Delineating the role of IL-21 in different phases of $CD8^+$ T cell immune response

Hong Sheng Ong

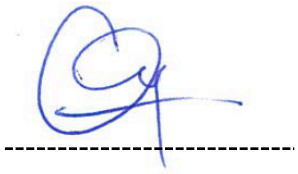
A thesis submitted for the degree of Doctor of Philosophy of The Australian National University

March 2019

Declaration

The experimental work presented in this thesis constitutes original work by myself under the supervision of A/Prof Di Yu unless otherwise stated in the methods, texts or figures. This thesis conforms to the Australian National University guidelines and regulations.

The work contained within has not been submitted for the purpose of obtaining any other degree at this or other universities.



Hong Sheng Ong
March 2019

Acknowledgments

I thank Di for the opportunity to be part of his lab. He is a supervisor who strives to understand the plight of students, and I deeply appreciate his effort in listening to my worries and problems – both scientific and personal. I have always been impressed by his passion in science – his wisdom and insight never fail to leave me in awe.

I would like to express my gratitude to my co-supervisor Ben and other members of my thesis advisory committee – Chris Parish, Ross Hannan and Anne Bruestle – for giving me invaluable advice on project matters. I also wish to thank Harpreet, Mick, Cathy and Anne for their professional help, and the Animal Technicians especially Joel, Samantha, Vidushi, Christine, Debbie, Daniel and Aaron for taking care of my mice. Special thanks to Tara and Marco for being there for us HDR students.

A huge thank you to members of the lab. To my Number One mentor – Yew Ann, thank you for imparting me with the skills to be successful in my PhD. Thanks for your patience and being ever so ready to help! You truly deserve all the success and achievements. I also thank Crystal and Anthony for being my other sources of knowledge and for providing me guidance whenever needed. My gratitude also goes to Kezhuo, Yin, Howard, Victor, PC, Eric and Anurag for their technical assistance, and Joseph, Michael, Andy, Raymond, Ting, Jie, Megan, Ryan and Jerry for helping me out in one way or another. I am honoured to have worked alongside such wonderful and outstanding people.

This PhD would not have been possible without my family and friends. I would like to thank my parents and my siblings – Ying and Boon – for their love and care. I also thank my Aunt Gina Ong; your support means so much to me. To my beloved friends – Jerry Lin, Joey, Anthony Liu, Jezza, Swen, Kristina, Jin, Jason Ng, Diana, Jason Ho, Auntie Helen, Kristy, Anna, Yin, Kezhuo, Wayne, Chinh, Jenni, Lithin, Yoshika, Dandan and Michelle, thank you for keeping me sane and alive in Australia. To my dearest buddies – Xiling, Deric and Weilong, thank you for always being there for me.

Last but not least, to my partner HY, I dedicate this PhD to you. Your unconditional love and support have given me the strength to complete something I once thought was insurmountable. Thank you for everything, you are the best!

Publications during enrolment

- **Ong HS**, Yim HCH. Microbial Factors in Inflammatory Diseases and Cancers. *In: Xu D. (eds)* ***Regulation of Inflammatory Signaling in Health and Disease. Advances in Experimental Medicine and Biology****, vol 1024. 2017. Springer, Singapore.*

- Leong YA, Chen Y, **Ong HS**, Wu D, Man K, Deleage C, Minnich M, Meckiff BJ, Wei Y, Hou Z, Zotos D, Fenix KA, Atnerkar A, Preston S, Chipman JG, Beilman GJ, Allison CC, Sun L, Wang P, Xu J, Toe JG, Lu HK, Tao Y, Palendira U, Dent AL, Landay AL, Pellegrini M, Comerford I, McColl SR, Schacker TW, Long HM, Estes JD, Busslinger M, Belz GT, Lewin SR, Kallies A, Yu D. CXCR5(+) follicular cytotoxic T cells control viral infection in B cell follicles. ***Nat Immunol.*** *2016 Oct;17(10):1187-96. doi: 10.1038/ni.3543.*

Abstract

Anti-tumour immune responses are largely carried out by cytotoxic immune cells including $CD8^+$ T cells and natural killer (NK) cells. Studies have shown that interleukin-21 (IL-21) can work synergistically with IL-7 or IL-15 to induce proliferation of both memory and naive $CD8^+$ T cells. On its own, IL-21 enhances the production of interferon-gamma (IFNγ), perforin and granzyme B (Gzm B), which are the key effector molecules sustaining the cytotoxic activity of $CD8^+$ T cells, via the induction of the transcription factor Tbet. Importantly, IL-21 prevents the exhaustion of $CD8^+$ T cells and is required to control chronic viral infection. In addition, IL-21 was shown to induce the proliferation of NK cells. It also plays a vital role in driving the maturation of NK cells, and thereby enhancing its effector cytotoxic function and IFNγ production. Furthermore, IL-21 has been shown to reverse the suppressive effects of regulatory T cells (Treg) on other T cells, ultimately improving the function of effector killer cells. All these pieces of evidence suggest that IL-21 could be a promising target for cancer immunotherapy.

Indeed, IL-21 treatment has been tested in various mouse tumour models and several human clinical trials for cancer immunotherapy as single agent or in combination. The results demonstrated that IL-21 treatment boosted the function of $CD8^+$ T cells and NK cells to better control cancers. Though encouraging, further development of IL-21 treatment is limited by our knowledge of how IL-21 treatment exactly regulates anti-tumour immune responses. For example, it remains unclear whether IL-21 treatment primarily enhances the priming of $CD8^+$ T cells or it mostly acts to sustain the function of $CD8^+$ T cells in the post-priming phase. My PhD project focuses on IL-21-mediated regulation on $CD8^+$ T cells and aims to delineate the role of IL-21 in different phases of immune responses. A better understanding of phase-specific function of IL-21 will greatly improve the design and application of IL-21 treatment.

By comparing the effect of IL-21 on $CD8^+$ T cell for priming and post-priming function, I found that IL-21 plays a more significant role on post-primed $CD8^+$ T cells by enhancing the effector function and also inducing the expression of memory and stem cell-like markers. The phenotypic and metabolic changes induced by IL-21 are distinct from the canonical effector T cell subset induced by IL-2, or memory T cell subset induced by IL-15. I revealed that IL-21-

induced $CD8^+$ T cells undergo major metabolic changes towards features reported to be associated with stem cells, which is likely mediated by a signalling pathway via the transcription factor hypoxia-inducible factor 1α (HIF-1α).

In summary, IL-21 primarily acts on the post-priming phase of $CD8^+$ T cells and induces a phenotype distinct from canonical effector or memory cells and associated with stem cell-like metabolic change. This discovery opens a new direction for the research of $CD8^+$ T cell differentiation and the application of rationale-based IL-21 therapy.

Table of contents

Abbreviations

%	Percentage
γ_c	Common gamma-chain
µg	Micrograms
µg/mL	Micrograms per millilitres
2B4	Signalling lymphocytic activation molecule 4
ACT	Adoptive cell transfer
AKT1	Protein kinase B
APL	Altered peptide ligand
AT3	C57BL/6-derived mammary gland carcinoma
B16F10	C57BL/6-derived skin melanoma
BCL2	Apoptosis regulator BCL2
BCL6	B-cell lymphoma 6 protein
CAR-T	Chimeric antigen receptor-T
CCR7	C-C chemokine receptor 7
CD107a	Lysosomal-associated membrane protein 1
CD122	Interleukin-2 receptor beta
CD127	Interleukin-7 receptor
CD25	Interleukin-2 receptor alpha
CD28	T-cell-specific surface glycoprotein
CD44	Extracellular matrix receptor III
CD62L	L-selectin
CD69	Early activation antigen CD69
CFSE	Carboxyfluorescein succinimidyl ester
CPT1	Carnitine palmitoyltransferase I
CTLA4	Cytotoxic T lymphocyte antigen 4
CTV	CellTrace Violet
DC	Dendritic cell
dLN	Draining lymph node
EC_{50}	Half-maximum effective killing
ECAR	Extracellular acidification rate

EGFR	Epidermal growth factor receptor
Eomes	Eomesodermin
ETC	Electron transport chain
FAO	Fatty acid oxidation
FTA	Fluorescent Target Array
G6P	Glucose-6-phosphate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLUT1	Glucose transporter 1
GMFI	Geometric mean fluorescence intensity
GzmB	Granzyme B
HI-FCS	Heat-inactivated fetal calf serum
HIF-1α	Hypoxia-inducible factor 1 alpha
hIL-2	Human interleukin-2
HIV	Human immunodeficiency virus
HK2	Hexokinase 2
HSC	Hematopoietic stem cell
i.p.	Intraperitoneal
i.v.	Intravenous
IFNγ	Interferon-gamma
IL	Interleukin
IL-21R	Interleukin-21 receptor
IRF4	Interferon regulatory factor 4
JAK	Janus kinase
Ki67	Proliferation marker protein Ki67
Lag3	Lymphocyte-activation gene 3
LCMV	Lymphocytic choriomeningitis virus
mAb	Monoclonal antibody
MC38	C57BL/6-derived colon carcinoma
MCA	Methylcholanthrene
MHC-I	Major histocompatibility complex class I
MHC-II	Major histocompatibility complex class II
MM	Metastatic melanoma
MRCC	Metastatic renal cell carcinoma

mTOR	Mammalian target of rapamycin
ng/mL	Nanogram per millilitres
NK	Natural killer
N4	OVA affinity peptide SIINFEKL
OCR	Oxygen consumption rate
ORR	Objective response rate
OT-I	Transgenic $CD8^+$ T cells that recognise chicken ovalbumin antigen peptide residues 257-264, SIINFEKL
OVA	Chicken ovalbumin
OXPHOS	Oxidative phosphorylation
PD1	Programmed cell death protein 1
PD-L1	Programmed death ligand 1
PD-L2	Programmed death ligand 2
Pmel-1	Transgenic $CD8^+$ T cells that recognise melanocyte differentiation antigen gp100 peptide residues 25-33, EGSRNQDWL
pMHC	Peptide-major histocompatibility complex
pmol/min	Picomole per minute
Q4	OVA affinity peptide SIIQFEKL
s.c.	Subcutaneous
s.e.m.	Standard error mean
Sca1	Stem cell antigen 1
SRC	Spare respiratory capacity
STAT	Signal transducer and activator of transcription
TAA	Tumour-associated antigen
Tbet	T-cell-specific T-box transcription factor
TCA	Tricarboxylic acid
TCF1	T-cell specific transcription factor 1
Tcm	$CD8^+$ T central memory cells
TCR	T cell receptor
Tem	$CD8^+$ T effector memory cells
Tfh	T follicular helper cells
TGFβ	Transforming growth factor beta

Th	T helper
TIL	Tumour-infiltrating lymphocytes
Tim3	T-cell immunoglobulin mucin receptor 3
TNFα	Tumour necrosis factor alpha
Treg	Regulatory T cells
Trm	Tissue-resident memory $CD8^+$ T cells
TRUCK	T cell redirected for universal cytokine-mediated killing
TSA	Tumour-specific antigen
Tscm	$CD8^+$ T memory stem cells
V4	OVA affinity peptide SIIVFEKL

Chapter 1

General Introduction

1.1 Cancer – the global pandemic

Cancer has remained as one of the leading causes of death in the past decade. According to the World Health Organisation, cancer cases worldwide are predicted to soar by 70% over the next two decades, from 14 million in 2012 to 25 million new cases a year [1]. In Australia alone, it is projected that the number of diagnosed cancer cases will reach about 150,000 in 2020, which is close to a 40% increase from 2007 [2]. On that note, major improvements in cancer treatment will be needed to tackle the increased cancer burden.

With the potential to promote tumour-specific immune response and generate immunological memory to tumour cells, immunotherapy is quickly developing into a new class of treatment that offers long lasting protection for cancer patients. By systematically targeting tumour cells in all parts of the body, the immune system acts through two mechanisms – innate and adaptive immunity [3], and can be applicable for almost all cancer types. Many immune cell types such as dendritic cells (DCs), macrophages, natural killer (NK) cells, $CD4^+$ T cells and $CD8^+$ T cells work collaboratively to eradicate tumour cells.

1.2 The role of the immune system in cancer – a double-edged sword

1.2.1 Inflammation and cancer development

Inflammation has been shown to play a role in cancer development. For example, low grade chronic inflammation caused by infections have been found to cause cancer – *Helicobacter pylori* infection causes gastric cancer [4, 5]; hepatitis B causes hepatocellular cancer [6]. This could be due to the effector mechanism regulated by the inflammatory pathway that generates free radicals to fight infections. These free radicals lead to oxidative DNA damage and on prolonged exposure can cause DNA mutations which promote cancer formation [7].

Following cancer formation, cancer cells can exploit inflammation to their favour by directly recruiting leukocytes that further results in the remodelling of the tumour microenvironment in

solid cancers [8, 9]. Tumour-associated macrophages and myeloid-derived suppressor cells, as well as helper T (Th) cell subset such as Th2 and Th17 then continue to promote inflammation and tumour growth, and inhibit anti-tumour responses [10-15].

Pro-inflammatory cytokines produced by tumour-infiltrating immune cells can further promote cancer development. For example, IL-6 has been shown to increase the survival and proliferation of intestinal epithelial cells, via the activation of signal transducer and activator of transcription 3 (STAT3), thereby resulting in the development of colitis-associated cancer [16]. Similar observations were also made in hepatocellular carcinoma where increased levels of IL-6 and TNF activate STAT3 resulting in hepatoma cell proliferation and survival [17]. In addition, IL-23 was found to upregulate the matrix metalloprotease MMP9 which is a key mediator of angiogenesis in tumours [14]. TNF-α-induced stabilisation of Snail, a transcription factor regulating epithelial-mesenchymal transition, was revealed to play a role in cancer cell migration and invasion [18].

1.2.2 Immune surveillance

The concept of cancer immune surveillance was first conceived by Ehrlich in 1909 [19]. Though not experimentally proven at that time, he proposed that the immune system can identify and remove nascent transformed cells. Subsequent discoveries using tumour transplantations further supported the hypothesis of cancer immune surveillance [20, 21]. Since then, the roles of immune cells in controlling cancer has been widely accepted and heavily studied.

T cells from the adaptive arm of the immune system recognise cancer antigens that are presented on major histocompatibility complex class I and II (MHC-I/II). Killing of cancer cells is a result of the release of cytotoxic molecules – Perforin, Interferon-gamma (IFNγ), and Granzyme B (GzmB) – leading to the death of cancer cells. $Rag2^{-/-}$ mice that are deficient in T cells were found to form tumours earlier than wild-type mice and in higher frequency when injected with methylcholanthrene (MCA) to induce tumour formation [22]. Similarly, the ability to control tumour was also ablated in IFNγ-insensitive mice that are either deficient in IFNγ receptor or STAT1, a transcription factor that is responsible in downstream signalling of IFNγ receptor [22], thus supporting the importance of T cells in anti-tumour response.

Unlike T cells, NK cells do not recognise their targets based on antigen specificity. Instead, NK cells identify and kill their target cells through an integrated balance of activating and inhibitory signals. NKG2D receptors found on NK cells interact with stress molecules such as MHC class I chain-related genes (MICA and MICB), expressed on a broad range of cancer, to activate target cell killing via IFNγ production [23]. NK cells also express the killer immunoglobulin-like receptors (KIRs) which interact with MHC-I to trigger inhibitory signals. Cancer cells have the ability to downregulate MHC-I [24], and as a result remove the inhibition on NK cells leading to NK cell activation when activating signals are present [25]. Evidently, the depletion of NK cells through the use of anti-NK1.1 or anti-asialo-GM1 in mice resulted in increased tumour formation after MCA injection [26].

1.3 $CD8^+$ T cells – the educated cancer killers

The discovery of tumour-associated antigens (TAAs) by van der Bruggen et. al. in 1991 first shed light on cancer-specific $CD8^+$ T cell response [27]. This is supported by the presence of cancer-specific $CD8^+$ T cells in the peripheral blood or tumour of patients that indicate signs of anti-tumour $CD8^+$ T cell response [28-30]. Studies have also shown that a high prevalence of intratumoural $CD8^+$ T cells in patients often correlate with better prognosis [31-34].

The employment of $CD8^+$ T cells to fight cancer is not a simple process **(Figure 1.1)**. In solid tumours, DCs phagocytose apoptotic cancer cells and migrate into the draining lymph node (dLN) to present TAA or tumour-specific antigen (TSA) to cognate $CD8^+$ T cells via the MHC-I. Recognition of the antigen by the T cell receptor (TCR) together with the costimulation of CD28 result in T cell activation and differentiation into effector T cells with potent anti-tumour function. Based on differences in chemokine expressions, these cells then egress the dLN and home into the tumour sites. Interaction with cancer cells via MHC-I will stimulate the release of cytotoxic molecules that eventually kill the cancer cells [35, 36]. Each step plays an important role in ensuring a potent anti-tumour $CD8^+$ T cell response.

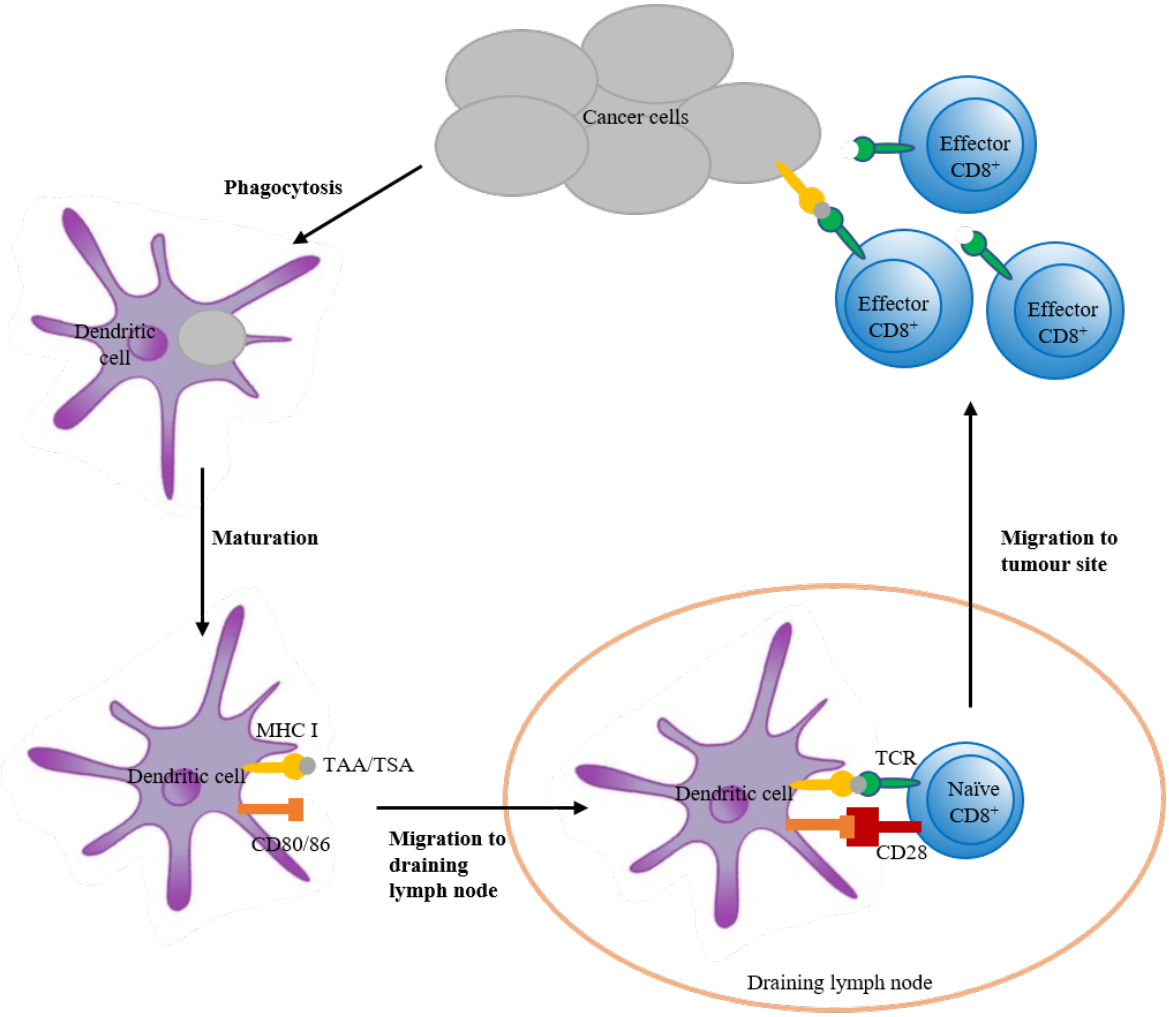


Figure 1.1. Activation of CD8$^+$ T cells in cancer. DCs capture cancer antigens, home into draining lymph node, and present the antigens through MHC-I to activate cancer-specific CD8$^+$ T cells. Effector CD8$^+$ T cells then migrate to tumour site to mount an immune response against the cancer cells.

There are multiple ways that the abovementioned process could go wrong which will result in the escape of cancer cells from immune recognition. Post selection in the thymus, surviving mature CD8$^+$ T cells have tolerance towards self-antigen. As basal expression of TAA also occurs in normal tissues, these antigens are subjected to central and peripheral tolerance mechanisms [37]. As such, CD8$^+$ T cells targeting TAA often have low avidity and could not mount a strong anti-tumour response. Another source of tumour antigen comes from TSA, also known as neoantigens, which are aberrant proteins that have arisen from mutations in the cancer cells. Though neoantigens are not subjected to central tolerance [37, 38], not all mutations are able to generate neoantigens recognisable by T cells [39, 40].

After $CD8^+$ T cells have been successful in launching an anti-tumour response, they face another challenge known as the immune checkpoints which is yet another safety mechanism of the immune system. When immune checkpoint receptors bind to their ligands, which can be expressed by immune cells such as dendritic and myeloid cells [41, 42], and can be upregulated by cancer cells [43], they initiate a chain of downstream inhibitory pathways that eventually downregulate $CD8^+$ T cell response [44]. The elevated expression of immune checkpoints on T cells is widely acknowledged to be a hallmark of exhausted T cells.

1.4 Cancer immunotherapy that boosts $CD8^+$ T cell responses

Since $CD8^+$ T cells are one of the main players in eradicating cancer cells, methods to circumvent those problems should favourably promote $CD8^+$ T cell anti-tumour activity and improve overall anti-tumour response.

1.4.1 Adoptive cell transfer and chimeric antigen receptor-T cells

With limited clones of $CD8^+$ T cells that are actually responding to tumour antigens, *ex vivo* expansion of these cells with suitable growth factors is used to promote better proliferation and survival after re-infusion *in vivo*. Adoptive cell transfer (ACT) therapy can be achieved by first isolating autologous or allogenic $CD8^+$ T cells from patients or donors respectively. Based on their ability to respond to cancer targets, cancer-specific cells are then selected from this pool, expanded *ex vivo*, and then introduced into patients **(Figure 1.2)**. This method was first pioneered by the Rosenberg's group in 1988 [45], and has been evolving ever since.

