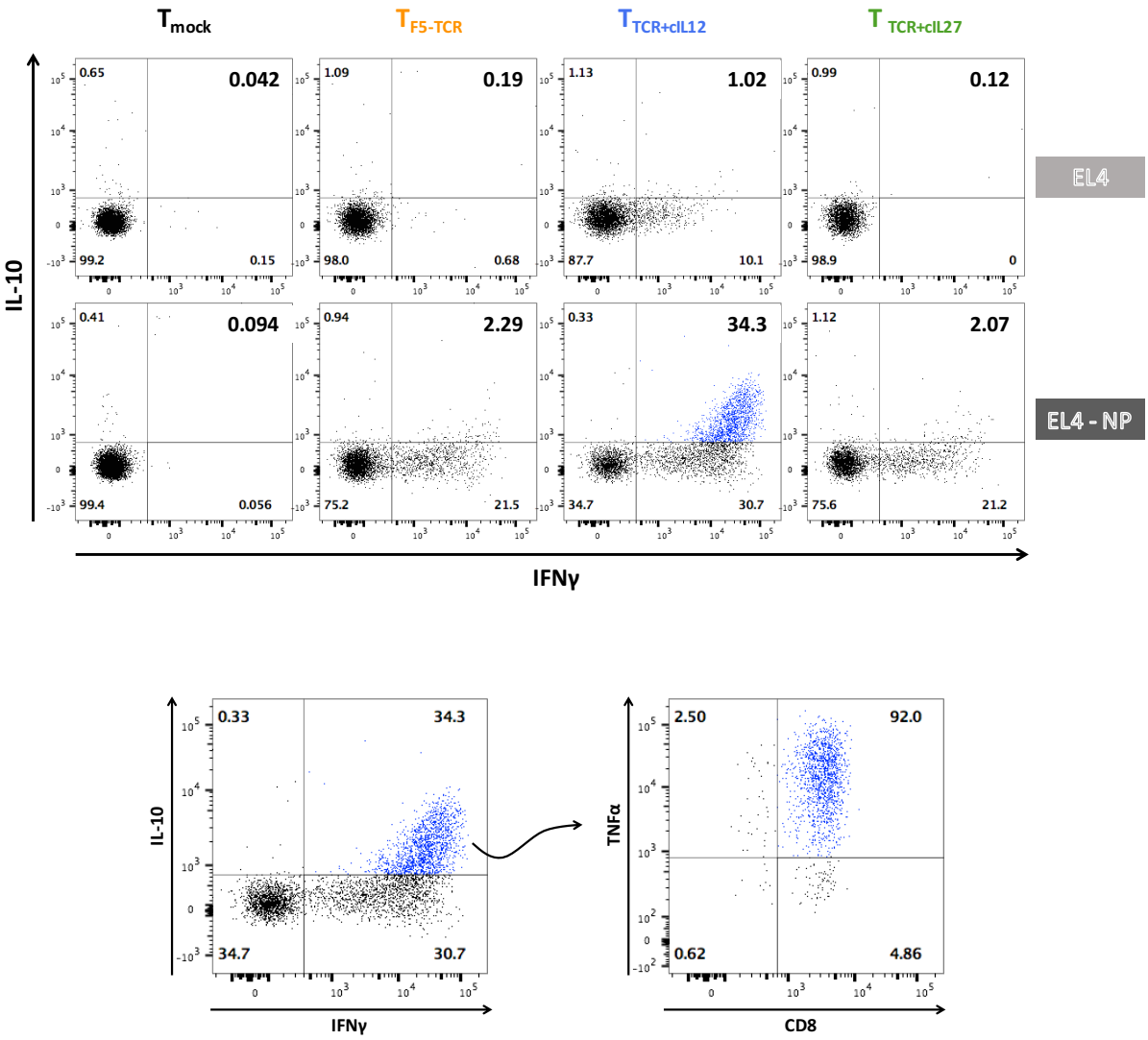
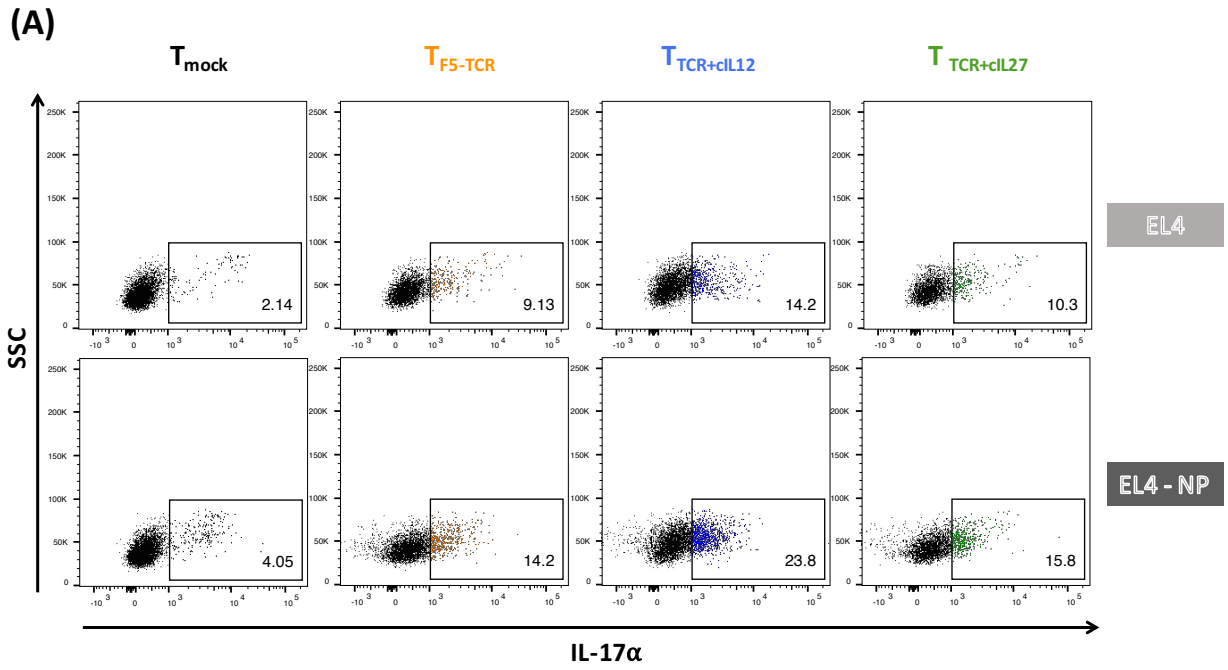
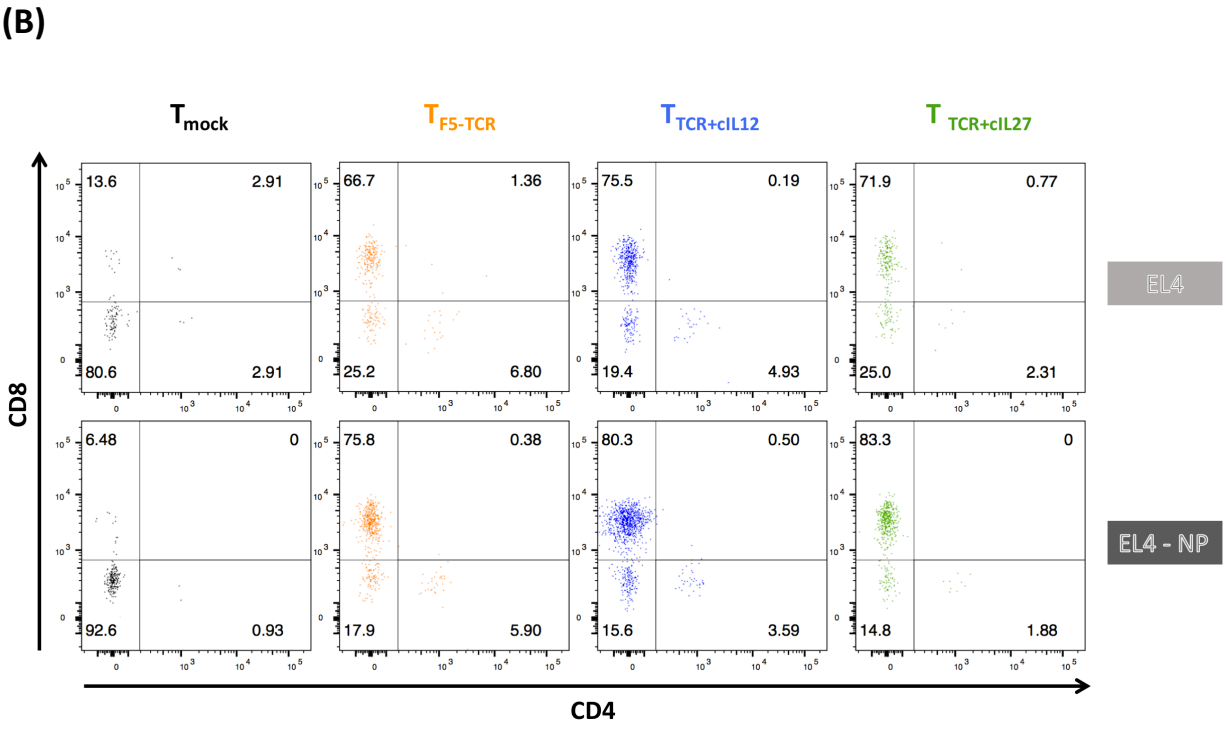


Figure 3.12 | IL-12-induced IL-10 production by highly activated engineered CD8$^+$ T cells. Splenocytes from wild type C57BL/6 mice were activated with CD3/CD28 beads. 24hrs following activation, cells were transduced with influenza-specific F5 TCR (T_{TCR}), F5 TCR and IL-12 ($T_{TCR+cIL12}$), F5 TCR and IL-27 ($T_{TCR+cIL27}$) or mock-transduced (T_{mock}). 4-5 days post-transduction, transduced T cells were stimulated with tumour cells expressing cognate antigen (EL4-NP) or with EL4 tumour cells. Stimulated cells were stained and assessed for cytokine expression including: IFNγ, TNFα, IL-4, IL-10, IL17α. Representative flow cytometry plots showing IL-12-induced antigen-specific IL-10 production by high IFNγ$^+$ cells in response to stimulation with EL4-NP tumour cells. Dot plots show live-gated TCR-expressing cells (CD19$^+$) ***(top)***. IFNγ$^+$IL-10$^+$ co-expressing cells were found to be CD8$^+$ T cells that were also expressing TNFα ***(bottom).*** Data shown represents 3 independent experiments.

3.3.7 IL-12 expression by engineered T cells enhances antigen-specific IL-17 production

As previously mentioned, I also sought to assess the expression of IL-17A which is the classical signature cytokine for Th17 cells in the same *in vitro* stimulation experiment described in section 3.2.4 (Figure 3.10 A). This demonstrated that IL-17A expression was slightly increased by $T_{TCR+IL12}$ compared to T_{TCR} cells in the presence and absence of antigen-specific stimulation. By contrast, IL-17A expression was observed to be largely unchanged in $T_{TCR+IL27}$ compared to T_{TCR} cells (Figure 3.13 A). Staining profile of CD4 and CD8 of IL-17A-expressing cells showed that the majority of these cells were $CD8^+$ T cells (Figure 3.13 B). IL-17^+CD8^+ T cells (Tc17) in mice are usually referred to as non-cytotoxic subset of Tc17 cells. However, Tc17 cells expressing IFNγ have been shown to gain cytotoxic activity and mediate antitumor activity in an IFNγ-dependant fashion [314-316]. Therefore, the observed fraction of IL-17^+CD8^+ T cells were further analysed for their expression of IFNγ. This indicated that these cells were also co-expressing IFNγ in T_{TCR}, $T_{TCR+IL12}$ and $T_{TCR+IL27}$ cells in response to EL4-NP but not EL4. In addition, a remarkable increase in the frequency of IFNγ-expressing Tc17 cells was observed in $T_{TCR+IL12}$ (77.5%) but not in $T_{TCR+IL27}$ cells (37.8%) compared to T_{TCR} cells (42.9%) (Figure 3.13 C). In summary, these data demonstrate that IL-12 can drive antigen-specific production of IFNγ, TNFα, IL-10 and IL-17 by engineered T cells.


(A)
T mock
T F5-TCR
T TCR+cIL12
T TCR+cIL27
EL4
EL4 - NP
SSC
IL-17α
2.14
9.13
14.2
10.3
4.05
14.2
23.8
15.8


(B)
T mock
T F5-TCR
T TCR+cIL12
T TCR+cIL27
EL4
EL4 - NP
CD8
CD4
13.6
2.91
80.6
2.91
66.7
1.36
25.2
6.80
75.5
0.19
19.4
4.93
71.9
0.77
25.0
2.31
6.48
0
92.6
0.93
75.8
0.38
17.9
5.90
80.3
0.50
15.6
3.59
83.3
0
14.8
1.88

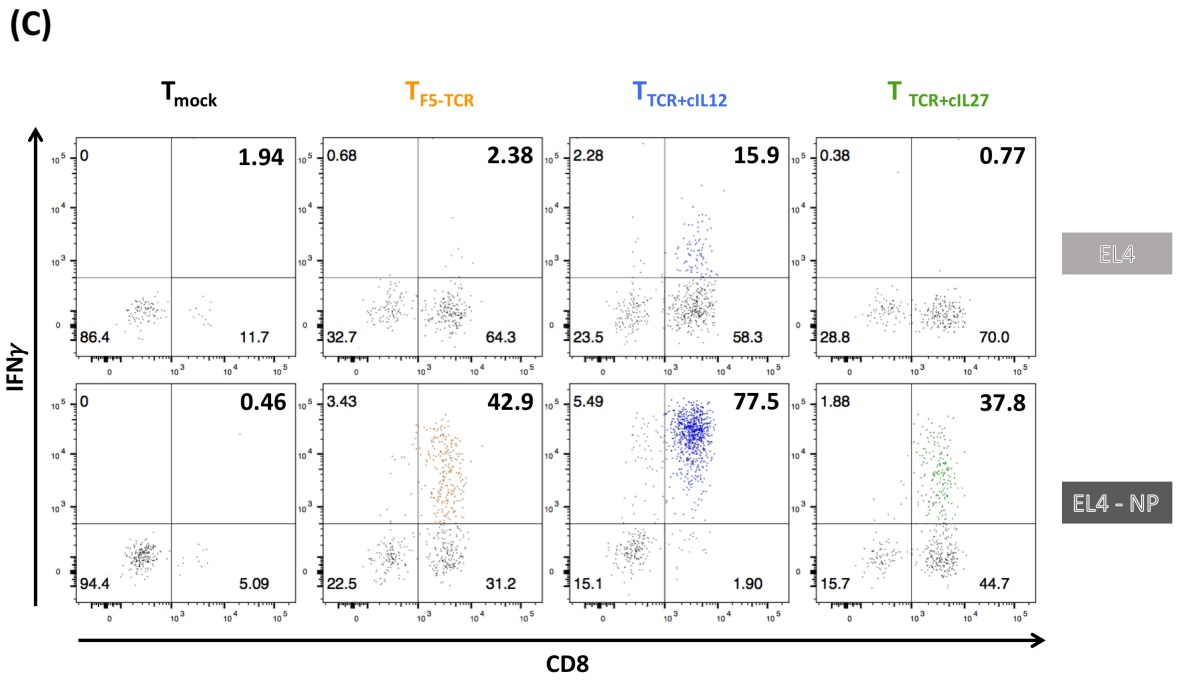


Figure 3.13 | Intracellular staining for IL-17A expression in engineered T cells upon *in vitro* stimulation. Bulk splenocytes from wild type C57BL/6 mice were activated with CD3/CD28 beads and transduced with influenza specific F5 TCR (T_{TCR}), F5 TCR and IL-12 ($T_{TCR+cIL12}$), F5 TCR and IL-27 ($T_{TCR+cIL27}$) or mock-transduced (T_{mock}). 4-5 days later, transduced cells were stimulated with tumour cells expressing cognate antigen (EL4-NP) or with EL4. Stimulated cells were stained and assessed for cytokine production including: IFNγ, TNFα, IL-4, IL-10, IL17A. **(A)** Representative flow cytometry plots illustrating an increased IL-17A expression by T_{TCR}, $T_{TCR+IL12}$ and $T_{TCR+IL27}$ cells upon antigen-specific (EL4-NP) and non-antigen-specific (EL4) stimulation. Dot plots show live-gated TCR-expressing cells ($CD19^+$). **(B)** CD4 and CD8 staining profile of IL-17A expressing cells showing that $CD8^+$ T cells is the major producer of IL-17A regardless of antigen-specificity. **(C)** IFNγ staining profile of IL-17A$^+$CD8$^+$ T cells pre-gated on live singlet TCR-expressing cells ($CD19^+$). Data shown represents 3 independent experiments.

3.3.8 Cytotoxic activity of TCR-redirected T cells secreting IL-12 or IL-27 upon antigen-specific stimulation

To further assess the functional characteristics of IL-12 and IL-27 expression in TCR-redirected T cells upon *in vitro* stimulation, CD107 expression was assessed along with CD62L expression. CD107, also known as lysosomal-associated membrane protein 1 (LAMP-1), is a degranulation marker expressed on the surface of antigen-specific $CD8^+$ T cells following their activation, and it has been proposed to be a potential marker that can correlate with cytolytic potential of activated T cells. Degranulation induced by activated T cells occurs rapidly following TCR triggering [317-319]. Therefore, CD107a antibody was added at the beginning of the stimulation, which has been previously described in figure 3.9 A, in order to pick up any transient expression of CD107a on the cell surface. Flow cytometric analysis indicated that CD107a expression is reduced in $T_{TCR+IL12}$ cells (36.1%) compared to T_{TCR} (48.8%) and $T_{TCR+IL27}$ cells (44.1%) in response to antigen-specific stimulation with EL4-NP tumour cells. Furthermore, CD107a was mostly expressed by antigen-specific $CD8^+$ effector T cells expressing low levels of CD62L (figure 3.14 A, B). Together, these data suggest that IL-12 reduces CD107-mediated degranulation upon antigen encounter *in vitro*.

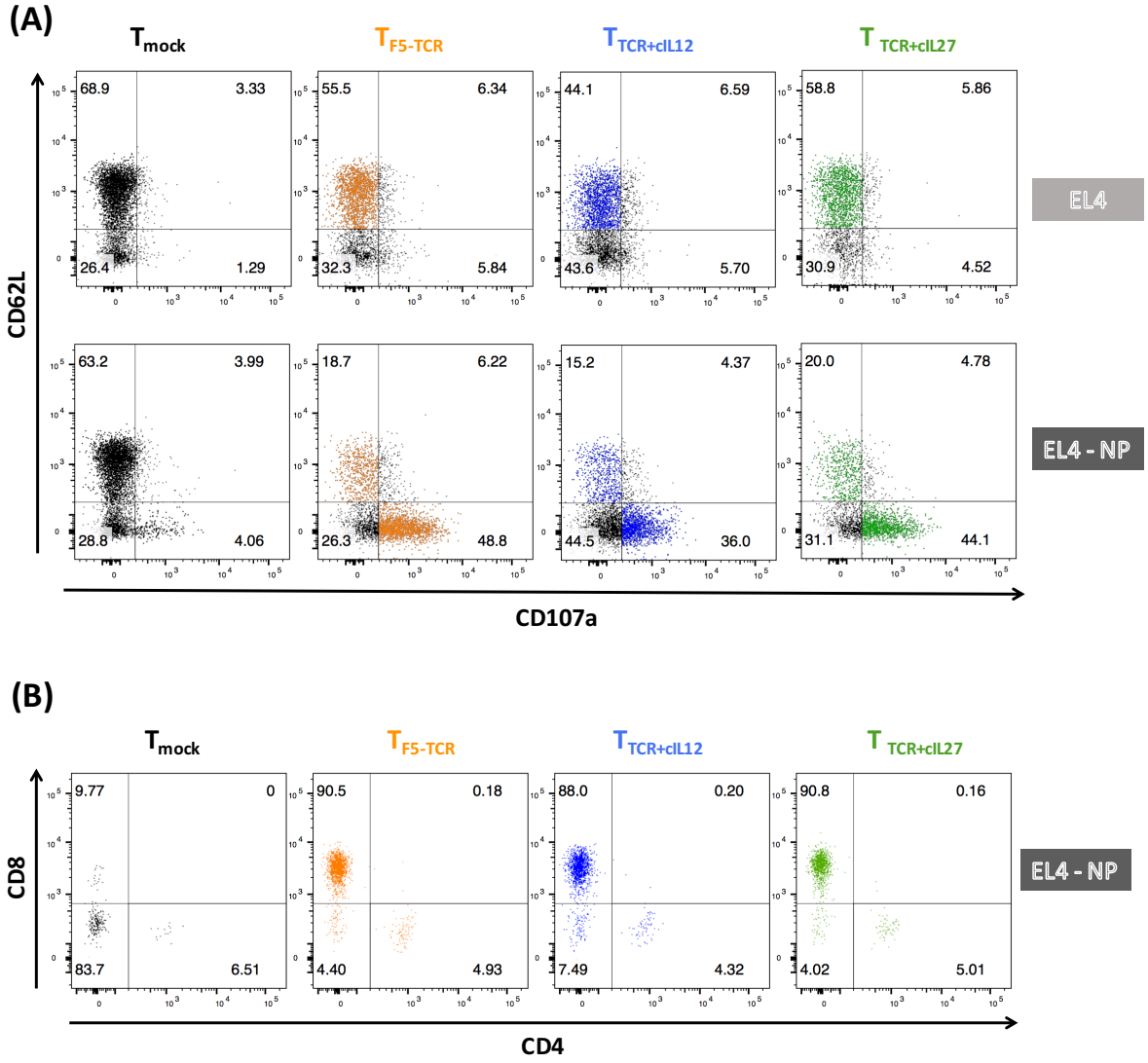
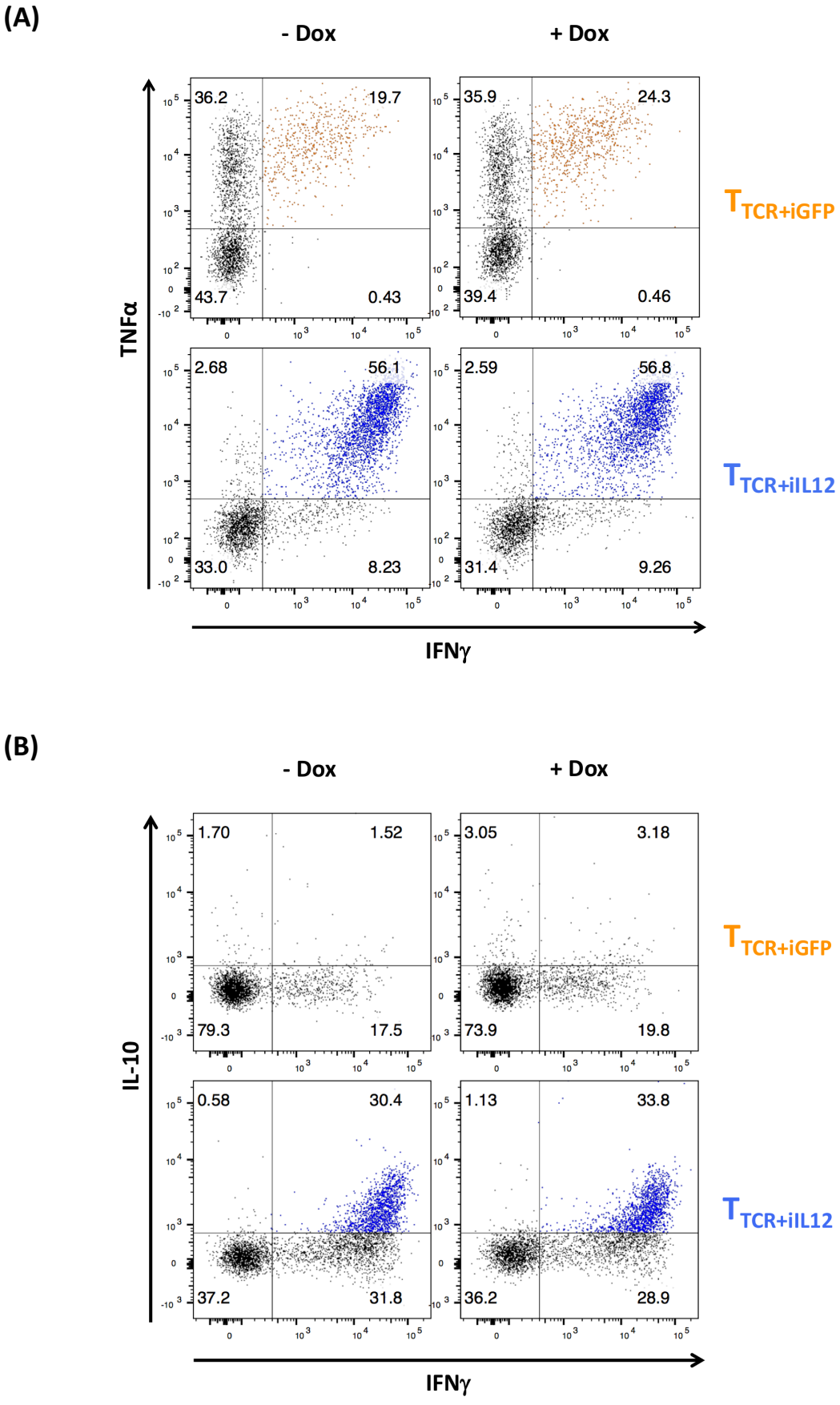


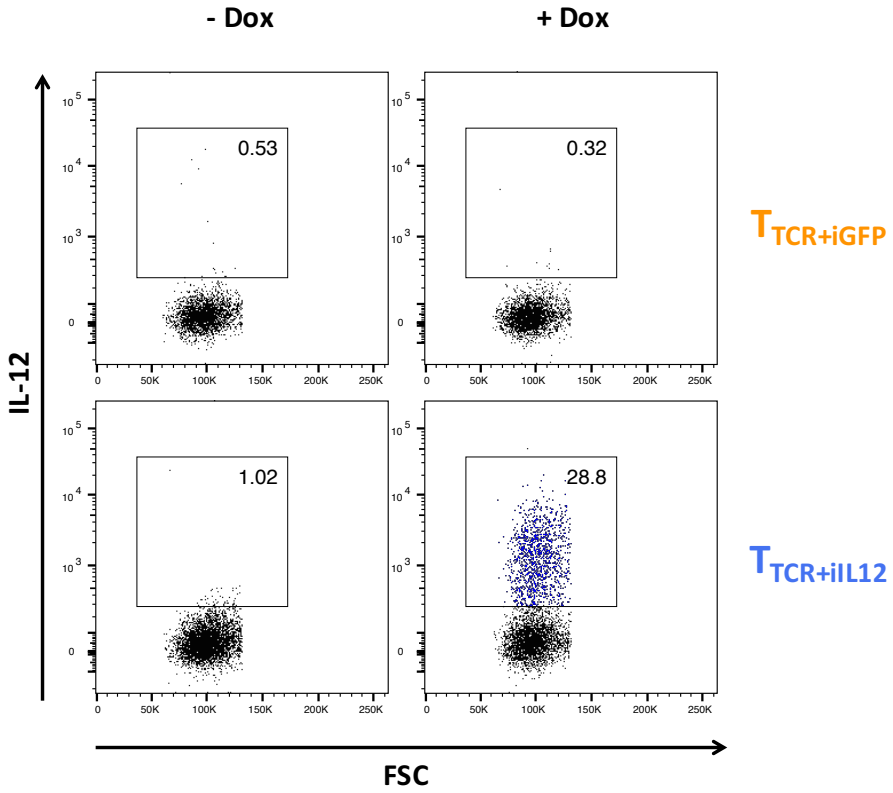
Figure 3.14 | CD107a expression on engineered T cells secreting IL-12 and IL-27 upon *in vitro* stimulation. Harvested splenocytes from wild type C57BL/6 mice were activated with CD3/CD28 beads and transduced with influenza-specific F5 TCR (T_{TCR}), F5 TCR and IL-12 ($T_{TCR+cIL12}$), F5 TCR and IL-27 ($T_{TCR+cIL27}$) or mock-transduced (T_{mock}). 4-5 days later, transduced cells were stimulated with tumour cells expressing cognate antigen (EL4-NP) or with EL4. CD107a was added at the beginning of the stimulation. The stimulated cells were stained and assessed for CD107a and CD62L expression 4hrs following stimulation. The cells were also stained with CD4 and CD8 antibodies. **(A)** CD107a expression increased by T_{TCR}, $T_{TCR+cIL12}$ and $T_{TCR+cIL27}$ cells upon encountering cognate antigen expressed by tumour cells (EL4-NP). Dot plots show live-gated TCR-expressing cells ($CD19^{+}$). **(B)** Representative plots showing CD4 and CD8 staining profile of the CD107a-expressing cells by T_{TCR}, $T_{TCR+IL12}$ and $T_{TCR+IL27}$ after stimulation. Data shown represents 3 independent experiments.

3.3.9 Regulated IL-12 expression enhances IFNγ production by engineered T cells in the absence of dox-induced IL-12

Having validated the inducible vectors *in vitro* previously (section 3.2.2), I next sought to determine the functional activities of T cells transduced with the inducible vectors. The previous results obtained from *in vitro* functional experiments using the constitutive vectors indicated that IL-27 expression in TCR-redirected T cells did not change their functional activity compared to TCR only. In contrast, IL-12 expression in TCR-redirected T cells has shown a functional impact by enhancing IFNγ, TNFα, IL-10 and IL-17A upon antigen-specific stimulation. Therefore, my functional analysis using the inducible constructs will be focused on regulated IL-12 expression in TCR-redirected T cells compared to TCR only. To do this, splenocytes from WT C57BL/6 mice were activated and transduced with F5 TCR and iIL-12 ($T_{TCR+iIL12}$) or with the vector control iGFP ($T_{TCR+iGFP}$). The cells were stimulated 4-5 days post-transduction; 24hrs prior to stimulation, transduced T cells were split into two groups, one was treated with dox (1μg/ml) and the other one was left untreated. The cells were then stimulated for 4hrs with EL4 and EL4-NP tumour cells in BFA-containing medium to block cytokine secretion. Intracellular cytokine staining of IFNγ, TNFα and IL-10 showed similar results obtained previously with the constitutive expression of IL-12, by which $T_{TCR+iIL12}$ cells showed enhancement of antigen-specific production of IFNγ and IL-10 compared to the antigen-specific T cells that do not express IL-12 ($T_{TCR+iGFP}$). However, $T_{TCR+iIL12}$ cells not only increased the production of IFNγ and IL-10 in the presence of Dox but also in the absence of Dox-induced IL-12 (figure 3.15 A, B). Although IL-12 expression was not evident by flow cytometry in the absence of Dox, an IL-12 ELISA of the cell culture supernatants has confirmed that there is a low level of IL-12 leakage from the tet-regulated vector. (Figure 3.15 C). Together, low level of IL-12 leaked from the inducible vector was enough to enhance the effector function of the TCR-modulated T cells in vitro.


(A)
- Dox
+ Dox
36.2
19.7
43.7
0.43
35.9
24.3
39.4
0.46
2.68
56.1
33.0
8.23
2.59
56.8
31.4
9.26
TNFα
IFNγ
T TCR+iGFP
T TCR+iIL12
(B)
- Dox
+ Dox
1.70
1.52
79.3
17.5
3.05
3.18
73.9
19.8
0.58
30.4
37.2
31.8
1.13
33.8
36.2
28.9
IL-10
IFNγ
T TCR+iGFP
T TCR+iIL12

(C)


- Dox
+ Dox
IL-12
FSC
0.53
0.32
1.02
28.8
$T_{TCR+iGFP}$
$T_{TCR+iIL12}$

(D)

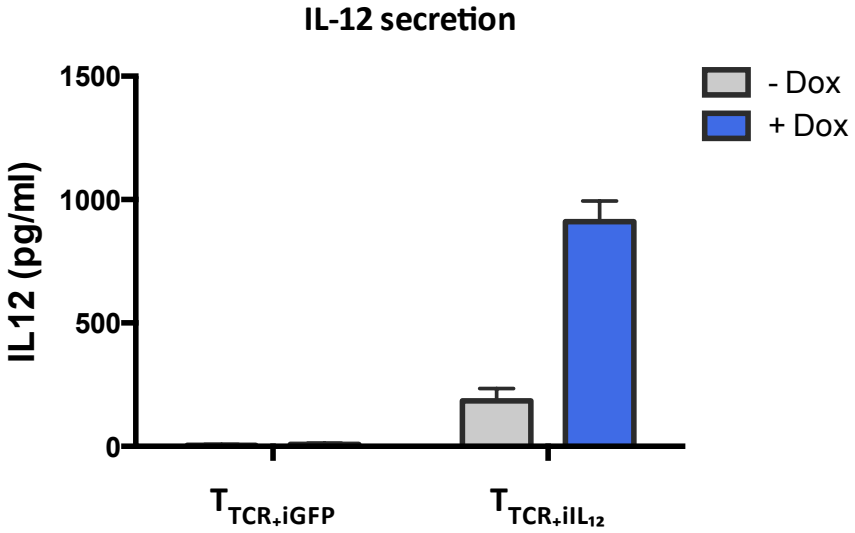

IL-12 secretion
IL12 (pg/ml)
1500
1000
500
0
- Dox
+ Dox
$T_{TCR+iGFP}$
$T_{TCR+iIL_{12}}$

Figure 3.15 | Low levels of IL-12 production in the absence of dox enhances engineered T cell effector functions *in vitro*. Splenocytes from WT C57BL/6 mice were activated with CD3/CD28 beads and retrovirally transduced with influenza-specific F5 TCR and iIL-12 ($T_{TCR+iIL12}$) or vector control iGFP ($T_{TCR+iGFP}$). 4-5 days post-transduction, cells were stimulated for 4hrs with EL4-NP tumour cells expressing cognate antigen in BFA-containing medium to block cytokine expression. 24hrs before stimulation, transduced T cells were split into two groups, one group was treated with dox (1µg/ml) and the other one was left untreated. **(A)** Enhanced IFNγ production-mediated by the $T_{TCR+iIL12}$ cells in response to antigen-specific stimulation in the absence and presence of dox. **(B)** Dot plots show increased IL10 expression by $T_{TCR+iIL12}$ cells expressing high levels of IFNγ in the absence and presence of dox. **(C)** No evidence for IL-12 expression by flow cytometry in the absence of dox. Data are representative of three independent experiments. **(D)** Measuring IL-12 secretion by ELISA has confirmed the low levels of IL-12 leakage from the tet-regulated vector. Data represents measurements in duplicate from two experiments.

3.4 Discussion

Adoptive transfer of TCR-redirected T cells has shown therapeutic potential in haematological malignancies and solid tumours. However, the effector function of these cells can be diminished by the local immune suppressive mechanisms in the tumour microenvironment. Cytokines present in the tumour microenvironment can play an important role toward tumour regression or tumour progression. For example, it has been shown that the adoptive transfer of TCR-transduced T cells in combination with TGFβ blockade enhanced the therapeutic efficacy of transferred T cells in transgenic adenocarcinoma mouse prostate model [93]. Moreover, delivery of immunostimulatory cytokines such as IL-12 was shown to improve adoptive T cell therapy and enhance tumour killing [181]. Thus, the therapeutic manipulation of cytokines in the tumour microenvironment can potentially enhance antitumor immunity.