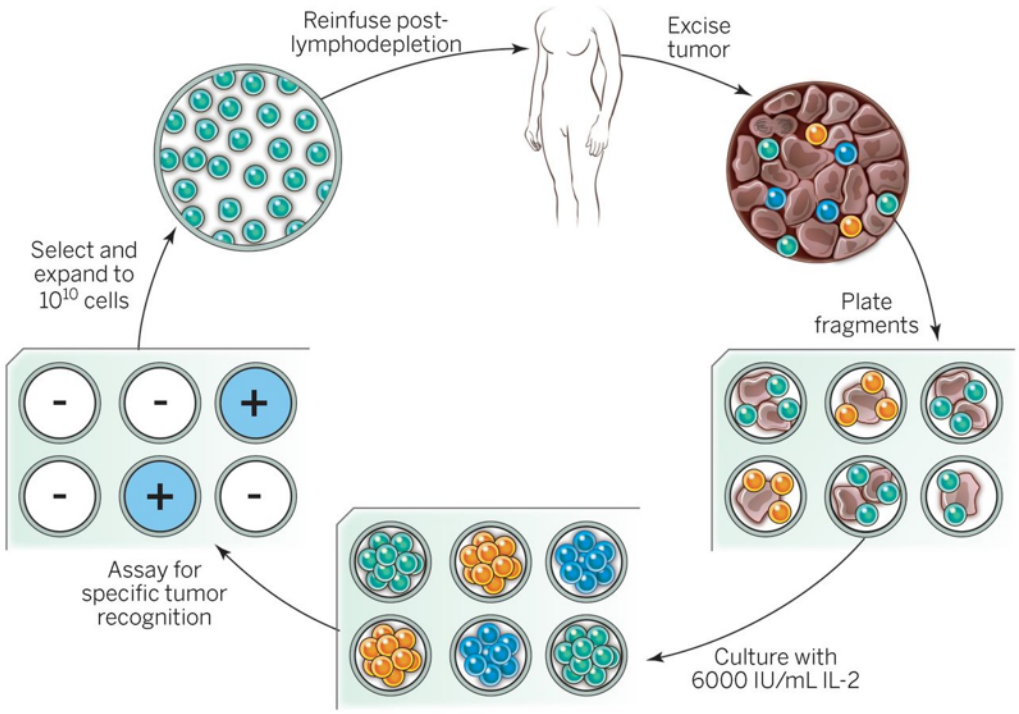


Figure 1.2. Adoptive cell transfer of tumour-specific CD8$^+$ T cells for cancer immunotherapy. Cells are harvested from tumours of patients and cultured *ex vivo* in IL-2 for two to three weeks to obtain pure lymphocyte cultures. Individual cultures are then tested for reactivity towards tumour. Those tested positive are selected and expanded before re-introducing them into patients. Figure from [46].

To enhance specificity towards cancer cells, CD8$^+$ T cells can be genetically modified to express high affinity TCRs. However, this did not significantly improve the outcome of cancer treatment, instead it resulted in an increase in side effects due to T cell recognition of the same antigen expressed in other tissues [47].

A recent advancement in ACT is the invention of chimeric antigen receptor (CAR)-T cells. CAR-T cells conventionally express a targeting motif which is a single chain variable region from a monoclonal antibody, linked to a transmembrane motif, and a TCR intracellular signalling activation motif – the ζ-chain. This design overcomes the problem of MHC-I downregulation found in certain cancer cells. Addition of costimulating motifs such as CD28 and 4-1BB in the second and third generation CAR-T has been found promote better efficacy **(Figure 1.3)** [48-50]. Further improvements include attempts to develop the fourth generation of CAR-T cells, also known as T cell redirected for universal cytokine-mediated killing

(TRUCKs). TRUCKs are modified to acquire cytokine-secreting ability to further improve CAR-T treatment outcome [51-53].

CAR-T treatment has achieved significant response but only in B cell haematological malignancies such as B cell acute lymphoblastic leukemia and chronic lymphocytic leukemia [54], whereas its results in solid tumours have been less promising [55]. This can be due to several factors – the lack of unique TAA and antigen heterogeneity; the inefficient trafficking to and penetration of tumour sites; and the hostile tumour microenvironment.

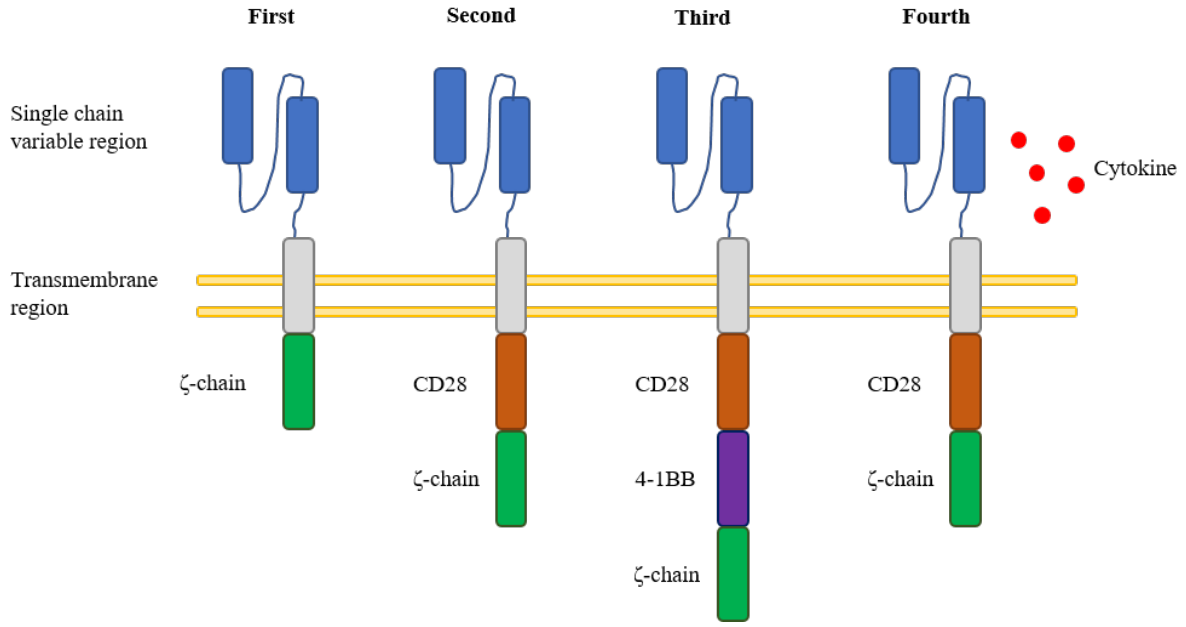


Figure 1.3. The advancement in chimeric antigen receptor (CAR). First generation CAR consists of a single chain variable region from a monoclonal antibody, linked to a transmembrane motif, and a TCR intracellular signalling activation motif – the ζ-chain. Second generation CAR consists of the addition of a costimulatory CD28 domain. Third generation consists of the addition of a transducer domain such as 4-1BB. Fourth generation consists of the addition of a cytokine-secreting gene such as IL-12 in the same CAR vector. Figure adapted from [56].

Ideally, the $CD8^+$ cell antigen should be strictly tumour-specific to avoid any off-tumour toxicity. For example, the epidermal growth factor receptor (EGFR) is an antigen expressed on normal cells. Even though its dysregulated expression was found to be associated with multiple cancers [57, 58], careful design of the CAR-T targeting motif is needed. One such target is a

truncated form of the extracellular EGFRvIII domain that results in a constitutive tyrosine kinase activity and thus promotes tumour growth and metastasis [59, 60]. In addition, high degree of tumour heterogeneity further complicates the use of CAR-T cells in treating solid tumours.

The lack of complementary chemokines CXCL9 and CXCL10 expression in tumours hampers the recruitment of $CXCR3^+$ effector $CD8^+$ T cells into tumour sites [61]. Upon successful migration to tumours, T cells will have difficulty penetrating the tumour endothelium as expressions of vascular endothelial growth factor-A and endothelin-1 can downregulate adhesion molecules [62, 63], which inhibits T cell infiltration. Furthermore, T cells will need to degrade components of the extracellular matrix such as the heparan sulphate proteoglycans in order to successfully penetrate the tumour bed [64]. Finally, the tumour microenvironment is inhospitable towards T cells. Hypoxic and nutrient-deficient, the tumour microenvironment decreases T cell proliferation and cytokine production [65, 66].

Long-term persistence of CAR-T cells have been shown to correlate with durable clinical remissions in patients [49, 67, 68], and could potentially be beneficial for treatment of solid tumours. Such long-term persistence could be related to the ability of CAR-T cells to form memory $CD8^+$ T cells *in vivo* [48, 68]. Indeed, modifications to cytokines used in *ex vivo* culture or forced expression of cytokines promoted the formation of the more undifferentiated memory subset and thus increased the eventual survival of these cells [69, 70]. Therefore, it is hopeful that further improvements to drive the development of CAR-T into undifferentiated memory such as the recently discovered T memory stem cells (Tscm) will enhance response rates in patients and prevent relapses.

1.4.2 Checkpoint blockade

The discoveries of checkpoint receptors and blockade of the checkpoint receptors have led to enormous breakthrough in cancer treatment. Notably, the researchers – Dr. James Allison and Dr. Tasuku Honjo – who discovered cytotoxic T lymphocyte antigen 4 (CTLA4) and programmed cell death protein 1 (PD1) respectively were awarded the Nobel Prize in Medicine in 2018.

CTLA4 shares similar ligands as the costimulatory receptor CD28 and has been shown to have an inhibitory effect on T cell response **(Figure 1.4)** [71-73]. Looking at its kinetics, CTLA4 is rapidly upregulated on the surface of T cells from intracellular protein stores as early as after one hour after antigen stimulation [74]. Though it seems that CTLA4 inhibition is mainly achieved by competing with CD28 for CD80 and CD86 binding, studies have shown that CTLA4 downstream signalling can directly interfere with TCR signalling [75-77]. Indeed, removal of CTLA4 in mice affects self-tolerance and eventually leads to immune hyperactivation [78, 79]. Preclinical studies in mice have revealed that CTLA4 blockade can inhibit tumour growth [80, 81]. In humans, administration of CTLA4 blockade can improve the overall survival of melanoma patients [82-84].

Till date, it remains relatively controversial if the effect of CTLA4 blockade acts directly by removing inhibitory signals in conventional T cells, or indirectly by suppressing Treg activity. Treg is a major cell type that expresses CTLA4 and there has been evidence suggesting that CTLA4 provides a form of activating signal for Treg [85, 86]. Recent studies also propose that CTLA4 on Tregs can induce the downregulation of CD80 and CD86 on antigen-presenting cells by physically removing the ligands via transendocytosis [87-90]. Through this process, Treg uptake the CD80 and CD86 ligands and target them for lysosomal degradation [90].

PD1 is another checkpoint receptor that can inhibit T cell response. Unlike CTLA4, PD1 expression takes a much longer time of about 48 hours for transcription to first occur [91]. As such, expression of PD1 is often referred to as the hallmark of T cell exhaustion. Binding to its ligands PD-L1 and PD-L2 found on immune cells and cancer cells [41-43], PD1 can directly inhibit TCR downstream signalling by SHP-2-mediated dephosphorylation of ZAP70 and PI3K [92]. Interestingly, a recent finding has elegantly proposed that PD1 actually favours the dephosphorylation of CD28 over TCR signalling components **(Figure 1.5)** [93]. Along the same line, knockout of CD28 has been shown to obliterate PD1 blockade-driven $CD8^+$ T cell proliferation, again indicating a vital role of CD28 signalling pathway in PD1 blockade [94]. When used in human clinical trials, PD1 blockade showed efficacy in treating diverse cancer types such as melanoma, kidney and colorectal cancers [95, 96], and have achieved durable responses in patients [97]. As the ligands of PD1 are mostly upregulated on cancer cells, blockade of PD1 is thought to be more specific and results in increased efficacy and decreased toxicity in humans as compared to CTLA4 blockade [98, 99].

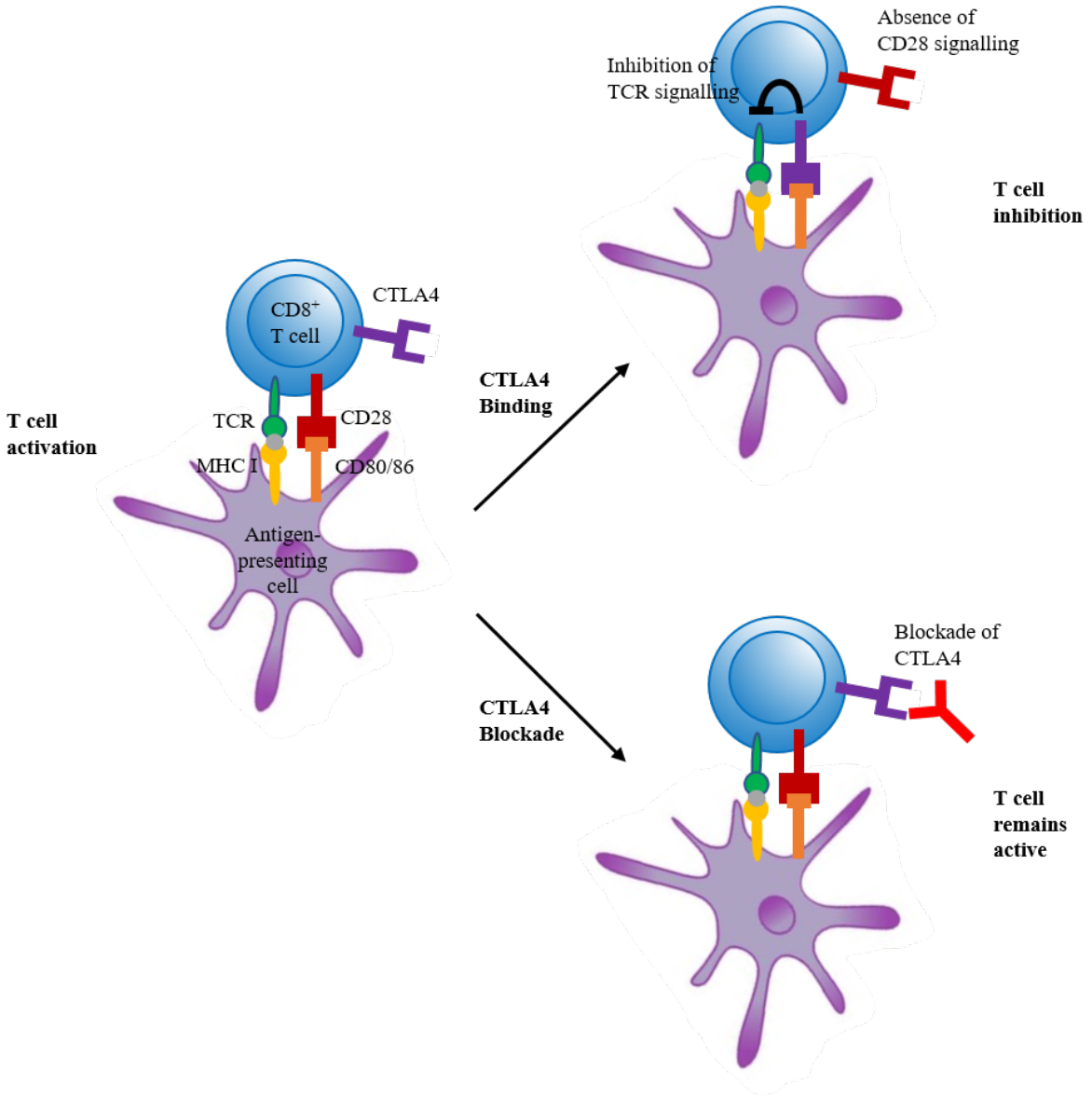


Figure 1.4. CTLA4 inhibits T cell response. $CD8^+$ T cells rapidly upregulates CTLA4 after activation. Binding of CD80/86 to CTLA4 inhibits T cell function by reducing CD28 costimulatory signalling and inhibiting TCR signalling. Blockade of CTLA4 using anti-CTLA4 antibodies prevents T cell inhibition.

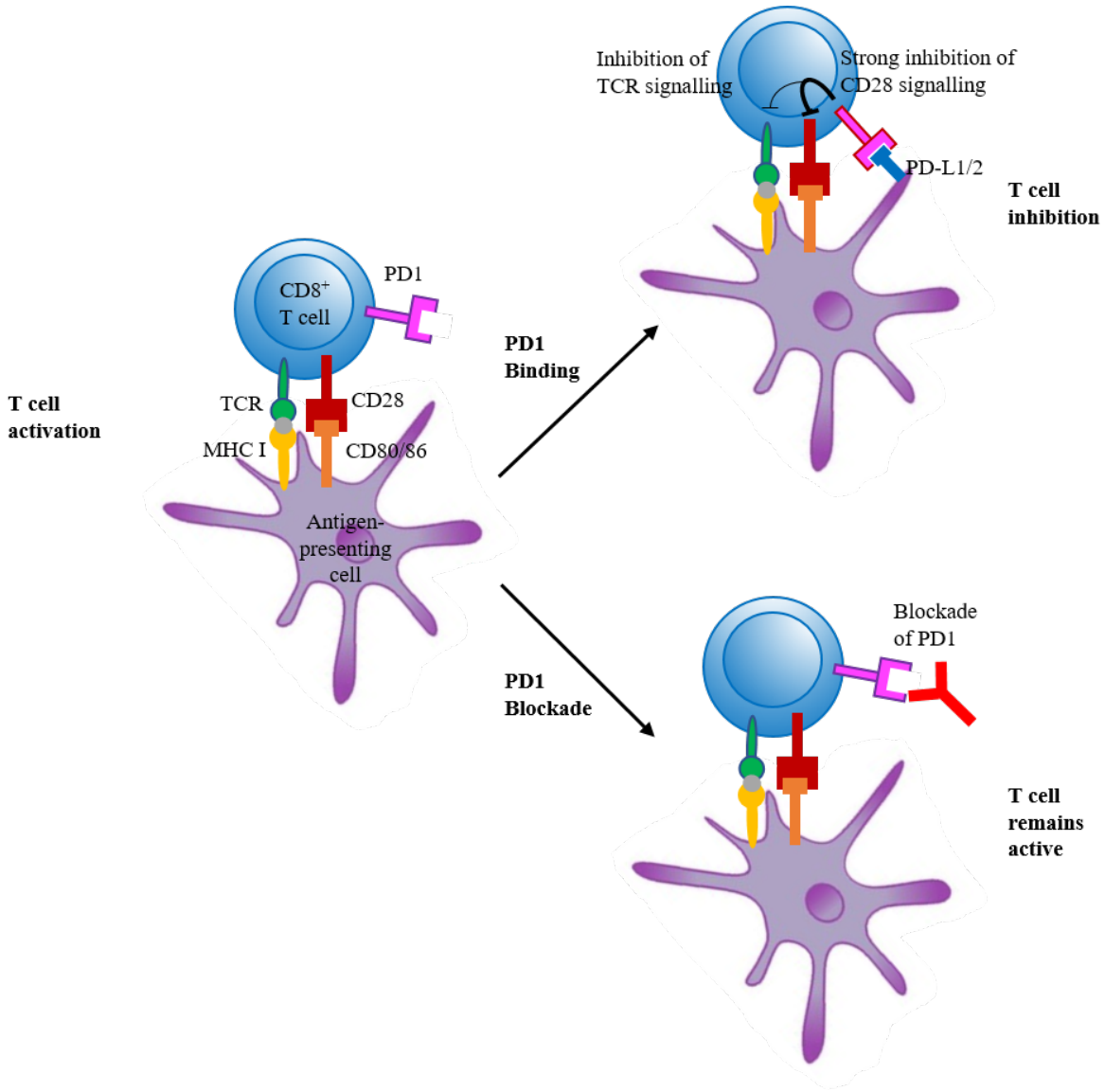


Figure 1.5. PD1 inhibits T cell response. $CD8^+$ T cells upregulates PD1 after activation. Binding of its ligand PD-L1/2 to PD1 inhibits T cell function by inhibiting predominantly CD28 costimulatory signalling and TCR signalling. Blockade of PD1 using anti-PD1 antibodies prevents T cell inhibition.

Despite notable success, the response rates in single agent checkpoint blockade treatment remained relatively low, and insights into accurate biomarkers providing early indication of positive response will be needed to steer decisions towards the correct treatment approach. PD-L1 expression within the tumour microenvironment was previously shown to be a favourable indication of patient response towards PD1 blockade treatment [95]. However, when PD1 blockade was compared across patients with PD-L1-positive versus PD-L1-negative tumours, patients with PD-L1-negative tumours still had an objective response rate (ORR) of 41.3%,

albeit it was lower than the 57.5% for patients with PD-L1-positive tumours [100]. This suggests that the use of PD-L1 expression level cannot confidently predict the success of PD1 blockade response.

Another proposed biomarker is the mutational burden of the cancer [101]. As PD1 blockade aims to re-invigorate cancer antigen-experienced T cells, absence of neoantigen or neoantigen presentation will indicate absence of such T cells, and thus the absence of "substrate" – the cancer antigen-experienced T cells – for the treatment to work on [101]. Higher mutation burden in tumours was found to be associated with improved ORR of 63%, durable clinical benefit and progression-free survival when patients were treated with PD1 blockade [102]. Consistent with this, higher mutational rate and neoantigen load were also found to correlate with clinical benefit in CTLA4 blockade treatment [103]. Intuitively, cellular defects such as DNA mismatch-repair deficiency that contribute to higher somatic mutations can also predict positive response to PD1 blockade treatment [104, 105].

A key question remains – how can we sensitise non-responders to checkpoint blockade? Understandably, this will mainly involve boosting the number of cancer antigen-experienced T cells, in other words, a way to increase T cell recognition of the cancer cells.

1.5 IL-21 treatment – the possible solution to boost $CD8^+$ T cell-targeted treatments?

1.5.1 Effects of IL-21 on immune cells

Interleukin-21 (IL-21) is the latest addition to the common-gamma chain cytokine family. Primarily produced by natural killer T, T follicular helper (Tfh) and Th17 cells [106], IL-21 mediates a diverse range of regulatory function on immune cells during allergy, cancer and viral infection **(Figure 1.6)**. IL-21 executes its anti-tumour function mainly through $CD8^+$ T cells, NK cells and regulatory T cells (Treg). Studies have shown that IL-21 can work synergistically with IL-7 or IL-15 to induce proliferation of both memory and naïve $CD8^+$ T cells [107]. On its own, IL-21 enhances the production of IFNγ, perforin and GzmB, which are the key effector molecules sustaining the cytotoxic activity of $CD8^+$ T cells, via the induction

of the transcription factor T-cell-specific T-box transcription factor (Tbet) [108]. In addition, IL-21 was shown to induce the proliferation of NK cells [109]. It also plays a vital role in driving the maturation of NK cells [110], and thereby enhancing its effector cytotoxic function and IFNγ production [111, 112]. Furthermore, IL-21 has been shown to reverse the suppressive effects of Treg cells on other T cells [113-115], ultimately improving the function of effector killer cells.

The broad downstream effects of IL-21 also mediate the activity of other cell types. In an autocrine manner, IL-21 induces BCL6 and RORγt, which in turn promotes the differentiation of Tfh and Th17 respectively [116-118]. For B cells, IL-21 not only affects the survival of B cell [109], but also plays a role in class switching of the immunoglobulin (Ig) particularly promoting IgG1 and IgG3 [119]. Direct regulation of BCL6 allows IL-21 to promote B cell proliferation and retention in germinal centres (GC) [120, 121].

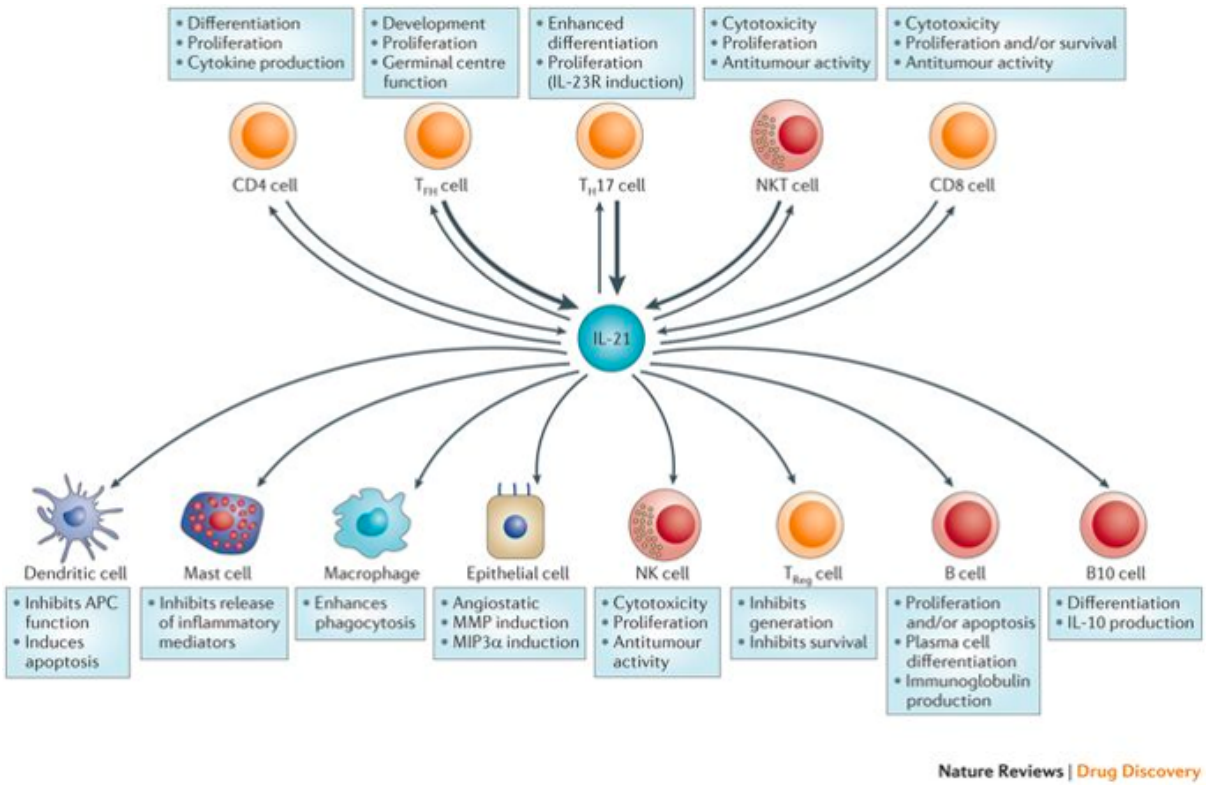


Figure 1.6. Sources of IL-21 and its effects. IL-21 is primarily produced by Tfh and Th17 $CD4^+$ T cells. IL-21 exerts its pleiotropic effects on a range of lymphoid and myeloid immune cells as well as epithelial cells. Figure from [122].

1.5.2 IL-21 signalling

IL-21 is a four α-helical bundle cytokine which belongs to the type-I cytokine family. The cytokines of this family have heterodimer receptors which share the common gamma-chain (γ_c). Binding of IL-21 to the receptor complex, formed by the IL-21 receptor (IL-21R) chain and the γ_c, recruits and activates Janus kinase 1 (JAK1) and JAK3 which then phosphorylate predominantly STAT3, and weakly STAT1 and STAT5 proteins **(Figure 1.7)** [123, 124]. As a result, STAT dimerises, translocates into the nucleus, binds to the target gene regulatory elements, and activates the target genes. While IL-21 is able to induce the phosphorylation of multiple STAT proteins, a reciprocal balance between STAT3 and STAT1 has been reported in $CD4^+$ T cells, that is, the activation of STAT1 is increased in the absence of STAT3 [125]. Additionally, PI3K/AKT and MAPK pathways have also been shown to be downstream targets of IL-21 signalling [124]. Interestingly, IL-21 has been shown to induce the expressions of genes needed for the differentiation and function of both effector $CD8^+$ T cells, such as *TBX21* and *IFNG* [108, 126], and memory $CD8^+$ T cells, such as *EOMES*, *TCF1* and *LEF1* [127, 128].

In addition, a recent genome-wide analysis using chromatin immunoprecipitation sequencing revealed that almost half of IL-21-regulated genes were also bound by interferon regulatory factor 4 (IRF4) in $CD4^+$ T cells and B cells [129]. IRF4 deficiency resulted in diminished STAT3 binding and STAT3-dependent gene expression [129], thus confirming cooperative binding between the two transcription factors. IRF4 also binds to AP-1–IRF composite elements in T cells, and ETS–IRF composite elements in B cells via the cooperative binding of BATF and JUN [130-132]. As a result, many IL-21-target genes are regulated by all four transcription factors.

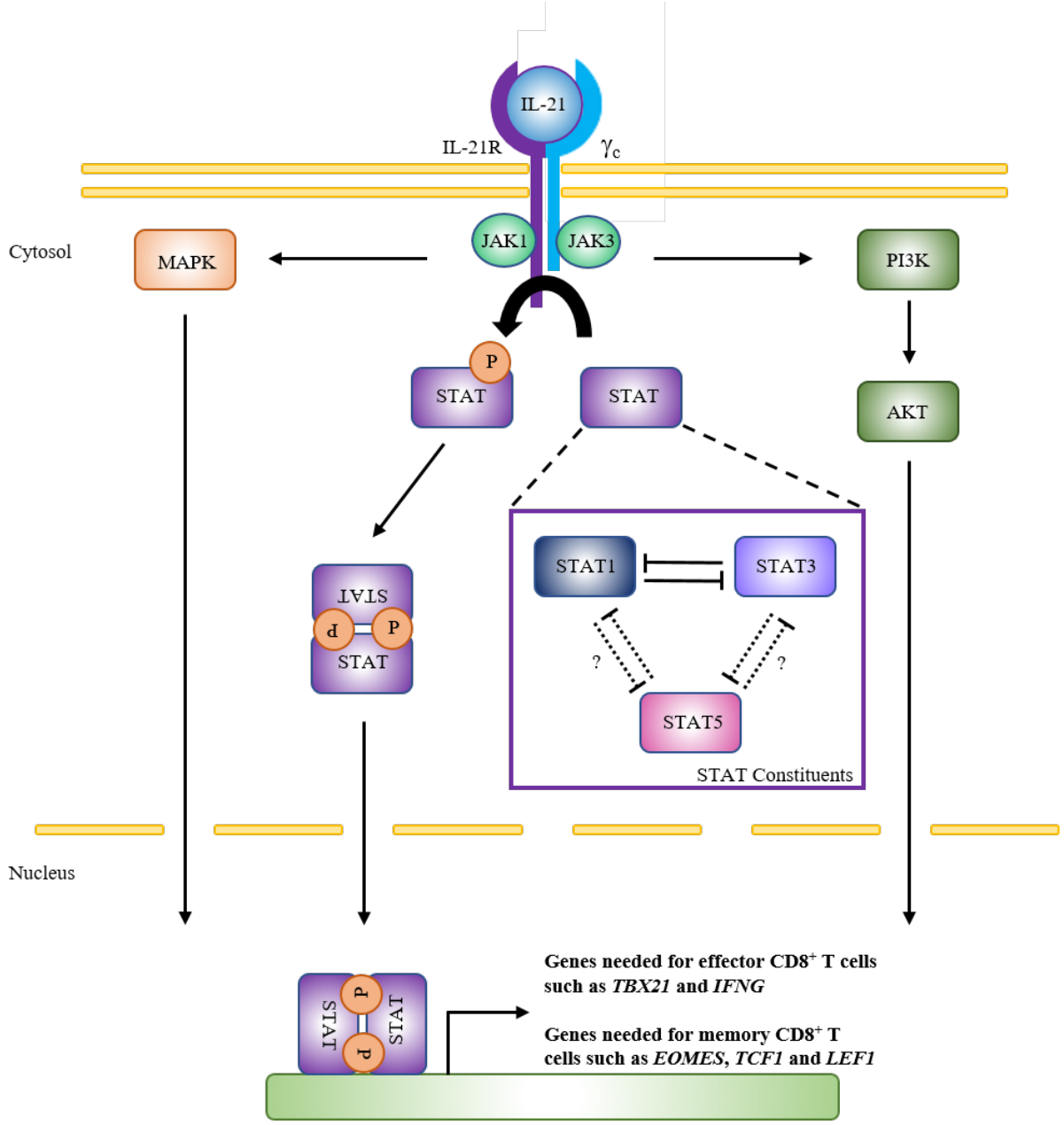


Figure 1.7. IL-21 signalling pathways. IL-21 binds to the IL-21R/γ_c receptor complex and triggers activation of JAK1 and JAK3, which in turn phosphorylates the STAT proteins. There is opposing action between STAT1 and STAT3, though that of STAT5 remains unknown. Phosphorylated STAT proteins dimerise and translocate into the nucleus to activate transcription of IL-21-target genes such as *TBX21*, *IFNG*, *EOMES, TCF1* and *LEF1*. IL-21 can also induce MAPK and PI3K signalling pathways. Figure adapted from [133, 134].

1.5.3 IL-21 in treating cancer

Cytokines have been known to direct T cell activation and function such as CD4 T cell polarisation and CD8 T cell effector function. In addition to signals from the TCR and the co-receptor CD28, a third signal is to induce the final T cell activity. It has since become a dogma that sequential exposure to the three signals is imperative for the full activation of T cells **(Figure 1.8)** [135, 136]. Furthermore, a recent study has found that the three signals are summed linearly to determine division fate of $CD8^+$ T cells [137]. As such, cytokine can be postulated to improve CD8 T cell activation and function when the TCR and costimulatory signals are weak like in cancer.

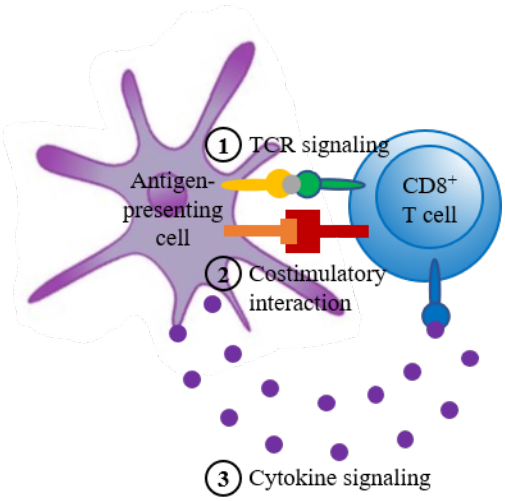


Figure 1.8. Three-signal model for T cell activation. Signal 1: Stimulation of the TCR by MHC/peptide complexes; Signal 2: Interactions between co-stimulatory ligands CD80/86 on the APC and CD28 on the T cell; Signal 3: The secretion of cytokines that polarise T cell responses.

Indeed, evidence of IL-21 in stimulating $CD8^+$ T cell anti-tumour response can be observed in several studies. When IL-21 was administered intraperitoneally, the survival of mice bearing the E.G7 thymoma tumour was improved compared to untreated, IL-2-treated and IL-15-treated mice [138]. Depletion of different cell types – NK cells, $CD4^+$ T cells and $CD8^+$ T cells – revealed that the anti-tumour immunity was mediated largely by $CD8^+$ T cells. Administration of IL-21 was found to augment the number of antigen-specific $CD8^+$ T cells through reducing apoptosis. In synergy with IL-15, IL-21 enhances the proliferation and effector function of $CD8^+$ T cells *in vitro*. Indeed, combination administration of IL-15 and IL-

21 was found to further increase tumour regression in B16 melanoma-bearing mice [107]. In the same study, a microarray analysis of naive $CD8^+$ T cells treated with cytokine *in vitro* revealed that combination of IL-15 and IL-21 synergistically upregulates the *GZMB* and *JUN* genes which are important for cytotoxicity and proliferation respectively. Interestingly, in another study, when pmel-1 $CD8^+$ T cell was treated with IL-21 while being primed by its cognate antigen, IL-21 appeared to confer a more memory $CD8^+$ T cell phenotype with reduced effector function – $GzmB^{lo}$, $CD44^{lo}$ and IL-$2R\alpha^{lo}$ [128]. When these cells were adoptively transferred into B16 melanoma-bearing mice, it resulted in increased tumour regression.

Despite positive outcomes in pre-clinical experiments, single agent IL-21 has shown mixed results in human clinical trials. In metastatic melanoma (MM), IL-21 treatment has resulted in ORRs of 34.5% and 50% in two phase I trials [139, 140], and 8.3% and 22.5% in two phase II trials [141, 142]. While in metastatic renal cell carcinoma (MRCC), IL-21 treatment has demonstrated an ORR of 89%. Changes in $CD8^+$ T cells have been observed in an IL-21 trial on patients with MM and renal cell carcinoma [143]. In that study, IL-21 upregulated cell proliferation, motility, activation and effector functions in $CD8^+$ T cells.

Combination of IL-21 with other cancer drugs has also been explored. Two phase I trials that utilised tyrosine kinase inhibitors together with IL-21 to treat MRCC showed inconsistent results. In an earlier study, combination of 10 μg/kg IL-21 with a tyrosine kinase inhibitor, sunitinib, resulted in high toxicities in patients and the study was subsequently discontinued [144]. On the other hand, the use of IL-21 with another tyrosine kinase inhibitor, sorafenib, showed anti-tumour activity with less toxicities in patients and achieved an ORR rate of 21% [145].

1.5.4 Combination of IL-21 with current cancer immunotherapy techniques

The ability of IL-21 to induce $CD8^+$ T cells of a more memory phenotype might be useful when propagating CAR-T cells *ex vivo*. This is likely to promote a $CD8^+$ T cell subset that can persist long-term *in vivo*. Indeed, CAR-T cells that were cultured in IL-21 and IL-2 have higher expression of memory markers – CD28, CD62L, CCR7 and CD45RO, and lower expression of effector markers – Eomesodermin (Eomes) and KLRG1 as compared to IL-2 alone [146].

When transferred into mice, CAR-T cells cultured in both IL-21 and IL-2 showed greater inhibition of tumour growth [146]. This result is consistent to the culture of cancer-specific $CD8^+$ T cells in IL-21 alone, which resulted in greater inhibition of tumour growth after IL-21-treated pmel-1 $CD8^+$ T cells were adoptively transferred into B16 melanoma-bearing mice [128].

Since IL-21 has the ability to increase PD1 expression on $CD8^+$ T cells *in vitro* [147], it could potentially sensitise non-responders to PD1 blockade therapy. Together with the ability to increase effector function of $CD8^+$ T cells, it can be postulated that IL-21 might achieve this by increasing the number of $CD8^+$ T cells that are activated through improving antigen sensitivity towards weak affinity peptides. *In vivo* studies using the combination of IL-21 and PD1 blockade were carried out by Bristol-Myers Squibb in 2013 [148], and showed enhanced efficacy in controlling tumour as compared to single agent treatments. This was supported by a separate study which attributed this observation to an increase in $CD8^+$ T cell infiltration, proliferation and an increase in $CD44^+$ $CD62L^-$ effector or effector memory phenotype over the $CD44^+$ $CD62L^+$ central memory phenotype [149].

1.5.5 The dilemma of IL-21 – $CD8^+$ T cell effector or memory?

IL-21 has been shown to elicit notable anti-tumour response in mice and humans. However, its actual mechanism of action still remains relatively conflicting with different studies reporting either an increase in effector or memory phenotypes. Two possibilities can be conceived – 1) IL-21 treatment primarily enhances the priming of $CD8^+$ T cells leading to higher number of activated $CD8^+$ T cells; 2) IL-21 promotes more memory $CD8^+$ T cell formation which eventually results in higher magnitude response upon rechallenge.

In the last decade, studies on chronic viral $CD8^+$ T cell response have shed light on the physiological role of IL-21. When comparing the level of IL-2- and IL-21-producing SMARTA T cells in lymphocytic choriomeningitis virus (LCMV)-infected mice, it was revealed that a higher percentage of cells were producing IL-21 as compared to IL-2 in chronic LCMV infection [150]. Conversely, there was more SMARTA T cells producing IL-2 in acute LCMV infection. This suggests that IL-21 response might be more dominant in chronic viral infection as opposed to acute which is more IL-2-oriented. Further comparison between early

(day 9) and late (day 30) phases of LCMV showed an almost four-fold increase in IL-21-producing SMARTA T cells in day 30 [150]. When IL-21 receptor was knocked-out in mice, it resulted in the inability to sustain virus-specific $CD8^+$ T cells during chronic viral infection, therefore leading to a reduced ability to control the chronic infection [150-152]. Collectively, these studies reveal that IL-21 is essential in the later phase of chronic viral response, and not in acute viral response. This indicates that IL-21, under physiological conditions, performs a more important role in the later stages of the adaptive immune response. On a similar note, human immunodeficiency virus (HIV) patients with dysfunctional IL-21 production or Tfh response have been found to correlate with disease progression [153-155], implying an inability to control the HIV infection and other opportunistic infections long-term in the absence of IL-21-mediated response.

Another role of IL-21 could be in $CD8^+$ T cell activation. As the three signal T cell activation theory suggests, cytokine might have the ability to contribute to $CD8^+$ T cell activation during priming [135, 156]. To elaborate, T cells interpret the sum of three signals – TCR signal, CD28 signal, and cytokine signal – in order to overcome a threshold to undergo activation. These three signals have been shown to be added linearly resulting in an overall signal strength [137]. For example, the addition of IL-2 was found to supplement the TCR signal required by $CD8^+$ T cell to proliferate [157]. It is thus plausible that IL-21 can perform a similar role.

Although both hypotheses have proposed the functions of IL-21 in two far spectrums of $CD8^+$ T cell response, they might not be mutually exclusive. Given the different effects that each cytokine has in different phases of immune response, the administration of IL-21 at a different phase of $CD8^+$ T cell response might bring about a completely different effect. This, therefore, warrants further investigation.

1.6 Hypothesis and Aims

Hypothesis

I hypothesise that the effect of IL-21 is different in the early and late phases of $CD8^+$ T cell response. Therefore, the timing of IL-21 administration is critical in determining the outcome of treatment.

To examine this hypothesis, this thesis has addressed several aims:

Aims

1. **To determine the effects of IL-21 on $CD8^+$ T cell activation and killing function when treated during priming or post-priming (Chapter 3)**

   This study will first attempt to understand if IL-21 can enhance $CD8^+$ T cell effector function by increasing $CD8^+$ T cell activation. A comparison between the timing of IL-21 administration – during $CD8^+$ T cell priming versus post $CD8^+$ T cell priming – on $CD8^+$ T cell killing will be made.

2. **To examine the effects of IL-21 in different phases of anti-tumour response (Chapter 4)**

   This study will once again compare if the timing of IL-21 administration has a different effect on anti-tumour response by using multiple solid tumour mice models. Changes in $CD8^+$ T cell phenotype will be examined.

3. **To compare the distinct $CD8^+$ T cell differentiation features induced by IL-2, IL-15 and IL-21 (Chapter 5)**

   This study will focus on one of the most important $CD8^+$ T cell post-priming step – $CD8^+$ T cell memory formation. Changes to $CD8^+$ T cell phenotype will be compared to IL-2 and IL-15 which are known to generate effector and memory $CD8^+$ T cells respectively.

4. **To understand the metabolic regulation of $CD8^+$ T cells by IL-21 (Chapter 6)**

This study will examine the changes in metabolism – glycolysis, oxidative phosphorylation, fatty acid oxidation, and more – in IL-21-treated cells. The metabolic profile of a cell can shed light on its function and role.

Findings from these studies will provide critical insights into how IL-21 regulate $CD8^+$ T cell response with emphasis on the changes in $CD8^+$ T cell phenotype and metabolism. On a larger scale, the use of IL-21 in cancer immunotherapy can be optimised to best exploit the function of IL-21 to enhance $CD8^+$ T cell function in anti-tumour response.

Chapter 2

Materials and Methods

2.1 Mice

All mice used were 7-14 weeks old female on wildtype C57BL/6 background and were maintained in specific pathogen free animal facilities in Monash University and the Australian National University. Wildtype mice and CD45.1 congenic mice were purchased from Monash Animal Research Platform, Australian Phenomics Facility, or the Animal Resources Centre. For transgenic strains, OT-I mice [transgenic expression of a T cell receptor specific for chicken ovalbumin (OVA) peptide residues 257-264, SIINFEKL] were used. In adoptive transfer experiments, animals were of matched sex and age. Animal experiments were approved by the Animal Ethics Committee of the institutes responsible for housing the mice.

2.2 Cell lines and tumour models

B16F10 melanoma were obtained from the Charles Mackay laboratory in Monash University. AT3 mammary gland carcinoma and MC38 colon carcinoma were obtained from the Phillip Darcy laboratory in Peter MacCallum Cancer Institute. B16F10 cells (1 x 10^5/mouse), AT3 cells (1 x 10^6/mouse), and MC38 cells (1 x 10^6/mouse) were inoculated s.c. into the right flanks of C57BL/6 mice. For tumour rechallenge, MC38 cells (10 x 10^6/mouse) were inoculated s.c. into the left flanks of C57BL/6 mice after primary tumour (on the right flank) had completely regressed. Tumour diameter was measured every other day for the first two weeks, twice weekly thereafter, and was used to calculate tumour volume (mm^3) using the formula 0.5 x length x breadth x breadth.

2.3 *In vivo* antibody treatment

Anti-CD4 (clone GK1.5) was purchased from the Antibody Services department in the Walter and Eliza Hall Institute. Anti-PDL1 (clone MIH5) was produced in-house and tested using the ToxinSensor Chromogenic LAL Endotoxin Assay Kit (GenScript) to have low endotoxin level (< 0.01 EU/mL). Antibodies were injected i.p. at a dose of 100 μg per mouse. Anti-CD4

monoclonal antibody (mAb) was administered on days 5, 9 and 13 after tumour inoculation. Anti-PDL1 was administered on days 5, 7, 9, 11, and 13 after tumour inoculation.

2.4 *In vivo* IL-21 treatment

Murine IL-21 was produced in-house [158] and tested using the ToxinSensor Chromogenic LAL Endotoxin Assay Kit (GenScript) to have low endotoxin level (< 0.01 EU/mL). Activity of in-house produced IL-21 was tested to be comparable to commercial IL-21 (Peprotech) **(Supplementary Figure S1)**. The 20, 50 and 80 μg dose were chosen on the basis of the dose-titration experiments performed by Sondergaard et al. that recommended 50 μg of IL-21 to be the optimal dose for i.p. injection [159]. 20 μg and 50 μg were then selected as the low and high dose respectively. For Fluorescent Target Array experiments, IL-21 was injected i.p. at 80 μg on days 1, 2 and 3, or at 50 μg on days 0 and 1. To treat solid tumours, IL-21 was injected i.p. at 20 μg every other day after solid tumour formation. To treat mice before tumour rechallenge, IL-21 was injected i.p. at 20 or 50 μg every other day from day 28 to 42.

2.5 Cell preparation

Mouse lymphocytes were prepared by gently mashing spleens or lymph nodes through 70 μm cell strainers. Red blood cells were then lysed with 1X Lysing Buffer (BD) for 3 minutes on ice. Single cell suspension was prepared by gently pipetting the tissues in complete RPMI [cRPMI, consists of 10 mM HEPES (Gibco), 1X non-essential amino acids solution (Gibco), 1 mM sodium pyruvate (Gibco), 1X Penicillin-Streptomycin-Glutamate solution (PSG, Gibco), 50 mg Normocin (Invivogen), 55 μM 2-mercaptoethanol and 10% heat-inactivated fetal calf serum (HI-FCS) in RPMI media (Sigma)]. Tumour-infiltrating lymphocytes (TIL) were harvested by cutting tumours into small pieces (<3mm), incubating tumours in dissociation solution (RPMI supplemented with 10% FCS, 100 U/mL Collagenase D, 100 μg/mL DNAse I), and gradient-separation in Lympholyte-M (Cedarlane).