In this study, I have sought to employ genetic engineering using retroviral gene transfer to manipulate T cell effector function by over expressing cytokines including IL-12 and IL-27. mscIL-12 or msc-IL-27 genes were successfully inserted into retrovirus vectors capable of constitutively expressing the cytokines or regulating their expression under the control of a tet-regulated promoter. Retroviral transduction of primary T cells with these vectors has shown an enhanced production of IL-12 and IL-27 by transduced T cells without altering the CD4:CD8 ratio. Expression of IL-12 and IL-27 under the tet-controlled gene expression was evident following dox-treatment. Ultimately, genetic engineering of cytokine production will be used in combination with TCR gene therapy to achieve cytokine expression in TCR-redirected T cells in an attempt to modulate the effector function of these antigen-specific T cells, and thereby enhance their therapeutic efficacy.

The effector function of engineered T cells including T_{TCR}, $T_{TCR+IL12}$ and $T_{TCR+IL27}$ cells was assessed *in vitro* upon stimulation with tumour specific antigen. Intracellular cytokine staining following *in vitro* stimulation demonstrated that engineered T cells displayed Th1-like cytokine profile including IFNγ, TNFα but not IL-4. IL-12 expression in TCR-redirected T cells ($T_{TCR+IL12}$) significantly increased the antigen specific production of IFNγ compared to T_{TCR} cells, whereas IL-27 expression did not significantly change the level of IFNγ production. This potent IFNγ production was mediated mainly by $CD8^+$ T cells, but only a small percentage of $CD4^+$ T cells were

found to produce IFNγ. Our results are consistent with previous studies, which demonstrated that IL-12 has a potent effect in enhancing IFNγ production by T cells upon antigen-specific stimulation [320, 321]. In a similar setting to us, it has also been shown that IL-12 expression in TCR-redirected T cells could enhance tumour recognition by inducing high levels of IFNγ production *in vitro* [302]. On the other hand, IL-27-mediated effect on $CD8^+$ T cell responses has been controversial due to its dual, pro- and anti-inflammatory, effects. Although there are some studies indicating that IL-27 enhances $CD8^+$ T cell effector function and IFNγ production in infection and cancer, other studies proposed that IL-27 is important in downregulating inflammatory responses [235, 244-246, 271, 278]. In accordance with this, we noted that the level of TCR expression increased *in vitro* upon IL-12 co-transduction with TCR, which is not the case when IL-27 is co-transduced with the TCR (data not shown). In a previous study, our lab demonstrated that enhanced TCR surface expression can improve the effector function of tumour-specific T cells [143]. Therefore, one possible mechanism is that IL-12 drives strong antigen-specific effector functions by improving the level of TCR expression on the T cell surface. Zhang *et al* also stated that, IL-12 co-transduction with TCR increased TNFα secretion compared to TCR-transduced T cells [302]. However, in our experiments, the level of TNFα expression was largely unchanged by $T_{TCR+IL12}$ cells.

Furthermore, intracellular cytokine staining revealed that IL-12 also induced IL-10 production by antigen-specific $CD8^+$ T cells producing high levels of IFNγ ($IFN\gamma^{hi}$-expressing cells). According to Assenmacher *et al.*, Th1 cells have the ability to induce sequential expression of cytokines during the primary immune response including IL-2 followed by late IL-10 expression to control their own IFNγ-mediated inflammation. In this context, IL-12, the potent inducer of IFNγ, has also been shown to induce IL-10 in primary T cells as part of a negative feedback loop [309, 312, 322]. Also, there is evidence that IL-12 has the ability to generate T cell clones co-expressing IFNγ and IL-10 upon polyclonal stimulation [310]. Therefore, it is conceivable that IL-12-induced IL-10 expression by highly activated T cells to dampen down the immune response intensity as a negative regulatory mechanism. Moreover, IL-27 can induce IL-10 production from antigen-specific $CD8^+$ T cells [260, 323]. Despite this, no IL-10 production was observed by engineered T cells expressing IL-27 upon *in vitro* stimulation.

As mentioned previously, one of the main immunobiological effects of IL-12 is to augment NK cell- and $CD8^+$ T cell-mediated cytotoxicity [324, 325]. IL-27 has also been shown to mediate enhancement of $CD8^+$ T cell cytotoxicity [241, 243]. However, in our experiments, CD107a expression profile following stimulation of engineered T cells in an antigen-specific setting showed a decrease of CD107a expression on $T_{TCR+IL12}$ cells compared with T_{TCR} and $T_{TCR+IL27}$ cells. Our findings contradict with the above studies by which neither IL-12 nor IL-27 expression improved cytotoxic activities of CTLs measured by CD107a expression. In addition, our results which are consistent to some extend with those of Change J *et al* contrast with the findings of Zhang *et al*, who found no difference in CD107a expression when T cells co-transduced with IL-12 and TCR [302, 320, 321, 326].

These data show that a small fraction of engineered $CD8^+$ T cells stimulated with tumour-specific antigen produce both IFNγ and IL-17. $IL\text{-}17^+CD8^+$ T cells, referred as Tc17 cells, were shown to be derived from naïve $CD8^+$ T cells and exhibit poor cytolytic activity in vitro. However, these cells can be plastically converted into IFNγ-expressing Tc17 cells which have been characterised to gain cytotoxic activity and mediate IFNγ-dependant immune response against infection and cancer [315, 316, 327]. It has been previously documented that IL-12 is capable to convert Tc17 cells into IFNγ-expressing Tc17. These converted cells have been shown to possess enhanced effector function and antitumorigenic activity similar to that seen with Tc1 cells [314]. Indeed, we also found that IL-12 expression by engineered T cells triggered higher levels of IFNγ with the majority of Tc17 cells were expressing IFNγ in response to antigenic stimulation. Work from El-behi *et al* showed that IL-27 does not induce Tc17 cells to produce IFNγ. In fact, IL-27 has a negative effect on the development of Tc17 cells and showed no or little effect on committed Tc17 cells [328]. This is consistent with the data presented here which revealed that IL-27 expression by engineered T cells did not increase levels of IFNγ production by Tc17 cell in vitro.

Taken together, these *in vitro* validation results demonstrated the feasibility to manipulate effector function of TCR-redirected T cells using retrovirus gene transfer. However, the most pronounced enhancement of antigen-specific T cell responses was seen by IL-12 co-expression, whereas IL-27 expression did not seem to change the functional profile of

these engineered T cells at least in our *in vitro* stimulation setting. Given that low levels of IL-12 leakage from the tet-regulated vector had a biological impact *in vitro*, I assessed in the next chapter the *in vivo* biological impact of leaked IL-12 production.

Chapter 4

Manipulation of cytokine production: *in vivo* validation

4.1 Introduction

The potency of IL-12 in tumour immunity has yielded promising results in pre-clinical and clinical studies. Anti-tumour activities of IL-12 have been broadly explored in transplantable murine models such as melanoma, C26 colon carcinoma, mammary carcinoma and sarcoma [182]. Attempts to translate IL-12 experimental results into clinical trials have involved many different approaches including recombinant IL-12, *in vivo* and *ex vivo* gene therapy [212]. Clinical application of IL-12 revealed minimal efficacy with an objective response rate of 0 – 7% against solid tumours such as melanoma, head and neck carcinoma and renal cell carcinoma [329]. In addition, clinical studies have been hindered by severe systemic toxicity with adverse effects in hematopoietic, hepatic, pulmonary and intestinal tissues, which is probably at least in part caused by high levels of IFNγ production [174, 175, 218, 281, 301]. To date, delivery of IL-12 without inducing lethal systemic toxicity *in vivo* remains challenging. As mentioned earlier, the controlled expression of IL-12 was developed as an alternative strategy using an NFAT promoter regulated genetic construct [194]. Yet, recent clinical trial of transferring T cells engineered to express IL-12 regulated by the NFAT promoter yielded disappointing results due to associated toxicity [302, 303]. Furthermore, the adoptive transfer of T cells containing the NFAT regulated IL-12 construct was toxic in the absence of antigen-specific T cell stimulation in lymphodepleted mice, but no observed toxicity when these cells were adoptively transferred into lymphoreplete mice (Personal communication; Gavin and Ben group). These results suggested a low affinity TCR interaction with self-antigens, which are known to play a role in homeostatic proliferation, leading to the activation of the NFAT promoter. In the present study, controlled expression using tet-promoter regulated genetic construct is achieved by Dox administration, and it could be speculated that such unpredictable induction seen with the NFAT vector is less likely. However, our *in vitro* data suggested that the tet-promoter was leaky, and accordingly, the low levels of IL-12 production by the leaky tet-promoter was enough to enhance T cell effector function in response to antigen stimulation. Therefore, it was important to assess toxicity issues related to the delivery of tet-regulated IL-12 expression *in vivo* in the absence and presence of antigen-specific stimulation. Unlike IL-12, *in vivo* delivery of IL-27 has shown to possess antitumor activity in the absence of adverse side effects. Since 2004, Hisada and colleagues have demonstrated

that IL-27 has potent antitumor activity mediated through different mechanisms, which depends also on the properties of tumour cells [271, 272]. Yet, no translational studies in IL-27-based immunotherapy have been established. IL-27 has been shown not only to enhance expansion and survival of antigen-specific T cells but also to differentiate them into memory precursor-like effector cells [283]. It also has the ability to inhibit differentiation of $Foxp3^+$ regulatory T cells (T_{reg}) and thereby decreases their numbers, which often accumulate in the suppressive tumour microenvironment [285]. Thus, IL-27 can be an attractive candidate in adoptive T cell therapy.

4.2 Aims

Here, I sought to establish engraftment and possible toxicity associated with our modified T cells when they are adoptively transferred *in vivo*. I have also sought to explore how IL-27 expression would affect the performance of TCR-modified T cells in tumour-bearing mice. In this chapter, I have tested the following hypothesis:

- Adoptive transfer of engineered T cells expressing constitutive IL-12 induce toxicity *in vivo*.
- Tet-regulated IL-12 expression can prevent toxicity *in vivo* in the absence and presence of antigen-specific stimulation.
- Adoptive transfer of engineered T cells expressing IL-27 can engraft *in vivo* without inducing toxicity.
- IL-27 expression promotes $CD4^+$ T cell engraftment *in vivo*.

4.3 Results

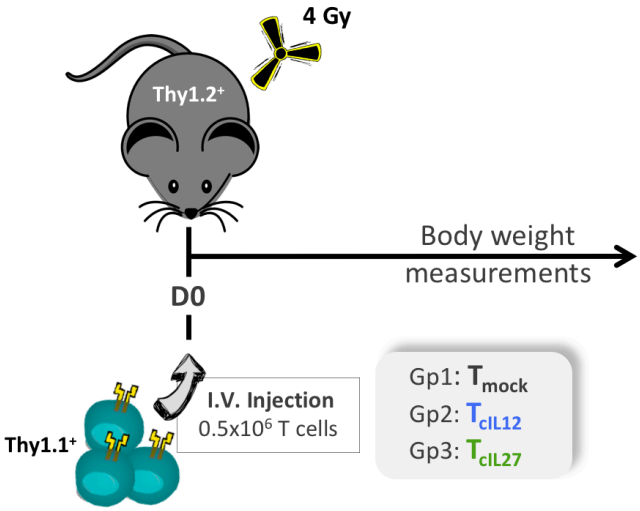
4.3.1 Constitutive expression of IL-12 by engineered T cells induces weight loss and lethal toxicity in mice

Based on data in the literature that IL-12 but not IL-27 can be toxic *in vivo*, I next sought to assess the adoptive transfer of gene-modified T cells *in vivo*. C57BL/6 recipient mice, expressing the congenic marker Thy1.2, were sublethally irradiated with 4Gy TBI and treated intravenously with $0.5x10^6$ T cells that were retrovirally transduced with constitutive vectors encoding IL-12 (T_{cIL12}), IL-27 (T_{cIL27}) or mock-transduced (T_{mock}). Adoptively transferred T cells were obtained from C57BL/6 donor mice expressing the congenic marker Thy1.1 to enable tracking of transferred cells in recipient mice (Figure 4.1 A). The pre-injection profile of the transduced T cells was assessed, and it showed good transduction efficiency and increased expression of IL-12 and IL-27 (Figure 4.1 B). Body weight measurements at different time intervals indicated that T_{cIL12} cell recipients showed weight loss of >20% of their initial body weight and had to be sacrificed according to Home Office regulations, but recipients of T_{cIL27} or T_{mock} cells did not (Figure 4.1 C). Increased mortality was seen in all mice received T_{cIL12} cells, whereas 100% survival was seen in the mice treated with T_{cIL27} or T_{mock} cells (Figure 4.1 D).

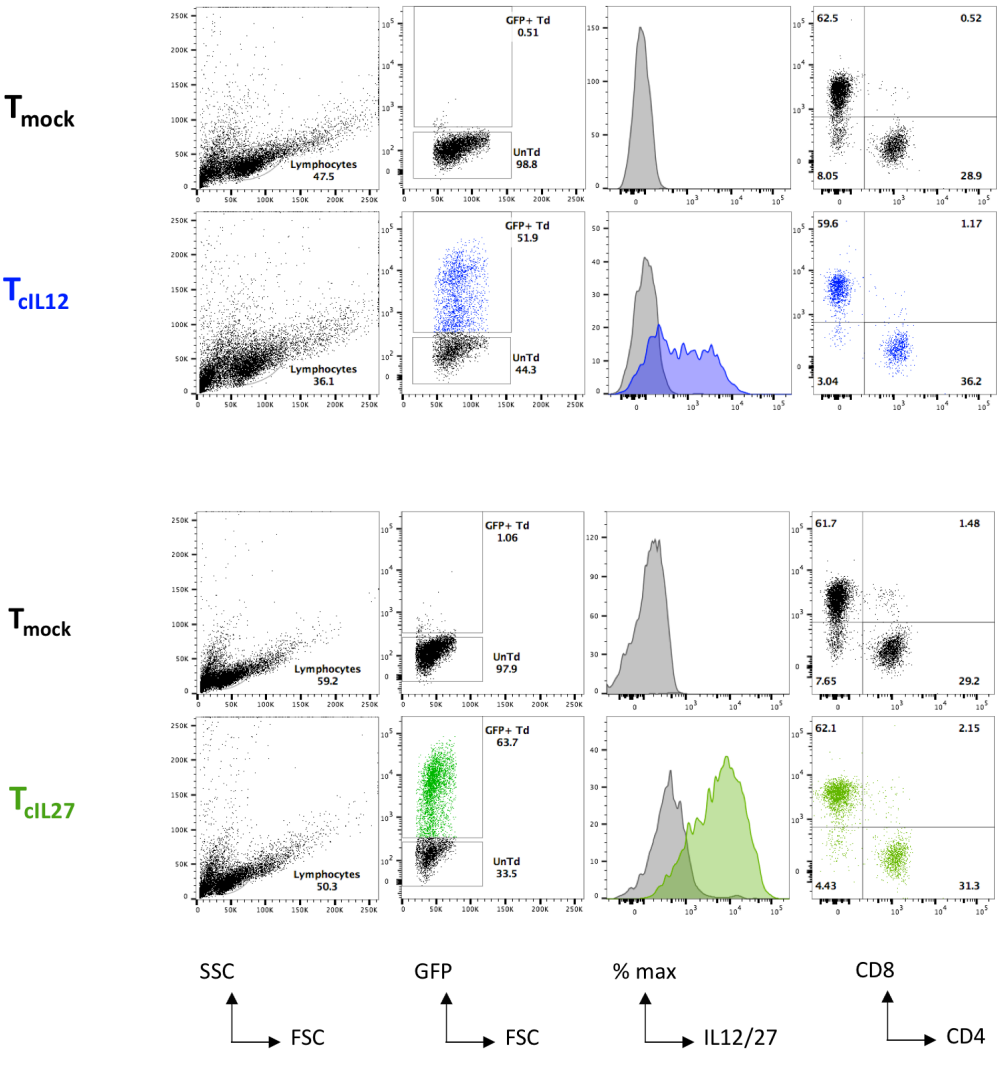
Based on our previous experimental data that low levels of IL-12 leakage from the tet-regulated vector had a biological impact *in vitro*, I assessed the biological impact *in vivo*. Thy1.1$^+$ T cells obtained from C57BL/6 mice were retrovirally transduced with tet-regulated control GFP (T_{iGFP}), IL-12 (T_{iIL12}) or IL-27 (T_{iIL27}) and adoptively transferred into irradiated Thy1.2$^+$ C57BL/6 mice. Transduction efficiency was assessed 3 days post-transduction and prior to T cell transfer, indicating similar transduction efficiency in all groups (Figure 4.2 A). Mice were split into two cohorts, one group of mice received dox in drinking water (2 mg/ml) at all time during the experiment and the other group did not. As seen with constitutive vectors, recipients of the T_{iIL12} cells in the presence of Dox showed severe toxicity measured by >20% body weight loss and had to be sacrificed within 10 days. By contrast, neither recipients of the T_{iGFP} cells nor the T_{iIL27} cells, in the presence of Dox, lost weight and all mice survived. Importantly, recipients of the T_{iIL12} cells in the absence of Dox showed no signs of IL-12-induced toxicity *in vivo* (Figure 4.2 B). Taken together, these data illustrate that constitutive expression of IL-12 is toxic *in vivo,* and that the

amount of leakage from the tet-regulated IL-12 vector has no detectable levels of toxicity as measured by weight loss.

(A)



(B) **Pre-injection profile …**



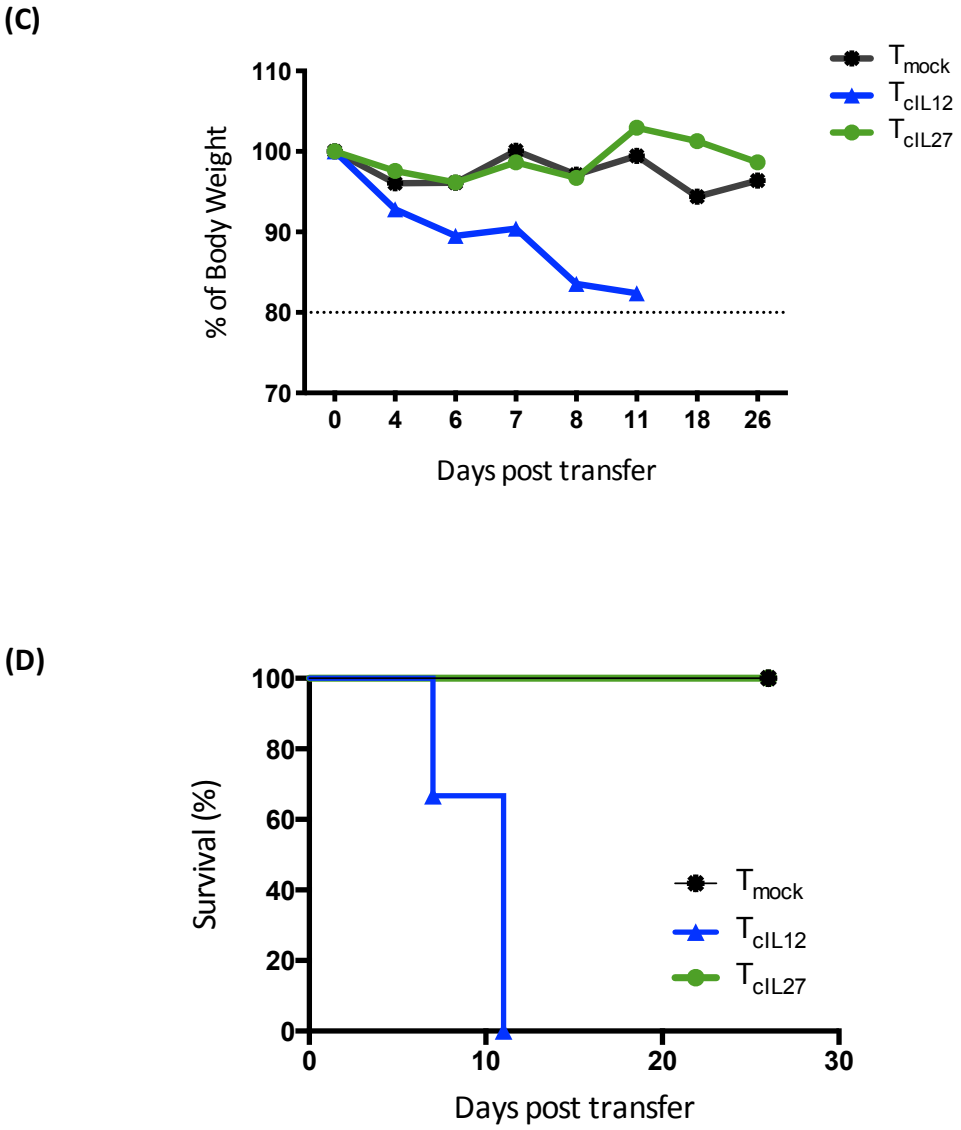


Figure 4.1 | The adoptive transfer of gene-engineered T cells expressing constitutive IL-12 or IL-27. (A) Experiment layout. **(B)** Pre-injection profile of transferred T cells. Representative flow cytometry blots showing transduction efficiency, CD4/CD8 ratio and the ability of transduced T cells to express elevated levels of IL-12 and IL-27 following 2hrs of BFA treatment. **(C)** Mean of body weight measurements over time post T cell transfer showing weight loss >20% of initial body weight in mice treated with T_{cIL12} cells. **(D)** Kaplan-Meyer survival plot displaying increased mortality in mice treated with T_{cIL12} cells. *n*=3 mice/group.

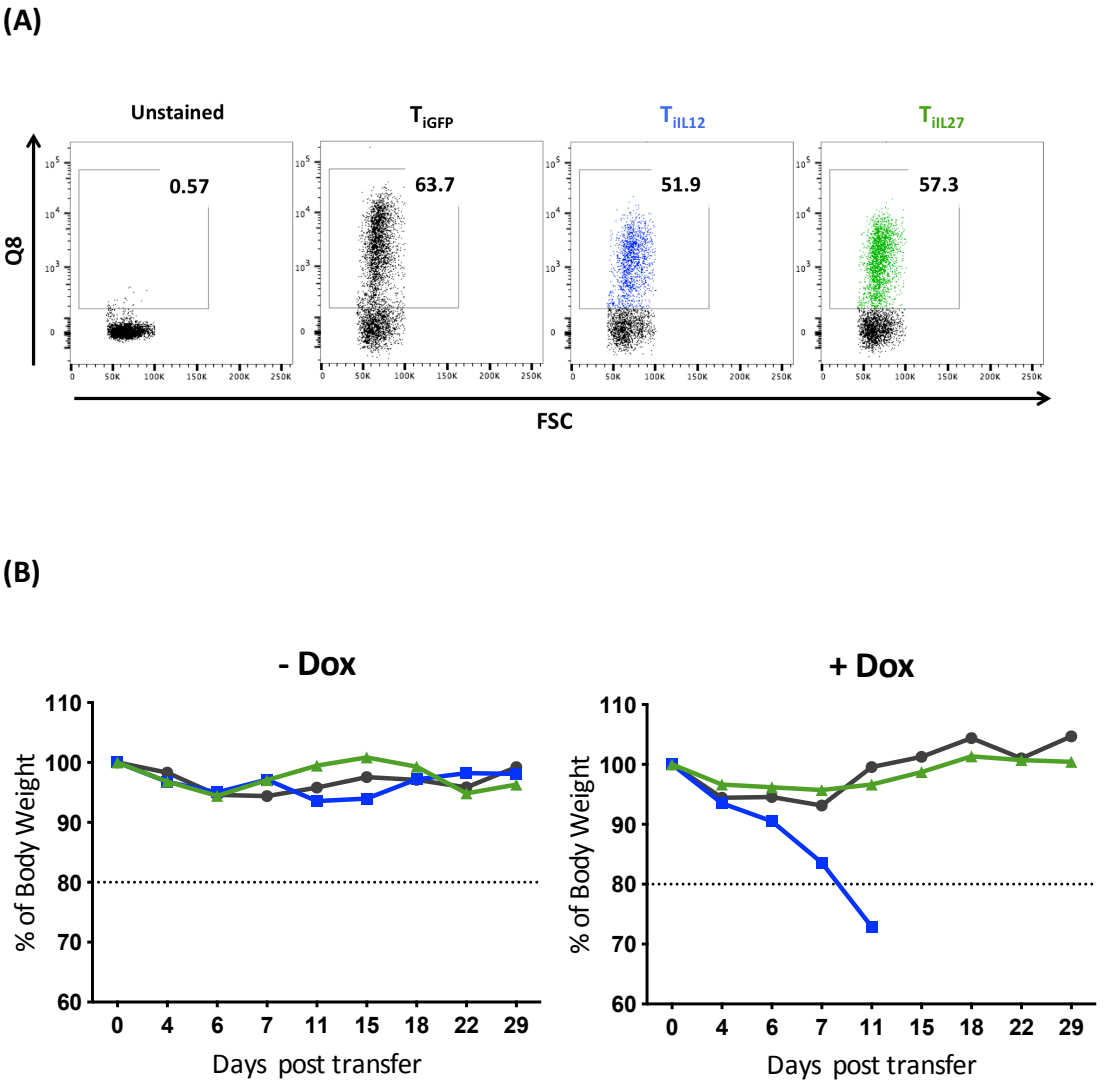


Figure 4.2 | The adoptive transfer of gene-engineered T cells expressing inducible IL-12 does not cause toxicity in the absence of Dox *in vivo*. Sub-lethally irradiated C57BL/6 mice with 4Gy TBI received tet-regulated IL-12-transduced T cells (T_{iIL12}), tet-regulated IL-27-transdcued T cells (T_{iIL27}) or tet-regulated control GFP-transduced T cells (T_{iGFP}) intravenously 3-4hrs after irradiation. Mice were split into two groups; one received Dox (2 mg/ml) in drinking water and the other group left untreated. **(A)** Transduction efficiency of the transferred T cells gating on $Q8^+$ cells showed good transduction efficiency. **(B)** Mean of body weight measurements over time following T cell transfer showed weight loss >20% of initial body weight in mice treated with T_{iIL12} cells in mice treated with Dox but not in untreated mice (*n*=3 mice/group).

4.3.2 low levels of IL-12 produced in the absence of Dox-induction does not translate in any detectable toxicity in the presence of antigen-specific stimulation

Given that we found no observable toxicity in mice receiving T cells transduced with tet-regulated IL-12 construct in tumour free mice, it was important to ensure that leaked IL-12 would not cause toxicity in the presence of antigen-specific stimulation. To test this, C57BL/6 (Thy1.2^{+}) mice were sublethally irradiated with 4Gy (TBI) and implanted with 1x10^{6} EL4-NP tumour cells subcutaneously or left free of tumour. 5 days later, mice were treated intravenously with T cells that had been transduced with F5 TCR and tet-regulated IL-12 ($T_{TCR+iIL12}$) or tet-regulated IL-27 ($T_{TCR+iIL27}$). Adoptive transfer of $T_{TCR+iIL27}$ was considered as a control because it has already shown in previous experiment that it does not cause toxicity. 10 days post T cell transfer, blood samples were taken from the tail vein to assess engraftment and expansion of the infused cells. Although the transferred T cells were detectable in the peripheral blood of all recipient mice, a high frequency of transferred T cells accumulated in the periphery of mice that received non-induced $T_{TCR+iIL12}$ cells in the presence of antigen-positive tumour cells compared to those transferred in tumour-free mice (Figure 4.3 A). Having shown that we found higher proportion of non-induced $T_{TCR+iIL12}$ cells in the peripheral blood in the presence of antigen but not in the absence of antigen, measurements of body weight indicated that this did not translate into any detectable toxicity, and 100% of the treated mice survived (Figure 4.3 B). Taken together, the data from these *in vivo* studies suggest that the tet-regulated gene expression system can be used to regulate the expression of IL-12 *in vivo*.

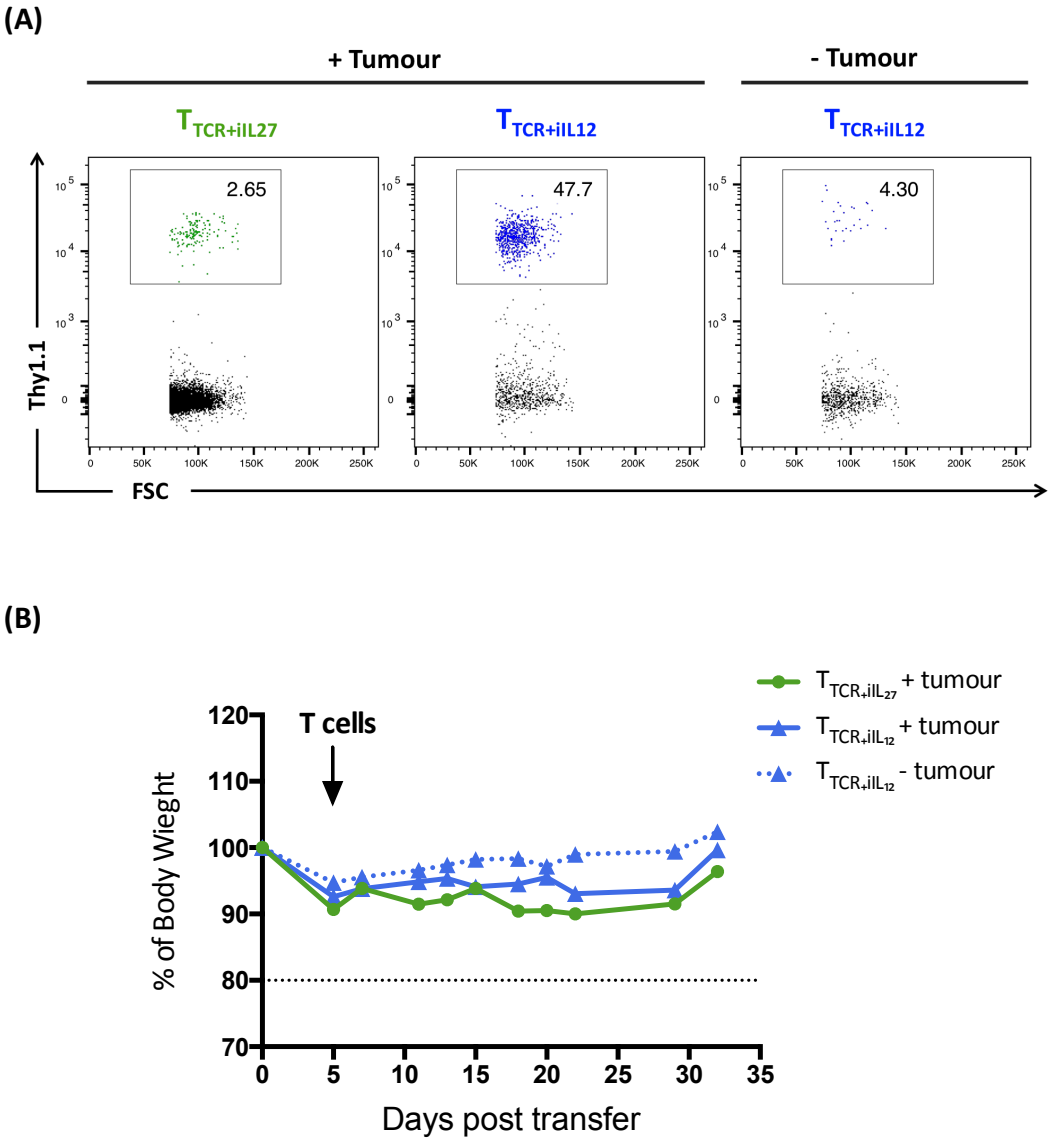
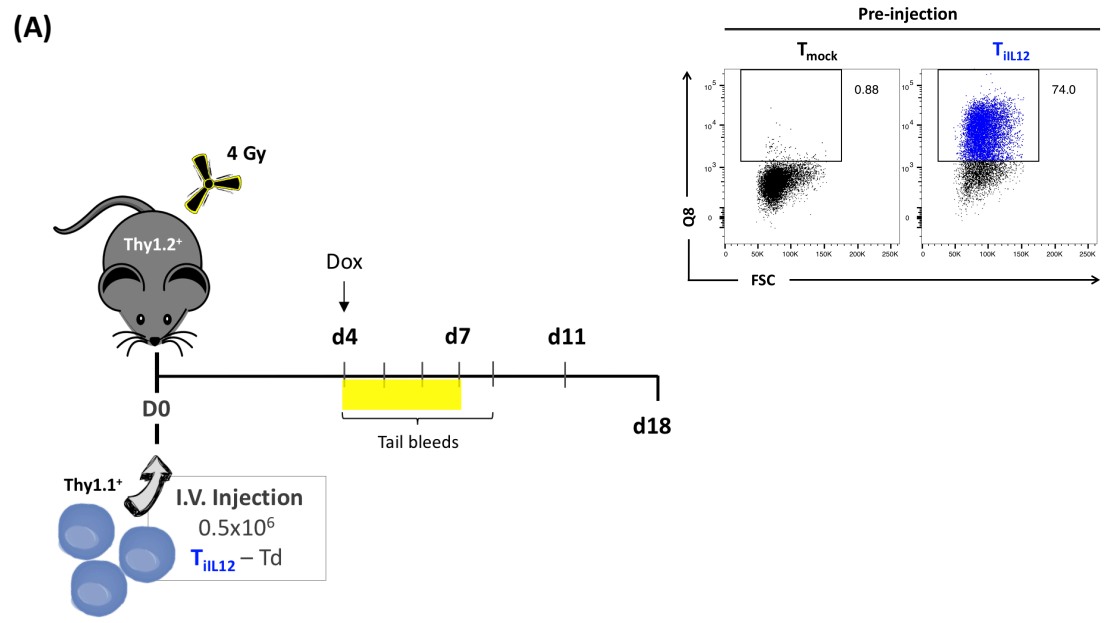


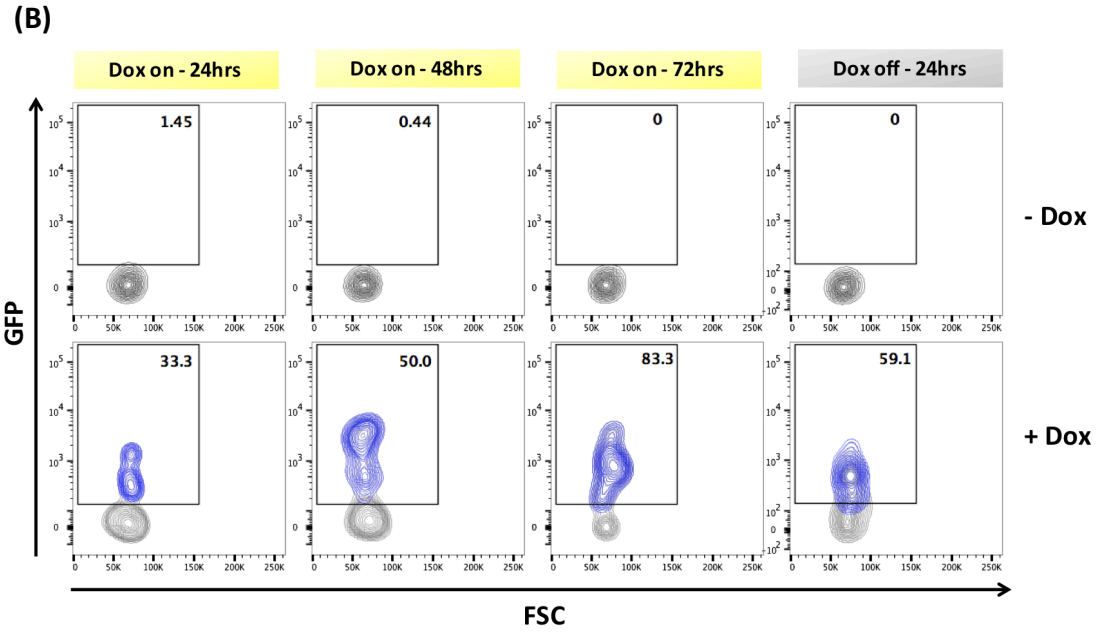
Figure 4.3 | low levels of IL-12 production in the absence of Dox-induction does not cause toxicity in the presence of antigen-specific stimulation. Sub-lethally irradiated Thy1.2^{+} C57BL/6 mice with 4Gy TBI were implanted with 1x10^{6} EL4-NP tumour cells subcutaneously or left free of tumour. 5 days later, mice received T cells (Thy1.1^{+}) transduced with influenza-specific F5 TCR and tet-regulated IL-12 ($T_{TCR+iIL12}$) or tet-regulated IL-27 ($T_{TCR+iIL27}$) intravenously. **(A)** Representative blots showing transferred cells, gated on Thy1.1^{+}, in the peripheral blood at d10 following T cell transfer. Cells were pre-gated on PI^{-} singlet lymphocytes. **(B)** Mean of body weight measurements over time following T cell transfer indicated no toxicity-mediated weight loss, and 100% survival of all treated mice was observed (*n*=3 mice/group).

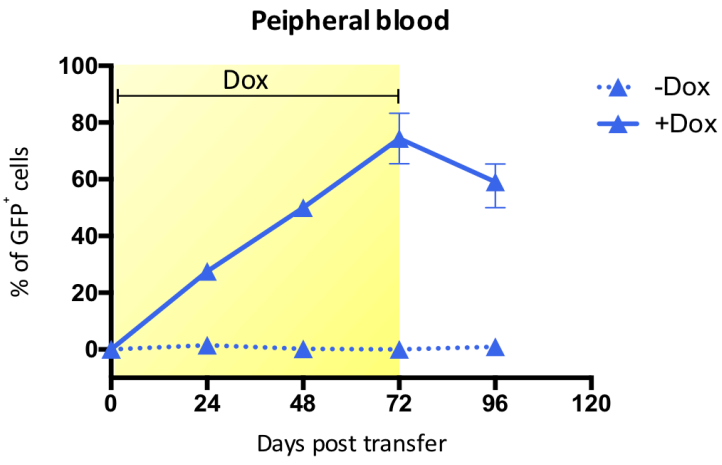
4.3.3 Induction kinetics of tet-regulated construct *in vivo*

The above results revealed that tet-regulated IL-12 expression is non-toxic *in vivo*. To understand the kinetic of Dox-induction *in vivo*, it was important to determine the kinetic of IL-12 expression following Dox administration and withdrawal. To do this, two cohorts of C57BL/6 (Thy1.2$^+$) mice that had been sublethally irradiated with 4Gy TBI were used. 3-4hrs after irradiation, 0.5x10^6 T cells (Thy1.1$^+$) that had been transduced with tet-regulated IL-12 construct (T_{iIL12}) were adoptively transferred. One group of mice received Dox in drinking water (2 mg/ml) at d4 post T cell transfer for 3 days and the other group was left untreated. Following Dox treatment, blood samples were obtained via tail vain bleed at 24hrs, 48hrs and 72hrs. Dox was then withdrawn and blood samples were collected again 24hrs later. 3 days later, half of the mice from each cohort were sacrificed and spleen tissues were analysed. The other half of the mice were monitored for another week for signs of toxicity, and then, they were sacrificed and spleen tissues were analysed. Prior to T cell transfer, transduction efficiency was assessed by flow cytometry by staining the cells for Q8 and this revealed good transduction efficiency, about 74% of gated Thy1.1$^+$ cells were transduced as determined by the Q8 expression (Figure 4.4 A). The transferred cells, gated on Thy1.1$^+$, were analysed for GFP expression to assess the level of induction in the peripheral blood and spleen at each designated time points. The decision to switch on IL-12 for 3 days following T cell transfer is because, considering the earlier experiments, it seems that after 3 days of induction we have not yet seen any toxicity. As indicated in figure 4.4 B, GFP expression was gradually increased in the peripheral blood over time and reached almost 80% induction 72hrs following Dox treatment. Taking into account that the transduction efficiency was 74% prior to adoptive T cell transfer, this seems to imply that actually all the transduced cells were expressing GFP, suggesting that the induction worked very efficiently *in vivo*.

As anticipated, GFP expression was not evident in the spleen at d4 and d11 following Dox withdrawal. No GFP expression was observed at any time point in the periphery or in the spleen of mice that did not receive Dox. In summary, these data suggest that the tet-regulated expression construct is well-regulated and can work efficiently *in vivo*.


(A)
Pre-injection
T mock
T iL12
0.88
74.0
Q8
FSC
4 Gy
Thy1.2+
Dox
d4
d7
d11
D0
d18
Tail bleeds
Thy1.1+
I.V. Injection
0.5x10^6
T iL12 – Td


(B)
Dox on - 24hrs
Dox on - 48hrs
Dox on - 72hrs
Dox off - 24hrs
1.45
0.44
0
0
33.3
50.0
83.3
59.1
- Dox
+ Dox
GFP
FSC


Peipheral blood
Dox
-Dox
+Dox
% of GFP+ cells
Days post transfer
0
20
40
60
80
100
0
24
48
72
96
120

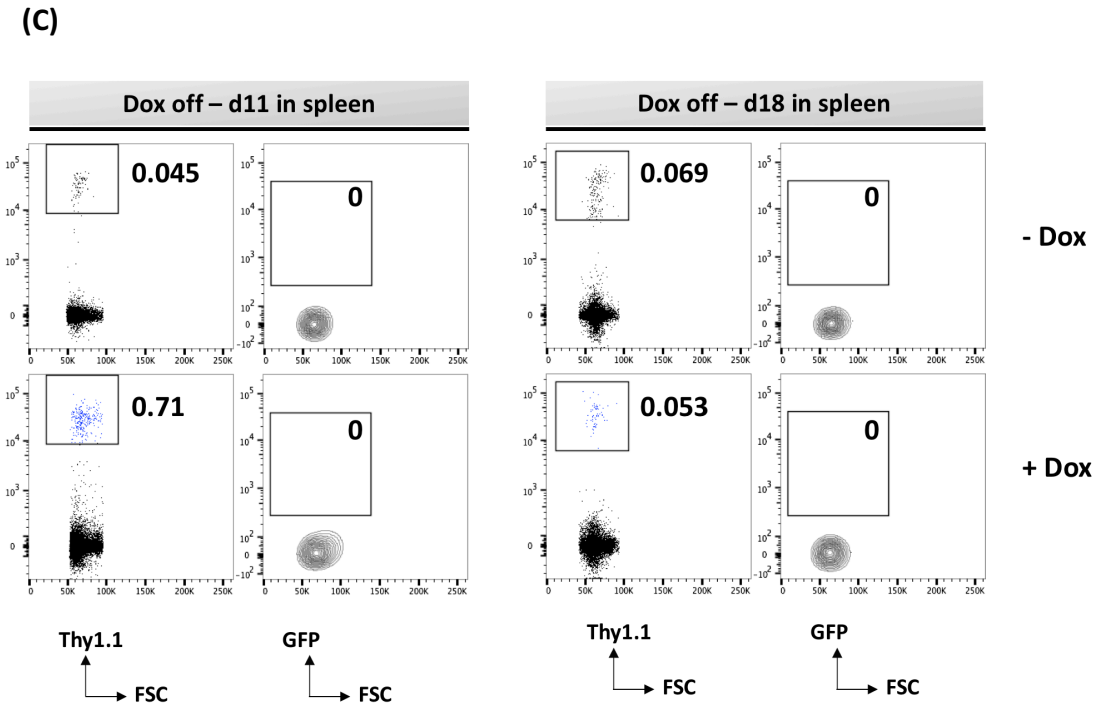


Figure 4.4 | Induction kinetics of tet-regulated expression construct *in vivo*. (**A**) Experimental setup. C57BL/6 mice were sublethally irradiated with 4Gy TBI 3-4hrs before the adoptive T cell transfer of tet-regulated IL-12 transduced T cells (T_{iIL12}). Mice were split into two groups, one group received Dox water (2 mg/ml) at d4 for 3 days and the other group left untreated. Blood samples were taken at 24hrs, 48hrs and 72hrs following Dox induction and 24hrs following Dox withdrawal. Half of the mice were sacrificed at d4 and the remaining mice at d11 post Dox withdrawal. **(B)** Representative blots showing the induction levels by GFP expression in the peripheral blood *(top)* and summary data *(bottom)*. Error bars showing mean ± SEM. **(C)** Representative blots showing GFP expression in the spleen at d11 and d18 following Dox withdrawal. Cells were pre-gated on PI^- singlet $Thy1.1^+$ lymphocytes. *n*= 4 mice (-Dox) *n*=6 mice (+Dox)

4.3.4 No significant difference in tumour rejection following adoptive transfer of T_{TCR} or $T_{TCR+cIL27}$ cells in EL4-NP tumour-bearing mice

Having shown that IL-27 is non-toxic *in vivo*, I next decided to gain an insight into the effect of IL-27 expression in TCR-modified T cells at the level of tumour rejection. EL4-NP tumour model, which is a well-established model in our lab, was used. This model is highly immunogenic and it has been shown that adoptive transfer of as low as $3x10^5$ of influenza (NP)-specific F5 TCR-transduced cells can potentially clear tumour cells [143]. To carry out a tumour challenge experiment, C57BL/6 mice were sublethally irradiated and subcutaneously inoculated with $1x10^6$ EL4-NP tumour cells. 5 days later, mice were treated by intravenous infusion of $0.5x10^6$ T cells that were transduced with F5 TCR (T_{TCR}), F5 TCR and cIL27 ($T_{TCR+cIL27}$) or mock-transduced (T_{mock}). Tumour size was measured at different time intervals post T cell transfer using a digital caliper (Figure 4.5 A). Mice treated with T_{mock} cells showed uncontrolled tumour growth and started to develop a lethal tumour burden by day 15. By contrast, mice treated with T_{TCR} or $T_{TCR+cIL27}$ cells showed controlled tumour growth and resulted in 100% tumour-free survival at day 25-35. However, the data suggested that there was no considerable difference between mice treated with $T_{TCR+cIL27}$ compared to T_{TCR} cells (Figure 4.5 B). In addition, bioluminescence imaging (BLI) was performed by challenging the mice with firefly luciferase-positive EL4-NP tumour cells and tumour burden was assessed in mice at different time intervals following tumour challenge. Representative imaging in figure 4.6 was in line with the caliper measurements and showed no difference in tumour growth between both groups of mice treated with T_{TCR} and $T_{TCR+cIL27}$ cells.

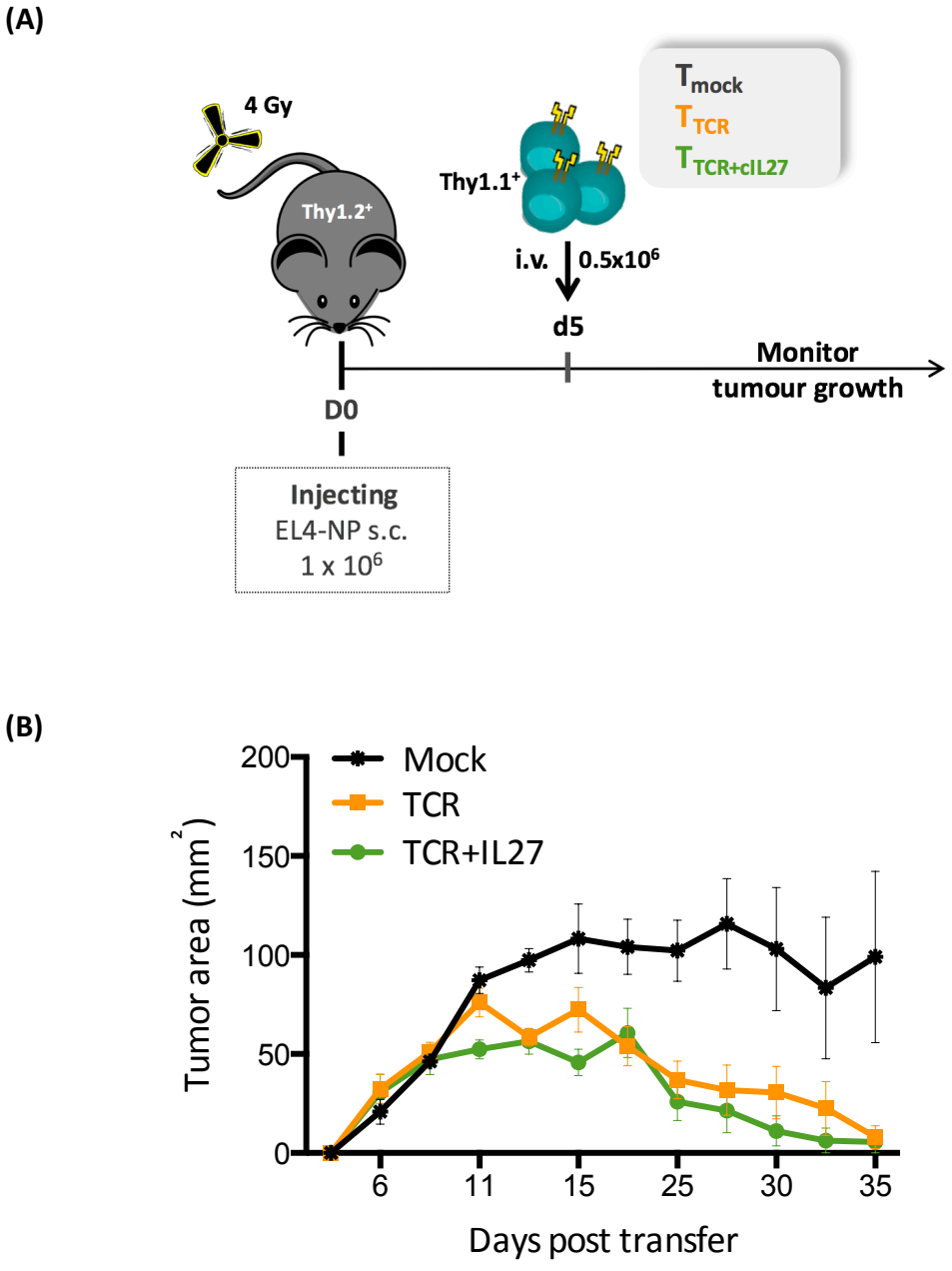


Figure 4.5 | No difference in tumour growth kinetics upon transferring T_{TCR} or $T_{TCR+cIL27}$ cells. (A) Experimental layout. Sublethally irradiated C57BL/6 mice were implanted with EL4-NP tumour cells subcutaneously. 5 days later, mice were treated with T cells transduced with F5 TCR (T_{TCR}), F5 TCR + cIL27 ($T_{TCR+cIL27}$) or mock-transduced T cells (T_{mock}). **(B)** Tumour size was assessed over time post T cell transfer. Statistical significance tested by two-tailed Mann Whitney test: T_{mock} versus T_{TCR} *P value* = 0.0069; T_{mock} versus $T_{TCR+cIL27}$ *P value* = 0.0037; T_{TCR} versus $T_{TCR+cIL27}$ *P value* > 0.05. Graph is showing mean ± SEM. Data shown represent two independent experiments (n=8 for T_{mock}, n=9 mice for T_{TCR}, n=9 mice for $T_{TCR+cIL27}$).

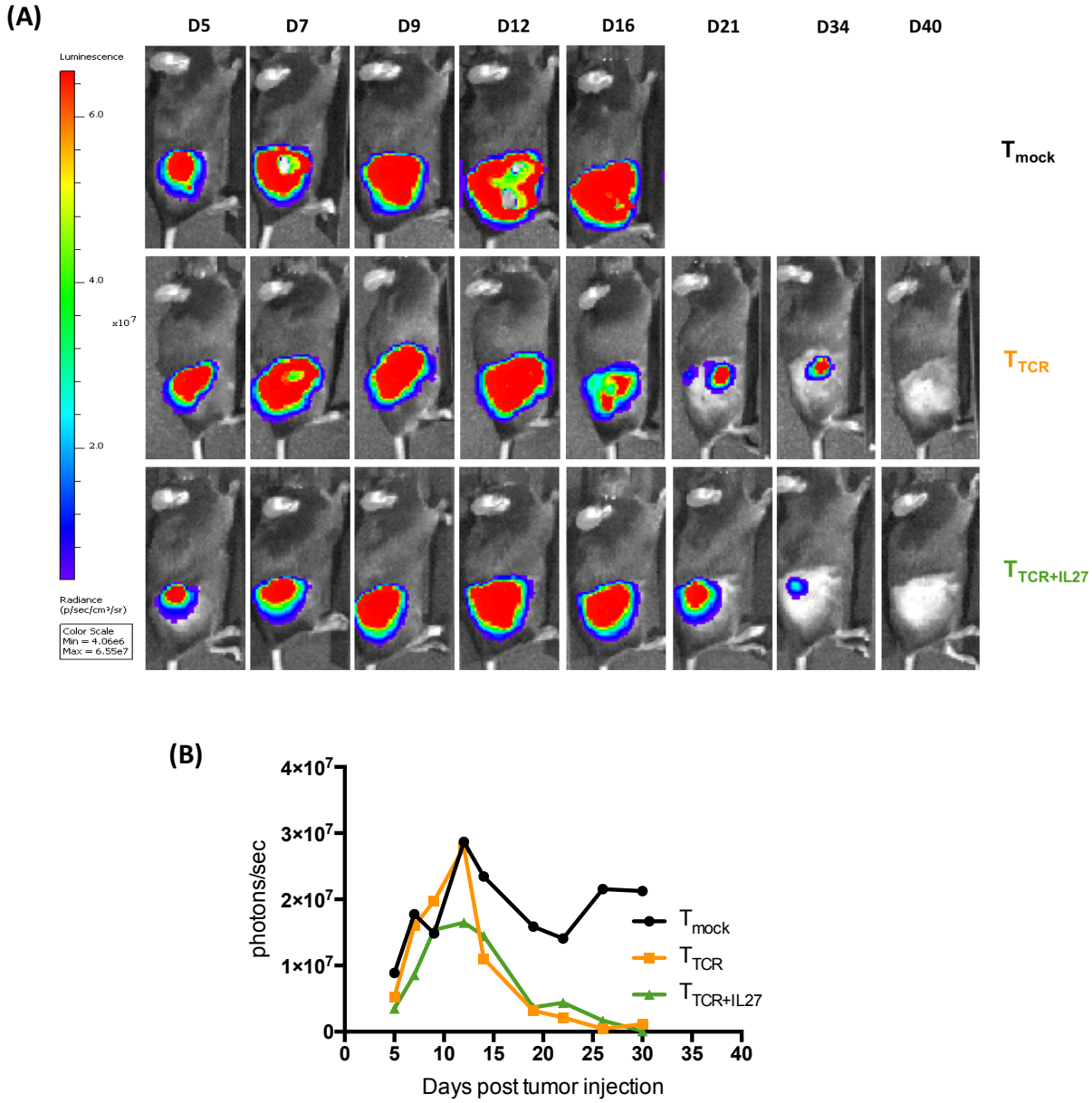


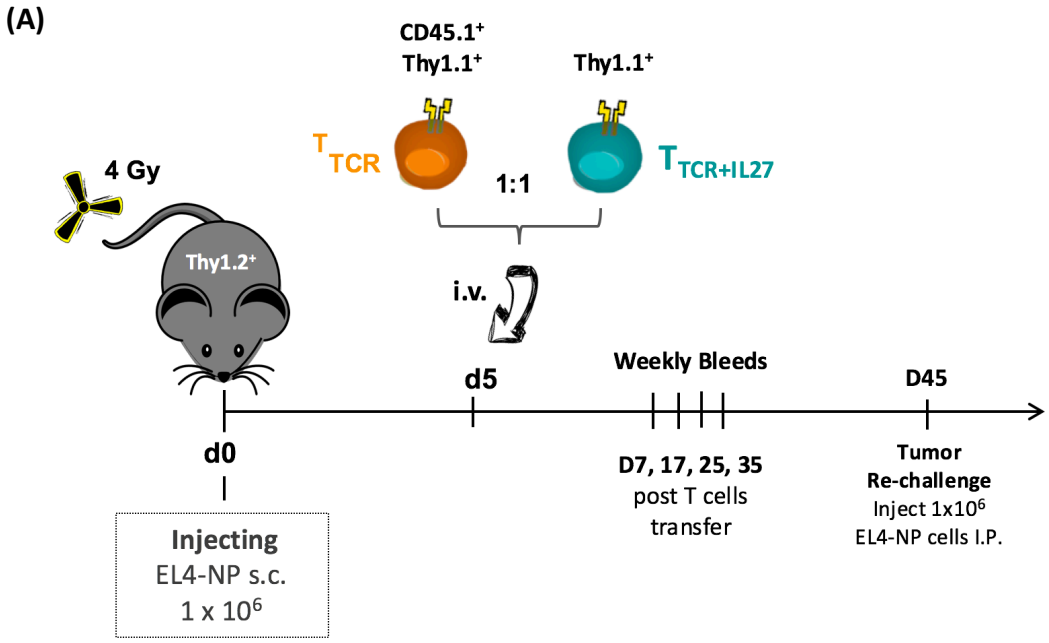
Figure 4.6 | Tumour growth kinetics following adoptive transfer of T_{TCR} and $T_{TCR+cIL27}$ cells. Sublethally irradiated C57BL/6 mice (4Gy TBI) were inoculated subcutaneously with 1x10^6 luciferase-positive EL4-NP tumour cells. 5 days later, mice were treated intravenously with T cells transduced with TCR (T_{TCR}), TCR+cIL27 ($T_{TCR+cIL27}$) or mock-transduced T cells (T_{mock}). 150 µg/g luciferin was administered at selected time points following tumour challenge and bioluminescence signals were measured. **(A)** Representative images showing uncontrolled tumour growth in mice received T_{mock} cells, and no remarkable difference in tumour protection in mice that received T_{TCR} or $T_{TCR+cIL27}$ cells. **(B)** The mean bioluminescent signals (photons/sec) measured in the different groups of treated mice at indicated time points. Data shown represent one experiment (n=5 for T_{mock}, n=5 mice for T_{TCR}, n=5 mice for $T_{TCR+cIL27}$).

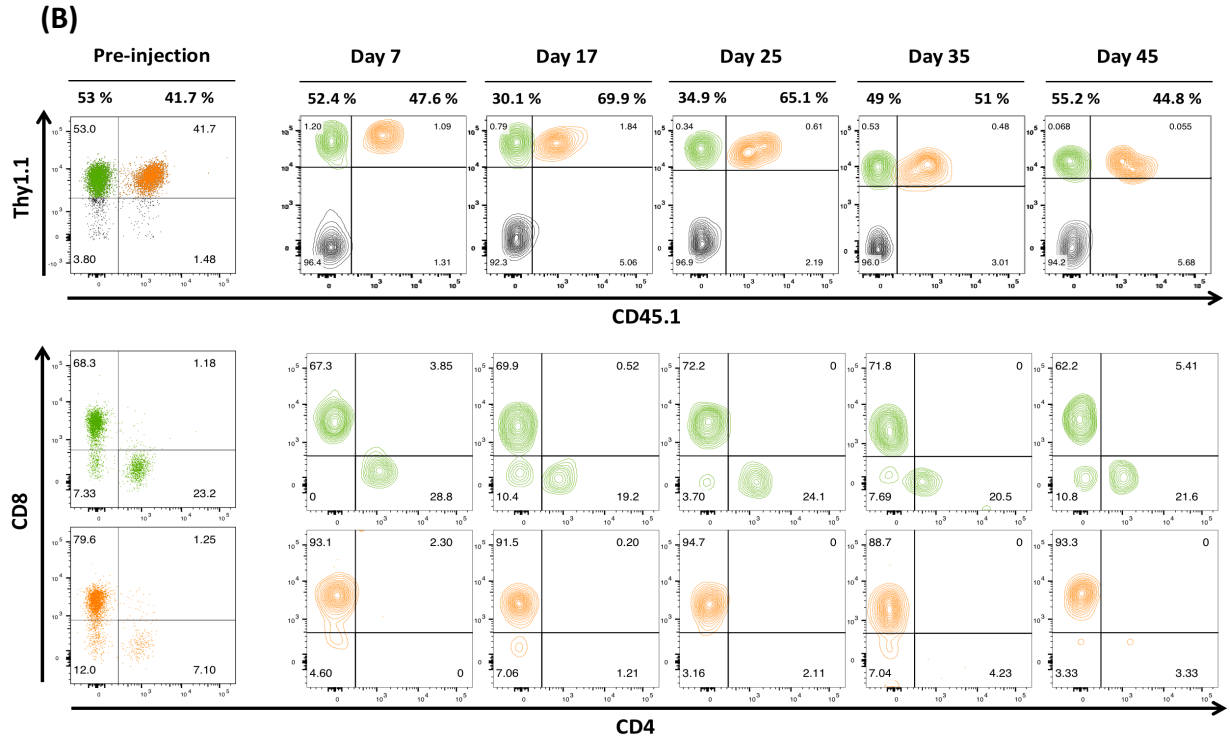
4.3.5 The effect of IL-27 expression in TCR-modified T cell performance *in vivo*

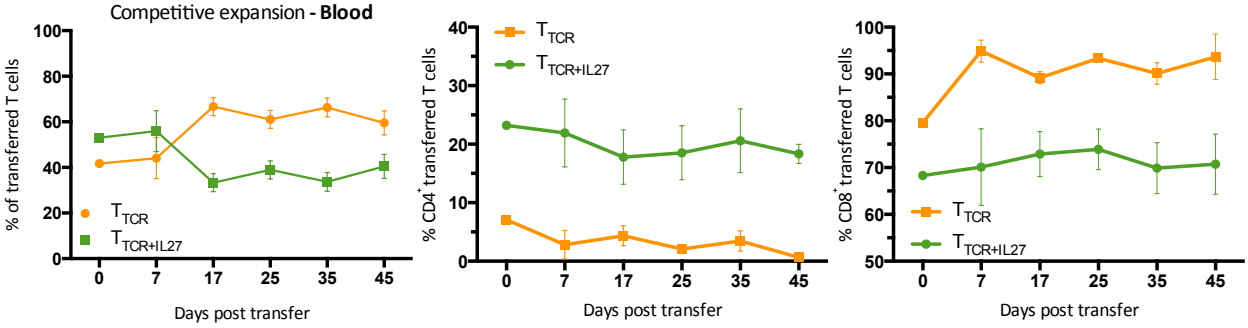
The above results demonstrated that IL-27 expression in TCR-redirected T cells had no significant impact on delaying tumour growth in established EL4-NP tumour model. Next, I sought to explore how IL-27 expression affects T cell expansion, phenotype and persistence. A competition experiment was designed to monitor TCR and TCR+IL-27 transduced T cell populations in the same animal. T cells that were transduced with F5 TCR only (T_{TCR}) or F5 TCR and cIL27 ($T_{TCR+IL27}$) were mixed together in 1:1 ratio (based on transduction efficiency) and were adoptively transferred into EL4-NP-tumour bearing mice as a source of antigenic stimulation. Transduced T cells were expressing congenic markers so they can be distinguished as Thy1.1$^+$CD45.1$^+$ (T_{TCR}) and Thy1.1$^+$CD45.1$^-$ ($T_{TCR+IL27}$). Following T cell transfer, blood samples were collected at d7, d17, d25, d35 and d45 to assess expansion, relative frequency and phenotype of the transferred T cells (Figure 4.7 A). The adoptive transfer of these two competing cells in tumour-bearing mice showed that T_{TCR} cells peaked at d17 in the peripheral blood followed by a sharp reduction. It also showed that T_{TCR} cells preferentially expanded over $T_{TCR+IL27}$ cells during the initial expansion phase.

In addition, CD4 and CD8 staining profile of the two competing cells revealed that the frequency of CD4$^+$ T cells dropped within the T_{TCR} population but they were maintained within the $T_{TCR+IL27}$ cells compared to their relative frequency before the adoptive transfer (Figure 4.7 B). Furthermore, differential phenotypic profile of the transferred T cells in the periphery was determined using CD62L and CD127 expression which subdivide the cells into effector (CD62L^{lo} CD127^{lo}), central memory (CD62L^{hi} CD127^{hi}) and effector memory (CD62L^{lo} CD127^{hi}) T cells. Flow cytometric analysis demonstrated that both T_{TCR} and $T_{TCR+IL27}$ cells had similar phenotype in the peripheral blood at all time points. During initial response, the transferred cells were found to exhibit an effector phenotype, had a greater proportion of CD62L^{lo} CD127^{lo}. Then, at day 17 post T cell infusion, the transferred T cells start to regain CD62L and CD127 expression and generated a high proportion of cells with central memory phenotype (CD62L^{hi} CD127^{hi}) by day 35 (when tumour was cleared) (Figure 4.7 C). Mice were re-challenged with the same antigen, irradiated EL4-NP at day 46 following T cell transfer. 3 days later, mice were sacrificed and tissue samples were analysed including spleen, lymph nodes and bone marrow to determine

frequency, total numbers and phenotypic profile of T_{TCR} and $T_{TCR+IL27}$ cells. A detectable high number of T_{TCR} and $T_{TCR+IL27}$ cells was observed in the spleen and lymph nodes but not in the bone marrow, indicating similar persistence of both T_{TCR} and $T_{TCR+IL27}$ cells (Figure 4.8 A). Phenotypic analysis revealed that both T_{TCR} and $T_{TCR+IL27}$ cells had similar expression of CD62L and CD127 in all tissues (Figure 4.8 B).


(A)
CD45.1+
Thy1.1+
Thy1.1+
TTCR
1:1
TTCR+IL27
4 Gy
Thy1.2+
i.v.
d5
Weekly Bleeds
D45
d0
D7, 17, 25, 35
post T cells
transfer
Tumor
Re-challenge
Inject 1x10^6
EL4-NP cells I.P.
Injecting
EL4-NP s.c.
1 x 10^6


(B)
Pre-injection
Day 7
Day 17
Day 25
Day 35
Day 45
53 %
41.7 %
52.4 %
47.6 %
30.1 %
69.9 %
34.9 %
65.1 %
49 %
51 %
55.2 %
44.8 %
Thy1.1
CD45.1
CD8
CD4


Competitive expansion - Blood
% of transferred T cells
% CD4+ transferred T cells
% CD8+ transferred T cells
TTCR
TTCR+IL27
Days post transfer

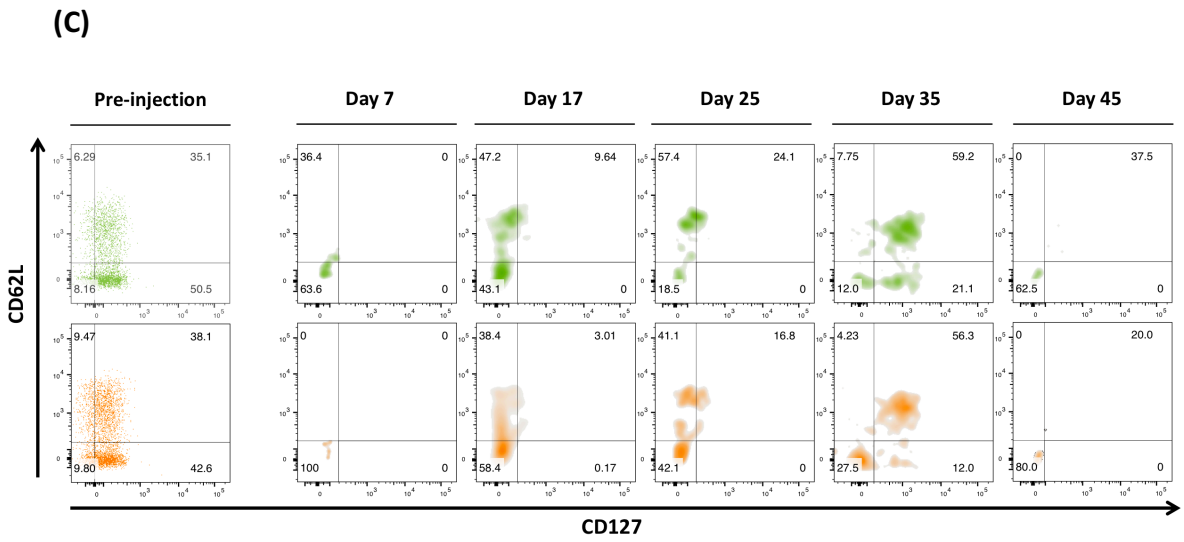


Figure 4.7 | The effect of IL-27 expression in TCR-modified T cells upon *in vivo* expansion and phenotype. (A) Experimental layout. **(B)** Flow cytometry blots representing Thy1.1 and CD45.1 staining to demonstrate competitive expansion between T_{TCR} (Thy1.1$^+$) and $T_{TCR+IL27}$ (Thy1.1$^+$CD45.1$^+$) cells in peripheral blood following adoptive T cell transfer in EL4-NP tumour-bearing mice *(top)* and CD4/CD8 staining profile *(bottom)* compared to their pre-injection relative frequency. Following graphs are showing summary data (error bars represent mean ± SEM). Cells were pre-gated on PI$^-$ singlet lymphocytes. **(C)** Flow cytometry blots represent staining of CD62L and CD127 surface expression in the peripheral blood compared to the pre-injection profile. (*n*= 6 mice).