Buffer used for magnetic separation is DPBS containing 2% HI-FCS and 1mM EDTA (Sort buffer). Buffer used for mAb staining for flow cytometry is DPBS containing 2% HI-FCS, 1mM EDTA and 10% sodium azide (FACS buffer).

B16F10, AT3 and MC38 cells used for tumour inoculation were dissociated using 0.25% Trypsin-EDTA (Gibco), neutralised in cold complete DMEM [cDMEM, consists of PSG and 10% HI-FCS in DMEM media (Sigma)], spun down at 1500 rpm 5 minutes, and resuspended in cold PBS for injection.

2.6 Flow cytometry

For surface staining of splenocytes and TIL, cells were incubated with anti-mouse antibodies for 30 minutes at 37°C.

For staining of intracellular cytokines, mouse splenocytes were stimulated with 50 ng/mL PMA (Sigma) and 1 μg/mL ionomycin (Sigma) in the presence of monensin (eBioscience) and brefeldin A (eBioscience) for four hours at 37°C. Stimulated cells were then stained with surface antibodies for 30 minutes at 4°C, washed twice after surface staining and permeabilised using Cytofix/Cytoperm (BD) for 30 minutes on ice. Antibodies specific for intracellular proteins were diluted in Perm/Wash Buffer (BD) and incubated for 30 minutes at 37°C.

For staining of intracellular transcription factors, cells were stained with surface antibodies, washed twice after surface staining, and permeabilised using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) for 30 minutes on ice. Antibodies specific for intracellular transcription factors were diluted in the Wash Buffer (eBioscience) and incubated for one hour at 37°C. When secondary staining was needed, cells were stained with fluorescent-conjugated anti-rabbit IgG for 30 minutes on ice.

For staining of mitochondria, cells were cultured in cRMPI with 50 nM of Mitotracker Green (Molecular Probes) for 20 minutes at 37°C.

All samples were resuspended in FACS buffer and acquired using BD LSRFORTESSA X-20 flow cytometer. Data were analysed with FlowJo. Antibodies used in experiments are listed in **Table 2.1**.

2.7 Cell culture

For $CD8^+$ T cell activation culture, naïve $CD8^+$ T cells were isolated using the naïve $CD8a^+$ T cell isolation kit (Miltenyi Biotec). Polyclonal naïve $CD8^+$ T cells were cultured in flat-bottom 96-well plates that were coated with anti-CD3 antibody (clone 145-2C11, WEHI) overnight at 4°C. Transgenic OT-I naïve $CD8^+$ T cells were cultured in round-bottom 96-well plate. Cells were cultured in cRPMI supplemented with soluble anti-CD28 antibody (clone 37.51, WEHI) and different concentration of murine IL-21 (Peprotech). Transgenic OT-I naïve $CD8^+$ T cells were cultured in the presence of different OVA affinity peptides (Mimotopes) – SIINFEKL (N4), SIINQFEKL (Q4), SIINVEFL (V4) added to the culture. Cells were harvested after one day and two days of culture for FACS analysis.

For $CD8^+$ T cell post priming culture, total splenocytes were culture in cRPMI with 100 ng/mL SIINFEKL and 100 U/mL human IL-2 (Peprotech) for three days. Cells were then washed twice in RPMI media supplemented with 5% HI-FCS (5% RPMI) before being resuspended in cRPMI only containing 50 ng/mL murine IL-2, IL-15, or IL-21 (Peprotech) and cultured for another three days. A cytokine concentration of 50 ng/mL was chosen based on previous dose-response experiments with 10 to 100 ng/mL concentrations and published literature [107, 160, 161].

For the *ex vivo* priming of $CD8^+$ T cells to be used for FTA, total splenocytes were cultured in cRPMI with 10 ng/mL SIINFEKL (Mimotopes) and 10 ng/mL murine IL-2 (Peprotech) for two days. Cells were then rested in 10 ng/mL murine IL-2 for a week before being injected into mice.

cDMEM was used to culture B16F10 and AT3 cell lines. cDMEM supplemented with 10 mM HEPES (Gibco), 1X non-essential amino acids solution (Gibco) was used to culture MC38 cell

line. Cells were maintained to a confluency of less than 80% in culture, for less than eight passages prior to use for tumour inoculation.

2.8 Target cell preparation for Fluorescent Target Array

Cells were prepared as previously described [162]. Briefly, total splenocytes were stained with CFSE (Molecular Probes) and CTV (Molecular Probes) in 5% RPMI at 37°C for 20 minutes. Cells were then coated with different concentration of OVA affinity peptides – N4, Q4 and V4 and control peptide KAVYNFATM (GP33) at 37°C for one hour. Cells were washed in 5% RPMI, underlaid with cold HI-FCS, and centrifuged with slow acceleration and braking to ensure that the interface of FCS and cell suspension solution was maintained. The RPMI and FCS were slowly aspirated with the washed pellets left undisturbed. Cells were washed again in 5% RPMI, and resuspended in HI-FCS for injection.

2.9 Adoptive transfer and priming for Fluorescent Target Array

5 x 10^6 total $CD8^+$ T cells, isolated from spleen of OT-I mice via magnetic separation, were injected i.v. into wildtype recipient mice. Priming was performed by s.c. injection of 25 μg OVA and 1 μg lipopolysaccharide (LPS) two hours after OT-I cell transfer. 5 x 10^6 *ex vivo* primed OT-I cells were injected i.v. into wildtype recipient mice, with no further priming *in vivo*. 50-70 x 10^6 peptide-coated, CFSE- and CTV-stained target cells were injected i.v. into wildtype recipient mice.

2.10 Real-time PCR

Cells were lysed in TRIzol (Invitrogen) and lysate was stored in -80°C until further processing. RNA isolations were done using the RNeasy kit (Qiagen) and single strand cDNA was synthesised using AccuPower RocketScript Cycle RT Premix (Bioneer). cDNA was stored in -20°C until it was ready to be used. Primers were purchased from Sigma and real-time PCR

was performed by the SYBR Green (Applied Biosystems) method using an Applied Biosystems 7900HT system. The mRNA expression of the genes of interest was normalised to the expression of the housekeeping gene GAPDH. Primers used in experiments are listed in **Table 2.2**.

2.11 Seahorse assay

Wells of the XFe96 plate were coated with 1.3% Cell-Tak (Falcon) diluted in 0.1M $NaHCO_3$ for 30 minutes in room temperature, washed once with water, left to dry for 30 minutes in room temperature and used within the next one hour. Cultured OT-I cells were seeded at 1.2 x 10^5 per well in XF media [non-buffered RPMI 1640 (Agilent) containing 10 mM glucose, 2 mM L-glutamine and 1 mM sodium pyruvate], and gently spun down at 500 rpm 3 minutes with slow acceleration and braking to ensure adherence to bottom of the well.

Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were measured under basal conditions and in response to 200 μM etomoxir, 1 μM oligomycin, 1.5 μM fluoro-carbonyl cyanide phenylhydrazone (FCCP) and 0.5 uM rotenone/antimycin A using the Seahorse XFe96 Extracellular Flux Analyzer (Agilent).

2.12 Statistical analysis

Student's *t*-tests was used for comparisons between two groups. One-way ANOVA was used to calculate univariate data set with more than two groups. Two-way ANOVA was used to calculate bivariate data set with more than two groups. ANOVA analysis was corrected by Sidak's multiple comparison tests. The sigmodal peptide concentration-to-percentage killing curve was plotted in Prism GraphPad and half-maximum effective killing (EC_{50}) was calculated in the software. Animals were of matched sex and age. Blinding was not performed due to the objective measurement of the experimental data. All statistical analyses were performed using Prism GraphPad.

Table 2.1. Antibodies used for Flow Cytometry

Antigen	Conjugates	Type	Dilution	Clone	Vendor
2B4	PECy7	Mouse mAb	1:200	m2B4(B6)458.1	Biolegend
B220	Brilliant violet (BV) 786	Rat mAb	1:200	RA3-6B2	BD
CCR7	APC	Rat mAb	1:50	4B12	Biolegend
CD107a	BV421	Rat mAb	1:200	1D4B	Biolegend
CD122	BV421	Rat mAb	1:100	TM-β1	BD
CD127	BV605	Rat mAb	1:50	A7R34	Biolegend
CD25	APCCy7	Rat mAb	1:200	PC61	Biolegend
CD44	APCCy7	Rat mAb	1:200	IM7	Biolegend
CD44	V500	Rat mAb	1:200	IM7	BD
CD45.1	AF647	Mouse mAb	1:400	A20	Biolegend
CD45.2	PE	Mouse mAb	1:400	104	Biolegend
CD62L	Alexa Fluor (AF) 488	Rat mAb	1:400	MEL-14	Biolegend
CD62L	BV711	Rat mAb	1:800	MEL-14	Biolegend
CD69	BV605	Hamster mAb	1:200	H1.2F3	Biolegend
CD8	AF488	Rat mAb	1:200	53-6.7	Biolegend
CD8	AF700	Rat mAb	1:100	53-6.7	Biolegend
GLUT1	Unconjugated	Rabbit mAb	1:100	EPR3915	Abcam
Gzm B	Pacific Blue	Rat mAb	1:200	GB11	Biolegend
HIF-1α	Unconjugated	Rabbit polyclonal Ab	1:200	NB100-134	Novus
IFNγ	PECy7	Rat mAb	1:400	XMG1.2	BD
IRF4	AF647	Rat mAb	1:800	IRF4.3E4	Biolegend
Ki67	PECy7	Mouse mAb	1:200	B56	BD
Lag3	PE	Rat mAb	1:200	C9B7W	Biolegend
PD1	FITC	Hamster mAb	1:200	J43	eBioscience

PD1	BV421	Hamster mAb	1:200	J43	BD
Rabbit IgG	AF488	Donkey polyclonal Ab	1:400	Poly4064	Biolegend
Rabbit IgG	BV421	Donkey polyclonal Ab	1:400	Poly4064	Biolegend
Rabbit IgG	AF647	Donkey polyclonal Ab	1:400	Poly4064	Biolegend
Sca1	PECy7	Rat mAb	1:200	D7	Biolegend
Tbet	BV605	Mouse mAb	1:100	4B10	Biolegend
TCF1	Unconjugated	Rabbit polyclonal Ab	1:400	C63D9	Cell Signaling Technology
TCRb	Brilliant ultra violet (BUV) 737	Rat mAb	1:100	H57-59	BD
Tim3	PE	Rat mAb	1:200	RMT3-23	Biolegend
TNFα	PE	Rat mAb	1:2000	MP6-XT22	Biolegend

Table 2.2. Primers used for Real-time PCR

Gene	**Direction**	**Sequence (5' – 3')**
CPT1a	F	CTCCGCCTGAGCCATGAAG
	R	CACCAGTGATGATGCCATTCT
CPT1b	F	GCACACCAGGCAGTAGCTTT
	R	CAGGAGTTGATTCCAGACAGGTA
CPT1c	F	GTTTCCTCTGGAGGTGGATTT
	R	GCTGGAGATATGGAAGGTGATT
GAPDH	F	TGAAGCAGGCATCTGAGG
	R	CGAAGGTGGAAGAGTGGGAG
HIF-1α	F	GAAACGACCACTGCTAAGGCA
	R	GGCAGACAGCTTAAGGCTCCT
HK2	F	GCCTCGGTTTCTCTATTTGGC
	R	CTGGTCAACCTTCTGCACTTGG

Chapter 3

The effects of IL-21 on CD8$^+$ T cell activation and killing function when treated during priming or post-priming

3.1 Introduction

Cytokines belong to a group of immune cell-secreted proteins that are capable of mediating cell signalling within its target cells. By doing so, cytokines regulate important cellular events of various immune cells. For example, it acts as a critical signal in the process of T cell activation, polarisation and differentiation [135, 163, 164].

T cell activation is a tightly regulated process which requires sequential exposure to three signals in the order of T cell receptor, costimulatory receptor, and cytokine to trigger a proper activation. An initial recognition of its cognate antigen by the TCR is the first signal for T cell activation and provides specificity for the T cell response. The second signal required comes from the binding of CD28 with CD80 and CD86 expressed on antigen-presenting cells, and the absence of this costimulatory signal after TCR stimulation has been shown to give rise to anergic T cells [165, 166]. To complete the process, a third signal from cytokine is needed. In CD4$^+$ T cells, cytokines direct the polarisation of different helper T cell subsets – IL-12 and IFNγ promote Th1 [167, 168]; IL-4 promotes Th2 [169, 170]; TGFβ and IL-6 promote Th17 [171, 172]; TGFβ and IL-2 promote Treg [171, 173]; IL-6 and IL-21 promote Tfh [174, 175]. While in CD8$^+$ T cells, IL-12 and IFNα/β promote expansion and effector functions [176, 177].

Recently, it has been discovered that the three signals are summed linearly to determine the division fate of CD8$^+$ T cells [137]. Exposure of OT-I CD8$^+$ T cells, when cultured with TCR stimulation, to a range of cytokines including IL-2, IL-4, IL-6, IL-12 and IL-21 have been shown to augment CD8$^+$ T cell proliferation in a dose-dependent manner. However, it remains unknown if the cytokine-induced increase in proliferation was, in fact, a consequence of an amplification of TCR signal induced by cytokine downstream signalling.

Notably, interpretation of signal 1 by the T cell is far more sophisticated than the typical receptor-ligand-triggered signalling cascade. The TCR signal threshold needs to be met where a minimum of four to six peptide-major histocompatibility complexes (pMHC) in a single TCR cluster is needed to set off downstream Ca^{2+} signalling [178]. Interestingly, the presence of CD28 costimulation was found to reduce the minimum pMHC per TCR cluster to just two [178]. This suggests that signal 2 might function by directly reducing the amount of stimulus

needed for TCR signal, which can also be viewed as an amplification of TCR signal to achieve the threshold with lower pMHC.

Can signal 3 also perform a similar function? It has indeed been observed that exposure to IL-2, IL-7 and IL-15 can lower the TCR signal threshold [179, 180]. Also from the common-gamma chain family, IL-21 is a pleiotropic cytokine that acts on a broad range of immune cells including T cells [122]. Particularly, IL-21 has been shown to enhance proliferation, cytotoxicity and survival of CD8$^+$ T cells [107, 108, 181], which are all results of CD8$^+$ T cell activation.

In addition to the role of cytokine in CD8$^+$ T cell priming, their roles have also been noted in shaping CD8$^+$ T cell phenotype and response post priming. IL-2 induces cytotoxic molecules IFNγ, perforin, and granzymes [182-184], while other cytokines like IL-7 and IL-15 promote long-term survival of CD8$^+$ T cells [185-187]. In a model of chronic LCMV infection, IL-21 has been shown to be a cytokine released by the CD4 T cells only in late T cell response [188, 189], suggesting a more prominent role of IL-21 potentially in the post priming phase of the CD8$^+$ T cell response. However, no direct comparison of the role of IL-21 in the priming versus post-priming phases of CD8$^+$ T cells have been made.

To compare the role of IL-21 in the priming versus post-priming phases of CD8$^+$ T cells, I will investigate its effect on CD8$^+$ T cell activation and its overall functional avidity, in terms of effector and killing functions, under different TCR signal strengths. Two systems will be used *in vitro* – (1) the direct stimulation of CD3 on polyclonal CD8$^+$ T cells using an agonistic antibody; (2) the natural-state stimulation of TCR on transgenic OT-I CD8$^+$ T cells using its cognate antigen. IL-21 only enhanced CD8$^+$ T cell activation at low TCR signal. To study the effect *in vivo*, an assay called the Fluorescent Target Array (FTA) will be used to study the killing function of CD8$^+$ T cells [162]. The FTA technology utilises fluorescent dyes such as carboxyfluorescein succinimidyl ester (CFSE) and violet laser excitable dyes (CellTrace Violet: CTV) to label mouse splenocytes into multiple cell clusters depending on the combination of dyes used. After being pulsed with MHC-I-binding peptides, these cell clusters can then act as target cells for CD8$^+$ T cells. This assay allows the analysis of CD8$^+$ T cells killing in real time *in vivo* by flow cytometry. With more than 250 possible cell cluster combinations, analysis of

T cell responses against multiple antigen epitopes at different concentrations in multiple replicates can be performed.

3.2 Results

3.2.1 IL-21 has a modest effect on polyclonal CD8$^+$ T cell primary activation *in vitro*

To determine if IL-21 can compensate TCR signalling and enhance CD8$^+$ T cell activation, an *in vitro* CD8$^+$ T cell activation culture assay was performed. Naïve CD8$^+$ T cells were cultured in the presence of varying concentration of coated anti-CD3 antibody (1, 3, 10 μg/mL) for one to two days. The effect of IL-21 (0, 2, 10, 50 ng/mL) on naïve CD8$^+$ T cells was then compared across different TCR strengths (1, 3, 10 μg/mL anti-CD3) (**Figure 3.1a**). With the aim to compare the effect of IL-21 on CD8$^+$ T cell activation, which occur within the first two days of TCR engagement *in vitro* [190], CD8$^+$ T cells were compared only for the first two days of culture.

CD8$^+$ T cells were gated as shown in **Supplementary Figure S2** before further analyses of other markers were done. As CD8$^+$ T cells activate, they upregulate several key surface markers – early activation marker CD69, activation marker CD44, and IL-2 receptor alpha CD25. There was a dose-dependent increase in CD8$^+$ T cell activation with increasing TCR strength. However, increasing the dose of IL-21 only resulted in weaker changes in percentage of CD69$^+$, CD44$^+$ and CD25$^+$ CD8$^+$ T cell when compared to the changes induced by TCR strength (**Figure 3.2a**). This suggests that IL-21 does not affect CD8$^+$ T cell activation directly.

Next, I set to examine intracellular markers involved in CD8$^+$ T cell activation and effector function. Tbet and IRF4 are transcription factors downstream of TCR signalling [191-193], and an increase in the expression of these transcription factors could signify a compensatory effect of IL-21 in TCR signalling. The use of IL-21 alone without any TCR stimulation has resulted in poor survival of the CD8$^+$ T cells (data not shown), thus marker analysis of the IL-21 alone group was not included. Of note, 2 ng/mL of IL-21 increased Tbet and IRF4

expressions at low TCR strength of 1 μg/mL anti-CD3, but the effect of IL-21 was not observed at higher TCR strengths (**Figure 3.2b**), demonstrating the dominance of the TCR signal in T cell activation over the cytokine signal.

Other effector markers – IFNγ and Gzm B were also investigated. Consistent with the effector transcription factors, an increase percentage of CD8$^+$ T cells expressing IFNγ and Gzm B can be observed when CD8$^+$ T cells were cultured with low TCR stimulus (**Figure 3.3a, b**). Interestingly, dose-dependent increase in both cytokines were also seen in the conditions with higher TCR strengths **(Figure 3.3a, b)**. This suggests that IL-21 plays a major role in CD8$^+$ T cells which are already activated by further enhancing expression of cytotoxic molecules. This result is consistent with the observation made by Casey et. al. where they found that IL-21 increased CD8 T cell cytolytic activity in a dose-dependent manner [181].

a. Polyclonal	Wildtype naïve CD8⁺ T cells seeded in wells with • 1, 3, 10 µg/mL coated anti-CD3 • 1 µg/mL soluble anti-CD28 • 0, 2, 10, 50 ng/mL IL-21	FACS analysis for surface activation markers	FACS analysis for intracellular markers to determine effector function
b. Transgenic OT-I	Naïve OT-I cells seeded in wells with • 4 nM OVA peptides of varying TCR binding affinity • 1 µg/mL of soluble anti-CD28 • 0, 2, 10, 50 ng/mL IL-21	FACS analysis for surface activation markers	FACS analysis for intracellular markers to determine effector function

c.

Variant	OT-1 sensitivity EC50 APL/N4
SIINFEKL (N4)	1.0
SAINFEKL (A2)	2.7
SIYNFEKL (Y3)	4.1
SIIQFEKL (Q4)	18.3
SIITFEKL (T4)	70.7
SIIVFEKL (V4)	680

Figure 3.1. Schematic overview of the *in vitro* CD8⁺ T cell activation experiment. Naïve CD8⁺ T cells were isolated from spleen via magnetic separation. **(a)** 5 x 10^4 polyclonal CD8⁺ T cells were seeded in wells coated with varying concentration of anti-CD3. **(b)** 5 x 10^4 transgenic OT-I cells were seeded with different 4 nM OVA affinity peptides (low-mid-high, V4-Q4-N4). **(a, b)** Increasing concentration of 0, 2, 10 and 50 ng/mL IL-21 were used. Surface activation markers were analysed after one day of culture, while intracellular markers were analysed after two days of culture. **(c)** The ratios of the concentration of an altered peptide ligand (APL) divided by the concentration of native OVA peptide required for inducing a half-maximum IFNγ response (EC_{50}) in OT-1 T cells are shown. Figure (c) from [194].

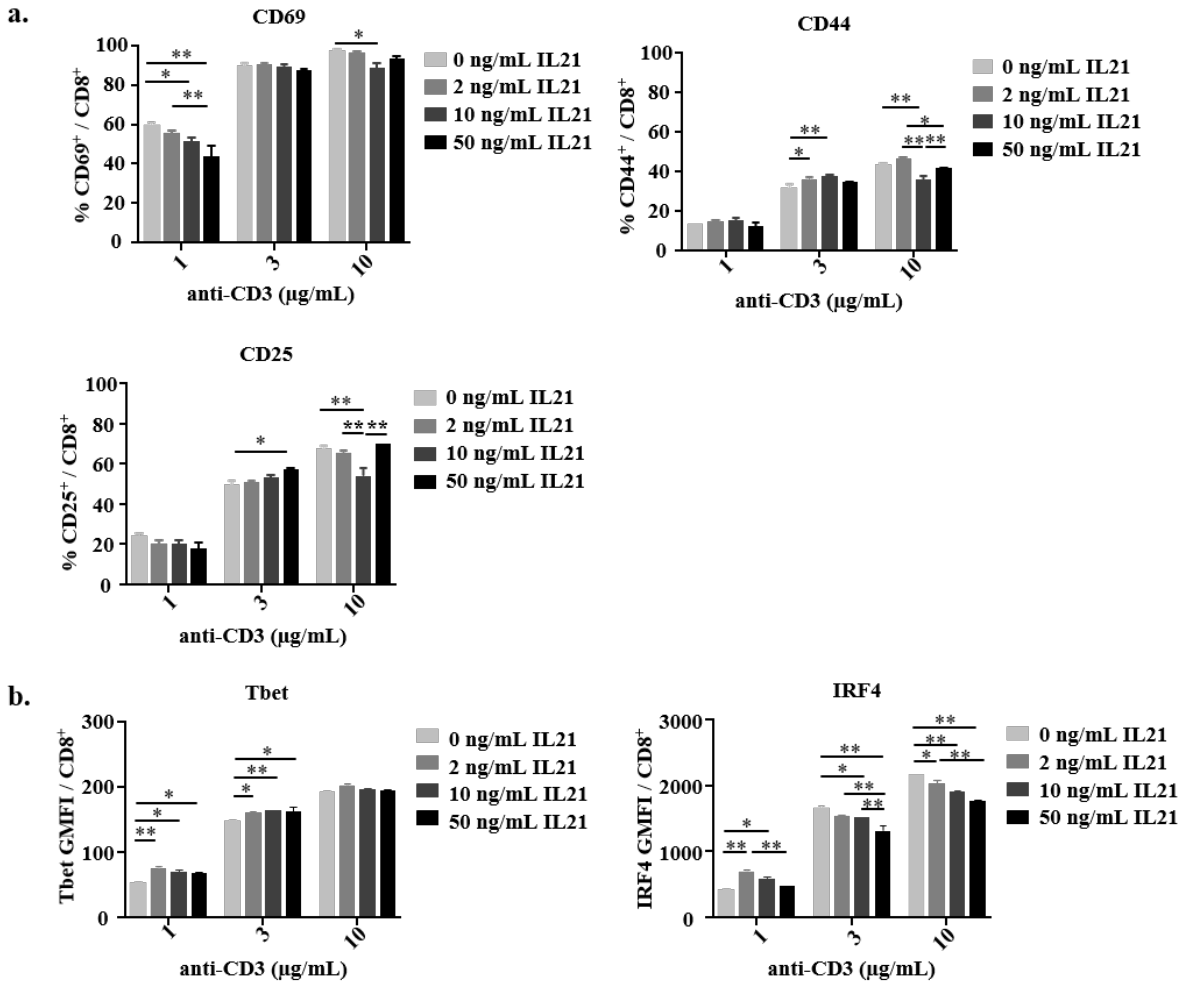


Figure 3.2. IL-21 does not increase polyclonal CD8⁺ T cell activation *in vitro*. *In vitro* culture was performed as described in Figure 3.1a. FACS analyses were performed on cytokine-treated CD8⁺ T cells after two days of culture. **(a)** Mean percentage of CD69⁺, CD44⁺ and CD25⁺ within CD8⁺ T cells. **(b)** Mean GMFI of Tbet and IRF4 expressions of CD8⁺ T cells. Data were analysed by Two-way ANOVA followed by Tukey's multiple comparison test. Small horizontal lines indicate the mean (± s.e.m.). **P* ≤0.05, ***P* ≤0.01. N = 3 (experimental replicate). Data are representative of two independent experiments.