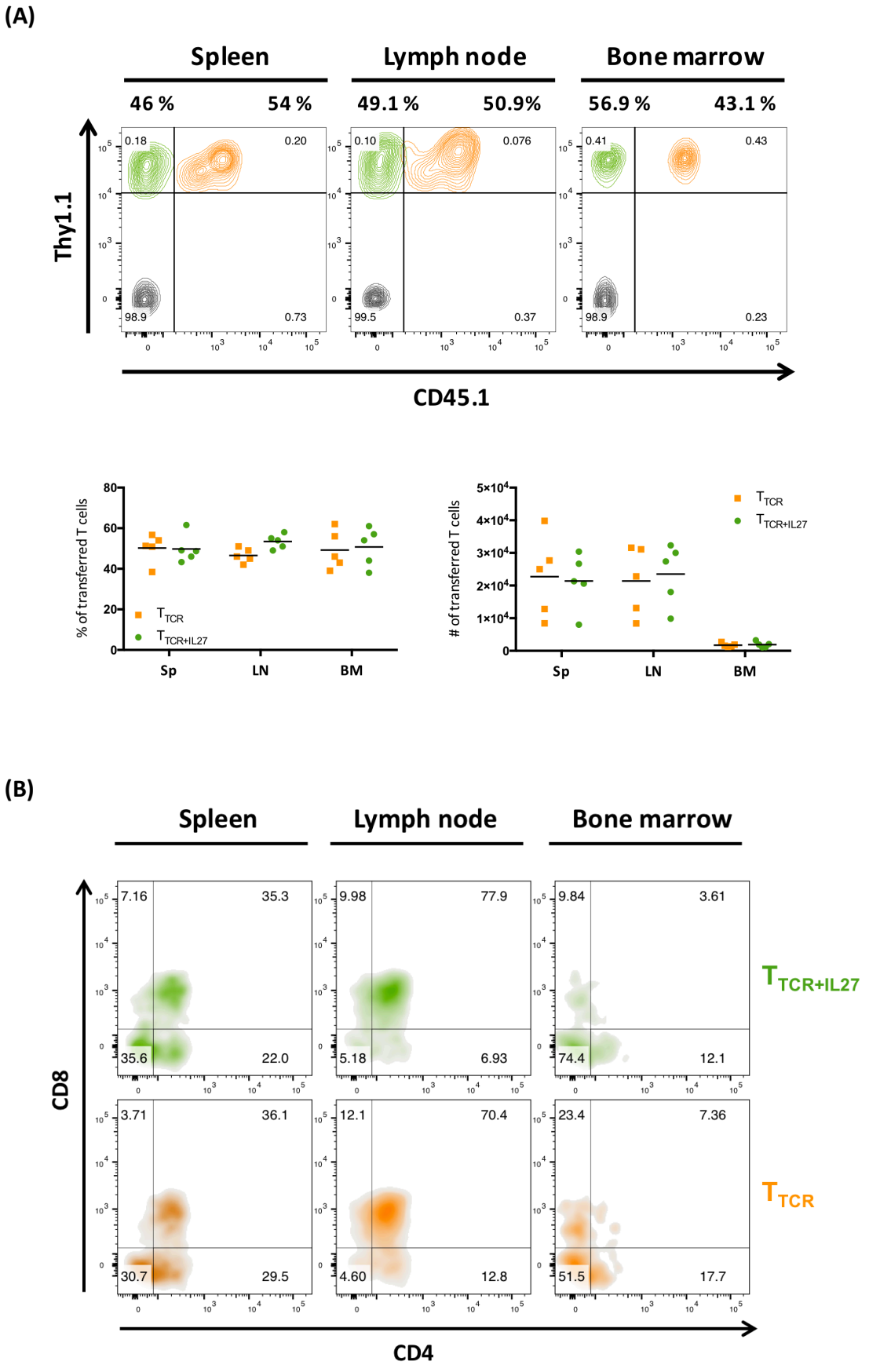

(A)
Spleen
Lymph node
Bone marrow
46 %
54 %
49.1 %
50.9%
56.9 %
43.1 %
Thy1.1
CD45.1
0.18
0.20
98.9
0.73
0.10
0.076
99.5
0.37
0.41
0.43
98.9
0.23
% of transferred T cells
# of transferred T cells
T TCR
T TCR+IL27
Sp
LN
BM
(B)
Spleen
Lymph node
Bone marrow
CD8
CD4
T TCR+IL27
T TCR
7.16
35.3
35.6
22.0
9.98
77.9
5.18
6.93
9.84
3.61
74.4
12.1
3.71
36.1
30.7
29.5
12.1
70.4
4.60
12.8
23.4
7.36
51.5
17.7

Figure 4.8 | The frequency of T_{TCR} and $T_{TCR+IL27}$ cells in tissues is similar following tumour re-challenge. Sublethally irradiated C57BL/6 mice bearing EL4-NP tumour for 5 days received a mix of 1:1 ratio of transduced T cells, T_{TCR} and $T_{TCR+IL27}$ cells. Mice were re-challenged with irradiated EL4-NP tumour cells on day45. **(A)** Representative flow cytometry plots depicting proportion of both T_{TCR} and $T_{TCR+IL27}$ cells *(top)* and summary data of frequencies and absolute numbers in all analysed tissues including spleen (Sp), lymph nodes (LN) and bone marrow (BM) *(bottom)*. Symbols show individual mice, and bars indicate group averages. Statistical significance tested by two-tailed Mann Whitney test: frequency of T_{TCR} versus T_{cIL27} in spleen and bone marrow *P value* > 0.05; frequency of T_{TCR} versus T_{cIL27} in lymph nodes *P value* = 0.0397; absolute numbers of T_{TCR} versus T_{cIL27} in spleen, lymph nodes and bone marrow *P value* > 0.05 (*n*=6 mice). **(B)** CD62L and CD127 surface expression in transferred T cells, T_{TCR} and $T_{TCR+IL27}$, in Sp, LN and BM 3 days following tumour re-challenge.

4.4 Discussion

Cytokine-based immunotherapy to treat cancer has shown an effective therapeutic potential to enhance antitumor immune responses and activate tumour-specific T cells in the suppressive tumour microenvironment [330].

When IL-12 first discovered to improve antitumor immunity and showed promising results in preclinical models either alone or in combination regimens, a number of clinical trials have tested the effectiveness of IL-12 in cancer patients [174, 331]. Different approaches to clinical trials have been used, such as recombinant IL-12 administration and gene transfer-based therapy. Systemic administration of recombinant IL-12 has been applied in different ways, including intravenous and subcutaneous routes. The former showed an objective response but was associated with severe toxicity, whereas the latter showed tolerable side effects but decreased therapeutic efficacy [218, 332, 333]. In addition, engineering immune cells such as DCs, MQs to express IL-12 has shown to trigger an antitumor immune response in different preclinical models but no therapeutic benefit in clinical trial for metastatic gastrointestinal cancers [334, 335]. Alternatively, adoptive transfer of tumour-specific T cells expressing IL-12 has shown remarkable efficacy of therapeutic T cells in murine models. In the context of TCR-modified T cells, constitutive expression of IL-12 resulted in two main drawbacks including lower proliferative capacity and severe toxicity induced upon transferring more than $0.5x10^6$ T cells [300, 302]. However, the continued interest in IL-12 as a candidate to enhance cancer immunotherapy is undeniable which led to develop ways to regulate IL-12 expression in order to avoid its associated toxicity. On the other hand, IL-27, the IL-12 family member, is an immune-regulatory cytokine that has been found to exhibit antitumor activity with no associated toxicity [282, 336].

Consistent with previous studies, the preliminary *in vivo* experiments presented in this chapter showed that transferring even $0.5x10^6$ T cells transduced with constitutive IL-12 construct in tumour free-mice induced toxicity as measured by weight loss, whereas IL-27 did not. Realising that IL-12 expression needs to be regulated, *Zhang et al* attempted IL-12 regulation using the NFAT-responsive promoter capable to express IL-12 upon TCR triggering. This was associated with cytokine-mediated toxicity in patients [302, 303]. In addition, low affinity TCR interaction with self-antigens associated with homeostatic proliferation has been suggested to activate the

NFAT promoter in adoptively transferred T cells comprising NFAT regulated IL-12 constructs and induce toxicity in lymphodepleted but not lymphreplete mice in the absence of antigen-specific stimulation (Personal communication; Gavin and Ben group).

In this study, I have sought to demonstrate that tet-controlled gene expression system can be used as an alternative approach to regulate the expression of IL-12 and IL-27 in adoptively transferred TCR-redirected T cells in order to avoid any associated adverse effects. Although *in vitro* data suggested that the tet-promoter is leaky, the low level of IL-12 production by the leaky tet-promoter in the absence of Dox induction showed no observable toxicity in the absence or presence of antigen-specific stimulation. This suggested that IL-12 expression can be regulated *in vivo* without mediating lethal systemic toxicity.

Exploring the *in vivo* effects of IL-27 expression on TCR-modified T cell performance was carried out in mice with established EL4-NP tumours. Upon transferring similar numbers of T_{TCR} and $T_{TCR+IL27}$ cells in EL4-NP tumour-bearing mice, similar kinetics of expansion and differentiation phenotype in the peripheral blood and similar persistence in the tissues was observed. However, IL-27 expression seems to maintain $CD4^+$ T cell population during the course of the experiment. In our study, following antigen encounter, both T_{TCR} and $T_{TCR+IL27}$ cells downregulated CD62L and CD127 expression and regained their expression following tumour clearance. This is in line with previous studies showing that CD127 expression is downregulated following antigen encounter on activated $CD8^+$ T cells as they differentiate and re-expressed after antigen clearance on selective effector $CD8^+$ T cells intended to differentiate into memory cells [337-339].

Adoptive transfer of $T_{TCR+IL27}$ cells in tumour-bearing mice displayed no significant difference in tumour rejection compared to T_{TCR} cells. Thus, no improved tumour protection was achieved by IL-27 in the EL4-NP system. The artificial nature of this model is that EL4-thymoma cells were transfected to stably express the influenza virus nucleoprotein (NP).

Chapter 5

Engineered T cells in B16F10 melanoma model

5.1 Introduction

Melanoma is a highly aggressive type of skin cancer, one of the most common types of cancer, which arises from pigment-containing cells called melanocytes in the skin. It is an immunogenic tumour but can be resistant to traditional regimens such as radiation and chemotherapy [281]. In 1988, Rosenberg *et al.* demonstrated cancer regression of metastatic melanoma using adoptive transfer of autologous TILs that were isolated, activated and expanded ex vivo before re-infusion back into the patient, together with IL-2 and pre-treatment with cyclophosphamide to achieve lympho-depletion [340]. However, the success of this approach has been limited to melanoma, while insufficient numbers of TILs were found in most malignancies. Alternatively, T-cell engineering approaches have provided a potential solution to redirect endogenous T cell specificity using retroviral or lentiviral gene transfer. As previously described, TCR gene therapy is based on re-directing T-cell specificity by expressing a specific TCR α and β chains, which mediate the process of antigen-recognition. The feasibility and therapeutic efficacy of TCR gene therapy has been shown in recent clinical trials for melanoma and synovial cells carcinoma [128].

Generating a successful tumour-specific TCR can be achieved by identifying an appropriate tumour antigen to target [106]. However, the most common tumour-associated antigens that can be recognised by CTLs are self-antigens, which are also expressed by normal tissues, and therefore, are subject to the mechanisms of central and peripheral tolerance. For example, melanocyte differentiation antigens, such as gp-100, TRP-1 and TRP-2, are expressed by both melanoma cells and normal melanocytes. TRP-2 is a weak antigen expressed in both human and murine B16 melanoma cells, and presented in the context of MHC class I HLA-A*0201 and H-2K^{b}, respectively [341, 342]. Studies in mice using vaccine regimens including TRP2 peptide-loaded dendritic cells and TRP2-encoding plasmid DNA suggested that tolerance toward TRP2 can be broken and TRP2 can elicit specific CTL response and antitumor immunity *in vivo* without developing autoimmune adverse events [341, 343, 344]. Therefore, TRP2 could be a good tumour associated antigen candidate for cancer immunotherapy. Recent studies using TRP2 TCR transgenic (TCR Tg) mouse model demonstrated that naïve TRP2 TCR Tg T cells primed *in vivo* can infiltrate the tumour and maintain effector function, despite being unable to control tumour growth of established primary melanoma [345].

As pointed out previously, tumour-specific T cells equipped with additional IL-12 have been shown to enhance the therapeutic efficacy of adoptively transferred T cells and increase tumour immunogenicity in pre-clinical models. IL-12 holds promise in cancer treatment due to its capacity to modulate antitumor immune response through different mechanisms, such as reversing dysfunctional myeloid-derived cells in the tumour by generating an acute inflammatory environment [195], and up-regulating Fas expression on myeloid-derived cells in the tumour that induces Fas/FasL mediated-killing [196]. However, the clinical application of IL-12 not only revealed minimal efficacy but also associated with severe systemic toxicity [218, 281, 301, 302]. Given the limitations of systemic IL-12 expression, controlled expression of IL-12 using an NFAT-responsive promoter resulted in intolerable side effects in patients [302, 303]. Based on our *in vivo* preliminary data presented in chapter 4, tet-regulated IL-12 expression did not cause toxicity *in vivo*.

Unlike IL-12, IL-27 is less toxic than IL-12; It has been demonstrated that IL-27 can boost antitumor immunity via different mechanisms, such as enhancing proliferation and effector functions of CTLs, and cytotoxic activity of NK cells [220, 227]. In the setting of B16F10 murine melanoma model, IL-27 has been demonstrated to possess anti-proliferative effect directly on tumour cells [273, 275].

5.2 Aims

Tumours can evade antitumor immune responses by mechanisms that may include suppression of TRP2-reactive T cells in the tumour microenvironment. In this chapter, the main aim is to test whether additional cytokine regimens can sufficiently boost the therapeutic efficacy of these TRP2-specific T cells and induce more potent tumour immunity while avoiding lethal systemic toxicity. Here, I have sought to assess the effect of regulated IL-12 and IL-27 expression in TRP2 TCR-redirected T cells in the setting of established B16F10 melanoma model. In this chapter, I have explored the following working hypothesis:

- Induced IL-12 and IL-27 expression in TRP2 TCR-redirected T cells results in increased tumour infiltration.
- Induced IL-12 and IL-27 expression changes the phenotype of tumour infiltrating T cells.
- Induced IL-12 and IL-27 expression improves tumour protection of TRP2-specific T cells.
- Improved tumour protection occurs in the absence of increased toxicity.
- Tumour escape is not associated with antigen loss.

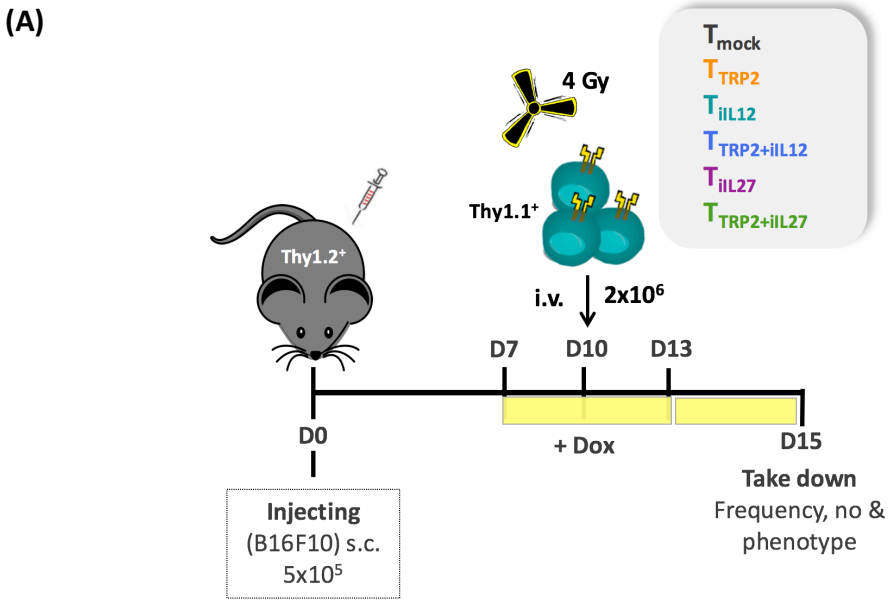
5.3 Results

5.3.1 Regulated IL-12 and IL-27 expression in TRP2 TCR-redirected T cells increases the accumulation of adoptively transferred T cells in the tumour

In vivo tumour challenge experiments were carried out to characterize the frequencies, total numbers, and phenotypic status of adoptively transferred T cells after infusion. C57BL/6 recipient mice, expressing the congenic marker Thy1.2, were subcutaneously injected with B16F10 melanoma cells (5 x 10^5 cells). 10 days later, mice had established tumours and were sublethally irradiated with 4Gy TBI prior to intravenous injection of 2 x 10^6 transduced T cells (Figure 6.1 A). The adoptive transferred T cells were obtained from C57BL/6 donor mice expressing the congenic marker Thy1.1 to enable transferred cell tracking in the recipient mice. These cells were transduced with TRP2 TCR only (T_{TRP2}), TRP2 TCR + iIL12 ($T_{TRP2+iIL12}$), TRP2 TCR + iIL27 ($T_{TRP2+iIL27}$) or mock-transduced T cells (T_{mock}). Additional control groups receiving either iIL12 (T_{iIL12}) or iIL27 (T_{iIL27}) transduced T cells (cytokine only) were also included in these experiments. All mice received Dox water (2 mg/ml) 2-3 days prior to receiving T cell infusions and kept on Dox for 3 days post-T cell injection for those groups received IL-12 treatment. Throughout the chapter, I am using colour-coding figures including: T_{TRP2} (orange), $T_{TRP2+iIL12}$ (blue), $T_{TRP2+iIL27}$ (green), T_{mock} (black), T_{iIL12} (teal), T_{iIL27} (purple). 3 days post T cell transduction and prior to T cell infusion, transduction efficiency was evaluated by CD19 (also by Vβ3) and Q8 (hCD34) expression to assess the transduction of TRP2 TCR and inducible vectors including iIL12 and iIL27, respectively. CD4:CD8 composition and phenotypic profile of the transduced cells were also identified prior to T cell transfer by staining the cells for the following markers; CD4 and CD8, CD62L and CD127, respectively. CD62L and CD127 markers were used to separate cells into end stage effector T cells ($CD62L^{lo}CD127^{lo}$), effector memory T cells ($CD62L^{lo}$ $CD127^{hi}$) and central memory T cells ($CD62L^{hi}$ $CD127^{hi}$). Flow cytometric analysis demonstrated similar transduction efficiency and a good level of double transduction with TCR+cytokine. Surface expression of CD62L and CD127 indicated that all transduced cells have similar phenotypes. Yet, it seems that $T_{TRP2+iIL12}$ and T_{iIL12} cells retain more of $CD62L^{hi}$ $CD127^{hi}$ (less differentiated cells) (Figure 6.1 B). At day 5 post-T cell transfer, mice were sacrificed and established tumours were excised and weighed to calculate the total number of cells per gram of tumour. Tissue samples

were also obtained from bone marrow, spleen and lymph nodes for further analysis. Single cell suspensions were prepared from all tissues and stained with anti-murine Thy1.1 to identify the transferred T cells. Gating on Thy1.1 revealed a marginal increase in the frequency of transferred T cells in the group of mice that received T_{TRP2} cells compared to recipients of T_{mock} cells. By contrast, a substantial increase in the frequency of adoptively transferred T cells was observed in the group of mice that received $T_{TRP2+iIL12}$ compared to mice that received T_{TRP2} cells (Figure 5.2 A). This increase in the percentage of $T_{TRP2+iIL12}$ cells was found in all analysed tissues including tumour, bone marrow, spleen and lymph nodes ($p<0.0001$). Interestingly, the group of mice that received $T_{TRP2+iIL27}$ cells showed an increase in the proportion of transferred T cells in the tumour, spleen and bone marrow ($p<0.05$) but not in the lymph nodes ($p>0.05$) compared to mice that received T_{TRP2} cells.

Furthermore, to identify whether the increased accumulation of transferred T cells is driven by TCR specificity or a cytokine-driven effect, I compared the group of mice that received $T_{TRP2+iIL12}$ and $T_{TRP2+iIL27}$ cells to the group of mice that received T_{iIL12} and T_{iIL27} cells, respectively. This demonstrated that the adoptive transfer of T_{iIL12} cells mediated a slight increase in the proportion of transferred T cells in all analysed tissues but not as much as $T_{TRP2+iIL12}$ cells, whereas the adoptive transfer of T_{iIL27} cells were barely detectable compared to $T_{TRP2+iIL27}$ cells, suggesting that the enhanced accumulation of transferred T cells is driven by TCR specificity. In addition, the total number of viable transferred T cells was also determined, indicating that the adoptive transfer of $T_{TRP2+iIL12}$ cells also showed an increase in the total cell numbers, although it was less pronounced than the relative frequency, whereas no significant increase was observed in $T_{TRP2+iIL27}$ cells (Figure 5.2 B) . This suggests that IL-12, and to a lesser extend IL-27, might had an effect on reducing other cells in the live lymphocyte gate resulted in a relative increase in the transferred T cell frequencies. Alternatively, it might be technically difficult to get a robust total number of cells especially when it comes to isolating 1 gram of tumour. Together, these data suggest that TCR expression is required for IL-12 and IL-27 to facilitate the accumulation of transferred T cells. The enhancement effect of IL-12 was seen in almost all tissues, whereas the effect of IL-27 was dominant inside the tumour.


(A)
Tmock
TTRP2
TiIL12
TTRP2+iIL12
TiIL27
TTRP2+iIL27
4 Gy
Thy1.1+
Thy1.2+
i.v.
2x10^6
D7
D10
D13
D0
+ Dox
D15
Take down
Frequency, no & phenotype
Injecting
(B16F10) s.c.
5x10^5

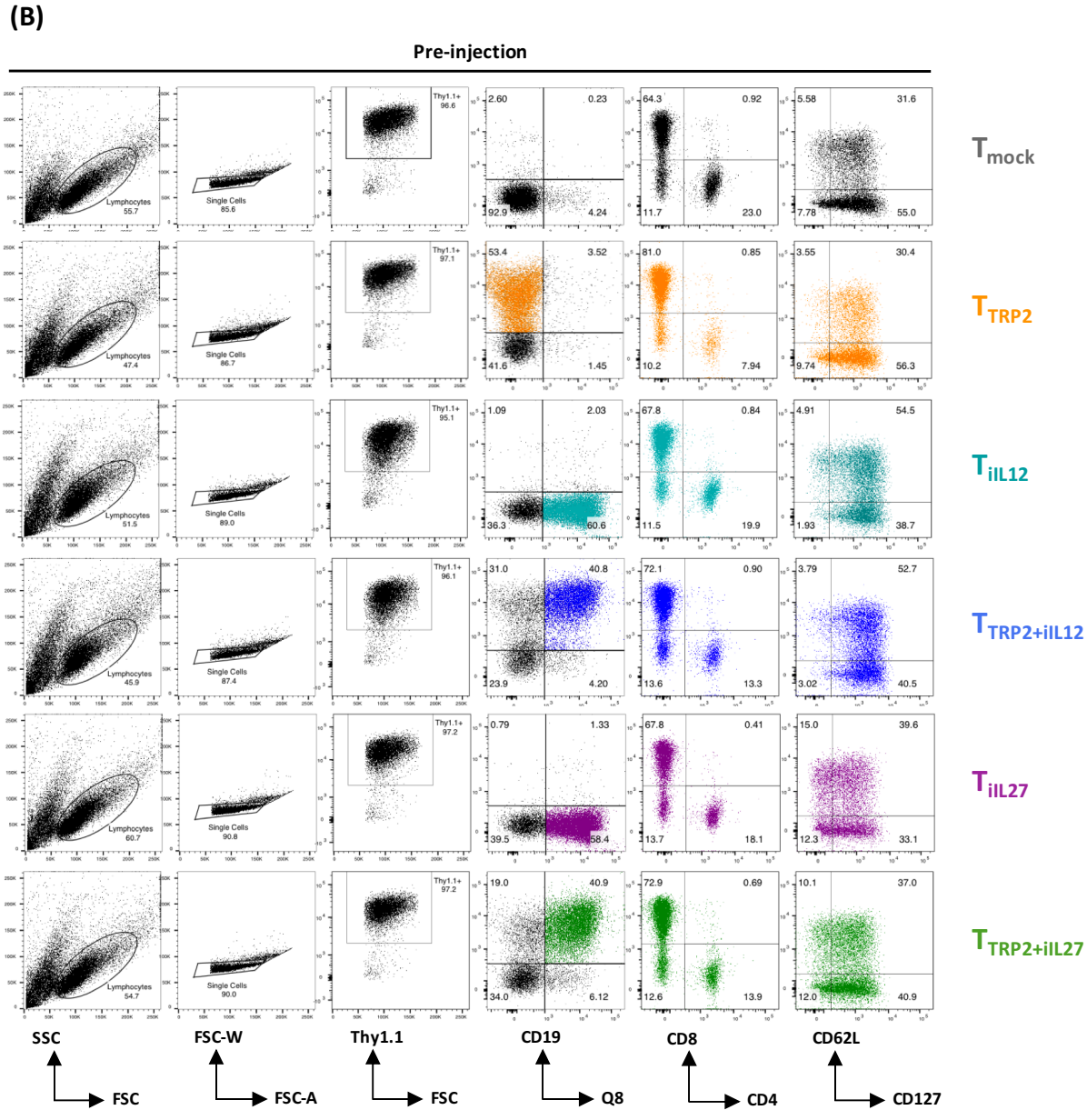
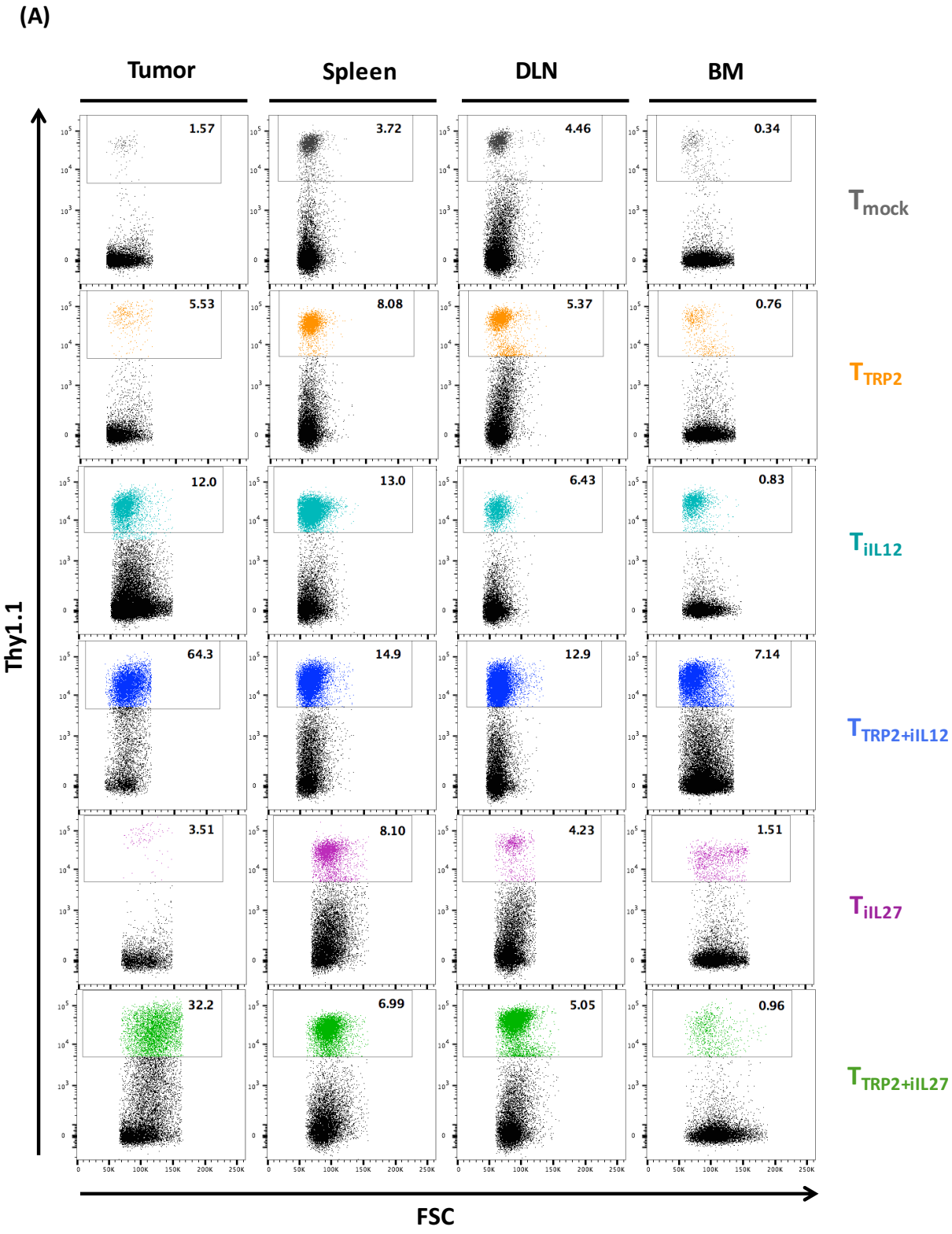

(B)
Pre-injection
Tmock
TTRP2
TiIL12
TTRP2+iIL12
TiIL27
TTRP2+iIL27
SSC
FSC
FSC-W
FSC-A
Thy1.1
FSC
CD19
Q8
CD8
CD4
CD62L
CD127

Figure 5.1 | Transduction efficiency, CD4:CD8 composition and phenotype of transferred T cells prior to injection. (A) Experimental layout. **(B)** Flow cytometric blots showing good transduction efficiency assessed by CD19 and Q8 expression including: T_{TRP2} ($CD19^+$ = 53.4%), T_{iIL12} ($Q8^+$ = 60.6), T_{iIL27} ($Q8^+$ = 58.4), $T_{TRP2+iIL12}$ ($CD19^+Q8^+$ = 40.8) and $T_{TRP2+iIL27}$ ($CD19^+Q8^+$ = 40.9). CD4/CD8 staining profile showed a slight skewing toward $CD8^+$ T cells when cells transduced with TCR (T_{TRP2}, $T_{TRP2+iIL12}$, $T_{TRP2+iIL27}$). CD62L and CD127 expression revealed similar differentiation status of transduced T cells. Yet, it seems that $T_{TRP2+iIL12}$ and T_{iIL12} cells retain a less differentiated phenotype, more of $CD62L^{hi}CD127^{hi}$ cells. Data shown are representative of two independent experiments.


(A)
Tumor
Spleen
DLN
BM
Thy1.1
FSC
1.57
3.72
4.46
0.34
5.53
8.08
5.37
0.76
12.0
13.0
6.43
0.83
64.3
14.9
12.9
7.14
3.51
8.10
4.23
1.51
32.2
6.99
5.05
0.96
Tmock
TTRP2
TiIL12
TTRP2+iIL12
TiIL27
TTRP2+iIL27

(B)

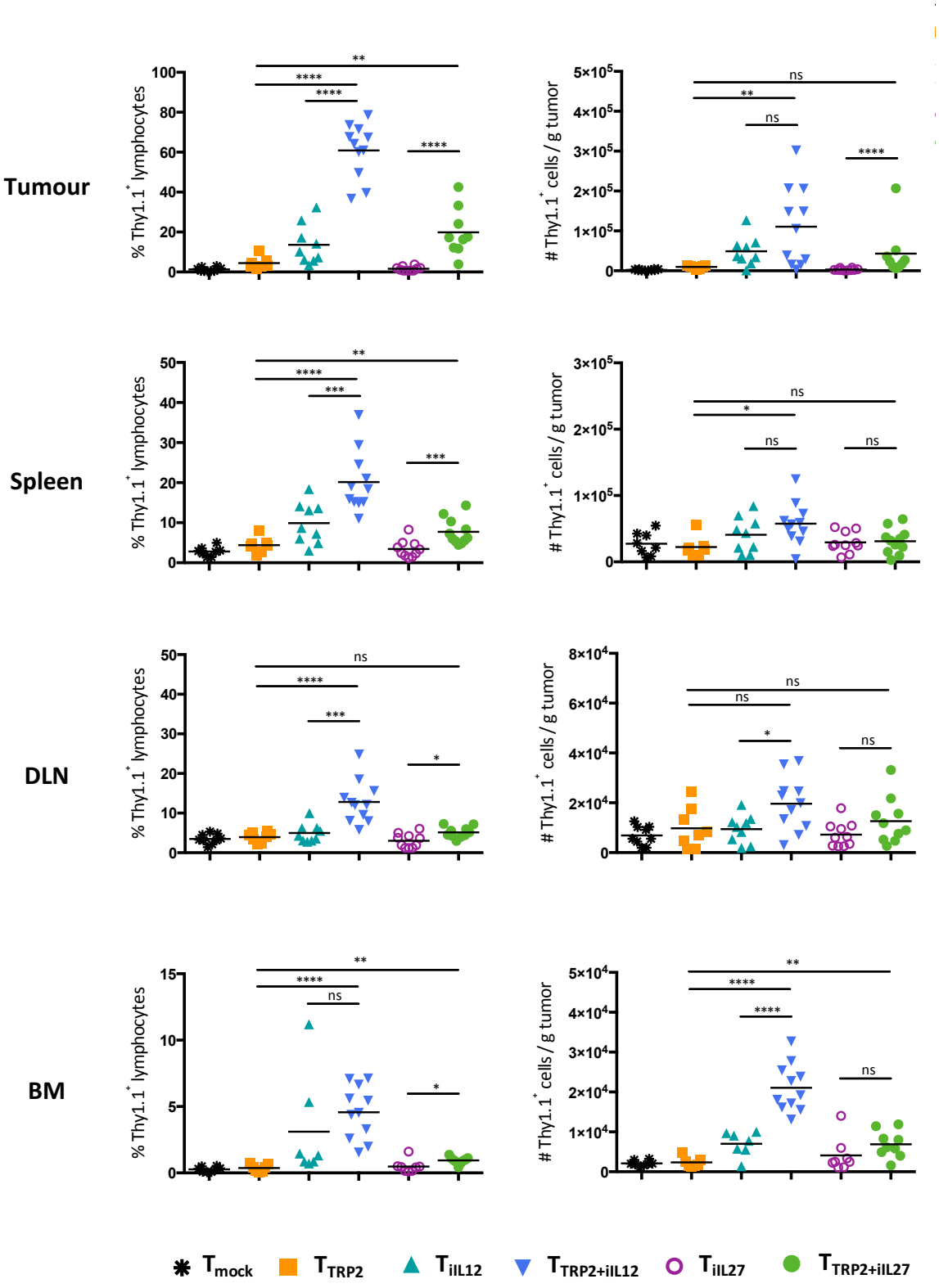
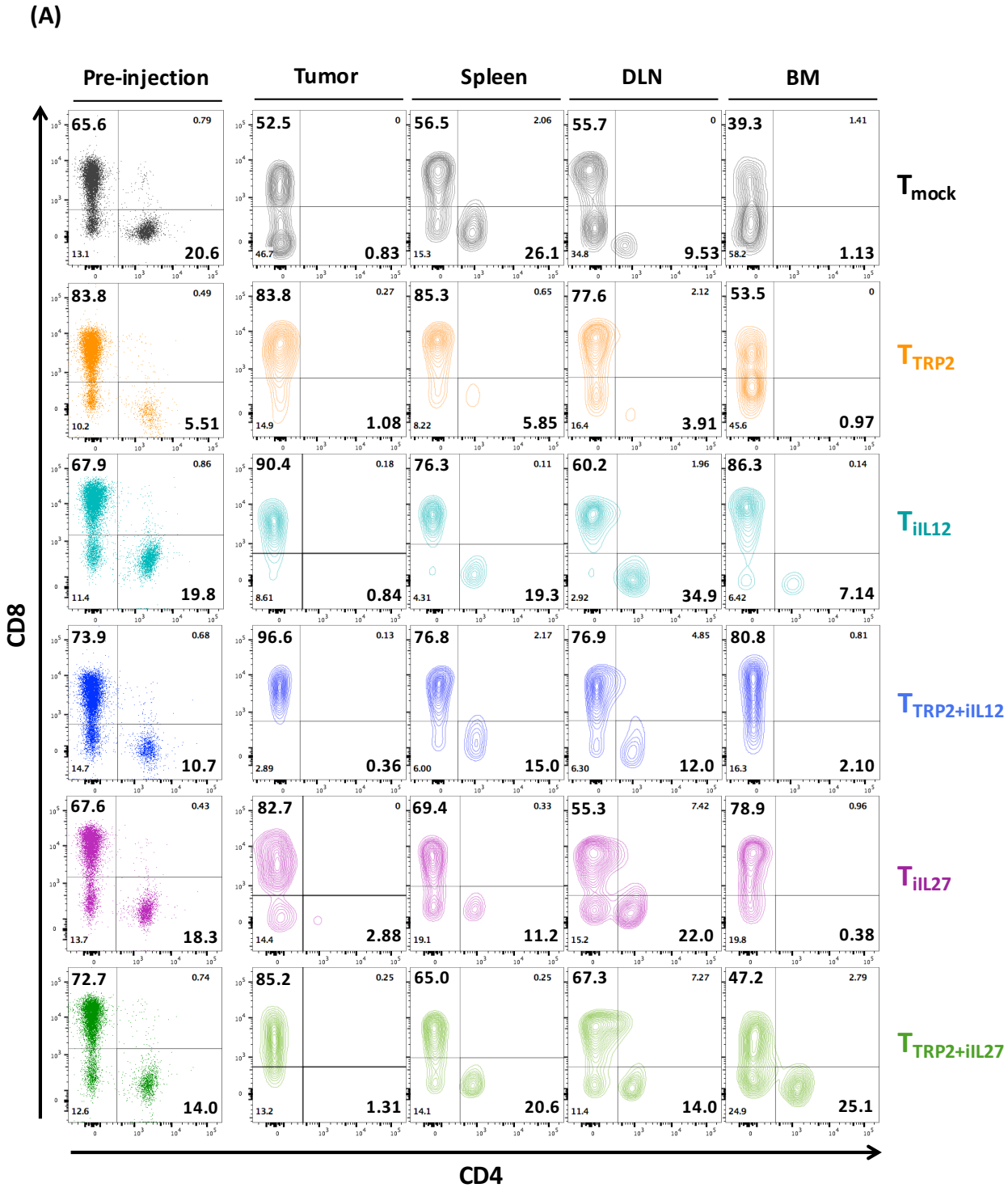

Tumour
Spleen
DLN
BM
% Thy1.1+ lymphocytes
# Thy1.1+ cells / g tumor
ns
*
**
***
****
T_{mock}
T_{TRP2}
T_{iIL12}
$T_{TRP2+iIL12}$
T_{iIL27}
$T_{TRP2+iIL27}$

Figure 5.2 | Adoptive transfer of TRP2 TCR-redirected T cells expressing regulated IL-12 and IL-27 facilitates the accumulation of transferred T cells in the tumour. C57BL/6 mice bearing subcutaneous B16F10 melanoma were sub-lethally irradiated with 4Gy TBI and treated with T_{TRP2}, $T_{TRP2+iIL12}$, $T_{TRP2+iIL27}$, T_{iIL12}, T_{iIL27} or T_{mock} cells 10 days post tumour injection. All mice received Dox water (2 mg/ml) 2-3 days prior to receiving T cell therapy and kept on Dox for 3 days. **(A)** Representative blots showing Thy1.1, marked the adoptively transferred T cells, surface expression at day 5 post T-cell injection in tumour, spleen, lymph nodes and bone marrow. **(B)** Graphs showing summary data of frequencies and absolute cell numbers of transferred cells ($Th1.1^{+}$) in all analysed tissues. Cells were pre-gated on PI^{-} singlet lymphocytes. Data shown are pooled results from two independent experiments. Symbols represent individual mice and bars show group averages. Statistical significance was tested by two-tailed Mann Whitney test: frequency of T_{TRP2} versus $T_{TRP2+iIL12}$ in all tissues *P value* < 0.0001; frequency of T_{TRP2} versus $T_{TRP2+iIL27}$ in tumour and bone marrow *P value* = 0.0012, in spleen *P value* = 0.0083, in DLN *P value* > 0.05; frequency of $T_{TRP2+iIL12}$ versus T_{iIL12} in tumour *P value* < 0.0001, in spleen *P value* = 0.0009, in DLN *P value* = 0.0004, in BM *P value* >0.05; frequency of $T_{TRP2+iIL27}$ versus T_{iIL27} in tumour *P value* < 0.0001, in spleen *P value* = 0.0008, in DLN *P value* = 0.0115, in BM *P value* = 0.0199; total number of T_{TRP2} versus $T_{TRP2+iIL12}$ in tumour *P value* = 0.0028, in spleen *P value* = 0.0114, in DLN *P value* >0.05, in BM *P value* < 0.0001; total number of T_{TRP2} versus $T_{TRP2+iIL27}$ in tumour, spleen and DLN *P value* > 0.05, in BM *P value* = 0.0033; Total number of $T_{TRP2+iIL12}$ versus T_{iIL12} in tumour and spleen *P value* > 0.05, in DLN *P value* = 0.0250, in BM *P value* < 0.0001; total number of $T_{TRP2+iIL27}$ versus T_{iIL27} in tumour *P value* < 0.0001, in spleen, DLN and BM *P value* > 0.05. Number of mice per group: T_{mock}, *n*= 10; T_{TRP2}, *n*= 8; $T_{TRP2+iIL12}$, *n*= 11; $T_{TRP2+iIL27}$, *n*= 10; T_{iIL12}, *n*= 9; T_{iIL27}, *n*= 10.