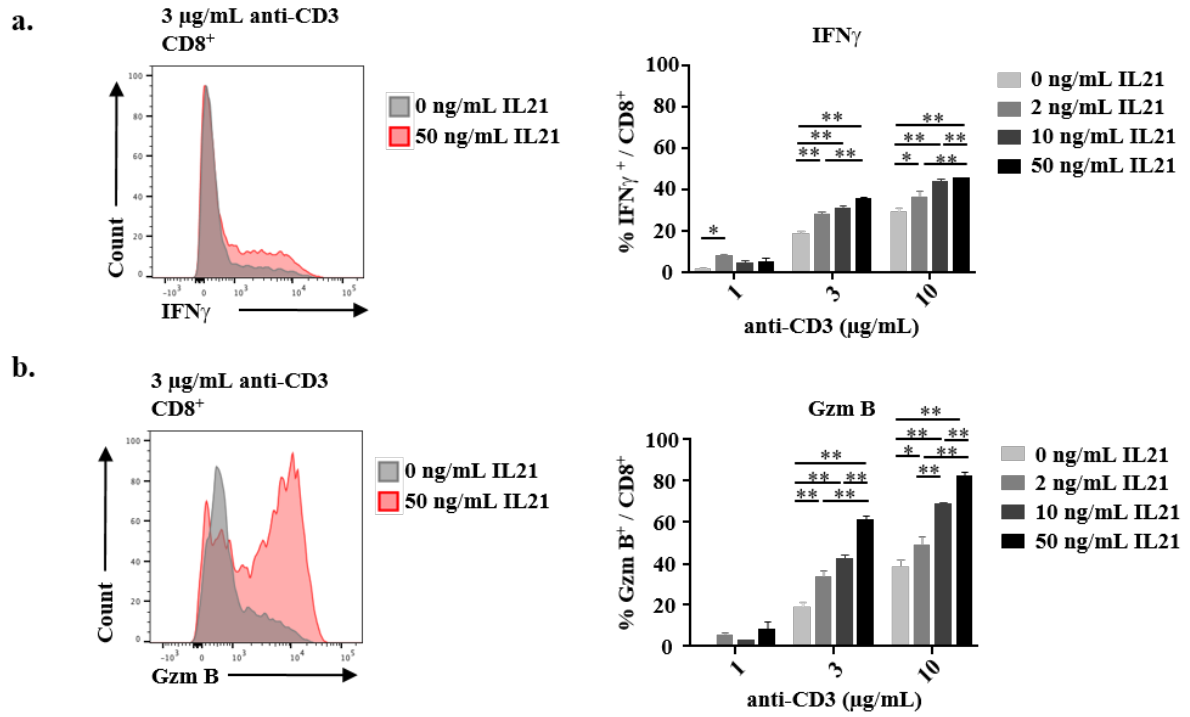


Figure 3.3. IL-21 increases effector marker expression on polyclonal CD8⁺ T cell *in vitro*. *In vitro* culture was performed as described in Figure 3.1a. FACS analyses were performed on cytokine-treated CD8⁺ T cells after two days of culture. **(a, b)** Intracellular marker analyses of IFNγ and Gzm B were then performed after cells were stimulated with PMA, ionomycin, brefeldin A and monensin for four hours. **(a)** Left: Flow cytometry plot displays intracellular IFNγ expression of cytokine-treated CD8⁺ T cells at 3µg/mL anti-CD3. Right: Mean percentage of IFNγ⁺ cytokine-treated CD8⁺ T cells. **(b)** Left: Flow cytometry plot displays intracellular Gzm B expression of cytokine-treated CD8⁺ T cells at 3µg/mL anti-CD3. Right: Mean percentage of Gzm B⁺ cytokine-treated CD8⁺ T cells. Data were analysed by Two-way ANOVA followed by Tukey's multiple comparison test. Small horizontal lines indicate the mean (± s.e.m.). *$P \leq 0.05$, **$P \leq 0.01$. N = 3 (experimental replicate). Data are representative of two independent experiments.

3.2.2 IL-21 has a modest effect on monoclonal CD8⁺ T cell primary activation *in vitro*

Functional avidity of CD8⁺ T cells is the complicated integration of the peptide-MHC-I binding affinity to the TCR, the expression levels of TCR and CD8 coreceptors, and the distribution of activating and inhibiting molecules. Therefore, instead of using anti-CD3 that directly stimulate TCR signalling, a more sophisticated affinity-based system might shed more light on the effect of IL-21 on the overall functional avidity of the CD8⁺ T cells. As such, the role of

IL-21 in the activation and overall functional avidity of OT-I CD8$^+$ T cells using its cognate OVA peptide was investigated. Naïve OT-I CD8$^+$ T cells were culture in the presence of its cognate OVA peptide for one to two days (**Figure 3.1b**). OVA peptides with different changes in their amino acid will affect its affinity to OT-I CD8$^+$ T cells (**Figure 3.1c**) [194]. Therefore, in order to cover a range of affinities from high to low, three OVA peptides – SIINFEKL (N4, high affinity), SIIQFEKL (Q4, mid affinity) and SIIVFEKL (V4, low affinity) – were chosen to be tested with different doses of IL-21. This culture system was adapted from Man et. al. [191], where CD8$^+$ T cells take on the role of the MHC-I-presenting cells and present the OVA peptides to themselves.

When tested on OT-I cells, IL-21 increased the percentage of CD69$^+$ CD8$^+$ T cells at low TCR strength V4 peptide (**Figure 3.4a**). On the other hand, IL-21 resulted in an overall decrease in the percentage of CD44$^+$ CD8$^+$ T cells and only modest change in the percentage of CD25$^+$ CD8$^+$ T cells (**Figure 3.4a**). Its effect on other markers also followed the observations made in polyclonal CD8$^+$ T cells, that is, increased Tbet, IRF4, IFNγ and Gzm B expressions with 2 ng/mL of IL-21 at low TCR strength V4 peptide (**Figure 3.4b** and **3.5a, b**). Dose-dependent increase in IFNγ and Gzm B were also seen in conditions of higher TCR strengths (**Figure 3.5a, b**). However, there was a decrease in the percentages of IFNγ$^+$ and Gzm B$^+$ CD8$^+$ T cells with concentrations of IL-21 exceeding 2 ng/mL.

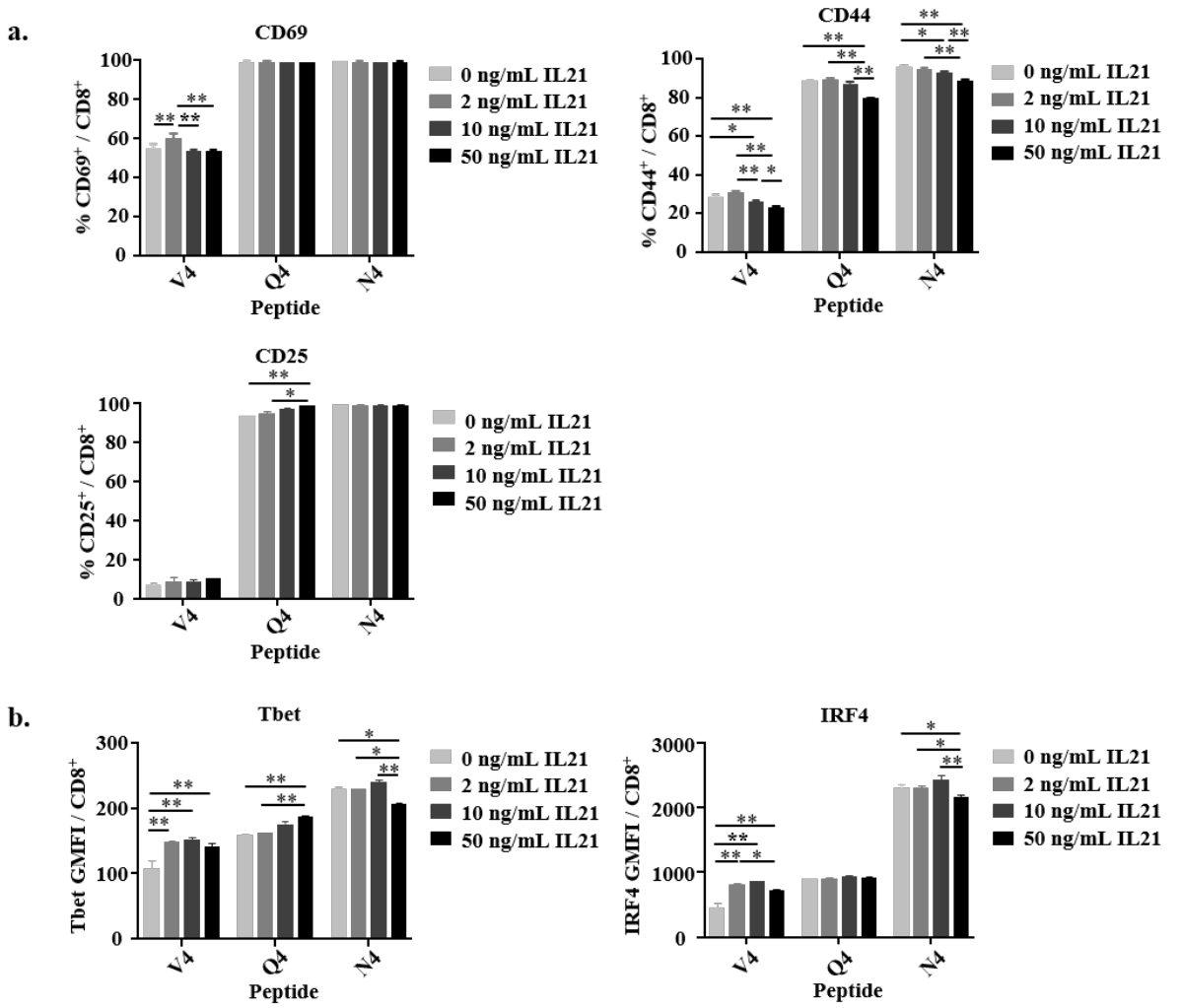


Figure 3.4. IL-21 does not increase transgenic OT-I CD8⁺ T cell activation *in vitro*. *In vitro* culture was performed as described in Figure 3.1b. FACS analyses were performed on cytokine-treated CD8⁺ T cells after two days of culture. **(a)** Mean percentage of CD69⁺, CD44⁺ and CD25⁺ within CD8⁺ T cells. **(b)** Mean GMFI of Tbet and IRF4 expressions of CD8⁺ T cells. Data were analysed by Two-way ANOVA followed by Tukey's multiple comparison test. Small horizontal lines indicate the mean (± s.e.m.). **P* ≤0.05, ***P* ≤0.01. N = 3 (experimental replicate). Data are representative of two independent experiments.

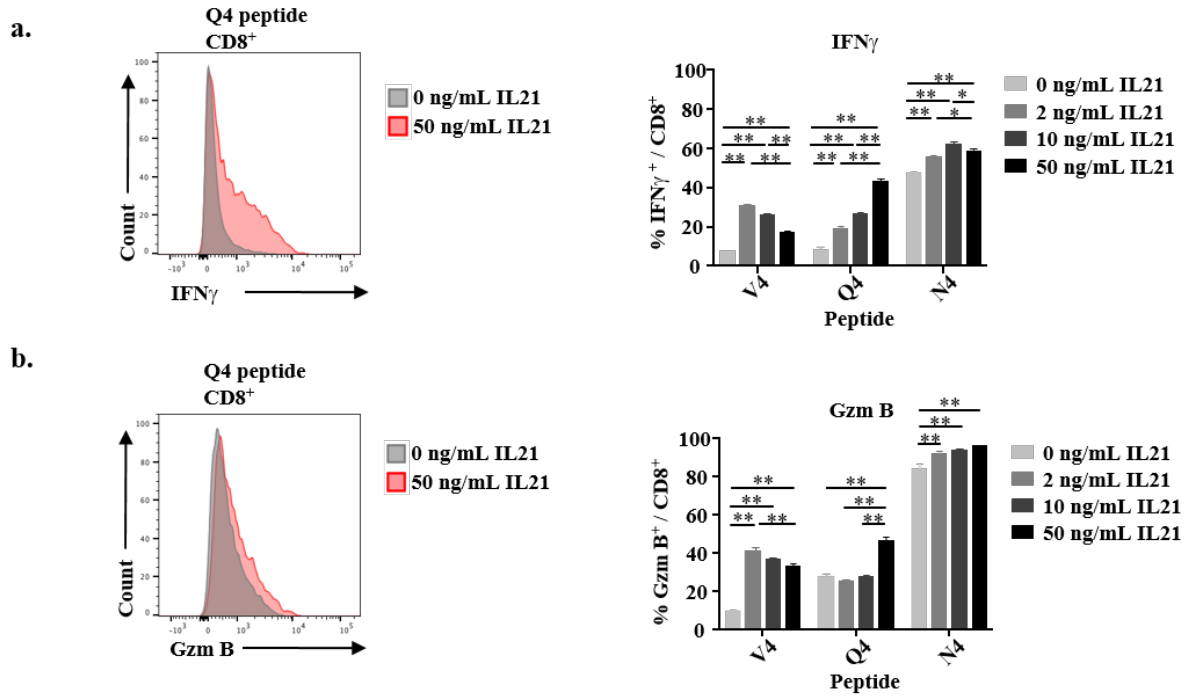


Figure 3.5. IL-21 increases effector marker expression on transgenic OT-I CD8⁺ T cell *in vitro*. *In vitro* culture was performed as described in Figure 3.1b. FACS analyses were performed on cytokine-treated CD8⁺ T cells after two days of culture. **(a, b)** Intracellular marker analyses of IFNγ and Gzm B were then performed after cells were stimulated with PMA, ionomycin, brefeldin A and monensin for four hours. **(a)** Left: Flow cytometry plot displays intracellular IFNγ expression of cytokine-treated CD8⁺ T cells at 3µg/mL anti-CD3. Right: Mean percentage of IFNγ⁺ cytokine-treated CD8⁺ T cells. **(b)** Left: Flow cytometry plot displays intracellular Gzm B expression of cytokine-treated CD8⁺ T cells at 3µg/mL anti-CD3. Right: Mean percentage of Gzm B⁺ cytokine-treated CD8⁺ T cells. Data were analysed by Two-way ANOVA followed by Tukey's multiple comparison test. Small horizontal lines indicate the mean (± s.e.m.). $*P \leq 0.05$, $**P \leq 0.01$. N = 3 (experimental replicate). Data are representative of two independent experiments.

3.2.3 IL-21 has modest effects on CD8⁺ T cell killing and antigen sensitivity when administered in the priming stage *in vivo*

To evaluate the effect of IL-21 in CD8⁺ T cell priming *in vivo*, I compared the treatment of IL-21 in the FTA killing assay. Briefly, as described in **Figure 3.6a**, the FTA killing assay involves three main steps. Firstly, OT-I CD8⁺ T cells are injected into mice and primed with LPS and OVA. Next, target cells are grouped and stained with varying concentration of CFSE and CTV (**Figure 3.6b**). Individual groups are then coated with OVA peptides of different binding

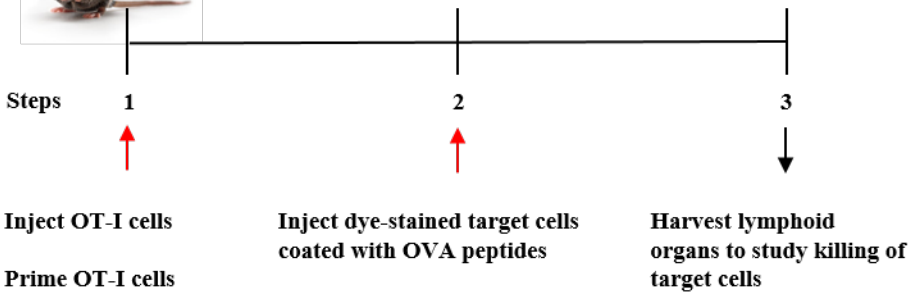
affinity at different concentration, and subsequently introduced into mice that already have the primed OT-I cells. Lastly, lymphoid organs are harvested and killing of target cells coated with specific OVA peptides can be identified under FACS (**Figure 3.6c**). Percentage specific killing can be calculated using the formula [1 – (Count of OVA peptide-coated target cells in primed mice/ Count of control peptide-coated target cells in primed mice)/(Count of OVA peptide-coated target cells in naive mice/ Count of control peptide-coated target cells in naive mice)] x 100% (**Figure 3.6d**).

In order to understand the effect of IL-21 on the priming phase of CD8$^+$ T cell, IL-21 treatment was administered one day after OT-I cells were primed *in vivo* and then daily for three days (**Figure 3.7a**). Three OVA peptides of varying affinity – N4 (high affinity), Q4 (mid affinity) and V4 (low affinity) – were chosen to coat target cells. The magnitude of killing measured signifies the strength of CD8$^+$ T cell effector response, while the EC_{50} calculated represents the amount of peptide to achieve 50% functional avidity, which also means the killing of 50% of the target cells. When compared between treatment groups, a lower EC_{50} signifies a higher antigen sensitivity, in other words, lesser amount of peptide to attain 50% functional avidity.

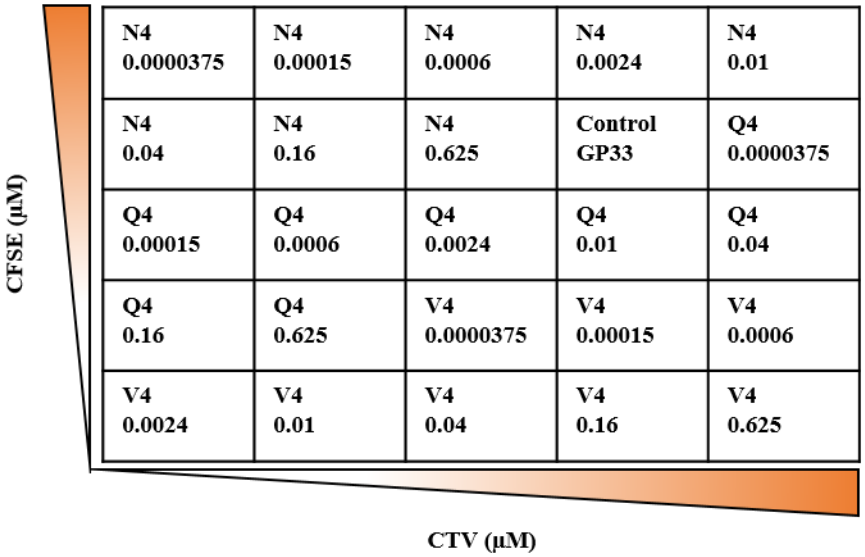
Looking at the magnitude of killing, IL-21 treatment did not have any effect towards N4 and Q4 peptides in the dLN and spleen **(Figure 3.7b)**. IL-21 treatment was found to significantly increase CD8$^+$ T cell killing towards 0.0024 μM V4 peptide in the dLN and spleen (**Figure 3.7b**). However, this increase was not observed with the other concentrations of V4 peptide tested. This suggests that a minimum level of TCR stimulation (0.0024 μM V4 peptide) is needed for CD8$^+$ T cell which is consistent to the *in vitro* observation where absence of TCR resulted in poor CD8$^+$ T cell survival and response. Since IFNγ and Gzmb were increased by IL-21 but IL-21 had only a modest effect on target killing, this also suggests that the killing might not be mediated by IFNγ and Gzmb.

When EC_{50} was compared, it was observed to have only increased the CD8$^+$ T cell antigen sensitivity towards the weak affinity V4 peptide by about two-fold (**Figure 3.8**). The antigen sensitivity towards higher affinity peptides N4 and Q4 was not affected. As the killing of Q4- and N4-coated target cells were already strong, the effect of IL-21 on the intensity of CD8$^+$ T cell effector response as well as CD8$^+$ T cell antigen sensitivity was less prominent (**Figure 3.8**).

a.

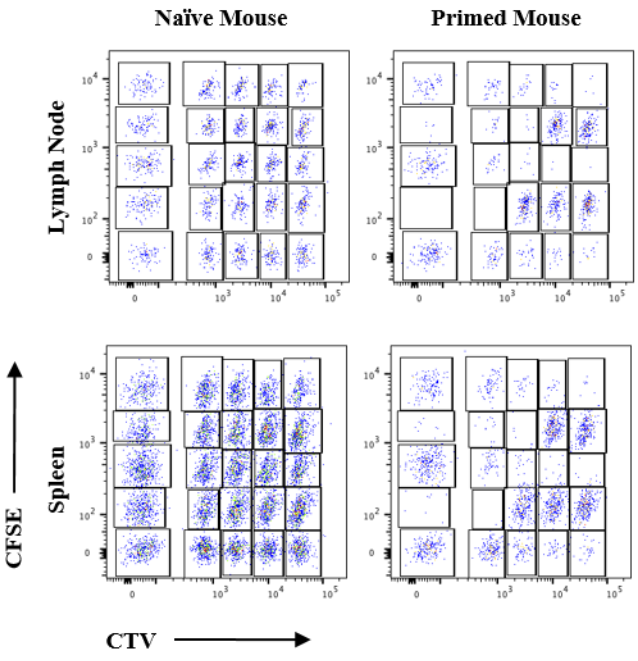


b.