5.3.2 Selective enrichment of adoptively transferred CD8$^+$ T cells in the tumour

Next, I asked what is the relative frequency of CD4$^+$ and CD8$^+$ T cells within the transferred T cells in each analysed tissue compared to the defined CD4$^+$:CD8$^+$ composition before infusing T cells infusion. Cells harvested from tumours, spleen, lymph nodes and bone marrow of B16F10 tumour-bearing C57B6/L mice 5 days after the adoptive cell transfer were stained with antibodies to CD4 and CD8 in addition to Thy1.1 and analysed by flow cytometry. The staining profile of CD4/CD8 was gated on live singlet Thy1.1$^+$ transferred T cells. Flow cytometric analysis revealed a selective accumulation of CD8$^+$ transferred T cells in all treated groups of mice in the tumour. This accumulation occurred irrespective of the TCR specificity or IL-12 and IL-27 expression, as it was also observed in the group of mice treated with T_{mock} cells. Similar selection of CD8$^+$ T cells was found in the bone marrow, with the exception that IL-27 seems to select for CD4$^+$ T cells in the bone marrow when combined with TCR ($T_{TRP2+iIL27}$), and IL-12 seems to select for CD4$^+$ T cells in the absence of TCR (T_{iIL12}). In contrast, the CD4/CD8 staining profile in the peripheral lymphoid organs, spleen and draining lymph nodes, did not significantly change compared to the pre-injection relative frequency (Figure 6.3).


(A)
Pre-injection
Tumor
Spleen
DLN
BM
CD8
CD4
T mock
T TRP2
T iIL12
T TRP2+iIL12
T iIL27
T TRP2+iIL27
65.6 0.79 13.1 20.6
52.5 0 46.7 0.83
56.5 2.06 15.3 26.1
55.7 0 34.8 9.53
39.3 1.41 58.2 1.13
83.8 0.49 10.2 5.51
83.8 0.27 14.9 1.08
85.3 0.65 8.22 5.85
77.6 2.12 16.4 3.91
53.5 0 45.6 0.97
67.9 0.86 11.4 19.8
90.4 0.18 8.61 0.84
76.3 0.11 4.31 19.3
60.2 1.96 2.92 34.9
86.3 0.14 6.42 7.14
73.9 0.68 14.7 10.7
96.6 0.13 2.89 0.36
76.8 2.17 6.00 15.0
76.9 4.85 6.30 12.0
80.8 0.81 16.3 2.10
67.6 0.43 13.7 18.3
82.7 0 14.4 2.88
69.4 0.33 19.1 11.2
55.3 7.42 15.2 22.0
78.9 0.96 19.8 0.38
72.7 0.74 12.6 14.0
85.2 0.25 13.2 1.31
65.0 0.25 14.1 20.6
67.3 7.27 11.4 14.0
47.2 2.79 24.9 25.1

(B)

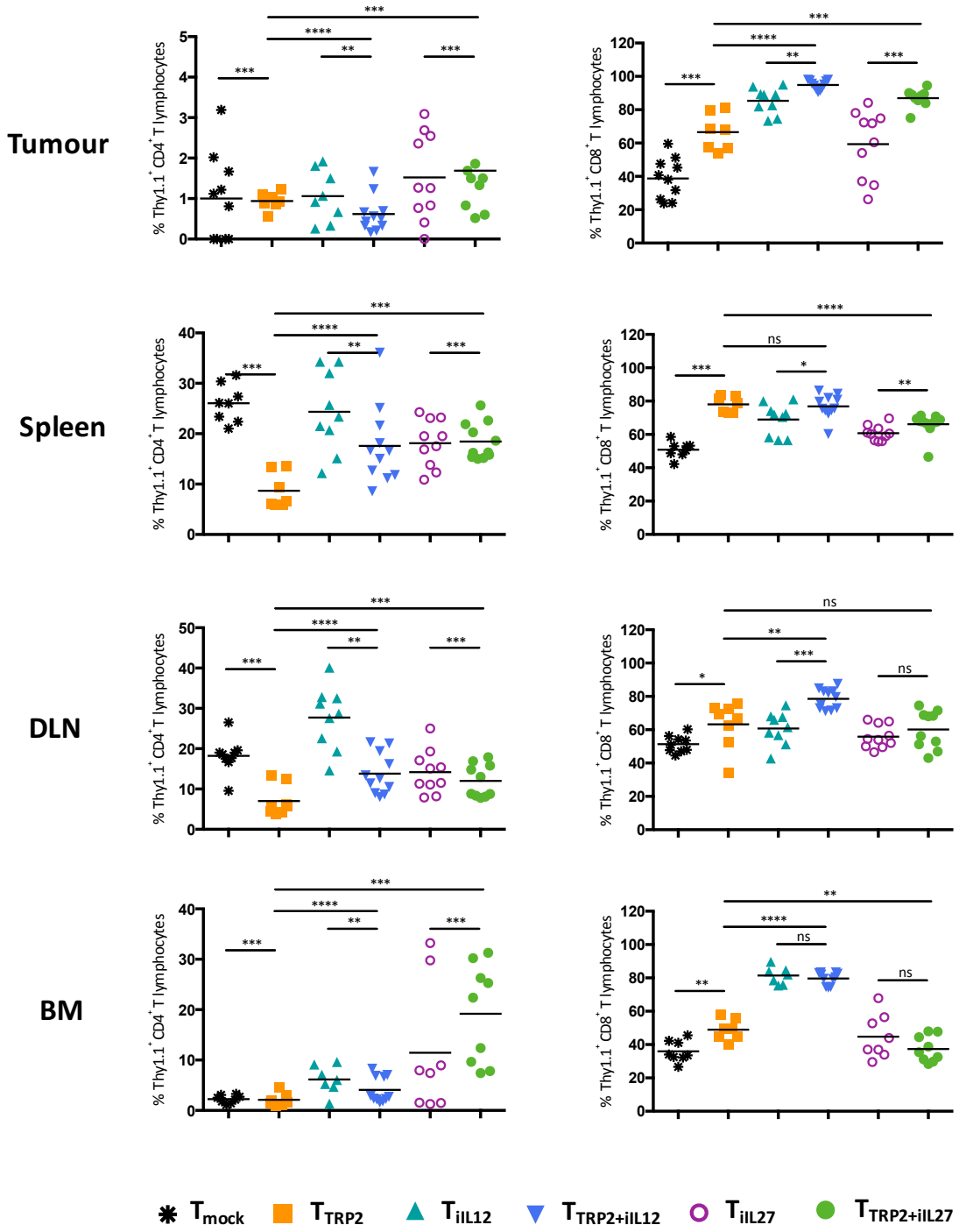
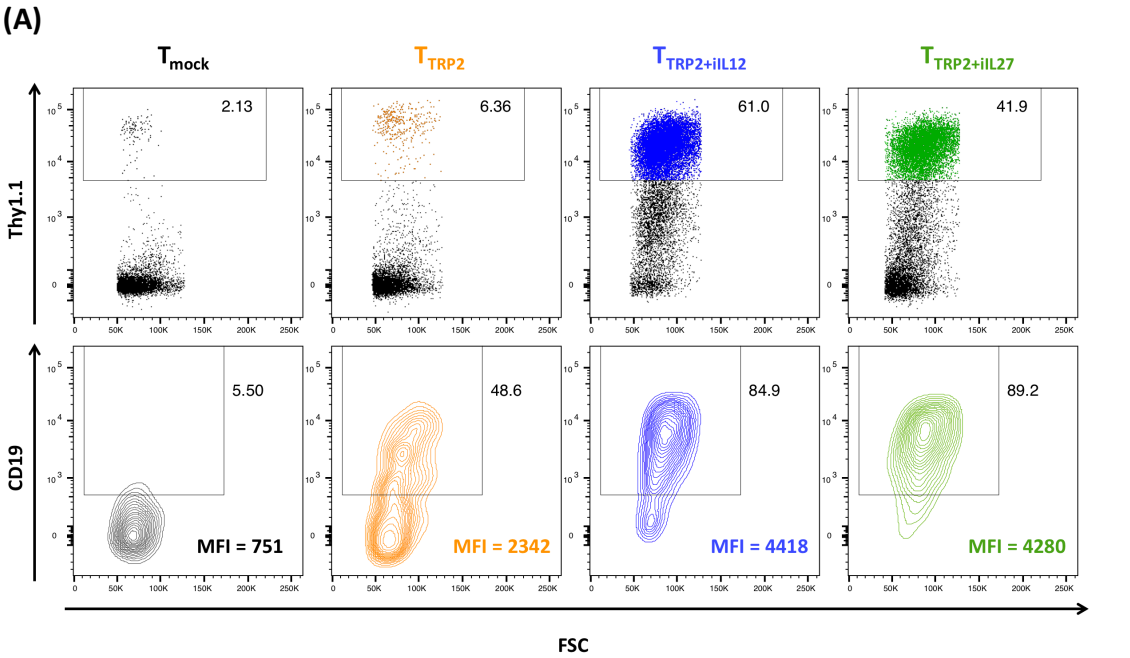

Tumour
Spleen
DLN
BM
% Thy1.1+ CD4+ T lymphocytes
% Thy1.1+ CD8+ T lymphocytes
Tmock
TTRP2
TiIL12
TTRP2+iIL12
TiIL27
TTRP2+iIL27

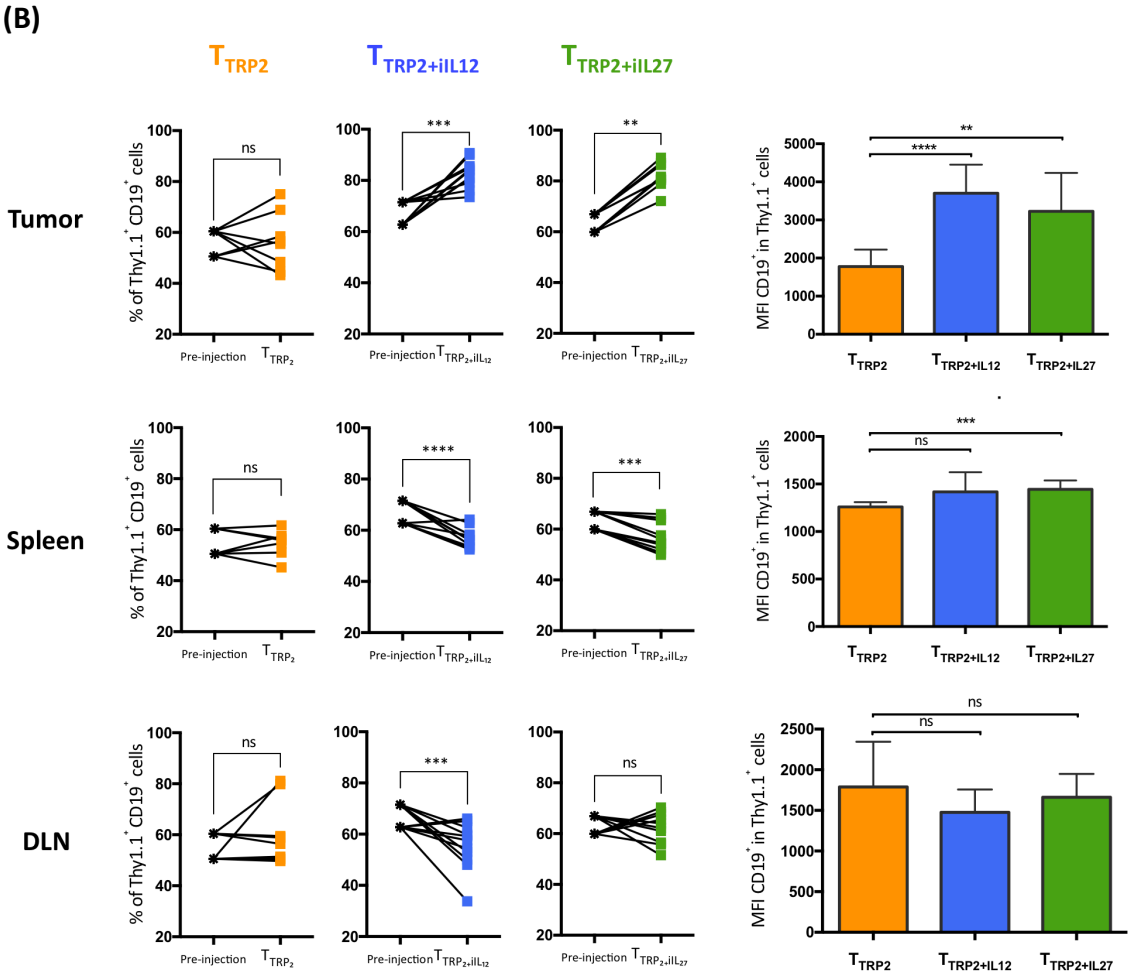
Figure 5.3 | Selective accumulation of transferred CD8$^+$ T cells in the tumour in all treated mice. C57BL/6 mice bearing subcutaneous B16F10 melanoma were sub-lethally irradiated with 4Gy TBI and treated with T_{TRP2}, $T_{TRP2+iIL12}$, $T_{TRP2+iIL27}$, T_{iIL12}, T_{iIL27} or T_{mock} 10 days after tumour injection. All mice received Dox water (2 mg/ml) 2-3 days before receiving T cell infusions and kept on for 3 days. **(A)** Flow cytometry blots representing the CD4 and CD8 expression profile at d5 post T cell transfer in tumour, spleen, DLN and BM compared to pre-injection relative frequency. **(B)** Summary graphs showing the frequencies of CD4$^+$ and CD8$^+$ T cells within transferred cells (Th1.1$^+$) in all analysed tissues. Cells were pre-gated on PI$^-$ singlet Th1.1$^+$ lymphocytes. Data shown are pooled results from two independent experiments. Symbols represent individual mice and bars show group averages. Statistical significance was tested by two-tailed Mann Whitney test: frequency of T_{mock} versus T_{TRP2} in tumour p >0.05, in spleen and DLN p =0.0003, in the BM p >0.05; T_{TRP2} versus $T_{TRP2+iIL12}$ in tumour p= 0.0468, in spleen p =0.0059, in DLN p =0.0053, in BM p =0.0548; T_{TRP2} versus $T_{TRP2+iIL27}$ in tumour p >0.05, in spleen p <0.0001, in DLN p =0.0085, in BM p =0.0002; $T_{TRP2+iIL12}$ versus T_{iIL12} in tumour, spleen and BM p >0.05, in DLN p =0.0005; $T_{TRP2+iIL27}$ versus T_{iIL27} in all tissues p >0.05. Number of mice per group: T_{mock}, n= 10; T_{TRP2}, n= 8; $T_{TRP2+iIL12}$, n= 11; $T_{TRP2+iIL27}$, n= 10; T_{iIL12}, n= 9; T_{iIL27}, n= 10.

5.3.3 Selective accumulation of TCR-expressing T cells in the presence of IL-12 and IL-27 in the tumour

Having shown that tumour infiltration by adoptively transferred T cells increases in mice treated with TRP2 TCR-transduced T cells secreting IL-12 or IL-27, I next determined the frequency and the levels of introduced TRP2 TCR within the transferred T cells in the tumour, spleen and draining lymph nodes compared to the pre-injection values. TCR-expressing T cells were identified by staining the cells for CD19, which is the reporter gene in the TRP2 TCR construct. These cells were also identified by staining the cells with anti-Vβ3 antibody, which binds to the TRP2 TCR Vβ region. The analysis was performed within the transferred T cells gated on Thy1.1^{+} T cells to test the relative accumulation of the TRP2 TCR-expressing T cells. As illustrated in figure 5.4 A, TCR expression within the transferred T cells in the tumour was higher in $T_{TRP2+iIL12}$ and $T_{TRP2+iIL27}$ cells compared to T_{TRP2} cells indicated by relative frequencies and MFI of CD19 expression (Figure 5.4 A). Data in figure 5.4 B reveal that there was no significant difference in the proportion of TCR^{+} cells when T_{TRP2} cells were adoptively transferred compared to their pre-injection frequency. In contrast, in mice treated with $T_{TRP2+iIL12}$ cells, there was a significant increase in the frequency of TCR-expressing cells in the tumour ($p=0.0010$) and a relative decrease, outside the tumor, in the secondary lymphoid organs, including the spleen and draining lymph nodes ($p<0.005$). This indicates that IL-12 mediates the accumulation of TCR-expressing cells in the tumour resulting in a lower frequency of these cells in the spleen and draining lymph nodes. Similarly, in the group of mice that received $T_{TRP2+iIL27}$ cells, IL-27 led also to a statistically significant increase in the frequency of TCR-expressing cells in the tumour ($p=0.0039$), which was associated with decreased frequency in the spleen but not the draining lymph nodes. Moreover, IL-12 and IL-27 not only increased the frequencies of TCR-expressing cells in the tumour but also increased the level of TCR expression per cell (MFI), indicating that co-transduction of TCR with IL-12 or IL-27 resulted in increased TCR surface expression (Figure 6.4 B). This observation was further confirmed by analysing the expression profile of Vβ3, as a surrogate marker of TCR expression (Figure 5.4 C). These data indicate the ability of IL-12 and IL-27 to mediate the selective accumulation of TRP2 TCR-expressing cells in the tumour, and that the TCR expression levels are considerably higher than the level seen in T_{TRP2} cells. Finally, I

have used Ki-67 (a nuclear proliferation marker) to analyse the proliferation profile of transferred T cells. This revealed a high proliferation rate of $T_{TRP2+iIL12}$ and $T_{TRP2+iIL27}$ cells with >80% were $V\beta3^{+}Ki\text{-}67^{+}$ compared to TCR-negative cells and endogenous T cells (Figure 5.5). Together, these data indicated that antigen specificity is required for TCR up-regulation, and enhancement of TCR expression is indeed driven by IL-12 and IL-27 cytokines.


(A)
T mock
T TRP2
T TRP2+iIL12
T TRP2+iIL27
Thy1.1
CD19
2.13
6.36
61.0
41.9
5.50
48.6
84.9
89.2
MFI = 751
MFI = 2342
MFI = 4418
MFI = 4280
FSC


(B)
T TRP2
T TRP2+iIL12
T TRP2+iIL27
Tumor
Spleen
DLN
% of Thy1.1+ CD19+ cells
MFI CD19+ in Thy1.1+ cells
Pre-injection
ns
***
**
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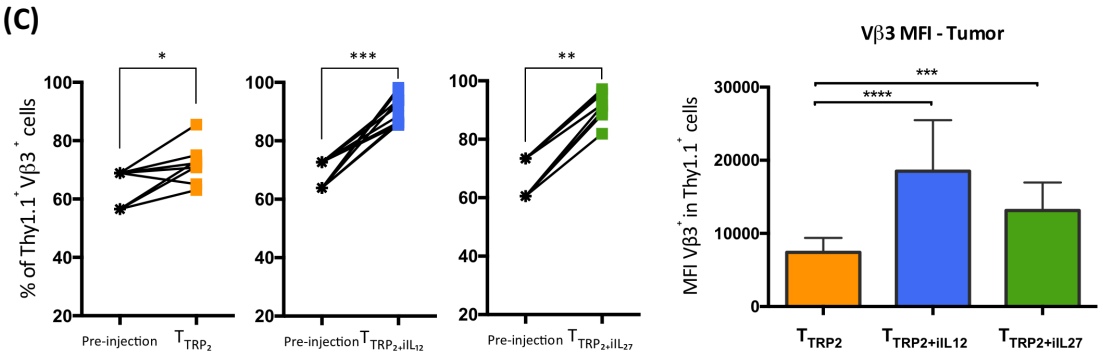
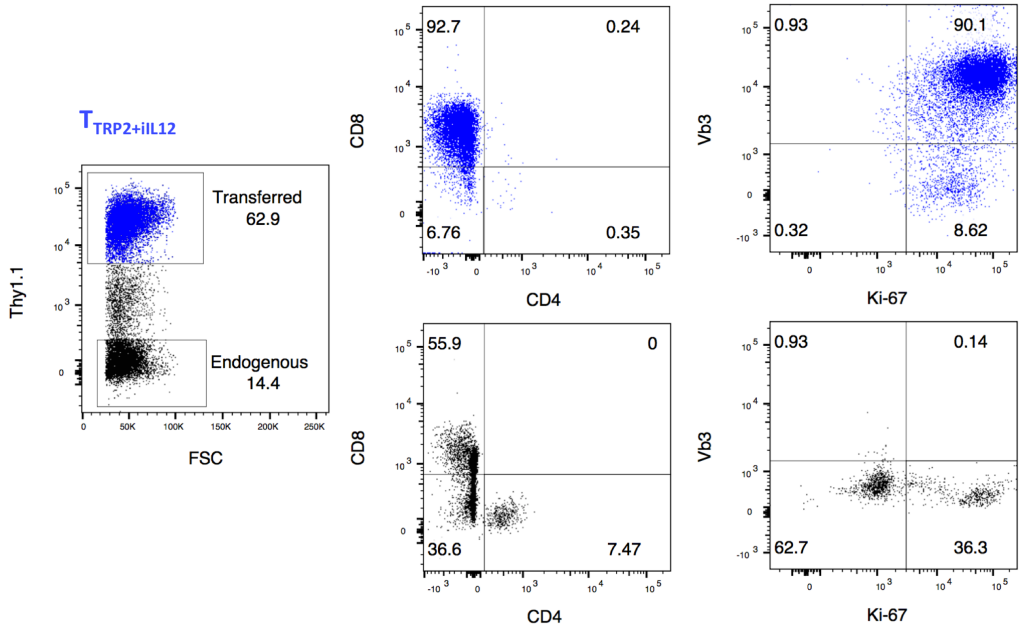
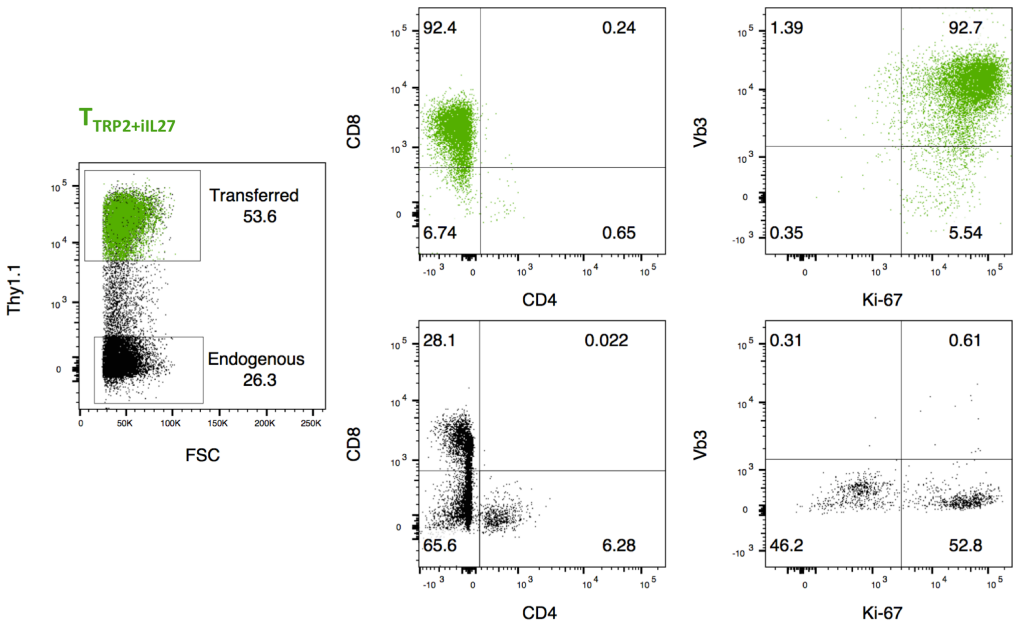


Figure 5.4 | IL-12 and IL-27 expression increases the introduced TRP2 TCR surface expression in the tumour. C57BL/6 (Thy1.2$^+$) mice were challenged subcutaneously with 5x10^5 B16F10 melanoma cells. 10 days later, mice had established tumours and were sub-lethally irradiated with 4Gy TBI before intravenous infusion of 2x10^6 transduced T cells (Th1.1$^+$) including: T_{TRP2}, $T_{TRP2+iIL12}$, $T_{TRP2+iIL27}$, T_{iIL12}, T_{iIL27} or T_{mock} cells. All mice received Dox water (2 mg/ml) 2-3 days prior receiving the T cells and kept on Dox for 3 days. **(A)** Representative flow cytometry blots displaying the frequency and MFI of CD19 expression, marked the TCR-expressing cells, within the transferred T cells (Thy1.1$^+$) including T_{TRP2}, $T_{TRP2+iIL12}$, $T_{TRP2+iIL27}$ cells in the tumour 5 days post injection. Cells were pre-gated on PI$^-$ singlet lymphocytes. **(B)** Summary graphs showing the frequency of CD19 expression in T_{TRP2}, $T_{TRP2+iIL12}$, $T_{TRP2+iIL27}$ cells relative to pre-injection *(left)* and CD19 MFI *(right)* in the tumour, spleen and draining lymph nodes. Statistical significance was tested by two-tailed Wilcoxon matched-pairs signed rank test: T_{TRP2} versus pre-injection in the tumour, spleen and DLNs *P value* > 0.05; $T_{TRP2+iIL12}$ versus pre-injection in the tumour *P value* = 0.0010, in the spleen *P value* = 0.0020 and in the DLNs *P value* = 0.0049; $T_{TRP2+iIL27}$ versus pre-injection in the tumour *P value* = 0.0039, in the spleen *P value* = 0.0010 and DLNs *P value* > 0.05. CD19 MFI (using two-tailed Mann Whitney test): T_{TRP2} versus $T_{TRP2+iIL12}$ in the tumour *P value* <0.0001, in the spleen and draining lymph nodes *P value* >0.05; T_{TRP2} versus $T_{TRP2+iIL27}$ in the tumour *P value* = 0.0072; in the spleen *P value* = 0.0001 and in the DLNs *P value* >0.05. **(C)** Increased TCR expression in the tumour was confirmed by Vβ3 staining profile. Summary graphs showing the frequency of Vβ3, also marked TCR-expressing cells, in T_{TRP2}, $T_{TRP2+iIL12}$, $T_{TRP2+iIL27}$ cells relative to the pre-injection frequency *(left)* and Vβ3 MFI *(right)* in the tumour. Statistical significance was tested by two-tailed Wilcoxon matched-pairs signed rank test: frequency of T_{TRP2} versus pre-injection *P value* = 0.0188; $T_{TRP2+iIL12}$ versus pre-injection and $T_{TRP2+iIL27}$ versus pre-injection *P value* < 0.0001. Vβ3 MFI (using two-tailed Mann Whitney test): T_{TRP2} versus $T_{TRP2+iIL12}$ *P value* <0.0001; T_{TRP2} versus $T_{TRP2+iIL27}$ *P value* = 0.0006. Symbols represent individual mice (T_{TRP2}, n= 8; $T_{TRP2+iIL12}$, n= 11; $T_{TRP2+iIL27}$, n= 10).

(A)


$T_{TRP2+iIL12}$
Thy1.1
FSC
Transferred
62.9
Endogenous
14.4
CD8
CD4
92.7
0.24
6.76
0.35
55.9
0
36.6
7.47
Vb3
Ki-67
0.93
90.1
0.32
8.62
0.93
0.14
62.7
36.3


$T_{TRP2+iIL27}$
Thy1.1
FSC
Transferred
53.6
Endogenous
26.3
CD8
CD4
92.4
0.24
6.74
0.65
28.1
0.022
65.6
6.28
Vb3
Ki-67
1.39
92.7
0.35
5.54
0.31
0.61
46.2
52.8

(B)

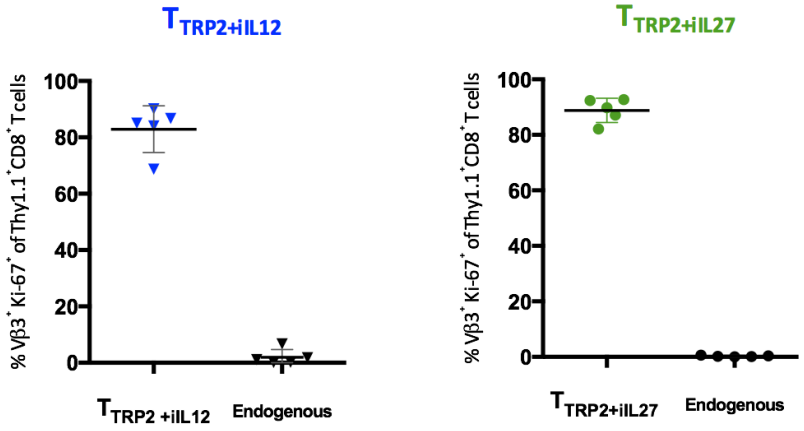
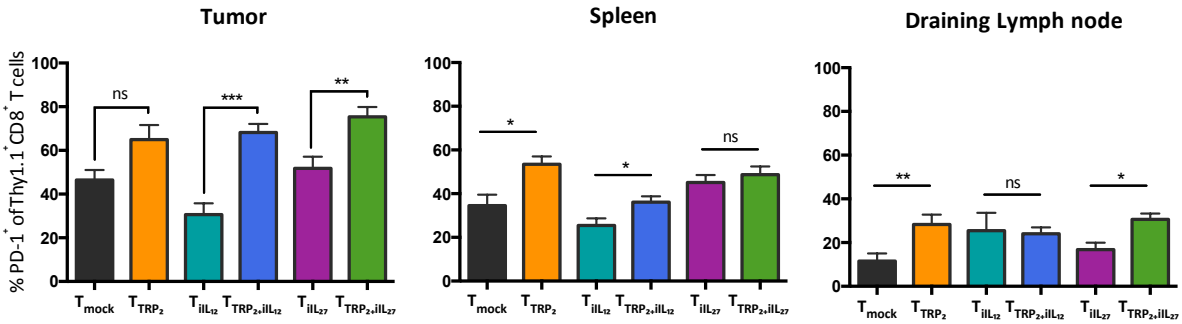


Figure 5.5 | High Ki-67 staining in adoptively transferred $T_{TRP2+iIL12}$ and $T_{TRP2+iIL27}$ cells compared to endogenous T cells in the tumour. C57BL/6 mice bearing subcutaneous B16F10 melanoma were sub-lethally irradiated with 4Gy TBI and treated with $T_{TRP2+iIL12}$ and $T_{TRP2+iIL27}$ cells 10 days following tumour injection. All mice received Dox water (2 mg/ml) 2-3 days prior receiving T cell infusions and kept on Dox for 3 days. **(A)** Flow cytometry blots showing Ki-67 expression in transferred $CD8^+$ $T_{TRP2+iIL12}$ and $T_{TRP2+iIL27}$ cells compared to endogenous T cells. **(B)** Summary data showing the frequency of $V\beta3^+Ki\text{-}67^+$ cells in transferred and endogenous $CD8^+$ T cells. Cells were pre-gated on live singlet lymphocytes. Symbols represent individual mice and bars show group averages. Statistical significance was tested by two-tailed Mann Whitney test: $T_{TRP2+iIL12}$ versus endogenous in the tumor $p= 0.0079$; $T_{TRP2+iIL27}$ versus endogenous in the tumor $p= 0.0079$. Number of mice per group: $T_{TRP2+iIL12}$, $n= 5$; $T_{TRP2+iIL27}$, $n= 5$.