N4 0.0000375	N4 0.00015	N4 0.0006	N4 0.0024	N4 0.01
N4 0.04	N4 0.16	N4 0.625	Control GP33	Q4 0.0000375
Q4 0.00015	Q4 0.0006	Q4 0.0024	Q4 0.01	Q4 0.04
Q4 0.16	Q4 0.625	V4 0.0000375	V4 0.00015	V4 0.0006
V4 0.0024	V4 0.01	V4 0.04	V4 0.16	V4 0.625

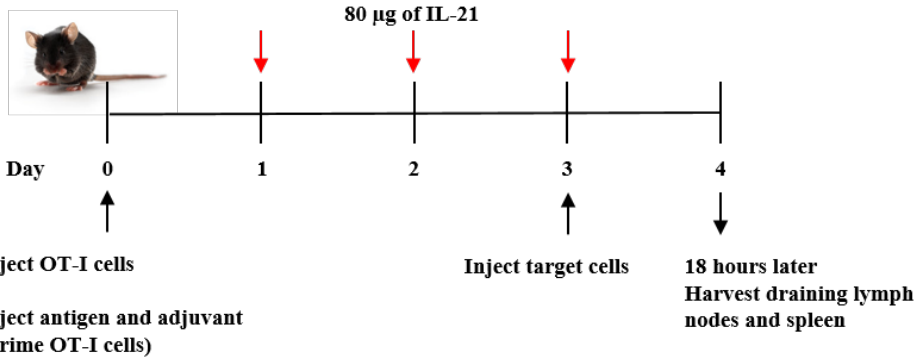
c.



d. $$\% \text{ Specific killing} = \left[1 - \left(\frac{\text{Targets}^{\text{OVA peptide}}_{\text{Primed}} / \text{Targets}^{\text{Control peptide}}_{\text{Primed}}}{\text{Targets}^{\text{OVA peptide}}_{\text{Naive}} / \text{Targets}^{\text{Control peptide}}_{\text{Naive}}}\right)\right] \times 100$$

Figure 3.6. **Schematic overview of FTA experiment. (a)** OT-I cells are intravenously injected and mice are then subcutaneously primed with OVA and LPS. Target cells (total splenocytes) are stained with CFSE and CTV and coated with known OVA peptide (low-mid-high affinity, V4-Q4-N4), and subsequently intravenously injected into mice. Lymphoid organs are harvested and killing of target cells are analysed using FACS. **(b)** A typical layout of target cells stained with different concentration of CSFE and CTV and coated with different affinity peptides at different concentration. **(c)** Clear groups of target cells from (b) identified on FACS. Reduction of cells belonging to specific group in primed mouse indicates killing. **(d)** Mathematical formula to calculate % specific killing.

a.


80 µg of IL-21
Day
0
1
2
3
4
Inject OT-I cells
Inject antigen and adjuvant
(Prime OT-I cells)
Inject target cells
18 hours later
Harvest draining lymph
nodes and spleen

b.

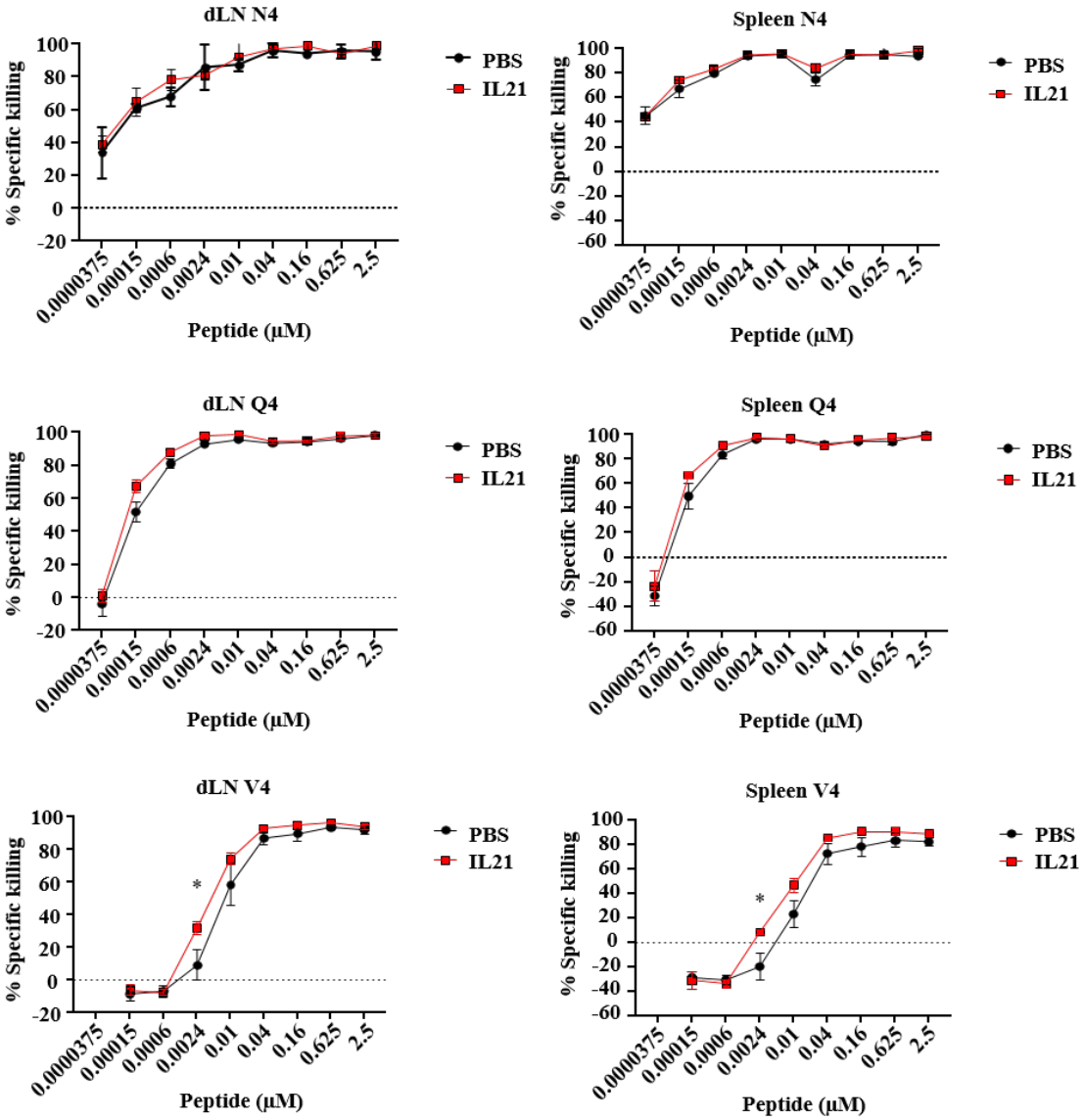

dLN N4
Spleen N4
dLN Q4
Spleen Q4
dLN V4
Spleen V4
% Specific killing
Peptide (µM)
0.0000375
0.00015
0.0006
0.0024
0.01
0.04
0.16
0.625
2.5
PBS
IL21
*

Figure 3.7. IL-21 increases magnitude of CD8$^+$ T cell killing when exposed to weak affinity peptide during priming. (a) OT-I CD8$^+$ T cells were isolated from spleen via magnetic separation. 5 x 10^6 cells OT-I cells were injected into recipient mice. OVA and LPS were injected to prime the OT-I cells. 80 µg of IL-21 was injected daily for three days during the priming stage of CD8$^+$ T cells. Total splenocytes from a congenic mouse were used as target cells, and were equally separated into 25 tubes, stained with 5 dilutions of CFSE and CTV as illustrated in Figure 3.6b, coated with different affinity OVA peptides at different concentration, and injected into recipient mice. Recipient mice were sacrificed on day 4, draining lymph nodes and spleens were harvested, and killing of target cells were analysed by FACS. **(b)** Mean percentage of specific killing of target cells by OT-I CD8$^+$ T cells at different concentrations of OVA peptides. High affinity OVA peptide N4; Mid affinity OVA peptide Q4; Low affinity OVA peptide V4. Data were analysed by Two-way ANOVA followed by Tukey's multiple comparison test. Small horizontal lines indicate the mean (± s.e.m.). *$P \leq$ 0.05. N = 3 (biological replicate). Data are representative of two independent experiments.

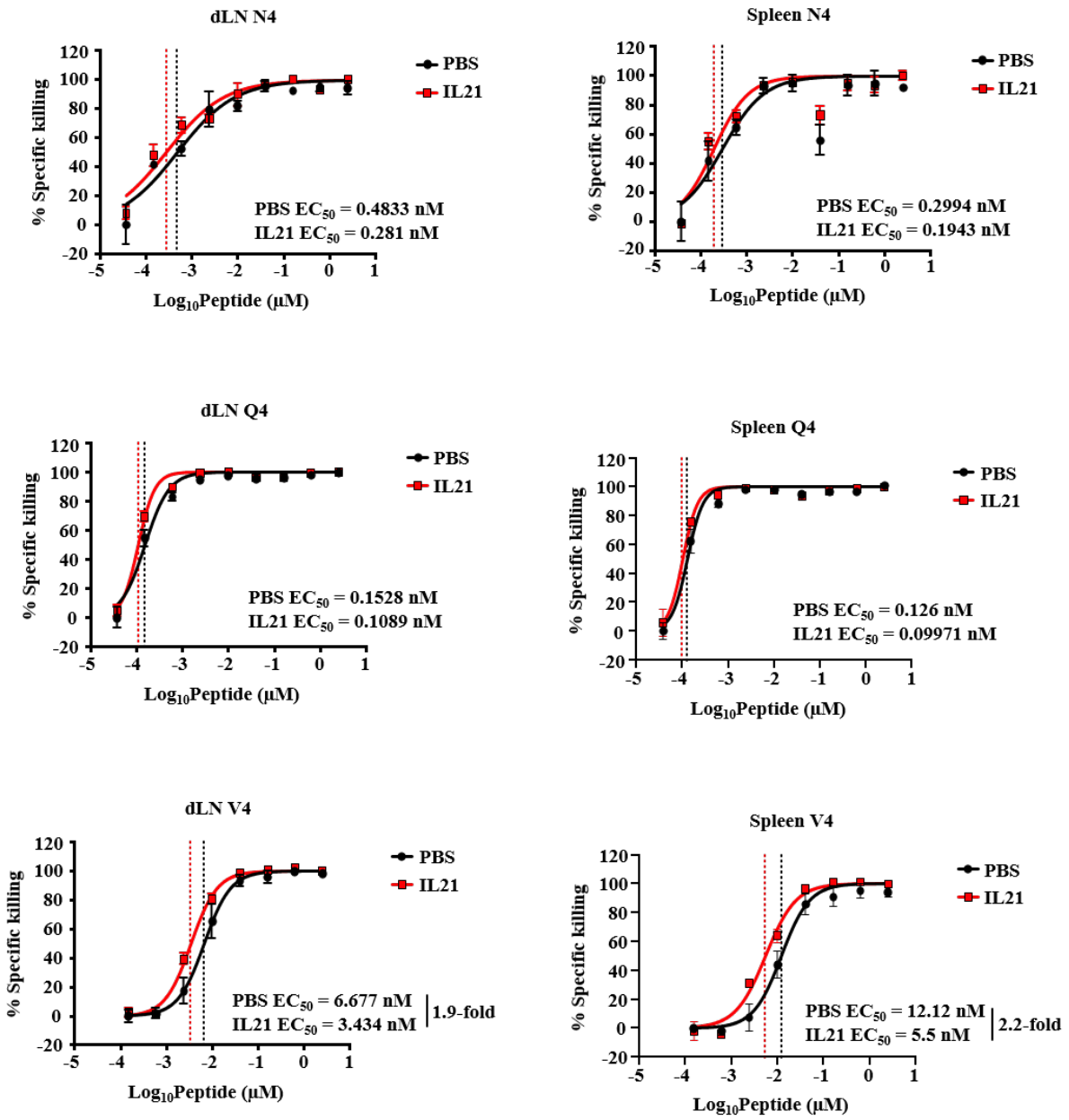


Figure 3.8. IL-21 increases CD8⁺ T cell antigen sensitivity when exposed to weak affinity peptide during priming. Experiment was carried out as illustrated in Figure 3.7a. Data in Figure 3.7b were plotted with peptide concentration in the scale of logarithm base 10. Graphs were normalised with 0% defined as the mean of the lowest peptide concentration in each data set, and 100% defined as the mean of the highest peptide concentration in each data set. EC_{50} was calculated and compared between PBS-treated and IL-21-treated mice.

3.2.4 IL-21 strongly enhances CD8$^+$ T cell killing and antigen sensitivity when administered in the post-priming stage *in vivo*

In chronic viral infection, knockouts of IL-21 and its receptor severely reduce the ability of mice to control the virus [151, 188, 195]. It can be inferred that CD8$^+$ T cells are strongly activated in the presence of foreign viral antigens, and the effect of IL-21 is related to the post-priming phase.

To validate the effect of IL-21 in the post-priming phase of CD8$^+$ T cell response, priming of OT-I cells was first done by culturing cells in N4 peptide and IL-2 *in vitro*, and rested for five days before being adoptively transferred into recipient mice (**Figure 3.9a**). N4 peptides were used to ensure that the OT-I cells were fully activated before allowing them to rest and to enter the post-priming phase. After which, IL-21 treatment was administered for four days. Here, IL-21 treatment appeared to increase CD8$^+$ T cell effector response at a limited range of peptide stimulation – higher concentration of the Q4 peptide and lower concentration of the N4 peptide – reaching significance at 0.0000375 and 0.0015 μM N4 in the dLN. A similar observation was made in the spleen where a significance was achieved at 0.0006 μM N4. Overall, there was an average increase of killing from 10-20% across N4 and Q4 peptide-coated cells in the spleen and dLN. (**Figure 3.9b**).

Strikingly, the effect of IL-21 on CD8$^+$ T cell antigen sensitivity was more drastic than the observation made during CD8$^+$ T cell priming. There was almost a four-fold increase in antigen sensitivity of CD8$^+$ T cell towards the N4 peptide, and an approximate 14-fold increase in antigen sensitivity of CD8$^+$ T cell towards the Q4 peptide in spleen (**Figure 3.10**). Similar observations can be made in the dLN with an approximate seven-fold increase towards N4 and three-fold increase towards Q4. Of note, killing of V4-coated cells were not observed as a minimum threshold requirement of TCR signal might not be met due to downregulation of TCR on primed CD8$^+$ T cells [196].

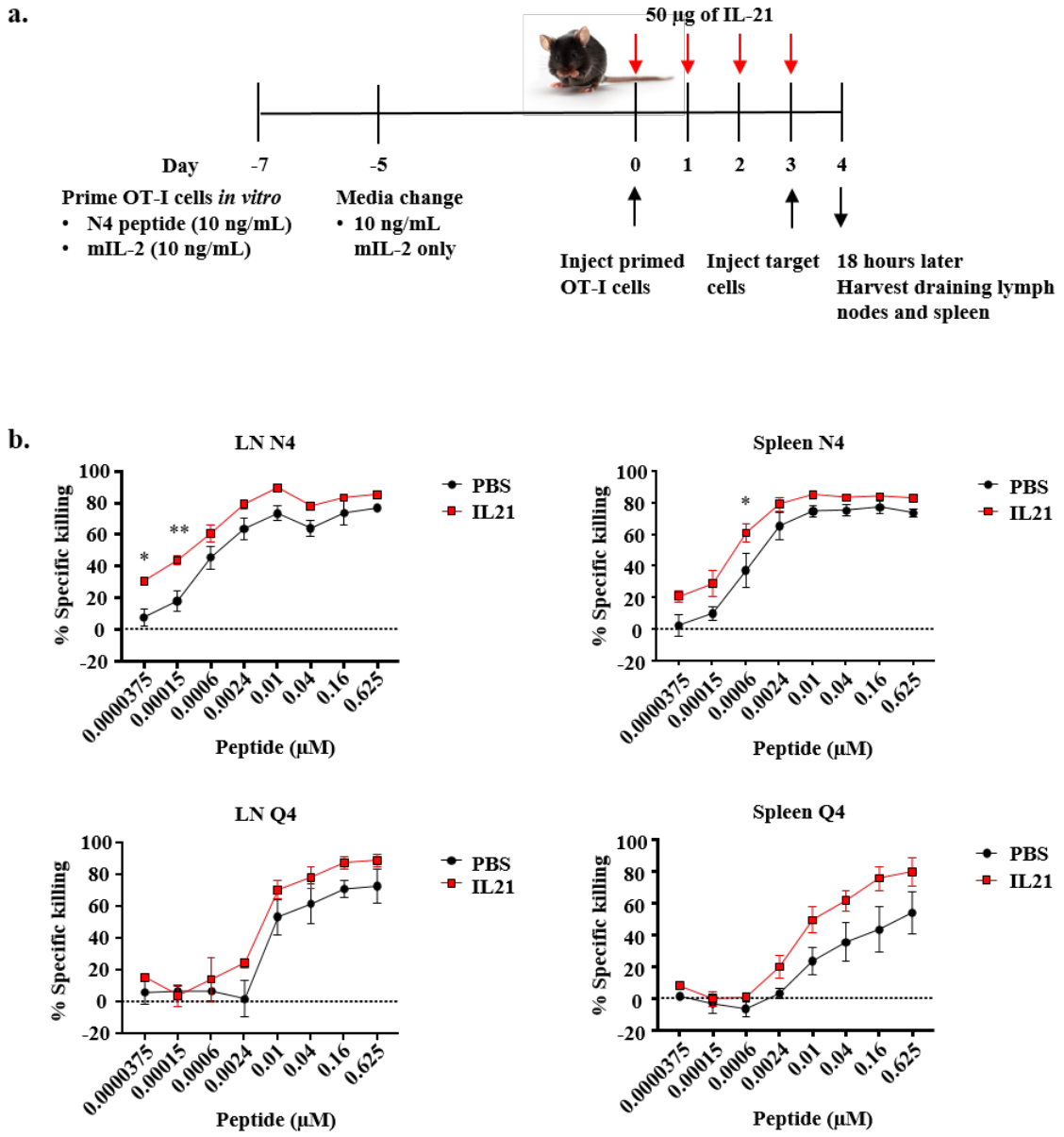


Figure 3.9. IL-21 increases magnitude of CD8⁺ T cell killing when exposed all affinity peptide post priming. (a) Total splenocytes were cultured in 1 x 10^6/mL with 10 ng/mL N4 peptide and 10 ng/mL mouse IL-2. After two days of priming, cells were transferred and rested in media only containing 10 ng/mL IL-2 for five more days. 5 x 10^6 cells primed OT-I cells were injected into recipient mice. 50 µg of IL-21 was injected daily for two days. Total splenocytes from a congenic mouse were used as target cells, and were equally separated into 36 tubes, stained with 6 dilutions of CFSE and CTV similar to that illustrated in Figure 3.6b, coated with different affinity OVA peptides at different concentration, and injected into recipient mice. Recipient mice were sacrificed on Day 4, lymph nodes and spleens were harvested, and killing of target cells were analysed by FACS. **(b)** Mean percentage of specific killing of target cells by OT-I CD8⁺ T cells at different concentrations of peptides. High affinity peptide N4; Mid affinity peptide Q4. Data were analysed by Two-way ANOVA followed by Tukey's multiple comparison test. Small horizontal lines indicate the mean (± s.e.m.). *$P \leq$

0.05, $^{**}P \leq 0.01$. N = 3 (biological replicate). Data are representative of two independent experiments.

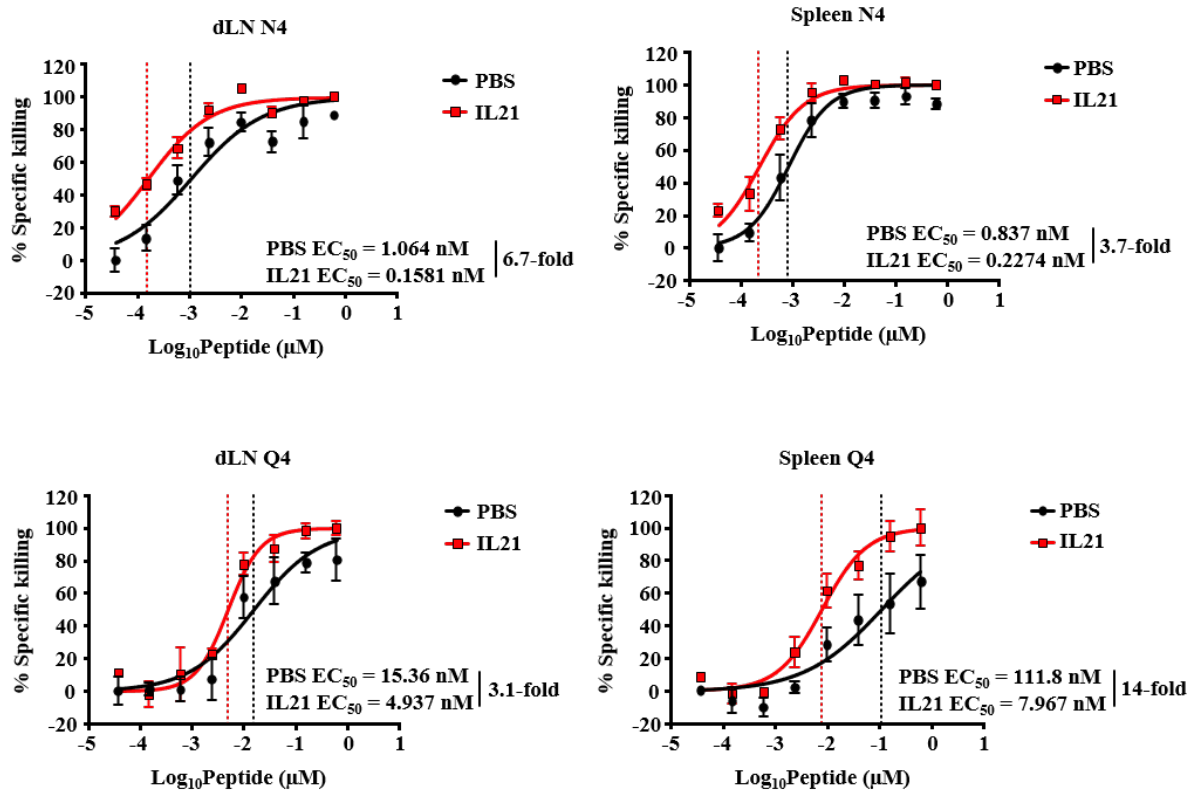


Figure 3.10. IL-21 increases CD8⁺ T cell antigen sensitivity when exposed to weak affinity peptide post priming. Experiment was carried out as illustrated in Figure 3.9a. Data in Figure 3.9b were plotted with peptide concentration in the scale of logarithm base 10. Graphs were normalised with 0% defined as the mean of the lowest peptide concentration in each data set, and 100% defined as the mean of the highest peptide concentration in each data set. EC_{50} was calculated and compared between PBS-treated and IL-21-treated mice.

3.3 Discussion

The timing of CD8⁺ T cell exposure to cytokine might have a role in determining the type, the magnitude and the memory capacity of the response. In this chapter, I investigated the differences in the use of IL-21 in the priming versus post-priming phases of CD8⁺ T cell responses using an *in vitro* CD8⁺ T cell activation culture system and an *in vivo* FTA killing assay.

The findings of Casey et al. support the role of IL-21 in enhancing CD8 T cell cytotoxicity and killing of its target [181]. Their results agree that IL-21 work in a dose-dependent manner to augment CD8 T cell cytolytic function but only in the presence of strong antigen stimulation. Here, our study went one step further to investigate if IL-21 can compensate for weak TCR stimulation by increasing CD8 T cell activation. The results will be potentially important in boosting CD8 T cell response in anti-tumour immunity.