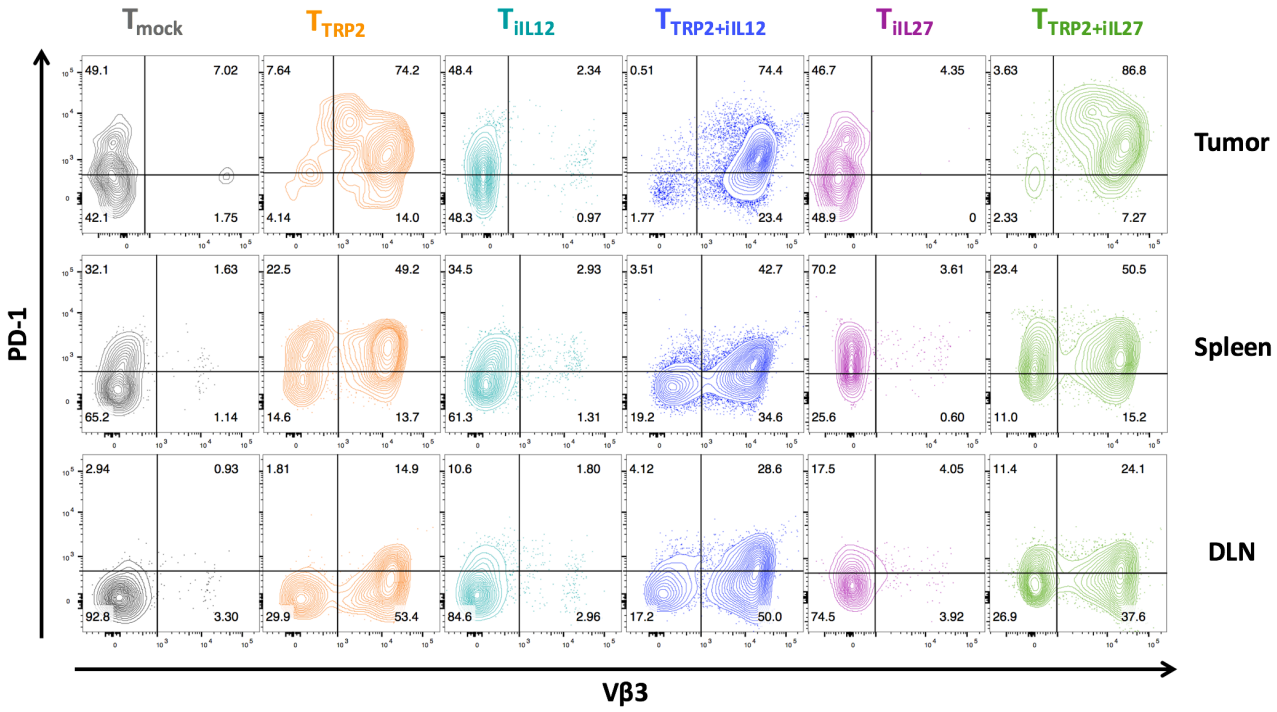
5.3.4 IL-12 prevents high PD-1 upregulation in TRP TCR-redirected T cells

PD-1 expression is induced not only by exhausted antigen-specific T cells but also by activated $CD4^+$ and $CD8^+$ T cells upon TCR engagement [346]. I therefore analysed the expression profile of PD-1 on adoptively transferred T cells at day 5 following T cell infusion. Harvested cells from tumour, spleen and draining lymph nodes (as described in previous section 5.2.1) were stained for PD-1 and analysed by flow cytometry. Having shown that most of the transferred T cells infiltrating tumors were $CD8^+$ T cells (as seen in figure 5.3, section 5.2.2), I assessed PD-1 expression on transferred $CD8^+$ T cells in the tumour compared to the spleen and draining lymph nodes. A large proportion of TCR-transduced $CD8^+$ T cells present in the tumour expressed PD-1 (Figure 5.6 A). This accumulation of PD-1^+ T cells was antigen-driven, because PD-1 upregulation was not observed in TCR-negative T cells including T_{mock}, T_{iIL12} and T_{iIL27} cells. The PD-1 upregulation in TCR-transduced T cells was particularly prominent in the tumour, and much less notable in the spleen and draining lymph nodes (Figure5.6 B). Given that high expression of PD-1 was more profound in mice that received antigen-specific T cells, PD-1 expression was analysed on tumour antigen-specific $CD8^+$ transferred T cells infiltrating the tumour, defined by Vβ3 expression. PD-1 staining profile divided the TCR-expressing cells ($V\beta3^+$) into two populations including $V\beta3^{hi}$PD-1^{low} T cells and $V\beta3^{low}$PD-1^{hi} T cells (Figure 5.7 A). In mice treated with T_{TRP2} or $T_{TRP2+iIL27}$ cells, there was a statistically significant increase in the proportion of $V\beta3^{low}$PD-1^{hi} T cells ($P \leq 0.0011$) compared to mice that received $T_{TRP2+iIL12}$ cells. In contrast, mice treated with $T_{TRP2+iIL12}$ cells had a significantly higher proportion of $V\beta3^{hi}$PD-1^{low} T cells compared to T_{TRP2} and $T_{TRP2+iIL27}$ cells ($P<0.0001$) (Figure 6.10 B). Taken together, these data suggest that IL-12 can prevent high PD-1 upregulation and the associated TCR down modulation.

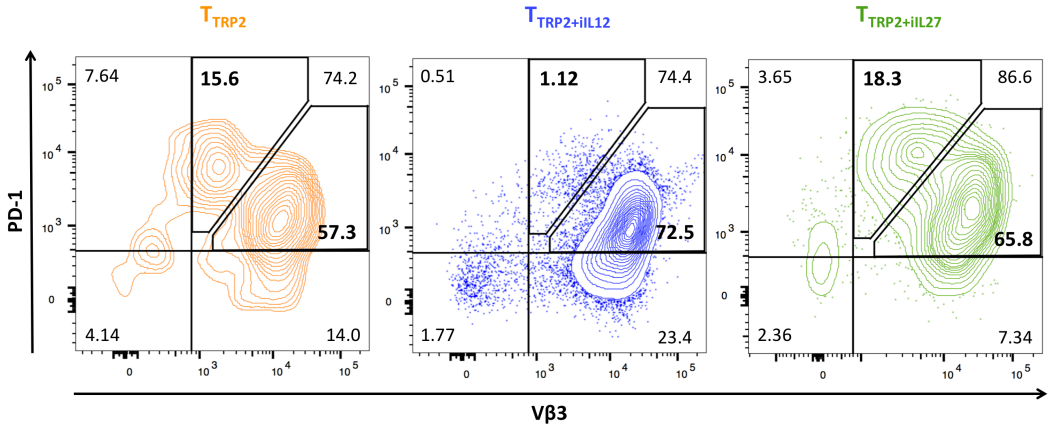
(A)

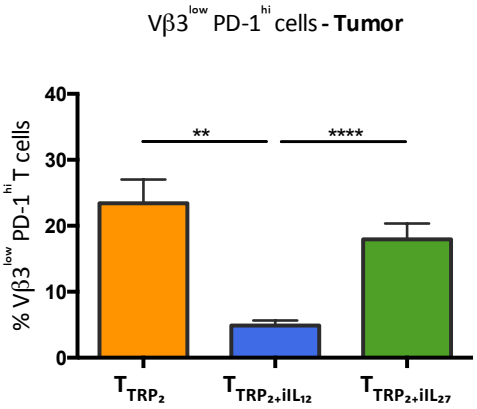

Tumor
Spleen
Draining Lymph node
% PD-1+ of Thy1.1+ CD8+ T cells
ns
***
**
*
*
ns
**
ns
*
Tmock
TTRP2
TiIL12
TTRP2,iIL12
TiIL27
TTRP2,iIL27

(B)


Tmock
TTRP2
TiIL12
TTRP2+iIL12
TiIL27
TTRP2+iIL27
PD-1
Vβ3
Tumor
Spleen
DLN
49.1 7.02 42.1 1.75
7.64 74.2 4.14 14.0
48.4 2.34 48.3 0.97
0.51 74.4 1.77 23.4
46.7 4.35 48.9 0
3.63 86.8 2.33 7.27
32.1 1.63 65.2 1.14
22.5 49.2 14.6 13.7
34.5 2.93 61.3 1.31
3.51 42.7 19.2 34.6
70.2 3.61 25.6 0.60
23.4 50.5 11.0 15.2
2.94 0.93 92.8 3.30
1.81 14.9 29.9 53.4
10.6 1.80 84.6 2.96
4.12 28.6 17.2 50.0
17.5 4.05 74.5 3.92
11.4 24.1 26.9 37.6

(C)


T TRP2
T TRP2+iIL12
T TRP2+iIL27
PD-1
Vβ3
7.64
15.6
74.2
57.3
4.14
14.0
0.51
1.12
74.4
72.5
1.77
23.4
3.65
18.3
86.6
65.8
2.36
7.34


Vβ3low PD-1hi cells - Tumor
% Vβ3low PD-1hi T cells
**
****
TTRP2
TTRP2+iIL12
TTRP2+iIL27

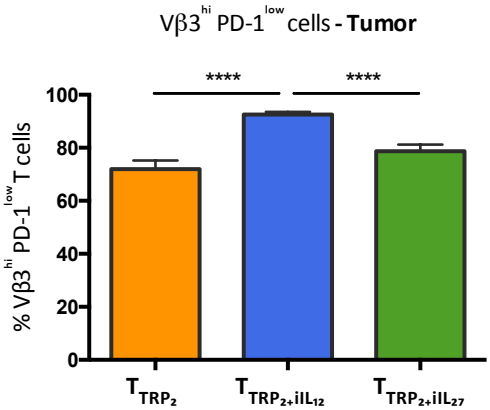

Vβ3hi PD-1low cells - Tumor
% Vβ3hi PD-1low T cells
****
****
TTRP2
TTRP2+iIL12
TTRP2+iIL27

Figure 5.6 | Level of PD-1 expression on transferred CD8$^+$ T cells in the tumour, spleen and draining lymph nodes. C57BL/6 mice implanted with B16F10 melanoma for 10 days were treated with transduced T cells including T_{TRP2}, $T_{TRP2+iIL12}$, $T_{TRP2+iIL27}$, T_{iIL12}, T_{iIL27} or T_{mock} cells following sublethal TBI irradiation (4Gy). All mice received Dox water (2mg/ml) 2-3 days before cell transfer and kept on Dox for 3 days. 5 days post T cell transfer, mice were sacrificed and tissue samples were obtained, including tumour, spleen and draining lymph nodes (DLN). Harvested cells were analysed for Vβ3 (marked TCR-expressing cells) and PD-1 expression on transferred (Thy1.1$^+$) CD8$^+$ T cells. **(A)** Bar charts showing the frequency of PD-1$^+$ cells of transferred CD8$^+$ T cells. Cells were pre-gated on PI$^-$ singlet lymphocytes. Statistical significance was tested by two-tailed Mann Whitney test: T_{TRP2} versus T_{mock} in tumour *P value* > 0.05, in spleen *P value* = 0.0188, in DLN *P value* = 0.0025; $T_{TRP2+iIL12}$ versus T_{iIL12} in tumour *P value* = 0.0001, in spleen *P value* = 0.0238, in DLN *P value* = 0.0025; $T_{TRP2+iIL27}$ versus T_{iIL27} in tumour *P value* = 0.0041, in spleen *P value* > 0.05, in DLN *P value* = 0.0115. Graphs are showing mean ± SEM. **(B)** Representative flow cytometry dot plots of PD-1 and Vβ3 expression on transferred (Thy1.1$^+$) CD8$^+$ T cells in the tumour, spleen and DLNs. Cells were pre-gated on PI$^-$ singlet lymphocytes. **(C)** Flow cytometry plots showing TCR-expressing cells (Vβ3$^+$) in the tumour divided into two populations by PD-1 status including: Vβ3^{hi} PD-1^{low} T cells and Vβ3^{low}PD-1^{hi} T cells *(top)* and bar charts showing summary data pooled from two independent experiments *(bottom)*. Statistical significance was tested by two-tailed Mann Whitney test: frequency of Vβ3^{hi} PD-1^{low} cells in T_{TRP2} versus $T_{TRP2+iIL12}$ *P value* < 0.0001, T_{TRP2} versus $T_{TRP2+iIL27}$ *P value* > 0.05; $T_{TRP2+iIL12}$ versus $T_{TRP2+iIL27}$ *P value* < 0.000; frequency of Vβ3^{low}PD-1^{hi} cells in T_{TRP2} versus $T_{TRP2+iIL12}$ *P value* = 0.0011, T_{TRP2} versus $T_{TRP2+iIL27}$ *P value* > 0.05; $T_{TRP2+iIL12}$ versus $T_{TRP2+iIL27}$ *P value* < 0.0001. Graphs are showing mean ± SEM. Data shown are representative of two independent experiments. Number of mice per group: T_{mock}, *n*= 10; T_{TRP2}, *n*= 8; $T_{TRP2+iIL12}$, *n*= 11; $T_{TRP2+iIL27}$, *n*= 10; T_{iIL12}, *n*= 9; T_{iIL27}, *n*= 10.

5.3.5 IL-12 retains expression of CD127 on adoptively transferred CD8$^+$ T cells in the tumour

The above data suggested that IL-12 might diminish exaggerated T cell activation in the tumour. The next set of experiments tested whether $T_{TRP2+iIL12}$ cells exhibited a less differentiated phenotype than T_{TRP2} and $T_{TRP2+iIL27}$ cells. As previously described, staining for CD62L and CD127 divides the cells into effector T cells ($CD62L^{low}CD127^{low}$), effector memory T cells ($CD62L^{low}$ $CD127^{hi}$) and central memory T cells ($CD62L^{hi}$ $CD127^{hi}$). $T_{TRP2+iIL12}$ cells were found to retain higher levels of CD127 expression in the tumour compared to T_{TRP2} and $T_{TRP2+iIL27}$ cells, which both had a greater proportion of $CD62L^{low}$ $CD127^{low}$ cells and a lower MFI (Figure 5.7 A, B). The proportion of $CD62L^{low}$ $CD127^{low}$ cells is greater when cells were co-transduced with TRP2 TCR and IL-27 ($T_{TRP2+iIL27}$) than in T_{TRP2} cells, suggesting that IL-27 may further push toward an end stage effector cells. Retaining CD127 expression driven by IL-12 is observed regardless of antigen specificity, because no significant difference was observed in the proportion of $CD127^+$ cells between $T_{TRP2+iIL12}$ and T_{iIL12} cells (P=0.1290) (Figure 5.7 C). In contrast, IL-27-driven effect was greater in $T_{TRP2+iIL27}$ cells than in T_{iIL27} cells. Finally, the analysis of spleen and draining lymph nodes revealed that the drive toward CD127-negative T cell differentiated phenotype was specific to the tumour environment. In fact, IL-12 retained CD127 in the tumour at levels similar to that seen outside the tumour.

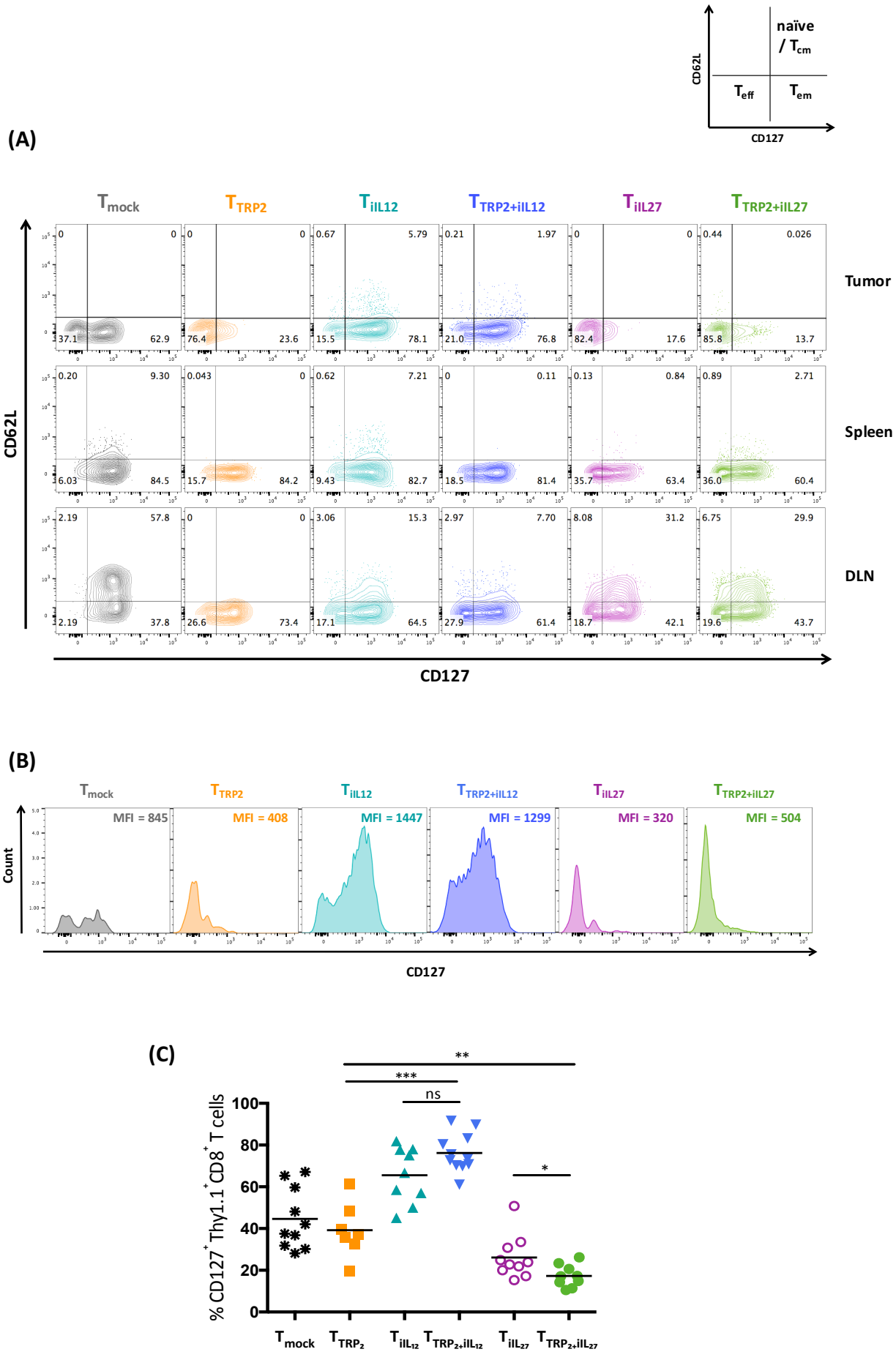

naïve / Tcm
Teff
Tem
CD62L
CD127
(A)
Tmock
TTRP2
TiIL12
TTRP2+iIL12
TiIL27
TTRP2+iIL27
Tumor
Spleen
DLN
CD62L
CD127
(B)
MFI = 845
MFI = 408
MFI = 1447
MFI = 1299
MFI = 320
MFI = 504
Count
CD127
(C)
% CD127+ Thy1.1+ CD8+ T cells
ns
*
**
***
Tmock
TTRP2
TiIL12
TTRP2+iIL12
TiIL27
TTRP2+iIL27

Figure 5.7 | Phenotypic analysis of adoptive transferred T cells in the tumour, spleen and draining lymph node. C57BL/6 mice challenged with $5x10^5$ B16F10 melanoma cells for 10 days were sub-lethally irradiated with 4Gy before the adoptive transfer of $2x10^6$ transduced (Thy1.1$^+$) T cells including: T_{TRP2}, $T_{TRP2+iIL12}$, $T_{TRP2+iIL27}$, T_{iIL12}, T_{iIL27} or T_{mock} cells. All mice were placed on Dox water (2 mg/ml) 2-3 days before T cell transfer and kept on for 3 days. **(A)** Representative flow cytometry blots showing surface expression of CD62L and CD127 on transferred Thy1.1$^+$CD8$^+$ T cells in the tumour, spleen and draining lymph nodes. **(B)** Histograms showing CD127 MFI on transferred Thy1.1$^+$CD8$^+$ T cells in the tumour. **(C)** Graph showing summary data pooled from two independent experiments. Statistical significance was tested using two-tailed Mann-Whitney test: T_{TRP2} versus $T_{TRP2+iIL12}$ *P value* = 0.0001; T_{TRP2} versus $T_{TRP2+iIL27}$ *P value* = 0.0012; $T_{TRP2+iIL12}$ versus T_{iIL12} *P value* >0.05; $T_{TRP2+iIL27}$ versus T_{iIL27} *P value* = 0.0279. Number of mice per group: T_{mock}, *n*= 10; T_{TRP2}, *n*= 8; $T_{TRP2+iIL12}$, *n*= 11; $T_{TRP2+iIL27}$, *n*= 10; T_{iIL12}, *n*= 9; T_{iIL27}, *n*= 10.

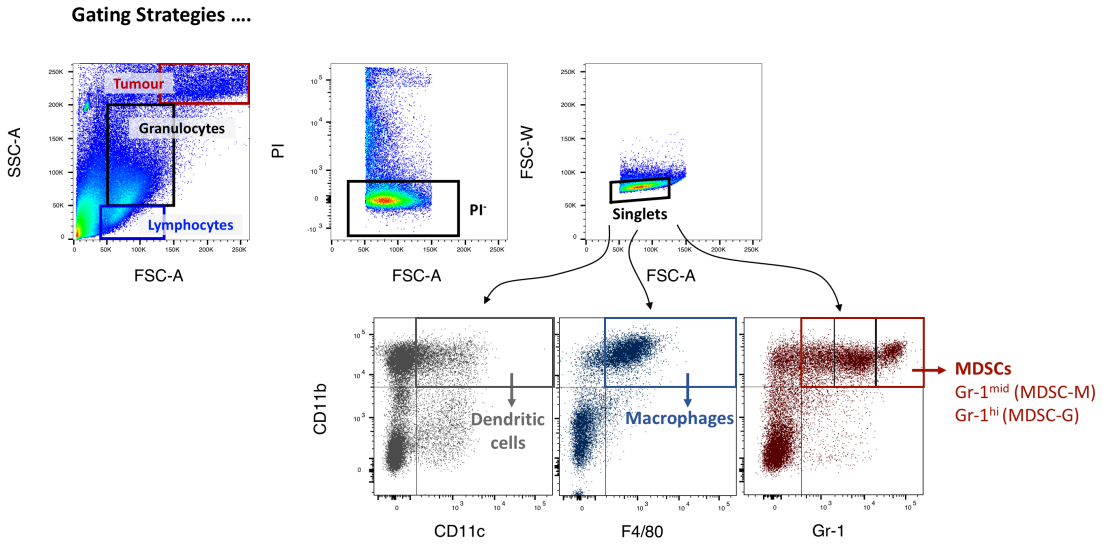
5.3.6 IL-12 and IL-27 effects on tumour-infiltrating myeloid-derived cells

Solid tumours are complex masses harbouring a heterogeneous population of endogenous cells such as myeloid-derived cells. These cells can establish an immunosuppressive networks in the tumour microenvironment, which can support tumour survival and progression. Given that myeloid-derived antigen-presenting cells possessing functional IL-12 receptor (IL-12R) and IL-27 receptor (IL-27R) can respond to IL-12 and IL-27 respectively, I sought to analyse the effect of transferring T cells engineered to secrete IL-12 and IL-27 on myeloid-derived cells in the tumour. In similar experiments described in section (5.2.1), single-cell suspensions prepared from tumours 5 days following adoptive T cell transfer were stained with a panel of antibodies including the following markers: CD3, CD11b, CD11c, F4/80, Gr-1, B220, CD95, MHCI and MHCII. This panel enabled us to identify myeloid cell populations based on their expression of classical myeloid markers. As can be seen in figure 5.8 A, forward scatter (FSC) and side scatter (SSC) gating was used to identify different cell populations including: tumour cells (FSC/SSC high), lymphocytes (FSC/SSC low) and granulocyte populations (SSC intermediate). In order to identify dendritic cells (DCs), macrophages (MQs) and myeloid-derived suppressor cells (MDSCs) that include two subsets: monocytic (MDSC-M) and granulocytic (MDSC-G), I used CD11b and CD11c, CD11b and F4/80, CD11b and Gr-1, respectively [100, 195]. Within the granulocyte gate, there was a marked decrease in the percentage of $CD11b^{+}CD11c^{+}$ (DCs), $C11b^{+}F4/80^{+}$ (MQs) and $CD11b^{+}Gr\text{-}1^{mid}$ (MDSCs-M) populations in the tumours of mice treated with $T_{TRP2+iIL12}$ cells compared to the tumour of mice that treated with T_{TRP2} cells ($P \leq 0.0003$). Reduced frequency of DCs, MQs and MDSCs-M populations correlated with reduced total numbers measured per gram of tumour. Mice received $T_{TRP2+iIL27}$ cells showed a significant decrease in the percentage of DC population ($p=0.0152$) in the tumour compared to those that received T_{TRP2} cells.

Interestingly, a fraction of myeloid cells ($CD11b^{-}Gr\text{-}1^{+}$) was significantly higher in the tumours of mice treated with either $T_{TRP2+iIL12}$ or $T_{TRP2+iIL27}$ cells compared to the tumour of mice that treated with T_{TRP2} cells. The proportion of $CD11b^{-}Gr\text{-}1^{+}$ was also increased, in a non-antigen specific setting, in the tumour of mice treated with T_{iIL12} cells. This fraction of cells, that were $CD11b^{-}Gr\text{-}1^{+}$, were further analysed, and a distinct population of cells expressing $B220^{+}CD11c^{lo/mid}$ was detected in mice treated with $T_{TRP2+iIL12}$ and $T_{TRP2+iIL27}$ cells. This distinct population of $CD11b^{-}$

$Gr1^{+}B220^{+}CD11c^{low/mid}$ cells has been characterised as murine equivalent of human plasmacytoid dendritic cells (pDCs) [347]. They generally express low levels of MHCII molecules which was reflected by looking at I-Ab staining profile. Moreover, the enrichment of pDCs appears to be antigen-dependant because a relatively modest effect can be seen in mice treated with T_{iIL12} but not T_{iIL27} cells compared to mice that treated with $T_{TRP2+iIL12}$ and $T_{TRP2+iIL27}$ cells, respectively. Taken together, IL-12 and IL-27 secretion by TCR-redirected T cells triggered an effect on tumour-infiltrating myeloid-derived cells, which was more pronounced by IL-12 in all subsets including; DCs, MQs, MDSCs and pDCs, whereas IL-27 showed a limited effect on two populations including; DCs and pDCs.

(A)


Gating Strategies ....
Tumour
Granulocytes
Lymphocytes
SSC-A
FSC-A
PI
PI-
FSC-A
FSC-W
Singlets
FSC-A
CD11b
Dendritic cells
Macrophages
MDSCs
Gr-1mid (MDSC-M)
Gr-1hi (MDSC-G)
CD11c
F4/80
Gr-1

(B)

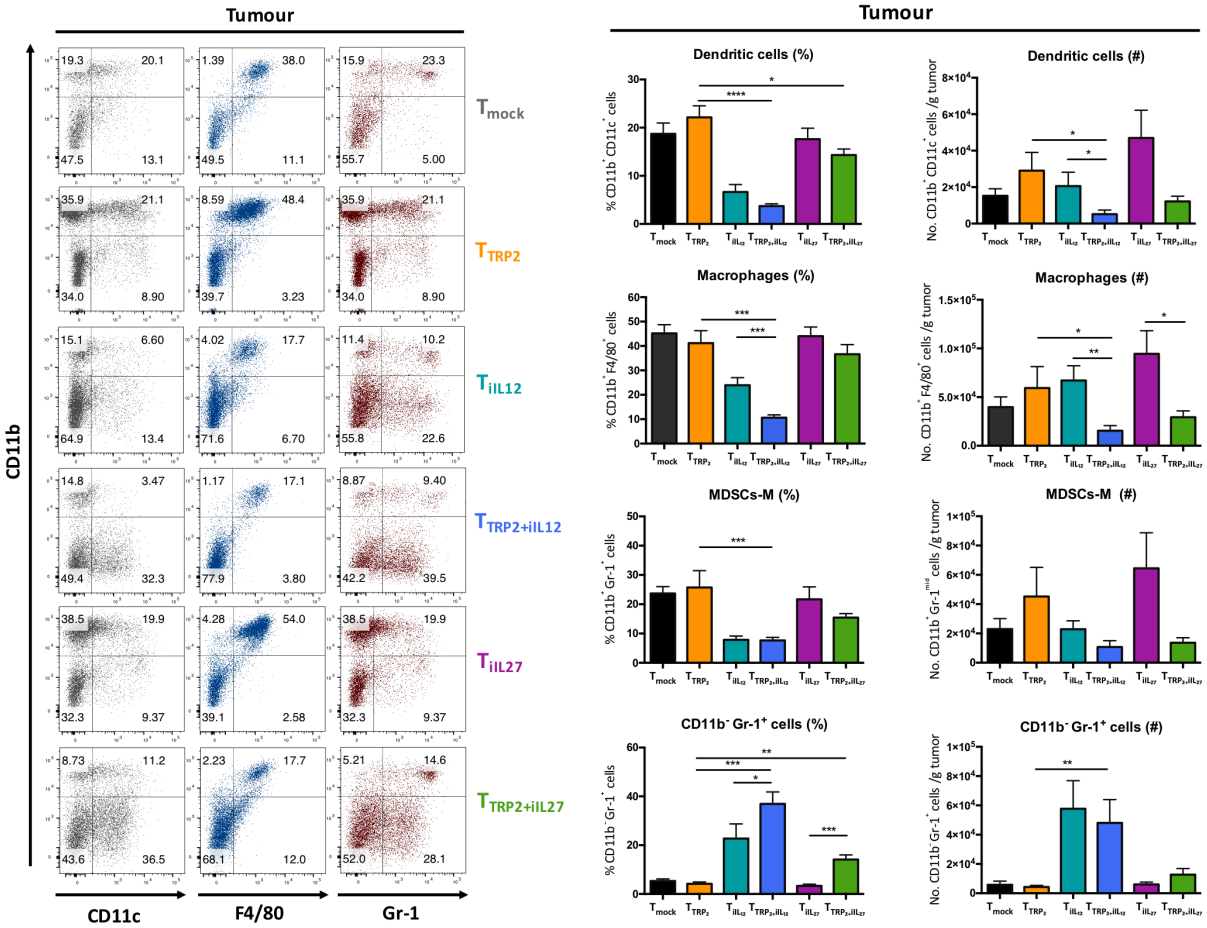
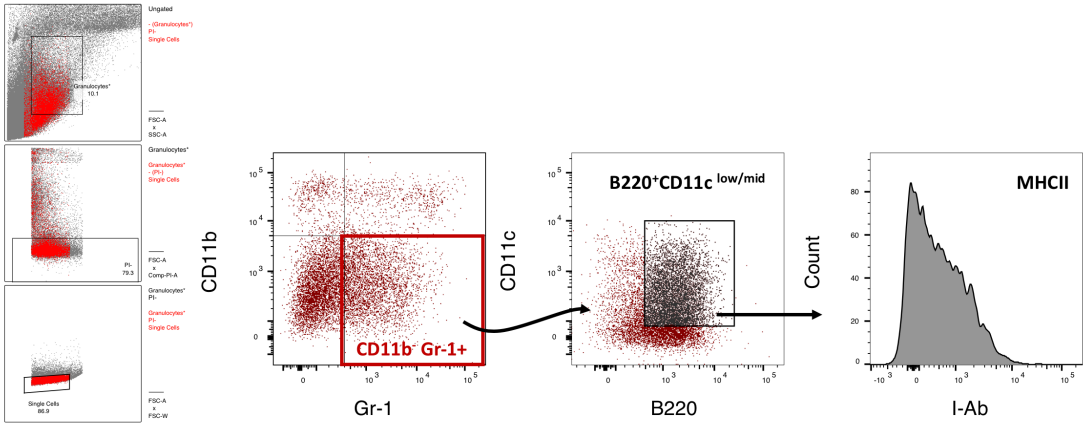

Tumour
CD11b
CD11c
F4/80
Gr-1
Tmock
TTRP2
TiIL12
TTRP2+iIL12
TiIL27
TTRP2+iIL27
Tumour
Dendritic cells (%)
Dendritic cells (#)
Macrophages (%)
Macrophages (#)
MDSCs-M (%)
MDSCs-M (#)
CD11b- Gr-1+ cells (%)
CD11b- Gr-1+ cells (#)

Figure 5.8 | The effect of IL-12 and IL-27 on tumour myeloid compartment. B16F10 melanoma-bearing mice for 10 days were sublethally irradiated with 4Gy TBI prior to adoptive T cell transfer of T_{TRP2}, $T_{TRP2+iIL12}$, $T_{TRP2+iIL27}$, T_{iIL12}, T_{iIL27} or T_{mock} cells. All mice were placed on Dox water (2 mg/ml) 2-3 days before T cell transfer and kept on Dox for 3 days. Mice were sacrificed and tumour-infiltrating cells were analysed. **(A)** Gating strategies. **(B)** Representative dot blots showing tumour-infiltrating myeloid-derived cells *(left)* and summary data pooled from two independent experiments *(right)*. Cells were pre-gated on PI^- singlet granulocytes. Graphs are showing mean ± SEM. Statistical significance was tested using two-tailed Mann-Whitney test: frequency of DCs in mice treated with T_{TRP2} versus $T_{TRP2+iIL12}$ *P value* = 0.0001; T_{TRP2} versus $T_{TRP2+iIL27}$ *P value* = 0.0152; frequency of MQs in mice treated with T_{TRP2} versus $T_{TRP2+iIL12}$ *P value* = 0.0001; $T_{TRP2+iIL12}$ versus T_{iIL12} *P value* = 0.0008; frequency of MDSCs in mice treated with T_{TRP2} versus $T_{TRP2+iIL12}$ *P value* = 0.0003; frequency of $CD11b^-Gr\text{-}1^+$ in mice treated with T_{TRP2} versus $T_{TRP2+iIL12}$ *P value* = 0.0001; T_{TRP2} versus $T_{TRP2+iIL27}$ *P value* = 0.0012; $T_{TRP2+iIL12}$ versus T_{iIL12} *P value* = 0.0432; $T_{TRP2+iIL27}$ versus T_{iIL27} *P value* = 0.0007; total number of DCs in mice treated with T_{TRP2} versus $T_{TRP2+iIL12}$ *P value* = 0.0195; $T_{TRP2+iIL12}$ versus T_{iIL12} *P value* = 0.0249; total number of MQs in mice treated with T_{TRP2} versus $T_{TRP2+iIL12}$ *P value* = 0.0441; $T_{TRP2+iIL12}$ versus T_{iIL12} *P value* = 0.0091; $T_{TRP2+iIL27}$ versus T_{iIL27} *P value* = 0.0399; total number of $CD11b^-Gr\text{-}1^+$ in mice treated with T_{TRP2} versus $T_{TRP2+iIL12}$ *P value* = 0.0031. Data shown are representative of two independent experiments. Number of mice per group: T_{mock}, *n*= 10; T_{TRP2}, *n*= 7; $T_{TRP2+iIL12}$, *n*= 11; $T_{TRP2+iIL27}$, *n*= 9; T_{iIL12}, *n*= 8; T_{iIL27}, *n*= 9.