Even though IL-21 had only a modest effect on CD8$^+$ T cell activation during the priming phase of the *in vitro* CD8$^+$ T cell assay, its effects were interestingly more profound in multiple intracellular effector markers – Tbet, IRF4, IFNγ and Gzm B. This was particularly true at low TCR strength (1 μg/mL or V4 peptide) where IL-21 treatment significantly enhanced expressions of those markers. The mechanism behind this is unclear but can be postulated to be STAT3-related. STAT3 is the major signalling pathway of IL-21 [123, 124]. In B cells and CD4$^+$ T cells, STAT3 can function cooperatively with IRF4 to upregulate downstream gene expression [129]. If a similar interaction applies in CD8$^+$ T cells, STAT3 signalling can potentially augment the effect of IRF4, a transcription factor that translates TCR strength into CD8$^+$ T cell effector function [191, 192]. Preliminary co-IP result shows that in CD8$^+$ T cells, phosphorylated or unphosphorylated STAT3 did not cooperatively bind to IRF4 (data not shown), suggesting that other additional factors might be needed for their interaction which needs to be explored in the future.

Consistent with the *in vitro* study, IL-21 was again observed to enhance CD8$^+$ T cells effector function at low TCR strength (V4 peptide) during CD8$^+$ T cell priming phase *in vivo*. It might be a result of the overlap in signalling pathways – STAT3 and IRF4 – downstream of IL-21 and TCR as discussed earlier. If this is true, it might explain why there was an approximate two-fold increase in the antigen sensitivity of CD8$^+$ T cells at low TCR strength (V4 peptide).

On the other hand, when treated post CD8$^+$ T cell priming, IL-21 strongly increased killing of target cells coated with Q4 and N4 peptides. Primed CD8$^+$ T cells require a stronger TCR strength to be re-stimulated due to the decrease in surface TCR expression and increase inhibition of TCR signalling from tyrosine phosphatases [196]. As a result, the weak TCR stimulation by V4 did not meet the minimum threshold requirement of TCR for CD8$^+$ T cells to activate. This further confirms that cytokine stimulation cannot completely replace TCR

stimulation in CD8$^+$ T cell activation. The increase in magnitude of killing might be a result of various factors such as stronger CD8$^+$ T cell cytotoxicity, increased survival of CD8$^+$ T cells, or increased CD8$^+$ T cell proliferation. Hence, it further confirms that though IL-21 does increase CD8$^+$ T cell antigen sensitivity towards weaker affinity peptides once the TCR stimulation has met the required minimum threshold to activate. Comparatively, the overall killing output of the CD8$^+$ T cells are strongly enhanced in post-primed CD8$^+$ T cells rather than in naïve CD8$^+$ T cells, suggesting a major role of IL-21 in post-primed CD8$^+$ T cells.

Furthermore, IL-21 was found to increase CD8$^+$ T cell antigen sensitivity towards N4 and Q4 peptides with a maximum of 14-fold for Q4 peptide in spleen. The overall strong increase in killing and antigen sensitivity only on primed CD8$^+$ T cell suggest an impact of IL-21 in the post-priming phase potentially altering the memory CD8$^+$ T cell formation or contraction phases. Does IL-21 mediate a conversion of primed CD8$^+$ T cells towards the central memory subset which has been proven to elicit high magnitude responses than effector memory CD8$^+$ T cells [197, 198]? Indeed, cytokines have been shown to play a major role in directing CD8$^+$ T cell memory subset formation [199-202].

Arguably, the change in CD8$^+$ T cell antigen sensitivity might not be a result of the IL-21-induced change in CD8$^+$ T cell subset but rather an increase in IL-21R expression in activated CD8$^+$ T cell. If this hypothesis is correct, the higher expression of IL-21R in activated CD8$^+$ T cell will lead to a stronger signal 3 and therefore higher magnitude of effector response. Though not investigated in this thesis, it remains a critical question to be addressed.

Taken together, both *in vitro* and *in vivo* data support the hypothesis that IL-21 plays a more significant role on CD8$^+$ T cell antigen sensitivity and effector response in the post-priming phase. How does the phase-specific administration of IL-21 differ in a disease state? In the next chapter, I applied this finding to two solid tumour models to further elucidate the application of IL-21 in different phases of CD8$^+$ T cell anti-tumour response.

Chapter 4

The effect of IL-21 treatment in different phases of anti-tumour response

4.1 Introduction

IL-21 has been shown to elicit anti-tumour response in mice via the induction of $CD8^+$ T cells. When directly administered to thymoma tumour-bearing mice, IL-21 treatment induced tumour inhibition by boosting $CD8^+$ T cell proliferation, survival and cytotoxic function [138]. In synergy with IL-15, IL-21 treatment can inhibit B16 melanoma growth by regulating granzyme B and boosting proliferation of $CD8^+$ T cells [203]. In addition, over-expression of IL-21 in mammary adenocarcinoma and bladder carcinoma resulted in upregulation of $CD8^+$ T cell cytotoxic function and inhibition of tumour growth [204, 205].

In human clinical trial, single agent IL-21 administration has also been used to treat various types of cancer. Treatment of metastatic melanoma using IL-21 achieved a 34.5% and 50% response rate in two phase I trials [139, 140], while lower responses of 8.3% and 22.5% were reported in two phase II trials [141, 142].

The inability of IL-21 single agent treatment to completely eradicate existing tumour in both mice and humans may suggest that the phase of $CD8^+$ T cells when IL-21 is administered has a crucial role to play in determining the outcome. When IL-21 is used to treat a primary tumour, $CD8^+$ T cells are constantly being primed by tumour antigen which indicates that IL-21 is acting on $CD8^+$ T cells in the priming phase. This observation is in line with the findings in Chapter 3 where IL-21 has been revealed to play a more important role in the post priming phase of $CD8^+$ T cells. As such, further optimisation of IL-21 treatment in cancer immunotherapy is required to elicit better treatment efficacy. This includes the use of IL-21 in boosting $CD8^+$ T cell response in the post priming phase as a form of secondary treatment to prevent cancer relapses instead of being used as a therapeutic agent to treat primary tumour.

To compare the role of IL-21 in $CD8^+$ T cell anti-tumour response during the phases of $CD8^+$ T cell priming and post-priming, I will investigate its effect on the control of solid tumours and understand the corresponding changes in $CD8^+$ T cells. As existing mice studies have mainly investigated the effect of treatment on anti-tumour response during the priming phase $CD8^+$ T cell, to study the post-priming phase of $CD8^+$ T cell, I will design a tumour model that first generates primed $CD8^+$ T cells prior to administration of treatment, followed by the study of treatment effect upon tumour rechallenge.

The mixed response of IL-21 treatment could also have stemmed from the upregulation of checkpoint receptors on $CD8^+$ T cells. γ_c family cytokines have been shown to upregulate PD1 expression on $CD8^+$ T cells [147]. The binding of PD1 with its ligands PD-L1 and PD-L2 will result in a decrease in $CD8^+$ T cell response and eventually lead to T cell exhaustion [206, 207]. Therefore, a combination therapy of IL-21 with PD-L1 blockade will be explored to remove this inhibition and potentially further augment the effect of IL-21 on $CD8^+$ T cells.

4.2 Results

4.2.1 Single agent IL-21 has a modest effect in inhibiting tumour growth in AT3, MC38 and B16F10 in the priming phase

To investigate the effect of IL-21 in the priming phase of $CD8^+$ T cells, tumour cells were first injected into mice subcutaneously to generate solid tumours. To ensure that subsequent tumour growth was not a result of difference in existing tumour growth rate, mice were grouped and randomised after tumour had been formed such that each group has a similar mean tumour size. Intraperitoneal IL-21 injections started after tumour formation and were performed every other day **(Figure 4.1a)**. IL-21 treatment was examined in three tumour types namely AT3 mammary adenocarcinoma, MC38 colon adenocarcinoma and B16F10 melanoma. These three tumour types were chosen based on their level of predicted neoantigen from high (AT3 and MC38) to low (B16F10) [208, 209]. Comparing tumour sizes, a trend of reduced tumour growth in IL-21-treated mice can be observed in all three tumours **(Figure 4.1b-d)**. This was potentially a result of the effect of IL-21 on $CD8^+$ T cells while in their priming phase.

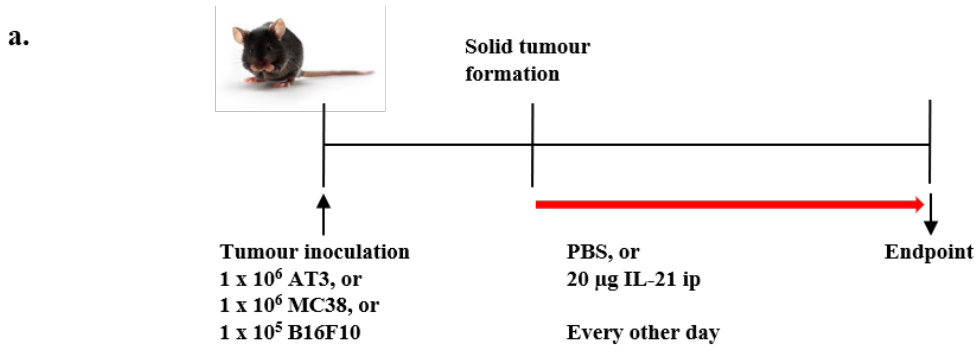
Mice were sacrificed day 28 for AT3, day 17 for MC38 and day 15 for B16F10. Tumours were harvested and TIL were isolated for FACS analysis. TIL were gated as shown in **Supplementary Figure S3** for the analyses done in this Chapter. It was observed that IL-21 increased $CD8^+$ T cell infiltration into tumour from 35% to 50% in AT3, 20% to 30% in MC38 and 20% to 30% in B16F10 **(Figure 4.1e)**. However, an increase in $CD8^+$ T cell infiltration does not always correlate with the strength of anti-tumour response as other factors are needed

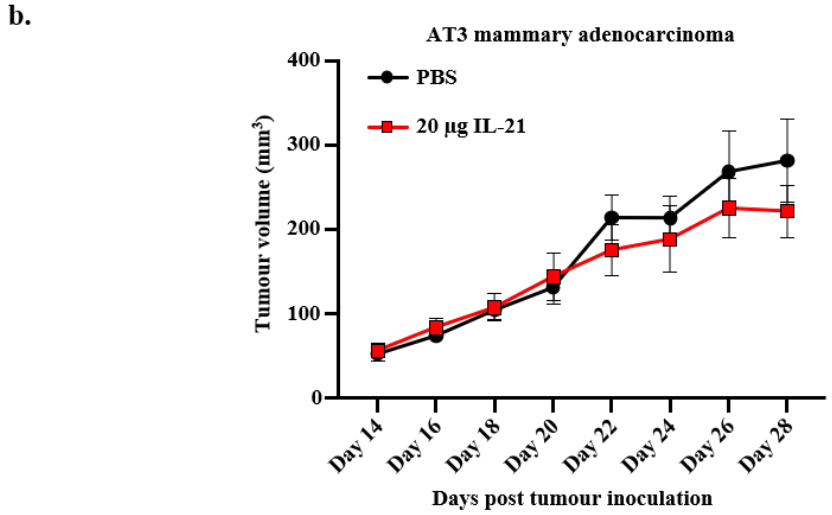
to be taken into account. One such is the presence of Treg that can inhibit $CD8^+$ T cell response. Indeed, previous studies have suggested that the $CD8^+$ T cell to Treg ratio correlate with treatment prognosis [210, 211]. Here, IL-21 treatment only augmented the ratio two-fold in AT3 tumour but not in MC38 and B16F10 **(Figure 4.1f)**, thus implying that factors other than Treg also have significant impacts on the tumour sizes.

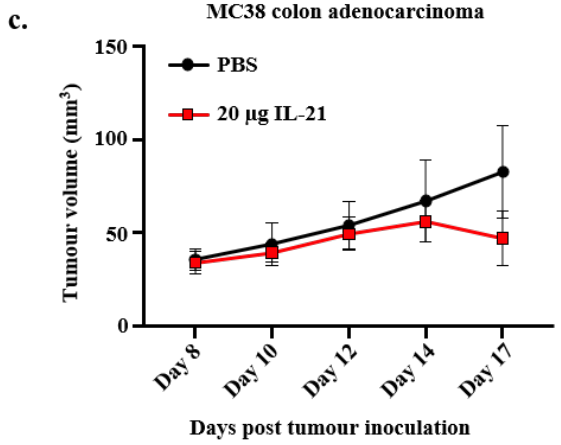
Looking at the expression of CD44 to identify activated $CD8^+$ T cell, there was an increase in the percentage of activated $CD8^+$ T cells in B16F10 from 70% to 85%, but not in AT3 and MC38 which already have strong activation in the untreated group **(Figure 4.1g)**. This is consistent with the finding in Chapter 3 where IL-21 can increase $CD8^+$ T cell antigen sensitivity towards weak affinity peptide during priming *in vivo*, which, in this case, can be observed in the poorly immunogenic B16F10 tumour.

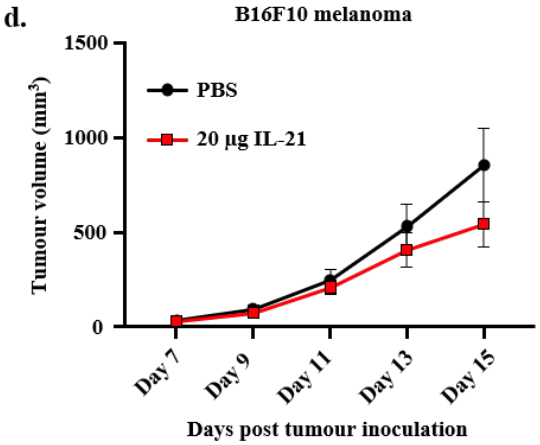
Due to limited number of tumour-infiltrating cells extracted from the MC38, only tumour-infiltrating cells from AT3 and B16F10 were used for PMA and ionomycin stimulation. It was revealed that IL-21 treatment increased IFNγ secretion of infiltrating $CD8^+$ T cells in B16F10 from 15% to 30%, but not in AT3 **(Figure 4.1h)**. The increase in B16F10 was not significant and this could potentially be a result of an increase in $CD8^+$ T cell inhibition based on the increase in the percentage of $PD1^+$ $CD8^+$ T cells from 50% to 70% in both AT3 and B16F10 tumours **(Figure 4.1i)**. Tumour cells are known to upregulate ligands of checkpoint receptors [212-214], or are capable of upregulating ligands in response to cytotoxicity [215, 216]. Binding of the ligands to their respective checkpoint receptors induces inhibitory signals such as inhibition of the TCR signalling and CD28 signalling [94, 217-219].

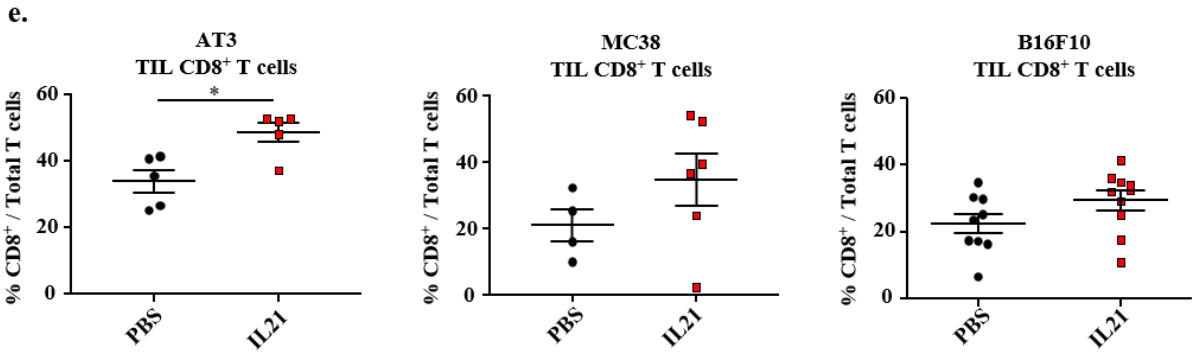
In line with the *in vitro* $CD8^+$ T cell activation assay and *in vivo* FTA killing assay, IL-21 single agent treatment did not further enhance $CD8^+$ T cell activation and killing during the priming phase of $CD8^+$ T cell in the presence of high TCR signal strength. With modest effect on tumour growth and $CD8^+$ T cell response, which could be a result of enhanced PD1 inhibition, I next explored the utility of IL-21 in combination with PD1/PD-L1 blockade.


a.
Solid tumour formation
Tumour inoculation
1 x 10^6 AT3, or
1 x 10^6 MC38, or
1 x 10^5 B16F10
PBS, or
20 µg IL-21 ip
Every other day
Endpoint


b.
AT3 mammary adenocarcinoma
PBS
20 µg IL-21
Tumour volume (mm3)
400
300
200
100
0
Day 14
Day 16
Day 18
Day 20
Day 22
Day 24
Day 26
Day 28
Days post tumour inoculation


c.
MC38 colon adenocarcinoma
PBS
20 µg IL-21
Tumour volume (mm3)
150
100
50
0
Day 8
Day 10
Day 12
Day 14
Day 17
Days post tumour inoculation


d.
B16F10 melanoma
PBS
20 µg IL-21
Tumour volume (mm3)
1500
1000
500
0
Day 7
Day 9
Day 11
Day 13
Day 15
Days post tumour inoculation


e.
AT3
TIL CD8+ T cells
MC38
TIL CD8+ T cells
B16F10
TIL CD8+ T cells
% CD8+ / Total T cells
60
40
20
0
*
PBS
IL21

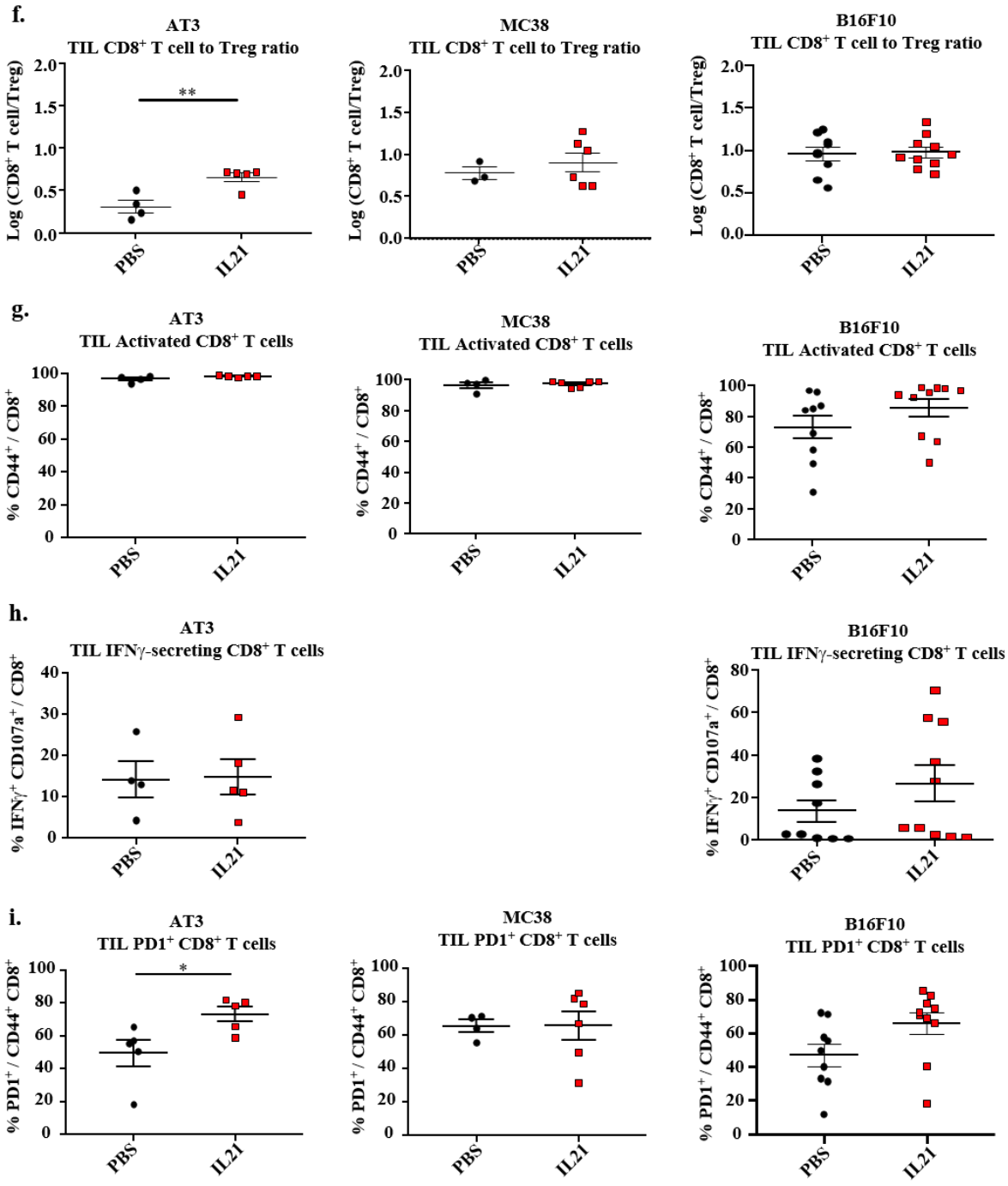


Figure 4.1. IL-21 modestly enhances anti-tumour response in the priming stage *in vivo* against AT3, MC38 and B16F10. (a-d) Mice were subcutaneously inoculated with tumour cells (1 x 10^6 AT3, 1 x 10^6 MC38 or 1 x 10^5 B16F10), 20 µg IL-21 intraperitoneal injection every other day commenced after solid tumours were observed (day 14 for AT3, day 8 for MC38 and day 7 for B16F10). Tumour size was measured and calculated by using the formula 0.5 x length x breadth x breadth in mm^3. **(e-i)** Mice were sacrificed day 28 for AT3, day 17 for MC38 and day 15 for B16F10. Tumours were harvested, and tumour-infiltrating lymphocytes were isolated for FACS analysis. For intracellular staining in (h) cells were stimulated with PMA, ionomycin, brefeldin A and monensin for four hours, then stained for IFNγ and CD107a. **(e)** Percentage of $CD8^+$ cells within total tumour-infiltrating T cells between PBS- and IL-21-

treated mice. **(f)** Ratio of $CD8^+$ T cells to $Foxp3^+$ $CD4^+$ Treg (in the scale of logarithm base 10) within tumour-infiltrating $CD8^+$ T cells between PBS- and IL-21-treated mice. **(g)** Percentage $CD44^+$ cells within tumour-infiltrating $CD8^+$ T cells between PBS- and IL-21-treated mice. **(h)** Percentage of $IFN\gamma^+$ $CD107a^+$ within tumour-infiltrating $CD8^+$ T cells between PBS- and IL-21-treated mice. **(i)** Percentage $PD1^+$ within tumour-infiltrating activated $CD8^+$ T cells Each symbol represents an individual mouse. (b-d) Data were analysed by Two-way ANOVA. (e-i) Data were analysed by unpaired t-test. Small horizontal lines indicate the mean (± s.e.m.). $*P \leq 0.05$, $**P \leq 0.01$. N = 4 to 10 (biological replicate). Data are representative of two independent experiments.