(A)


Ungated
Granulocytes*
Single Cells
FSC-A
SSC-A
Granulocytes*
PI-
Comp-PI-A
FSC-W
CD11b
Gr-1
CD11b⁻ Gr-1+
CD11c
B220
B220⁺CD11c low/mid
Count
I-Ab
MHCII

(B)

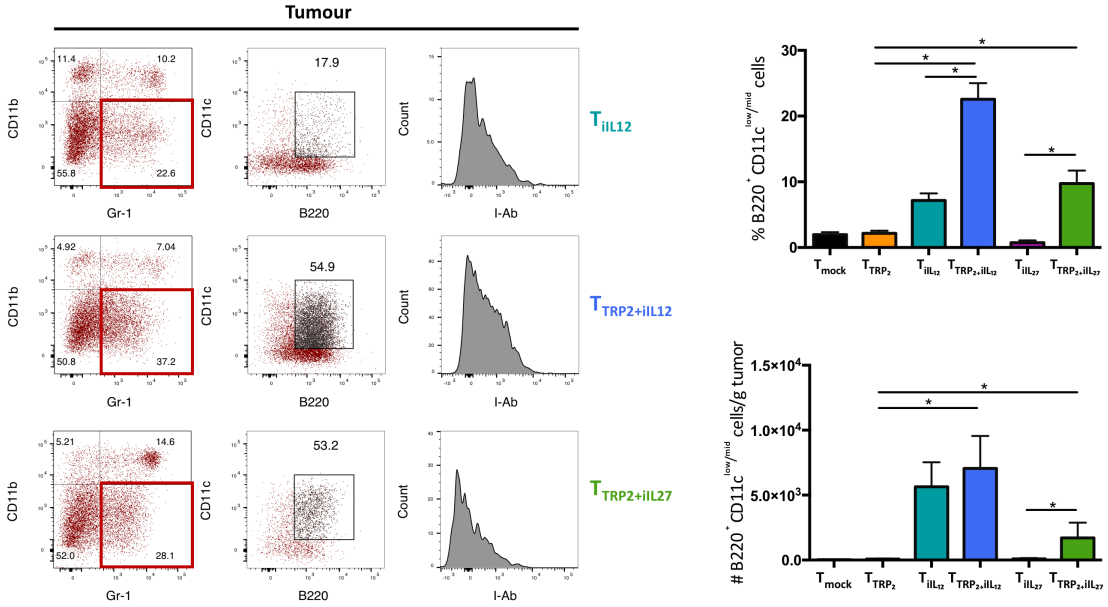

Tumour
CD11b
CD11c
Gr-1
B220
I-Ab
Count
11.4
10.2
55.8
22.6
17.9
T iIL12
4.92
7.04
50.8
37.2
54.9
T TRP2+iIL12
5.21
14.6
52.0
28.1
53.2
T TRP2+iIL27
% B220⁺ CD11c low/mid cells
# B220⁺ CD11c low/mid cells/g tumor
T mock
T TRP2
T iIL12
T TRP2.iIL12
T iIL27
T TRP2.iIL27

Figure 5.9 | Effects of IL-12 and IL-27 on pDC accumulation in the tumour. C57BL/6 mice challenged with B16F10 melanoma for 10 days were treated with transduced T cells including T_{TRP2}, $T_{TRP2+iIL12}$, $T_{TRP2+iIL27}$, T_{iIL12}, T_{iIL27} or T_{mock} cells following sub-lethal irradiation. All mice received Dox water (2mg/ml) 2-3 days before the adoptive T cell transfer and kept on Dox for 3 days. At day 5 post T cell transfer, mice were sacrificed and tumour-infiltrating cells were analysed. **(A)** Gating strategy for identification of pDC subsets. **(B)** Representative flow cytometry blots showing tumour inilftrating pDCs in mice treated with $T_{TRP2+iIL12}$ and $T_{TRP2+iIL27}$ cells, and to a lesser extend in mice treated with T_{iIL12} cells *(left)*. Summary data pooled from two independent experiments *(right)*. Graphs are showing mean ± SEM. Statistical significance was tested using two-tailed Mann-Whitney test: frequency of pDCs in mice treated with T_{TRP2} versus $T_{TRP2+iIL12}$ or $T_{TRP2+iIL27}$, $T_{TRP2+iIL12}$ versus T_{iIL12} and $T_{TRP2+iIL27}$ versus T_{iIL27} *P value* = 0.0159; total number of pDCs in mice treated with T_{TRP2} versus $T_{TRP2+iIL12}$ or $T_{TRP2+iIL27}$, and $T_{TRP2+iIL27}$ versus T_{iIL27} *P value* = 0.015

5.3.7 IL-12 and IL-27 overexpression in TRP2 TCR-redirected T cells delay the development of lethal tumour burden in mice bearing B16 melanoma

After characterizing the effect of IL-12 and IL-27 on TCR-redirected T cells early after the adoptive transfer in B16F10 melanoma mouse model, I next sought to determine their effect on tumour protection. *In vivo* tumour challenge experiments were carried out in C57BL/6 mice as previously described (section 5.2.1). Mice were inoculated subcutaneously with $5x10^5$ B16F10 melanoma cells. 10 days later, mice bearing B16F10 melanoma were sublethally irradiated with 4Gy TBI and, 3-4hrs later, they were treated with $2x10^6$ TRP2 TCR-transduced T cells (T_{TRP2}), iIL12-transuced T cells (T_{iIL12}), TRP2 TCR + iIL12 co-transduced T cells ($T_{TRP2+iIL12}$), iIL27-transduced T cells (T_{iIL27}), TRP2 TCR + iIL27 co-transduced T cells ($T_{TRP2+iIL27}$), or mock-transduced T cells (T_{mock}). All mice received Dox water (2 mg/ml) 2-3 days prior receiving the T cell treatment and kept on Dox water for another 3 days or 14 days in the case of IL-12 or IL-27, respectively. Mice were left until they reached lethal tumour burden. Measurements of tumour size, using a digital caliper every other day, showed differences in tumour growth kinetics over time (Figure 5.10 A). Tumour progression was fastest in mice received T_{mock} or T_{TRP2} cells. This demonstrated that T_{TRP2} cells did not affect tumour growth and failed to reduce tumour burden. In contrast, adoptive therapy with $T_{TRP2+iIL12}$ or $T_{TRP2+iIL27}$ cells resulted in delayed tumour growth and improved survival (Figure 5.10 B, *left*). In these *in vivo* tumour protection experiments, two additional groups of mice were treated with either T_{iIL12} or T_{iIL27} cells, without redirecting antigen specificity through TRP2-TCR gene transfer, to see non-TCR-related effects of IL-12 and IL-27 on the level of tumour protection. The transfer of T_{iIL12} cells showed a slight enhancement in antitumor immunity early after the adoptive T cell therapy, but there was no significant difference ($P>0.05$) in overall survival compared to mice treated with T_{mock} and T_{iIL27} cells (Figure 5.10 B, *right*). This demonstrated that antigen specific immune response is required for shaping the therapeutic benefit of IL-12 and IL-27 in the B16F10 melanoma model. Taken together, these data indicated that short burst of IL-12 and IL-27 can enhance the therapeutic efficacy of TRP2 TCR-redirected T cells, and thereby, contribute to tumour protection against the development of B16F10 melanoma.

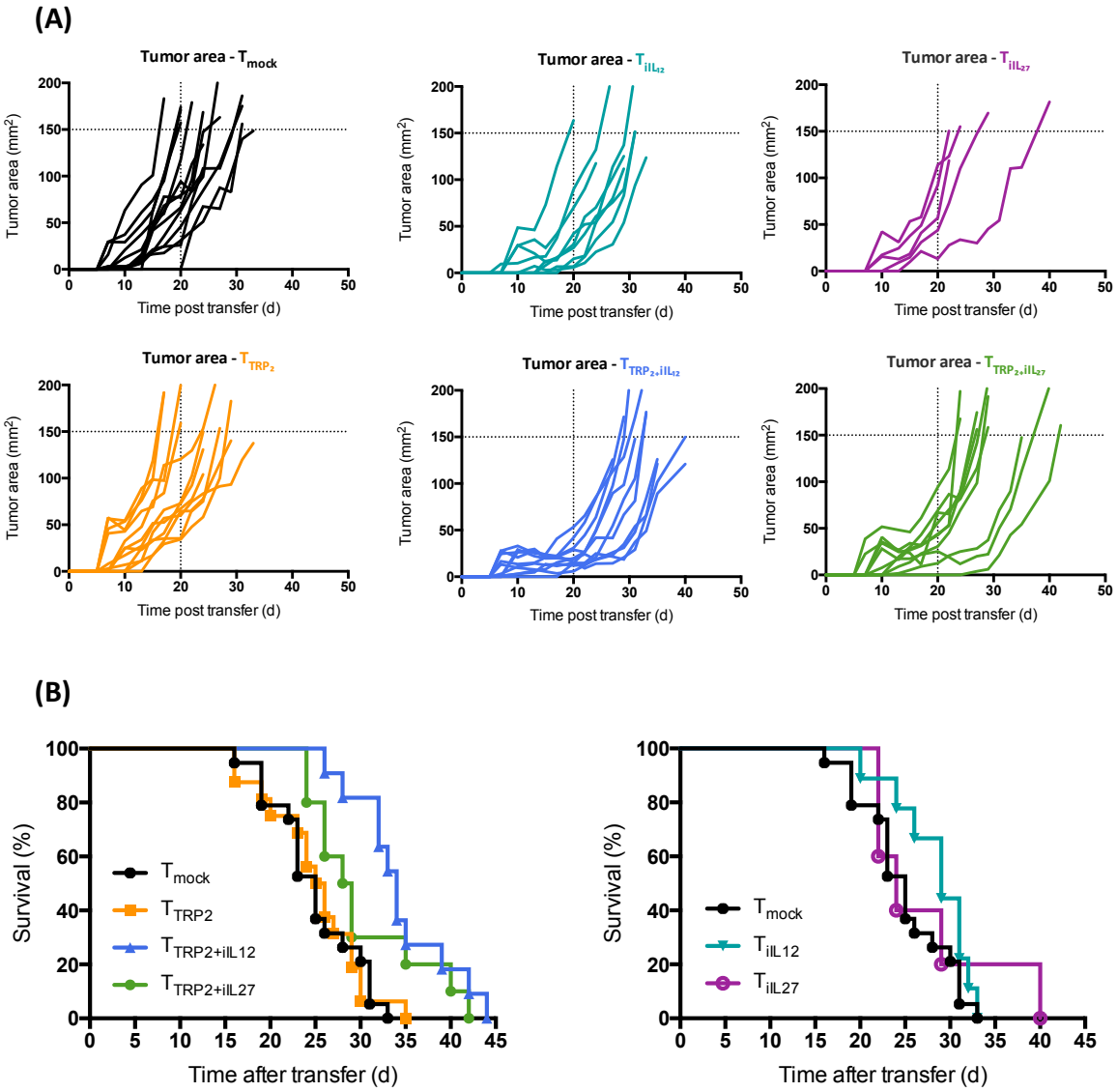


Figure 5.10 | Regulated IL-12 and IL-27 expression in TRP2 TCR-modified T cells affect tumour growth kinetics of B16F10 melanoma. C57BL/6 (Thy1.2^{+}) mice-bearing B16F10 melanoma for 10 days were sublethally irradiated with 4Gy TBI prior to intravenous infusion of transduced T cells including; T_{TRP2}, $T_{TRP2+iIL12}$, $T_{TRP2+iIL27}$, T_{iIL12}, T_{iIL27} and T_{mock} cells. **(A)** Tumour area over time post T cell transfer. **(B)** Kaplan-Meyer survival plots of mice treated with T_{RP2}, $T_{TRP2+iIL12}$, $T_{TRP2+iIL27}$ or T_{mock} cells *(left)* and mice treated with T_{iIL12}, T_{iIL27} or T_{mock} cells *(right)*. Statistical significance was tested using log-rank (Mantel-cox) test: T_{RP2} versus T_{mock} *p value* >0.05; T_{RP2} versus $T_{TRP2+iIL12}$ *p value* = 0.0004; T_{RP2} versus $T_{TRP2+iIL27}$ *p value* = 0.0004; T_{mock} versus T_{iIL12} or T_{iIL27} *p value* > 0.05. Data shown are cumulative results of two independent experiments. Number of mice per group: T_{mock}, *n*= 10; T_{TRP2}, *n*= 8; $T_{TRP2+iIL12}$, *n*= 11; $T_{TRP2+iIL27}$, *n*= 10; T_{iIL12}, *n*= 9; T_{iIL27}, *n*= 10.

5.3.8 Detectable levels of tumour-specific transferred T cells despite tumour progression

Given our previous observation that additional IL-12 and IL-27 expressed by TCR-transduced T cells result in a substantial accumulation of adoptively transferred T cells infiltrating the tumour early after the transfer, I assessed tumour-infiltrating T cells (TILs) when mice reached a lethal tumour burden. Single cell suspensions were prepared as previously described (materials and methods). Flow cytometric analysis of tumour tissues when mice reached lethal tumour burden demonstrated a similar frequency of Thy1.1$^+$ transferred T cells in mice treated with T_{TRP2}, $T_{TRP2+iIL12}$, $T_{TRP2+iIL27}$ cells (Figure 5.11 A). Thus, IL-12-mediated enhanced accumulation of transferred cells in the tumour early after the adoptive transfer is lost when mice reached lethal tumour burden. The majority of the Thy1.1$^+$ transferred T cells detected in the tumour were antigen-specific T cells according to Vβ3 expression, which marked the introduced TCR (Figure 5.11 B). A factor that complicates the comparison of the T_{TRP2}, $T_{TRP2+iIL12}$, $T_{TRP2+iIL27}$ groups is the fact that the analysis of tumour-infiltrating T cells was performed at different time points after the adoptive transfer when mice reached lethal tumour burden.

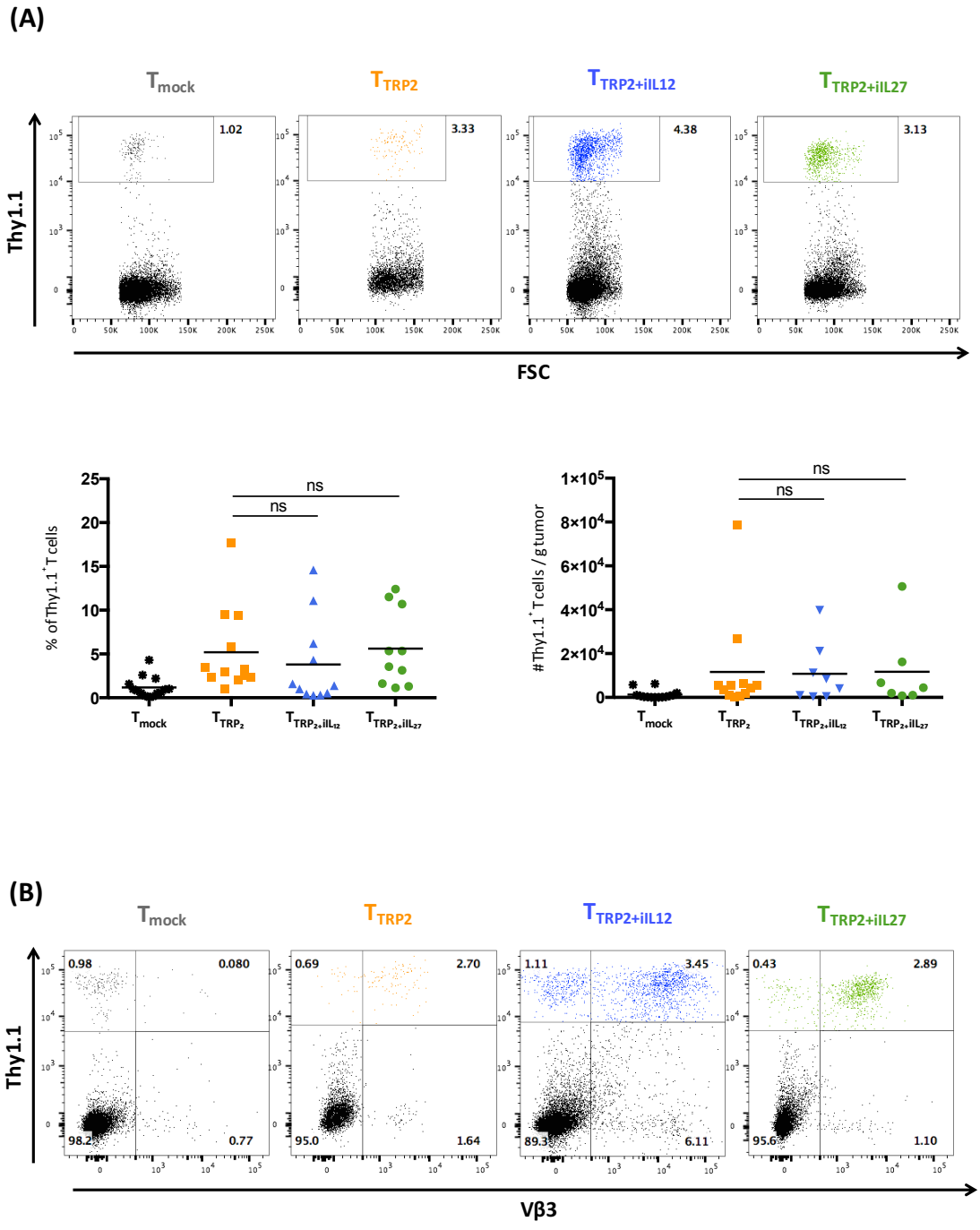

(A)
T mock
T TRP2
T TRP2+iIL12
T TRP2+iIL27
1.02
3.33
4.38
3.13
Thy1.1
FSC
% of Thy1.1+ T cells
ns
ns
Thy1.1+ T cells / g tumor
ns
ns
T mock
T TRP2
T TRP2+iIL12
T TRP2+iIL27
(B)
T mock
T TRP2
T TRP2+iIL12
T TRP2+iIL27
0.98
0.080
98.2
0.77
0.69
2.70
95.0
1.64
1.11
3.45
89.3
6.11
0.43
2.89
95.6
1.10
Thy1.1
Vβ3

Figure 5.11 | Adoptive transferred T cells display similar frequencies in the tumour when mice reached lethal tumour burden. B16F10 tumour challenged C57BL/6 mice for 10 days were sublethally irradiated before adoptive T cell transfer (T_{mock}, T_{TRP2}, $T_{TRP2+iIL12}$, $T_{TRP2+iIL27}$). All mice received Dox water for 2-3 days before T cell infusion and kept on Dox for 3 days or 14 days in the case of IL-12 or IL-27 treatment, respectively. **(A)** Representative blots showing similar frequencies of transferred T cells (gated on Thy1.1$^+$) in the tumour when mice reached lethal tumour burden *(top)*. Summary graphs showing data pooled from three independent experiments *(bottom)*. Cells were pre-gated on PI$^-$ singlet lymphocytes. Statistical significance was tested by two-tailed Mann Whitney test: T_{TRP2} versus $T_{TRP2+iIL12}$ or $T_{TRP2+iIL27}$ *P value* >0.05. Symbols represent individual mice ($T_{mock,}$ *n*=14, T_{TRP2}, *n*=12; $T_{TRP2+iIL12}$, *n*=11; $T_{TRP2+iIL27}$, *n*=10). Bars show group averages. **(B)** Representative blots showing the frequency of TCR-expressing cells (Vβ3$^+$) within the transferred cells in the tumour of mice reached lethal tumour burden. Cells were pre-gated on PI$^-$ singlet lymphocytes. Data shown are representative of 3 independent experiments.

5.3.9 TCR expression results in substantial up-regulation of MHC molecules on tumour cells

The murine B16F10 melanoma cell line (H-2^b) usually expresses no or low levels of MHC class I (K^b, D^b) and MHC class II (I-A^b) molecules, but IFNγ results in a remarkable up-regulation of these molecules, and thereby, render them susceptible to CTL-mediated specific lysis [49]. B16F10 tumour cell line used in these experiments expresses low levels of MHC molecules as demonstrated *in vitro* when B16F10 tumour cells were left untreated or pre-treated overnight with IFNγ-containing medium (Figure 5.12 A). Therefore, I sought to assess the expression levels of H2-K^b (class I) and I-Ab (class II) molecules on re-isolated tumours when mice reached lethal tumour burden. Flow cytometric analysis demonstrated that the expression levels of H2-K^b class I and I-Ab class II molecules were substantially up-regulated on re-isolated tumours from the groups of mice treated with either T_{TRP2}, $T_{TRP2+iIL12}$ or $T_{TRP2+iIL27}$ cells. However, the adoptive transfer of TCR-transduced cells expressing additional cytokine, especially IL-27, resulted in decreased frequency of tumour cells co-expressing class I and class II MHC molecules. In contrast, low surface expression of MHC molecules was detected on re-isolated tumours from mice treated with T_{mock}, T_{iIL12} or T_{iIL27} cells (Figure 5.12 B). In summary, these data demonstrate that adoptive transfer of tumour antigen-specific T cells results in a substantial up-regulation of MHC molecules on B16 tumour cells, and this enhanced up-regulation is seen despite of tumour progression.

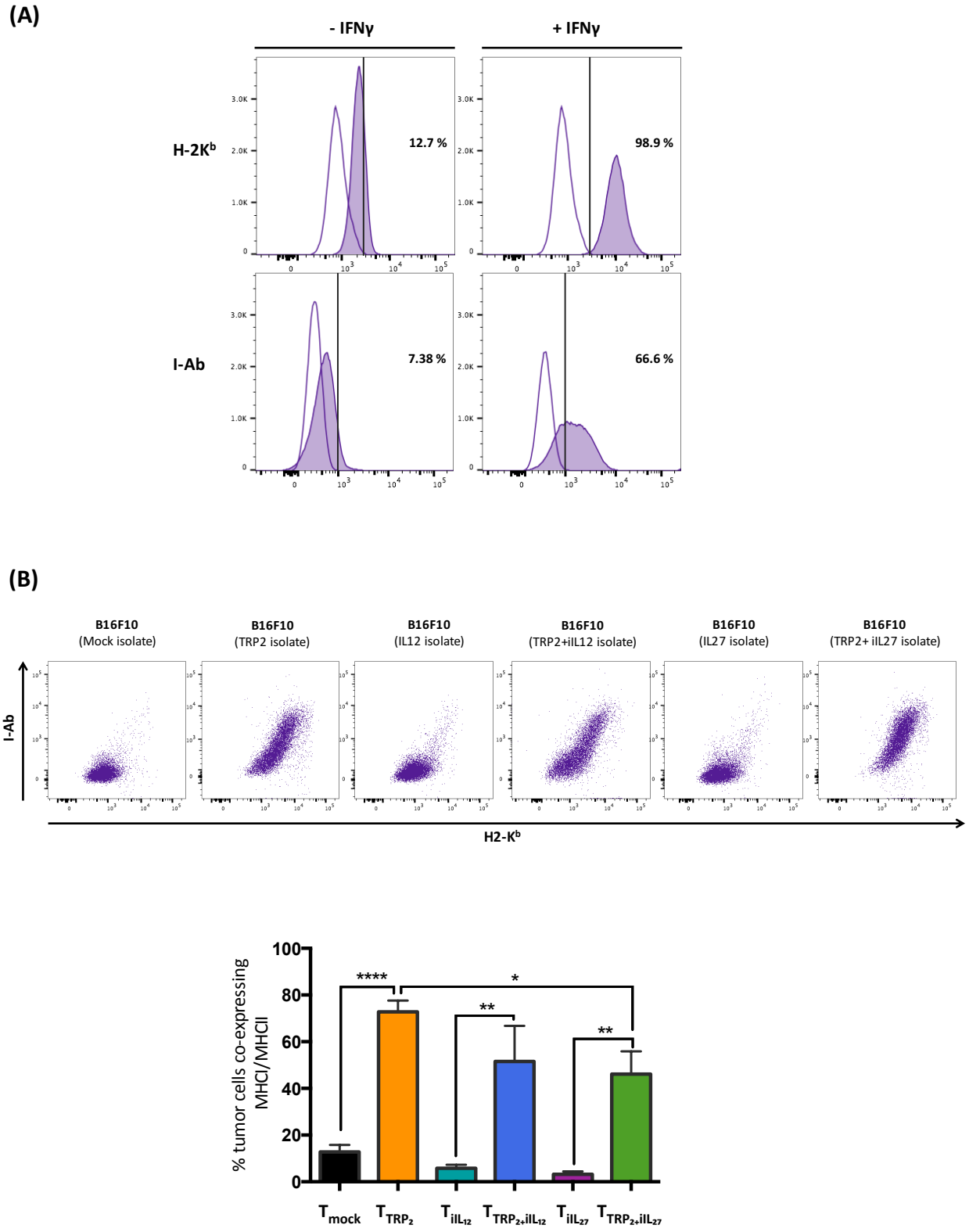

(A)
- IFNγ
+ IFNγ
H-2K^b
I-Ab
12.7 %
98.9 %
7.38 %
66.6 %
(B)
B16F10 (Mock isolate)
B16F10 (TRP2 isolate)
B16F10 (IL12 isolate)
B16F10 (TRP2+iIL12 isolate)
B16F10 (IL27 isolate)
B16F10 (TRP2+ iIL27 isolate)
I-Ab
H2-K^b
% tumor cells co-expressing MHCI/MHCII
****
*
**
**
T_mock
T_TRP2
T_iIL12
T_TRP2+iIL12
T_iIL27
T_TRP2+iIL27

Figure 5.12 | Surface expression of MHC molecules on B16F10 tumours isolated from mice reached lethal tumour burden. (A) *In vitro* surface expression of MHC class I (H-2k^b) and MHC class II (I-A^b) molecules on B16F10 melanoma cell line with or without overnight pre-treatment with IFNγ. **(B)** Dot blots depicting H-2k^b and I-A^b staining of isolated tumours from B16F10 melanoma-bearing mice treated with T_{TRP2}, $T_{TRP2+iIL12}$ or $T_{TRP2+iIL27}$, T_{mock}, T_{iIL12} or T_{iIL27} cells when they reached lethal tumour burden *(top)*. Summary graphs showing data pooled from at least two independent experiments *(bottom)*. Graphs are showing mean ± SEM. Statistical significance was tested using two-tailed Mann-Whitney test: frequency of tumor cells coexpressing MHCI and MHCII molecules in mice treated with T_{TRP2} versus T_{mock} *P value* <0.0001, $T_{TRP2+iIL12}$ versus T_{iIL12} *P value* = 0.0016, $T_{TRP2+iIL27}$ versus T_{iIL27} *P value* = 0.0070 and T_{TRP2} versus $T_{TRP2+iIL27}$ *P value* = 0.0177. Number of mice per group: T_{mock}, *n*= 10; T_{TRP2}, *n*= 8; $T_{TRP2+iIL12}$, *n*= 11; $T_{TRP2+iIL27}$, *n*= 10; T_{iIL12}, *n*= 9; T_{iIL27}, *n*= 10.

5.3.10 Partial tumour protection is not due to tumour escape variants

Despite the enhancement of antitumor immunity mediated by TCR-redirected T cells expressing additional IL-12 or IL-27, tumour protection was incomplete and tumours continued to grow until a lethal tumour burden was reached. I assessed whether tumours growth in treated mice was due to escape of tumour variants, which fail to trigger antigen-specific T cell responses. To test this, re-isolated tumours from treated mice that had reached lethal tumour burden were used *ex vivo* to stimulate TRP2-TCR-transdcued T cells (as described in material and methods), and IFNγ production was measured by flow cytometry. Parental B16F10 and EL4-NP tumour cells were also included as a positive and negative controls, respectively. This demonstrated that all re-isolated tumours can be recognised by TRP2-TCR-transduced T cells resulting in antigen-specific production of IFNγ (Figure 5.13). In summary, these results indicate that tumour escape variants are not responsible for the limited tumour protection observed in mice treated with $T_{TRP2+iIL12}$ or $T_{TRP2+iIL27}$ cells.

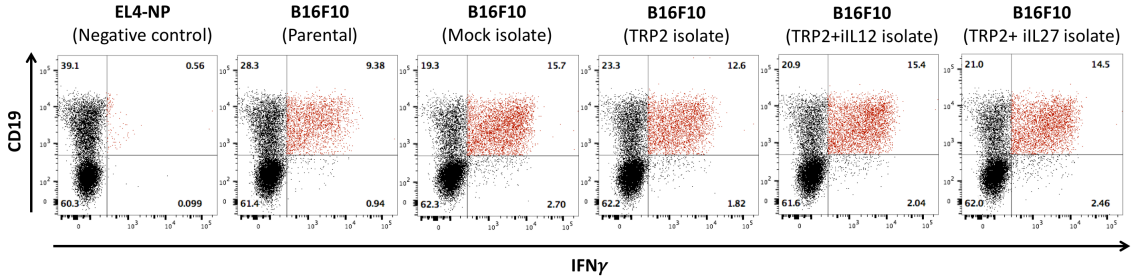
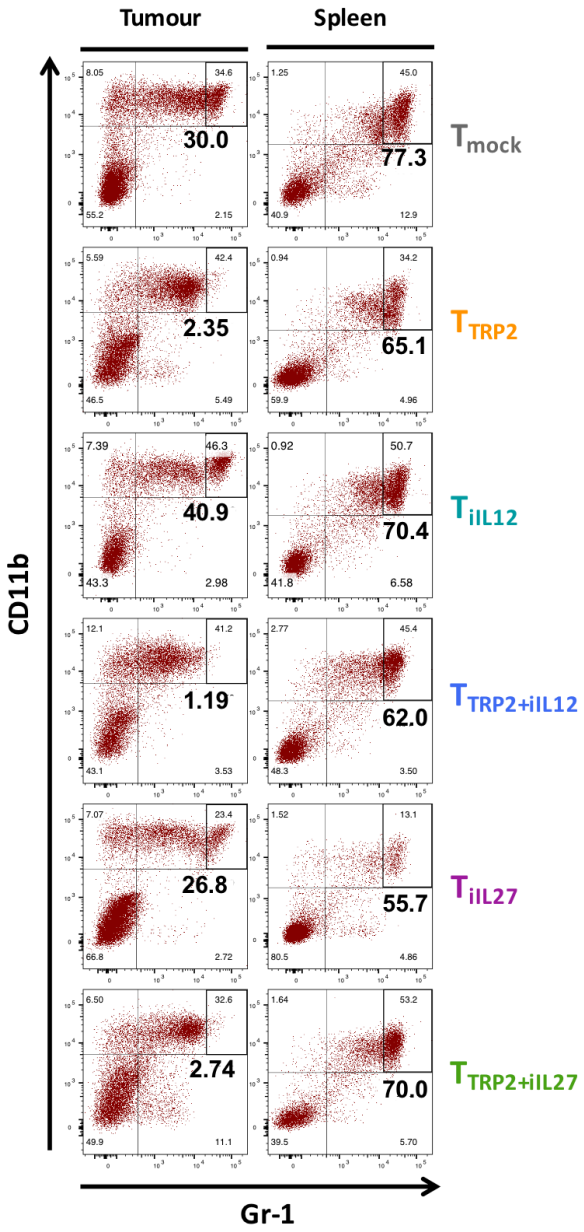
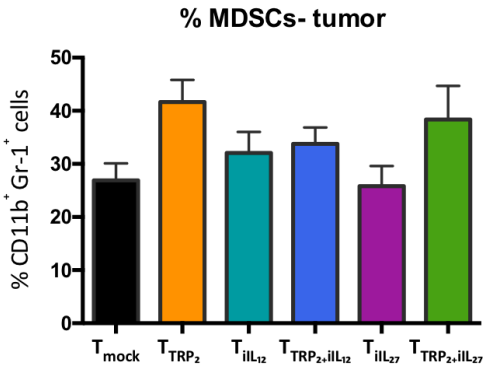


Figure 5.13 | Re-isolated tumours from mice reached lethal tumour burden can induce IFNγ production by TCR-transduced T cells upon *in vitro* co-culturing. Tumours were re-isolated from B16F10-bearing mice treated with T_{TRP2}, $T_{TRP2+iIL12}$, $T_{TRP2+iIL27}$, T_{mock} cells and failed to control tumour growth. Representative flow cytometry blots showing IFNγ production by TRP2-TCR-transduced T cells ($CD19^{+}$) co-cultured with re-isolated tumours, which were pre-treated overnight with IFNγ-containing medium prior to stimulation for 5hrs. Cells were pre-gated on live singlet lymphocytes.

5.3.11 The adoptive transfer of TCR-expressing cells decreases the frequency of CD11b^{+}Gr-1^{hi} cells in the tumour

Myeloid compartment was also assessed when animals reached lethal tumour burden using the multicolor flow cytometry panel previously described (section 6.3.7). Analysis of tumour-infiltrating myeloid cells revealed that the programmatic change induced by IL-12 expression in TCR-redirected T cells at d5 following adoptive cell transfer is not sustainable. However, the frequency of the MQ population was significantly lower in the tumour of mice that received $T_{TRP2+iIL12}$ and $T_{TRP2+iIL27}$ cells compared to mice that received T_{TRP2} cells. This reduction seems to be cytokine-driven because no significant differences in the proportion of MQs in the tumour of mice that received $T_{TRP2+iIL12}$ and $T_{TRP2+iIL27}$ cells compared to mice that received T_{iIL12} and T_{iIL27} cells, respectively (Data not shown). Although relative frequency of total MDSCs analysed in the tumour showed no significant differences, a remarkable decrease in the proportion of CD11b^{+}Gr-1^{hi} MDSCs was observed in the tumour of mice that treated with T_{TRP2}, $T_{TRP2+iIL12}$ or $T_{TRP2+iIL27}$ cells (Figure 5.14). MDSCs have been divided into two sub-populations according to the level of Gr-1 expression including: monocytic MDCSc (MDSCs-M; CD11b^{+}Gr-1^{mid}) and polymorphonuclear MDSCs (MDSCs-G; CD11b^{+}Gr-1^{hi}). According to Gabrilovich *et al,* MDSCs-G characterise the major circulating subset of MDSCs and the most predominant population of MDSCs in tumour-bearing mice. They are immunosuppressive and can suppress antigen-specific CD8^{+} T cell responses [100, 348, 349]. Taken together, these results indicate that tumour-specific TCR is required to remove the polymorphonuclear MDSCs from the tumour microenvironment.


Tumour
Spleen
CD11b
Gr-1
30.0
77.3
2.35
65.1
40.9
70.4
1.19
62.0
26.8
55.7
2.74
70.0
T mock
T TRP2
T iIL12
T TRP2+iIL12
T iIL27
T TRP2+iIL27


% MDSCs- tumor
% CD11b+ Gr-1+ cells
50
40
30
20
10
0
T mock
T TRP2
T iIL12
T TRP2+iIL12
T iIL27
T TRP2+iIL27

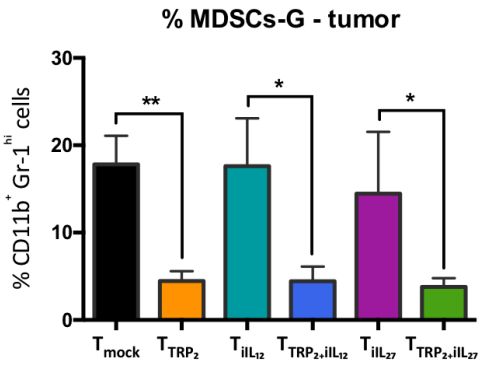
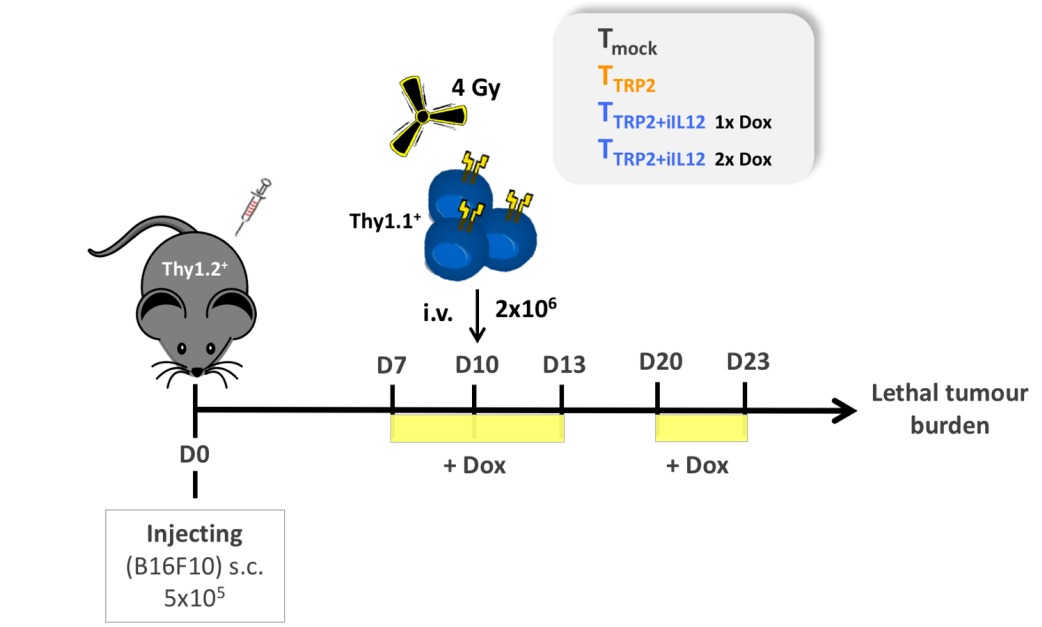

% MDSCs-G - tumor
% CD11b+ Gr-1hi cells
30
20
10
0
**
*
*
T mock
T TRP2
T iIL12
T TRP2+iIL12
T iIL27
T TRP2+iIL27

Figure 5.14 | The adoptive transfer of TCR-expressing cells reduces the frequency of MDSC-G population in the tumour. C57BL/6 mice bearing 10 days B16F10 melanoma were sublethally irradiated before they receive the infusion of T_{TRP2}, $T_{TRP2+iIL12}$ or $T_{TRP2+iIL27}$, T_{iIL12}, T_{iIL27} or T_{mock} cells. All mice received Dox water 2-3 days prior to adoptive T cell transfer and left on Dox for 3 days and 14 days in the case of IL-12 and IL-27 treatment, respectively. **(A)** Dot blots showing reduction in the frequency of $CD11b^{+}Gr\text{-}1^{hi}$ (MDSCs-G) in mice treated with antigen-specific T cells (T_{TRP2}, $T_{TRP2+iIL12}$ or $T_{TRP2+iIL27}$ cells) in the tumour but not in the spleen. Cells were pre-gated on PI^{-} singlet granulocytes. **(B)** Graphs represent cumulative results of two independent experiments. Graphs are showing mean ± SEM. Statistical significance was tested using two-tailed Mann Whitney test: frequency of MDSCs-G in the tumour of mice treated with T_{TRP2} versus T_{mock} *p value* = 0.0051; $T_{TRP2+iIL12}$ versus T_{iIL12} *p value* = 0.0121; $T_{TRP2+iIL27}$ versus T_{iIL27} *p value* = 0.0490. Number of mice per group: T_{mock}, *n*= 10; T_{TRP2}, *n*= 8; $T_{TRP2+iIL12}$, *n*= 11; $T_{TRP2+iIL27}$, *n*= 10; T_{iIL12}, *n*= 9; T_{iIL27}, *n*= 10.

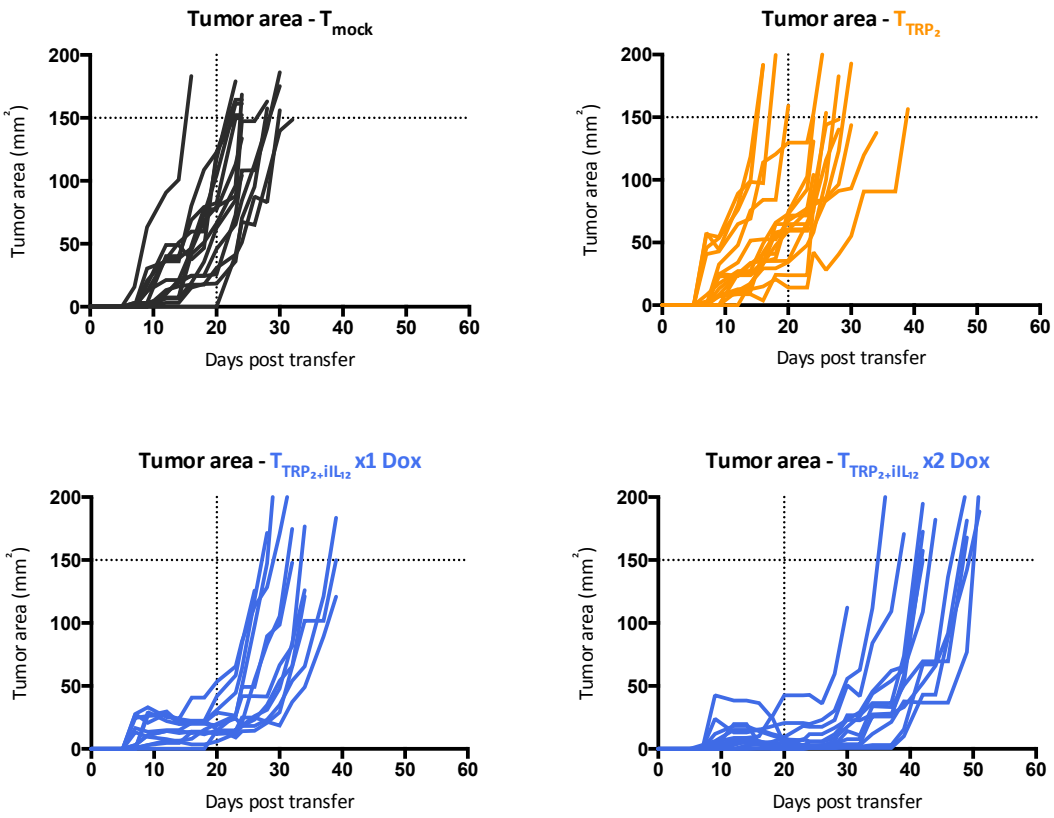
5.3.12 IL-12 double induction in TRP2 TCR-redirected T cells results in lasting tumour protection

Our data presented in section 6.3.9 showed that transient induction of IL-12 in TCR-redirected T cells early after the adoptive T cell transfer in tumour-bearing mice results in tumour protection compared to TCR-redirected T cells. Having shown that tumour escape variants are not responsible for the incomplete tumour protection, we hypothesized that a second induction of IL-12 can results in lasting tumour protection. To test this hypothesis, C57BL/6 mice bearing-subcutaneous B16F10 melanoma were sublethally irradiated and treated with T_{TRP2}, $T_{TRP2+iIL12}$ or T_{mock} cells 10 days after tumour challenge. All mice received Dox water (2 mg/ml) 2-3 days prior to receiving the T cell treatment and kept on Dox for 3 days (Figure 5.15 A). One additional group of mice, which was also treated with $T_{TRP2+iIL12}$ cells, had double induction of IL-12 at day 10 (at the time of T cell infusions) and day 20 post tumour injection. The time point at which IL-12 was induced again was selected based on the tumour growth kinetics of IL-12 single induction, shown previously in section 6.2.8, when the tumour starts to grow. Tumour size was measured 2-3 times per week and mice were sacrificed once they reach lethal tumour burden (Figure 5.15 B). The second induction of IL-12 led to a statistically significant delay in the development of lethal tumour burden (P= 0.0004) and no IL-12-related toxicity was observed (Figure 5.15 C). However, this prolongation in the survival of mice that received double induction of IL-12 was not a long-lived tumour protection.

(A)


T mock
T TRP2
T TRP2+iIL12 1x Dox
T TRP2+iIL12 2x Dox
4 Gy
Thy1.1+
Thy1.2+
i.v.
2x10^6
D7
D10
D13
D20
D23
Lethal tumour burden
D0
+ Dox
+ Dox
Injecting (B16F10) s.c. 5x10^5

(B)


Tumor area - T mock
Tumor area - T TRP2
Tumor area - T TRP2+iIL12 x1 Dox
Tumor area - T TRP2+iIL12 x2 Dox
Tumor area (mm2)
Days post transfer
0
50
100
150
200
0
10
20
30
40
50
60

(C)

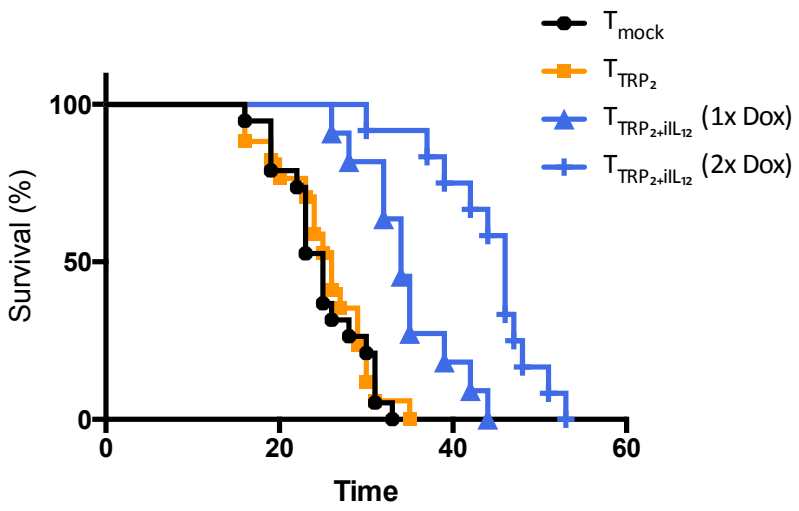


Figure 5.15 | Double induction of IL-12 in TRP2 TCR-redirected T cells increases the survival rate of B16F10 melanoma-bearing mice. (A) Experimental layout. **(B)** Tumour size over time post adoptive T cell transfer. **(C)** Kaplan-Meier survival plots of mice treated with T_{TRP2}, $T_{TRP2+iIL12}$ (single induction), $T_{TRP2+iIL12}$ (double induction) and T_{mock} cells. Statistical significance was tested using log-rank (Mantel-cox) test: T_{TRP2} versus $T_{TRP2+iIL12}$ (1x Dox) *p value* = 0.0002; T_{TRP2} versus $T_{TRP2+iIL12}$ (2x Dox) *p value* < 0.0001; $T_{TRP2+iIL12}$ (1x Dox) versus $T_{TRP2+iIL12}$ (2x Dox) *p value* = 0.0004. Data shown represent pooled data from three independent experiments. Number of mice per group: T_{mock}, *n*= 15; T_{TRP2}, *n*= 17; $T_{TRP2+iIL12}$ (1x Dox), *n*= 12; $T_{TRP2+iIL12}$ (2x Dox), *n*= 13.

5.4 Discussion

In this study, we demonstrate that regulated expression of IL-12 and IL-27 can enhance the therapeutic efficacy of TRP2-TCR-modified T cells in a mouse model of melanoma. This combination regimen of TCR gene therapy and regulated cytokine expression not only resulted in enhanced survival of treated mice, but also reduced the risk of associated toxicity seen with adoptive cell transfer especially in the case of IL-12. To our knowledge, this is the first time to show that IL-12 can be regulated *in vivo* without mediating lethal systemic toxicity, regardless of the number of transferred T cells.

Our *in vivo* experiments indicated that the adoptive transfer of TCR+cytokine modified T cells ($T_{TCR+IL12}$, $T_{TCR+IL27}$) in tumour-bearing mice can enhance the accumulation of antigen-specific transferred $CD8^+$ T cells in the tumour compared to TCR-only T cells (T_{TCR}). The enhanced tumour infiltration was particularly pronounced in mice treated with $T_{TCR+IL12}$ cells. Kerker *et al* have previously demonstrated that the adoptive transfer of tumour antigen-specific $CD8^+$ T cells secreting IL-12 improved the effectiveness of the treatment which correlated with increased infiltration of transferred T cells, as well as endogenous $CD8^+$ T cells and NK cells [300]. In the case of IL-27, Salcedo *et al* found that modifying TBJ neuroblastoma cell lines to express IL-27 resulted in a remarkable tumour growth delay which was associated with heavy infiltration of $CD8^+$ T cells and high IFNγ production [272]. Also, there is evidence to indicate that IL-27 can promote tumour-specific CTLs activation and proliferation and can enhance their survival [232, 270, 271, 283].

Interestingly, we also observed that IL-12 and IL-27 expression in TCR-redirected T cells resulted in a substantial increase in the expression level of the introduced TCR upon *in vivo* priming in the tumour. As previously noted, co-transduction of TCR and IL-12 genes increased the level of introduced TCR expression *in vitro*, whereas IL-27 co-transduction did not. It could be speculated that IL-27 might has an indirect effect on T cells to enhance TCR expression which was evident *in vivo*, whereas IL-12 has a direct effect. As mentioned previously, It has been shown that pro-inflammatory

cytokines such as IL-12 can have a direct effect on T cells during the activation phase by providing a third signal that can generate an optimal T cell response [171, 172].
Our group has previously shown that the level of TCR expression can be greatly enhanced in TCR-modified T cells when it co-transduced with CD3 genes. This strategy was associated with enhanced functional avidity by recognising lower concentration of peptide *in vitro*, and consequently, enhanced anti-tumour immune response *in vivo* [143]. A number of evidence has indicated that IL-12 is capable of increasing the functional avidity of human and mouse $CD8^+$ T cells, which enhances sensitivity to antigens and improves tumour recognition [350, 351]. In addition, previous studies in murine model have shown that inflammation induced by infection can increase TCR avidity [352, 353]. Therefore, it could be interpreted as that IL-12 and IL-27 induce a pro-inflammatory milieu accounted for the elevated levels of TCR expression observed in the tumour of mice that received $T_{TCR+iIL12}$ and $T_{TCR+iIL27}$ cells compared to T_{TCR}. It seems that this effect is induced due, in part, to the nature of the inflammation triggered in the tumour microenvironment where the antigen is highly expressed, since no increase in TCR expression was observed in secondary lymphoid tissues.

In this context, it has been highlighted that high-avidity TCRs are required to recognise cross-presented antigens by tumour stromal cells [110, 354-356]. On the other hand, Kerkar *et al* argued that TCR-modified T cells expressing IL-12 can facilitate the recognition of cross-presentated antigens without changing TCR avidity but by reprogramming dysfunctional myeloid derived cells in the tumour [195]. In good agreement with above statements, we found that IL-12 indeed can orchestrate tumour immunosuppression by dysfunctional myeloid-derived cells by which a significant decrease in the frequency of DCs, MQs and MDSCs was observed upon transferring $T_{TCR+iIL12}$ cells. One of the mechanisms that has been shown to account for the programmatic change triggered by IL-12 is the induction of Fas expression on tumour-infiltrated myeloid cells, and thereby activating Fas/FasL-mediated killing by T cells [196]. Unlike IL-12, the effect of IL-27 on tumour-infiltrating myeloid cells was limited to the DC population. IL-27 signalling in DCs has been found to suppress their antigen presentation capabilities [287-289].

Of note, we also found that IL-12 and IL-27 significantly increased pDCs in the tumour compared to TCR only. The pDCs role in cancer immunity is controversial and remains not well understood [357]. Nevertheless, a growing body of evidence suggests that pDCs may play a crucial role in the process of cancer immunoediting which is attributed to the ability of pDCs to bridge innate and adaptive immunity. It has been shown that activated pDCs are capable to cross-present antigens, and thereby, activate $CD8^+$ T cells which is an important process in tumour immunity [357-359]. Thus, the adoptive transfer of $T_{TCR+iIL12}$ cells and to a lesser extent $T_{TCR+iIL27}$ cells can generate a more immunogenic environment, and consequently, improve T cell-mediated tumour regression. This cytokine-driven effect clearly requires TCR specificity as demonstrated by the adoptive transfer of T_{iIL12} and T_{iIL27} cells. One possible reason is that redirecting T cell specificity can lead to high local cytokine concentration in the tumour. For example, although we observed an increase in the proportion of pDCs in the tumour of mice that treated with T_{iIL12} cells, this did not result in enhanced tumour protection. This idea can be supported by Leonard & colleagues studies which showed that systemic administration of pro-inflammatory cytokines has shown limited success [218, 331, 360].

Collectively, our data would seem to indicate that IL-12 expression in TCR-redirected T cells can enhance antitumor immune responses using several mechanisms, which not only re-educate tumour-associated myeloid cells but also alter the TCR avidity upon *in vivo* priming.

Furthermore, we also demonstrated that the majority of the antigen-specific $CD8^+$ T cells infiltrating the tumour at d5 following adoptive T cell transfer expressed high levels of PD-1 compared to outside the tumour including: spleen and draining lymph nodes, suggesting that PD-1 up-regulation occurs upon antigen re-encounter in the tumour microenvironment. Additionally, IL-12 was found to prevent high PD-1 upregulation and concomitant TCR down-modulation in $T_{TCR+IL12}$ compared to T_{TCR} and $T_{TCR+IL27}$ cells. The accumulation of $PD\text{-}1^+$ TILs seems to be antigen-driven because such an upregulation of PD-1 was not observed in TCR-negative cells (T_{mock}, T_{iIL12} and T_{iIL27} cells). Phenotypic profile of the activated T cells revealed that $T_{TCR+IL12}$ cells retained expression of CD127 associated with T_{em} phenotype ($CD62L^{low}CD127^{hi}$) in the tumour compared to T_{TCR} and $T_{TCR+IL27}$ cells, which both

expressed low levels of CD127 associated with T_{eff} phenotype ($CD62L^{low}CD127^{low}$). Two main models for T cell differentiation into effector and memory T cells have been described including: 'On-Off-On'/'Off-On-Off' model and linear/developmental model. The former model proposed that memory T cells can rise from effector T cells (T_{eff}) (effector→memory). According to the latter model, the linear model of T cell differentiation, T_{eff} cells depict the end stage of T cell development, which indicates that terminally differentiated T_{eff} cannot give rise to memory cells (memory→effector) [361]. Thus, in this case, it appears that IL-12 can prevent extravagant activation/terminal differentiation of T cells in the tumour. In human chronic infection, antigen-specific T cells expressing $PD\text{-}1^{hi}CD127^{low}$ exhibit exhausted phenotype and function [362-364]. Although exhausted cells display high PD-1 expression, not all $PD\text{-}1^{hi}$- expressing cells are exhausted [339].

From different perspective, the expression of CD127 (IL-7Rα) is generally known to enhance memory T cell survival/homeostasis. Kaech *et al* have demonstrated that the levels of CD127 expression on $CD8^+$ T cells during the effector phase might correlate with the capacity of survived effector T cells to develop into long-lived memory cells [338]. Moreover, studies by Jung Chang's group have shown that T cell primed in the presence of IL-12 greatly enhanced the generation of memory $CD8^+$ T cells which was characterised by expressing high levels of CD127 [326]. Therefore, it is reasonable to suppose that IL-12 can increase memory precursor effector cells. By contrast, this does not imply that IL-27 is unable to enhance T cell memory formation. A recent study that has evaluated the phenotype of tumour antigen-specific $CD8^+$ T cells stimulated in the presence of IL-27 *in vivo* revealed that IL-27 enhances long-term survival of CTLs and programs them into a unique effector T cells with a memory precursor phenotype [283].

In terms of tumour protection, our *in vivo* tumour challenge experiments indicated that the adoptive transfer of $T_{TCR+iIL12}$ and $T_{TCR+iIL27}$ cells improved tumour protection compared to T_{TCR} cells, which completely failed to control tumour growth. Noteworthy, IL-12 protective effect was superior compared to IL-27. Despite this, tumour protection was not sustained long term. Although transferred cells persist in the tumour of mice that reached lethal tumour burden, it is clear that they lose the ability to sustain a protective antitumor immunity. This could be partially due to the

transient nature of cytokine expression, particularly in the case of IL-12. Another possible explanation that could be associated with the observed limited tumour protection is the emergence of tumour antigen escape variants. They are most likely to develop as an immune escape mechanism following an active antitumor immune response [365]. Yet, analysis of B16F10 tumour cells isolated from mice reached lethal tumour burden indicated that escape variants are unlikely to be the case. Even though we cannot exclude the possibility that there was a transient loss of TRP2 antigen or MHC class I expression on tumour cells after adoptive T cell transfer *in vivo*. According to Landsberg *et al*, adoptive transfer of melanoma-specific T cells can stimulate a transient loss of melanoma tumour antigen expression derived by TNFα localization in the tumour. They also postulated that induction of inflammatory microenvironment following adoptive T cell transfer led to the acquisition of a reversible dedifferentiation phenotype by tumour cells which is attributed to the plasticity of melanoma cells [366].

Furthermore, we found that double induction of IL-12 in B16F10 tumour-bearing mice treated with $T_{TCR+iIL12}$ cells resulted in a significant delay in development of a lethal tumour burden compared to the single induction of IL-12. Even though the second induction of IL-12 has provided some additional benefit and prolonged the survival of the treated mice, long-lasting tumour protection was not achieved. Therefore, these results imply that IL-12 induces a potent antitumor immunity by reprogramming the tumour microenvironment, but it seems to be transient.

In conclusion, the data presented in this chapter showed that the combination of cytokine and TCR gene therapy can enhance the fitness of engineered T cells and enhance antitumor immunity. The data suggest that tet-regulated IL-12 expression in TCR-redirected T cells can be used to regulate the expression of IL-12 *in vivo*, and it can be exploited to regulate cytokine expression while avoiding treatment-associated toxicity. In the next step, it is important to dissect the therapeutic window of IL-12 to mediate tumour protection in the absence of toxicity and dissect the mechanism by which this can be achieved. It is also necessary to explore the mechanism by which IL-27 can enhance TCR-mediated tumour immunity.

Chapter 6
General discussion

T cell engineering is an exciting, promising and fast-growing area of research. Unlike drugs, monoclonal antibodies and small-molecule inhibitors, the potency of T cells is due to a number of biological characteristics that can be harnessed to generate a more efficient therapeutic product to treat cancer. T cells are characterised by their ability to recognise a specific tumour antigen and successfully destroy tumour cells, to proliferate and differentiate into effector and memory T cells. Therefore, therapeutic T cells can act like a "living drug". Genetic engineering of T cells has provided an efficient strategy to generate large number of therapeutic T cells with defined specificity toward a tumour target antigen of choice, which solved the deficit of TIL therapy to which the frequency of tumour-reactive T cell is low in many cancer patient [106]. The feasibility and clinical potential of engineered T cells has been demonstrated in recent clinical trials for treating patients with metastatic melanoma, synovial sarcoma and colorectal carcinoma, with an objective response rate up to 67% [127-130, 367]. In addition, the potency of T cell therapy to treat metastatic tumours was further highlighted by the ability of TCR-engineered T cells to eliminate brain metastasis in melanoma patients by trafficking to the central nervous system (CNS) [368]. However, clinical studies have also revealed limited therapeutic efficacy as demonstrated by treatment-related toxicity and transient tumour regression. Treatment-related toxicity was evident in clinical trials using TCRs with high-affinity directed against TAAs. For example, treatment that involved 'On-target' toxicity by melanocyte destruction resulted in severe inflammation, albeit treatable in some cases, in skin, eye and ear in patients who received MART1-specific T cells [127]. Sever inflammation of the colon has also been reported in colorectal carcinoma patients who received CEA-specific T cells [367]. The observation that transferred T cells often fail to maintain effector function resulted in a transient tumour regression, which is considered as one of the major current limitations of T cell engineering [298]. The effector function of tumour-reactive T cells can be tuned down by many factors that involved the mechanisms of tumour evasion. Therefore, most of the developing strategies nowadays aimed to enhance the therapeutic T cell fitness using current variety of genetic tools. One of the new important developments is to engineer T cell specificity as well as effector function.

In this project, the main aim was to equip TCR-redirected T cells with additional effector cytokines, IL-12 and IL-27, to enhance T cell effector function and modify the immunosuppressive tumour microenvironment.

Cytokines have provided compelling evidence for their ability to enhance antitumor activity. It has been initially demonstrated in metastatic melanoma patients treated with immunomodulatory cytokines, such as IFNα or high-dose of IL-2, that cytokine-based therapy resulted in complete responses but were more likely to develop autoimmune vitiligo [369]. Due to the fact that the half-life of IL-2 in the serum is particularly short, high doses of IL-2 has been systemically administered to achieve a therapeutic effect, but this was associated with treatment-related toxicity in many cases [370-372]. For this reason, local delivery of cytokines to the tumour site utilizing engineered T cells as vehicles has shown promising results [369].

Engineering T cells to secret IL-12 has greatly enhanced the therapeutic efficacy of TCR-redirected T cells by facilitating the accumulation of the tumour-reactive $CD8^+$ T cell, improving T cell effector function and modulating the tumour suppressive microenvironment (also recently reviewed in [161]). Although IL-12 is particularly effective, its associated toxicity is still considered as the main unresolved drawback of IL-12-based therapy. In this project, considering this issue, a regulated expression system has been utilized aiming at regulating cytokine expression without causing systemic toxicity. This was achieved by using retroviral vector expressing IL-12 under the control of tet-regulated promotor which drives IL-12 expression upon Dox administration. This indeed demonstrated a fine-tuning of the relative cytokine expression by achieving enhanced efficacy of the transferred T cells while avoiding systemic toxicity.

The data in this project also indicate that although tumour-specific T cells equipped with additional effector cytokines enhanced overall survival and induced tumour growth delay, they were unable to provide long-term tumour protection. Recent work has highlighted one possible explanation for T cell therapy resistance in melanoma tumour, which is due to the emergence of immunosuppressive pathways triggered by inflamed $CD8^+$ T cells in the tumour microenvironment as a negative feedback mechanism [90]. In this study, three key mechanisms have been witnessed upon characterising the tumour microenvironment in B16F10 melanoma; including PD-L1/B7-H1 (inhibitory receptor), IDO (metabolic dysregulation) and $Foxp3^+$ T_{reg} cells (immune suppressive cells) [90]. Therefore, targeting negative regulators of T cells might be an alternative/combined strategy to enhance the therapeutic efficacy of engineered T cells.

PD-L1, a ligand for the co-inhibitory receptor PD-1, can be induced in tumour cells and APCs. Because PD-1 is expressed on activated T cells, engagement of this negative regulatory receptor can mediate inhibition of T cell effector function, especially in the tumour microenvironment [373, 374]. It has been shown that blocking PD-1/PD-L1 interaction with monoclonal antibody (mAb), such as anti-PD-L1 can restore T cell effector function and suppress tumour growth [375, 376]. Combining anti-PD-L1 mAb with adoptive cell therapy was also found to suppress squamous cell carcinoma growth in syngeneic mice [377]. Anti-PD-1/PD-L1 therapy has also achieved compelling benefits in clinical studies by mediating tumour regression and enhancing survival [378, 379]. To date, a number of therapeutic mAbs targeting the PD-1/PD-L1 signalling pathways have been approved by the FDA to treat human cancers, including two anti-PD-1 mAbs (called nivolumab and pembrolizumab), and more recently, another mAb targeting PD-L1 [380]. However, systemic administration of these blocking antibodies can disrupt peripheral tolerance due to the fact that restraining T cell responses by most tissues rely on their expression of PD-L1 [381]. Therefore, selective inhibition of the co-inhibitory receptors using gene-editing tool has been utilized to selectively down-modulate these negative signals in tumour-reactive T cells, such as ZFNs and TALEN, but they have their own limitation [137, 382]. More recently, a new technology called CRISPR/Cas9 has shown its feasibility in knocking out the PD-1 gene in primary human T cells, which could provide a new approach to enhance the therapeutic efficacy of adoptive cell therapy [383].

Apart from PD-1, Fas molecules are constitutively expressed on activated T cell surface, and therefore, they may be susceptible to Fas-mediated apoptosis upon binding to its receptor FasL. It has been reported that some tumour cells express FasL as a strategy to escape tumour immunity, such as melanoma, colon carcinoma, lung carcinoma and hepatocellular carcinoma; thus, it is reasonably expected that $FasL^+$ tumour cells can counterattack tumour infiltrating T cell expressing Fas and limit antitumor immunity [384]. Engineering T cells to become insensitive to Fas/FasL-mediated killing by knocking down Fas gene in T cells could serve as a strategy to enhance T cell-based therapy. In this context, EBV-specific CTLs have been engineered to become insensitive to FasL-induced apoptosis by transduction with a retrovirus vector encoding siRNA-targeting Fas, as an approach to enhance ACTs for EBV-associated tumours [385]. Notably, these modified T cells showed an increased resistance to apoptosis induced by

FasL, and they were dependent on antigen and growth factors to maintain their proliferation and survival. However, the role of FasL in immune evasion by tumours is not yet crystal clear [386]. Accordingly, it has been suggested that other immunosuppressive molecules expressed by tumours could be targeted, such as galectin-1 [387]. Hence, engineering T cells to become unresponsive to galectin-1 could be another strategy to generate therapeutic T cells resistant to tumour-induced cell death.

Another negative regulator of T cells that has been targeted to enhance the effectiveness of ACT is TGFβ, the T cell suppressive cytokine. There is good evidence to show the role of TGFβ in tumour-induced immunosuppression in humans [91, 94, 388, 389]. In the setting of ACT, interfering with TGFβ signalling by transducing tumour-specific T cells with a dominant-negative TGFβ receptor-II (dnTGFβRII) has significantly improved the therapeutic efficacy of engineered T cells in B16 mouse melanoma model, suggesting a strategy by which the performance of T cells can be greatly enhanced in the immunosuppressive tumour microenvironment [390, 391].

The COX2 enzyme and its metabolite PEG2 secreted by some tumours are also work as negative regulators of T cells. These inhibitors support T cell dysfunction and inhibit their antitumor reactivity. PEG2 enhances the production of the suppressive cytokine IL-10 and alters the balance between IL-10 and IL-12 production. Blocking the activity of COX2 and PEG2 by COX2 inhibitors was shown to increase pro-inflammatory cytokines, such as IFNγ and IL-12, associated with a decrease in the levels of IL10. COX blockade has also enhanced T cell infiltration into the tumour [107]

The second mechanism of immunosuppression that has been defined in the melanoma tumour microenvironment is high IDO expression [73]. IDO catalyses tryptophan, an essential amino acid, which limits its utilization by T cells, and thereby, influences T cell metabolism and effector function [392]. Suppressing IDO with tryptophan analogues showed enhanced T cell infiltration into tumours and delayed tumour growth [393]. In addition, IDO has been found to activate $Foxp3^+$ T_{reg} cells, which is also one of the defined immunosuppressive mechanisms. Blocking IDO in combination with antitumor vaccine have been shown to induce reprogramming of T_{reg} cells to Th17-like cells, which further enhanced antitumor immunity [394].

Therefore, metabolic disruption is another challenge that tumour-reactive T cells face in the tumour microenvironment, particularly in solid tumours. Under normal condition, activated T cells are subject to a metabolic shift toward aerobic glycolysis and amino acid metabolism, as both glucose and amino acids are important supportive nutrients for T cell proliferation and effector function [392]. Thus, metabolic stress in the tumour microenvironment and nutrient deprivation can impair antitumor immune responses. For example, insufficient glucose can affect T cell effector function partly via altering metabolic signalling pathways that are sensitive in T cells, such as mTORC1 [392]. Hence, metabolic engineering of T cells is an attractive strategy to support their effector function and metabolic fitness, which can enhance T cell-based therapy. Our group has shown that engineering tumour-reactive T cells to overexpress RAS homolog enriched in brain (RHEB), a key positive regulator of the mTORC1 pathway, can modulate T cell metabolism by increasing aerobic glycolysis, leading to enhanced T cell expansion and effector function and imporved tumour protection in murine tumour model [395].

Apart from IL-12 and IL-27, a number of other cytokines have been assessed in the setting of T cell engineered including IL-15 and IL18 [298, 396]. Tumour-site delivery of IL-15 has been shown to enable adoptively transferred cells to eliminate both antigen-positive and -negative tumour cells [396].

Furthermore, choosing an appropriate target antigen is one of the important factors influencing the safety and efficacy of T cell therapy. There are a number of criteria should be considered for selecting candidate target antigens including: i) no expression or low levels of expression by normal tissues and ii) high immunogenicity to trigger an efficient immune response against tumour [298]. The targeted antigen in this study is TRP2, which is a melanoma differentiation antigen. Our observation that TRP2-redirected T cells failed to control tumour growth suggests that TRP2 antigen is a poor target for T cell therapy. Nevertheless, the combination of redirecting T cell specificity to recognise TRP2 as well as effector function by overexpressing IL-12 has improvemed therapeutic efficacy in the absence of increased toxicity.

Concluding remarks

In this project, engineering T cell specificity and function by combining TCR gene transfer with genetic engineering to achieve effector cytokine production in therapeutic T cells represents an effective strategy to enhance cancer immunotherapy. The aim of delivering IL-12 safely at the tumour site using engineered T cells has been achieved by utilising tet-regulated gene expression system, resulting in enhanced antitumor immunity without any observable severe side effects. Exploring IL-27 delivery in engineered T cells has shown some antitumor effectiveness when combined with TCR gene transfer. Yet, many open questions still remain to be addressed to understand the mechanism by which IL-27 can enhance antitumor immunity in the context of adoptive cell therapy.

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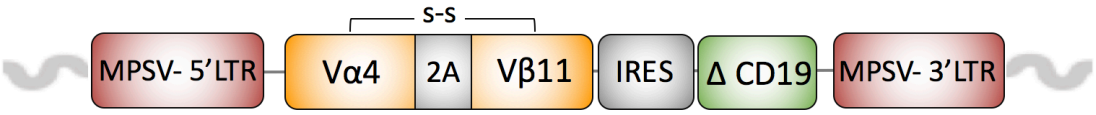
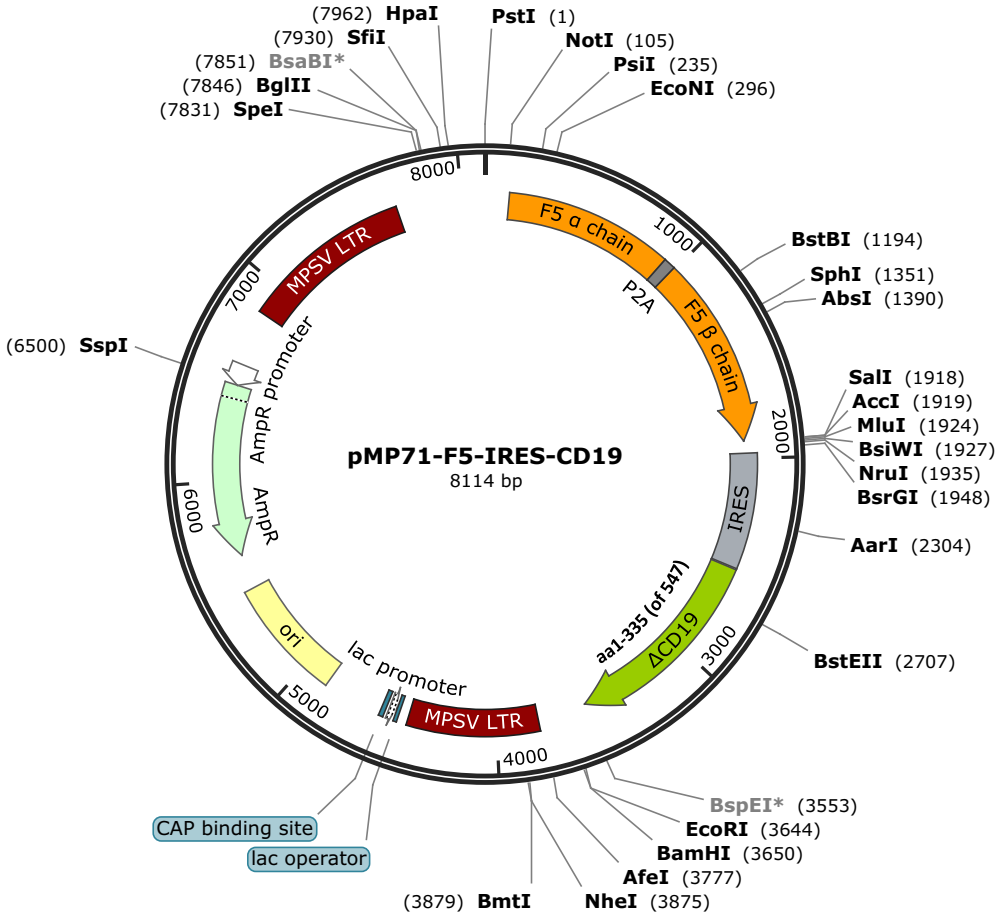
Appendix

Schematic diagram of TCRs used in this thesis

F5 TCR (pMP71_F5_IRES_CD19 retroviral construct)





TRP2 TCR (pMP71_TRP2_IRES_CD19 retroviral construct)