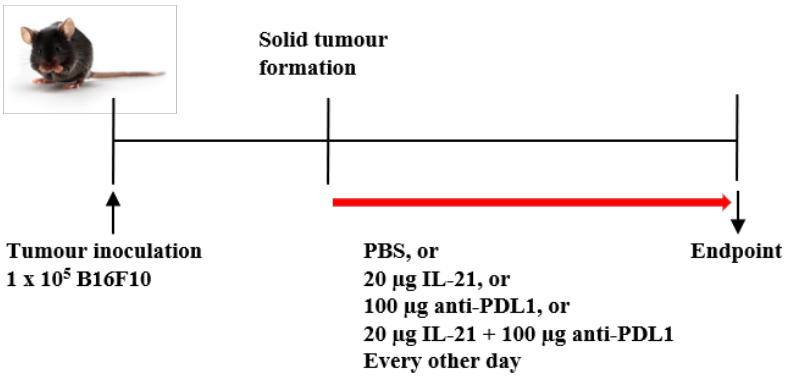
4.2.2 IL-21 treatment in combination with PD-L1 blockade has a modest effect in inhibiting tumour growth in B16F10 in the priming phase

While PD1 blockade can be the solution to limited response of IL-21 treatment, the reverse might also hold true. Checkpoint blockade has been a breakthrough in cancer immunotherapy, but not all patients respond to it. In these patients, the tumour is often characterised by poor $CD8^+$ T cell infiltration, which may be due to immunological ignorance [220]. Recent studies suggest that this is a result of low mutational burden in tumour which do not sufficiently prime $CD8^+$ T cells [102, 221, 222]. Hence, with lower activation, $CD8^+$ T cells in those patients tend not to express PD1, and thus do not respond to PD1 blockade.

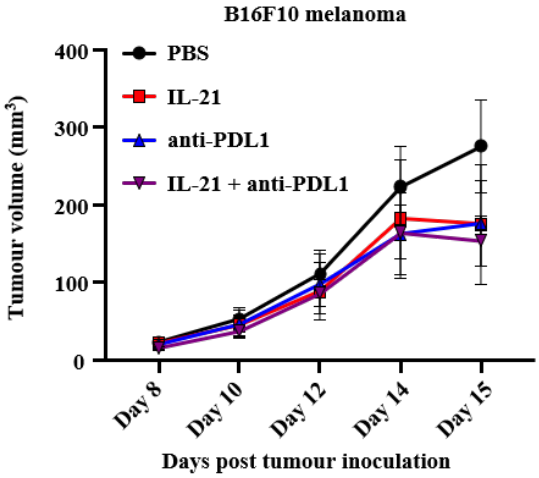
Since IL-21 can increase the percentage of $PD1^+$ $CD8^+$ T cells in tumours, it can sensitise $CD8^+$ T cells towards PD1 blockade. To test this, combination treatment of PD1 blockade and IL-21 was performed. Intraperitoneal IL-21 and/or anti-PDL1 injections started after tumour formation and were performed every other day **(Figure 4.2a)**. Mice were sacrificed on day 15. Tumours were harvested and tumour-infiltrating lymphocytes were isolated for FACS analysis. Based on tumour sizes, it appeared that combination treatment of IL-21 and PD1 blockade further inhibited tumour growth as compared to single agent treatment, albeit statistically insignificant **(Figure 4.2b)**. Combination treatment resulted in further increase in $CD8^+$ T cell infiltration from about 30% $CD8^+$ T cell in single agent treatment to about 40% **(Figure 4.2c)**. There was also an increase in $CD8^+$ T cell to Treg ratio from below 1.0 in single agent treatment to about 1.2 in mice under the effect of combination treatment **(Figure 4.2d)**. Interestingly, $CD8^+$ T cell activation and IFNγ secretion were not further enhanced **(Figure 4.2e, f)**. As a

result, the inhibition of tumour growth was only modest, hence suggesting other inhibitory mechanisms were in place. Indeed, other known inhibitory receptors such as 2B4, Lag3 and Tim3 were increased in both single agent and combination treatments **(Figure 4.2g)**.

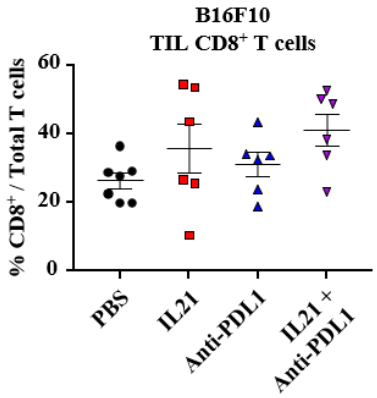
a.


Solid tumour formation
Tumour inoculation
1 x 10^5 B16F10
PBS, or
20 µg IL-21, or
100 µg anti-PDL1, or
20 µg IL-21 + 100 µg anti-PDL1
Every other day
Endpoint

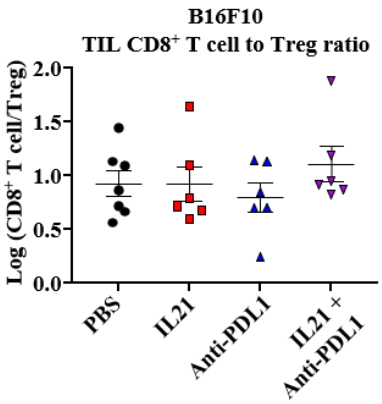
b.


B16F10 melanoma
PBS
IL-21
anti-PDL1
IL-21 + anti-PDL1
Tumour volume (mm3)
400
300
200
100
0
Day 8
Day 10
Day 12
Day 14
Day 15
Days post tumour inoculation

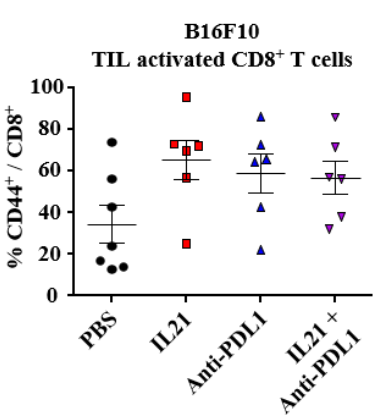
c.


B16F10
TIL CD8+ T cells
% CD8+ / Total T cells
60
40
20
0
PBS
IL21
Anti-PDL1
IL21 + Anti-PDL1

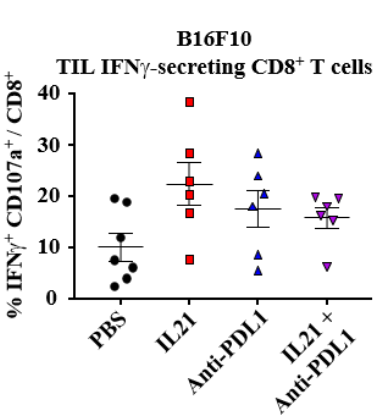
d.


B16F10
TIL CD8+ T cell to Treg ratio
Log (CD8+ T cell/Treg)
2.0
1.5
1.0
0.5
0.0
PBS
IL21
Anti-PDL1
IL21 + Anti-PDL1

e.


B16F10
TIL activated CD8+ T cells
% CD44+ / CD8+
100
80
60
40
20
0
PBS
IL21
Anti-PDL1
IL21 + Anti-PDL1

f.


B16F10
TIL IFNγ-secreting CD8+ T cells
% IFNγ+ CD107a+ / CD8+
40
30
20
10
0
PBS
IL21
Anti-PDL1
IL21 + Anti-PDL1

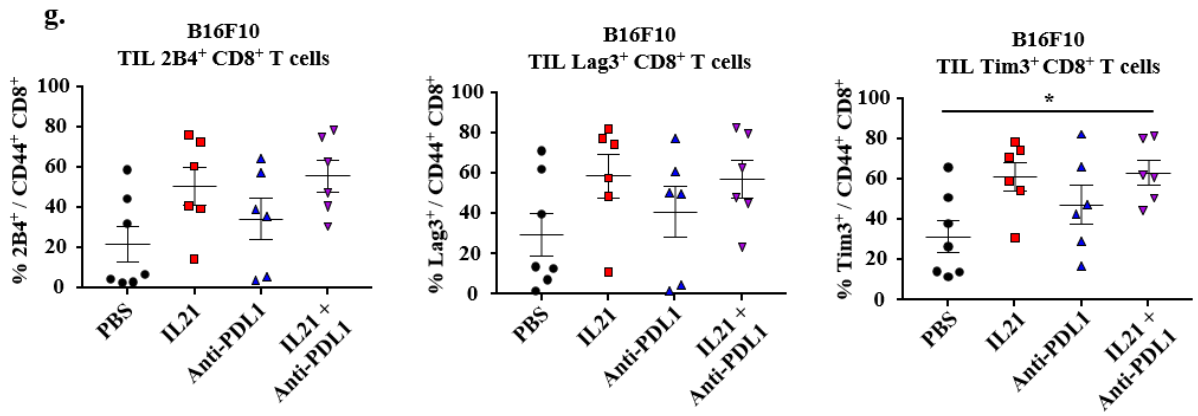


Figure 4.2. IL-21 alone and in combination with PDL1 blockade modestly enhance anti-tumour response in the priming stage *in vivo* against B16F10. (a-e) Mice were subcutaneously inoculated with tumour cells (1 x 10^5 B16F10), 20 µg IL-21 and/or 100 µg anti-PDL1 via intraperitoneal injection every other day commenced after solid tumours were observed (day 8). Mice were sacrificed on day 15. Tumour size was measured and calculated by using the formula 0.5 x length x breadth x breadth in mm^3. **(c-g)** Tumours were harvested, and tumour-infiltrating lymphocytes were isolated for FACS analysis. For intracellular staining in (e), cells were stimulated with PMA, ionomycin, brefeldin A and monensin for four hours, then stained for IFNγ and CD107a. **(c)** Percentage of $CD8^+$ cells within total tumour-infiltrating T cells between different treatment groups. **(d)** Ratio of $CD8^+$ T cells to $Foxp3^+$ $CD4^+$ Treg (in the scale of logarithm base 10) within tumour-infiltrating $CD8^+$ T cells between different treatment groups. **(e)** Percentage of $CD44^+$ cells within tumour-infiltrating $CD8^+$ T cells between different treatment groups. **(f)** Percentage of $IFN\gamma^+$ $CD107a^+$ within tumour-infiltrating $CD8^+$ T cells between different treatment groups. **(g)** Percentage of $2B4^+$, $Lag3^+$ and $Tim3^+$ within tumour-infiltrating activated $CD8^+$ T cells between different treatment groups. Each symbol represents an individual mouse. (b) Data were analysed by Two-way ANOVA. (c-g) Data were analysed by One-way ANOVA followed by Tukey's multiple comparison test. Small horizontal lines indicate the mean (± s.e.m.). $*P \leq 0.05$. N = 6 to 7 (biological replicate). Data are representative of two independent experiments.

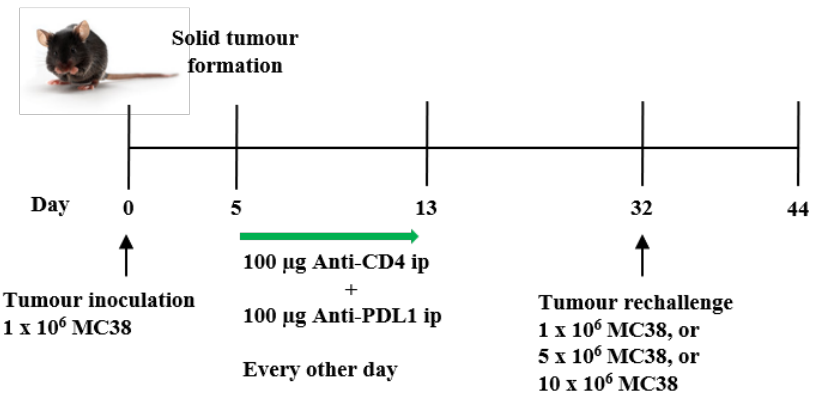
4.2.3 The establishment of a recurrent tumour model to investigate the role of IL-21 on memory $CD8^+$ T cell responses

To examine the effect of IL-21 in the post-priming phase of $CD8^+$ T cells anti-tumour response, primed $CD8^+$ T cells have to be generated *in vivo* prior to IL-21 treatment and tumour challenge. Based on the information from the FTA killing assay in Chapter 3, the effect of IL-21 on primed $CD8^+$ T cells can only be observed in strong or mid TCR strengths. Hence, MC38, a tumour that strongly stimulates $CD8^+$ T cells, was chosen for this study,

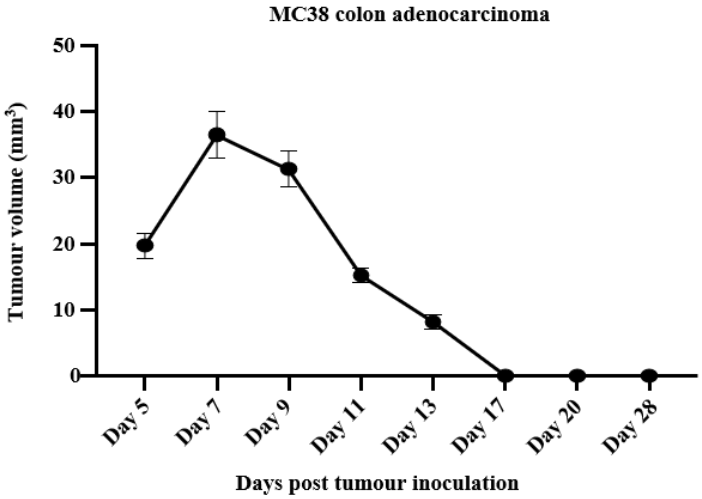
To induce complete remission of the primary tumour, MC38 tumour-bearing mice were treated with a combination of anti-PD-L1 and anti-CD4 **(Figure 4.3a)**. As adapted from Ueha et. al. [223], mice treated with this combination of antibodies have complete tumour remission that are long-lasting. PD-L1 blockade revigorated exhausted $CD8^+$ T cells, while CD4 blockade removes immunosuppressive immune cell populations like Treg, Th2 and myeloid-derived suppressor cells [224, 225]. Another advantage of CD4 blockade is the removal of $CD4^+$ T cells. Removing the generation of primed $CD4^+$ T cells will ensure that the eventual anti-tumour response is a more direct result of $CD8^+$ T cell response. MC38 tumour cells were used as it is known to be responsive to PD1 and PD-L1 blockades [226, 227], and thus ensure complete tumour removal. Optimisation using the MC38 tumour will form a basis of this study before proceeding to less immunogenic tumours like the B16 melanoma. Consistent with the publication, combination of PD-L1 and CD4 blockades resulted in complete tumour remission by day 17, and the effect was long lasting **(Figure 4.3b)**.

Due to the presence of background memory response, rechallenging of mice with similar number of tumour cells resulted in rapid tumour regression **(Figure 4.3c)**. Hence, the number of tumour cells to be used in rechallenge has to be titrated such that the rate of regression is slow enough for the effect of IL-21 to be later observed. As such, 1, 5, and 10 million tumour cells were injected into three groups of mice. Rechallenge of mice with 10 million cells showed a slower rate of background tumour regression **(Figure 4.3c)** and was selected to study the effect of IL-21.

a.



b.



c.

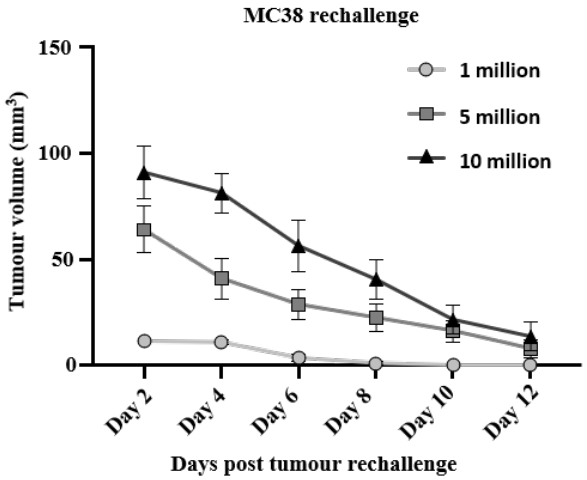


Figure 4.3. Rechallenging mice with 10 x 10^6 MC38 cells shows a slower rate of tumour regression. (a-c) Mice were subcutaneously inoculated with 1 x 10^6 MC38 cells, 100 µg anti-CD4 and anti-PDL1 intraperitoneal injection every other day commenced after solid tumours were observed on day 5. Mice were rechallenged subcutaneously with 1 x 10^6, 5 x 10^6, or 10 x 10^6 MC38 cells on day 32 **(b, c)** Tumour size was measured and calculated by using the formula 0.5 x length x breadth x breadth in mm^3.

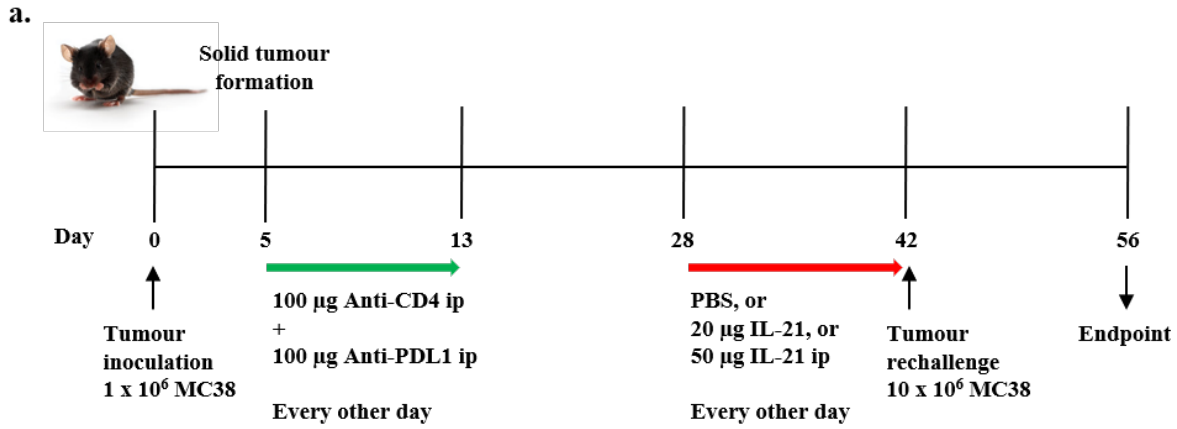
4.2.4 IL-21 treatment after the resolution of primary tumour strengthens the memory CD8$^+$ T cell response against tumour relapse

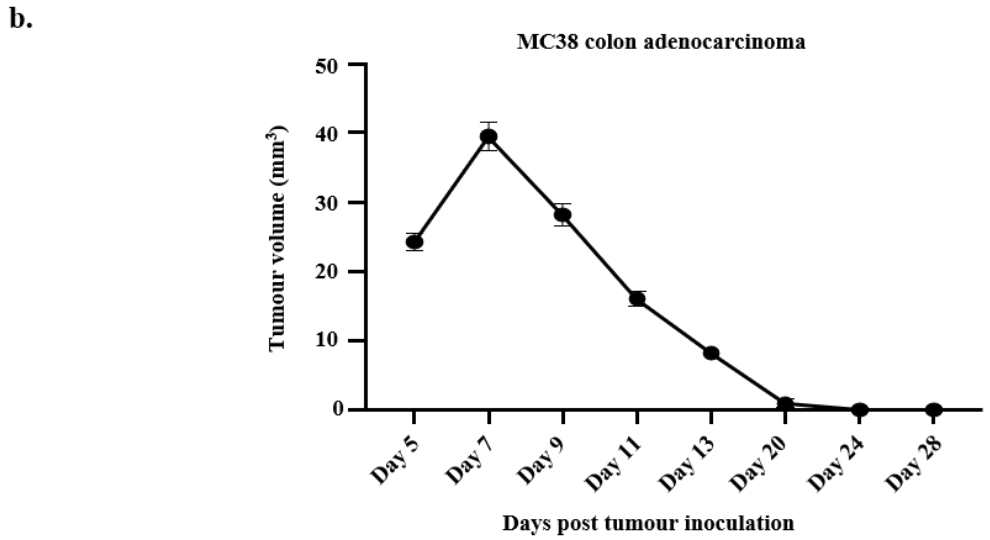
To study the effect of IL-21 on the anti-tumour response of primed CD8$^+$ T cells, as described in **Figure 4.4a**, mice were inoculated with tumour cells and subsequently treated with PD-L1 and CD4 blockades to induce complete tumour remission. This will generate primed CD8$^+$ T cells within the mice. After which, mice were left untreated for two weeks to ensure that there was no tumour regrowth **(Figure 4.4b)**. Following that, mice were treated with either PBS control, 20 μg IL-21, or 50 μg IL-21 every other day for two weeks. A low dose of 20 μg IL-21 and optimal dose of 50 μg IL-21 were selected to compare the dosage needed *in vivo* in order to achieve a response. At the end of the treatment, mice were rechallenged with 10 million MC38 tumour cells on the opposite flank to minimise the effect tissue-resident memory CD8$^+$ T cells (Trm) has on the tumour.

In this model, tumour-infiltrating lymphocytes could not be studied due to the size of tumour. Therefore, lymphocyte populations in the dLN were examined instead. Comparing the tumour size across treated and control mice, IL-21-treated mice saw a significant increase in tumour regression **(Figure 4.4c)**. This was potentially a result of increased CD8$^+$ T cell population in the dLN from about 45% to 55% **(Figure 4.5a)**, but not a change in CD8$^+$ T cell activation **(Figure 4.5b)**. Specifically, a higher percentage of 97% CD8$^+$ T cells in IL-21-treated mice expressed a TCF1$^+$ Tim3$^-$ phenotype as opposed to 92% in PBS-treated mice **(Figure 4.5c)**. The subset of CD8$^+$ T cells that is TCF1$^+$ Tim3$^-$ has been shown to exhibit stem cell-like properties, and was needed to sustain long term immune response against chronic viral infection [228-230]. When comparing effector and memory markers, IL-21 was found to increase T-bet and IRF4 at 20 μg but not 50 μg, while there was no change in CD127 expression (**Figure 4.5d**).

Taken together, IL-21 treatment significantly enhanced CD8$^+$ T cell response in the post-priming stage. Analysis of the CD8$^+$ T cell population in dLN suggests that IL-21 boosts the formation of the TCF1$^+$ Tim3$^-$ CD8$^+$ T cells which are proposed as the stem cell-like CD8$^+$ T

cells [228-230]. The $CD8^+$ T cells in IL-21-treated mice were also found to be $Tbet^{hi}$, $IRF4^{hi}$, and $CD127^{hi}$, which constitute an interesting mix of effector and memory phenotypes.





c.

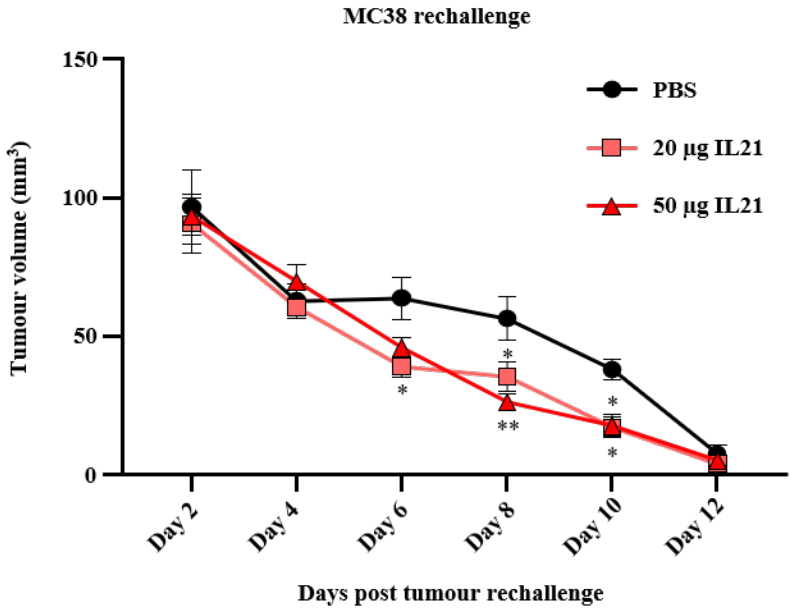
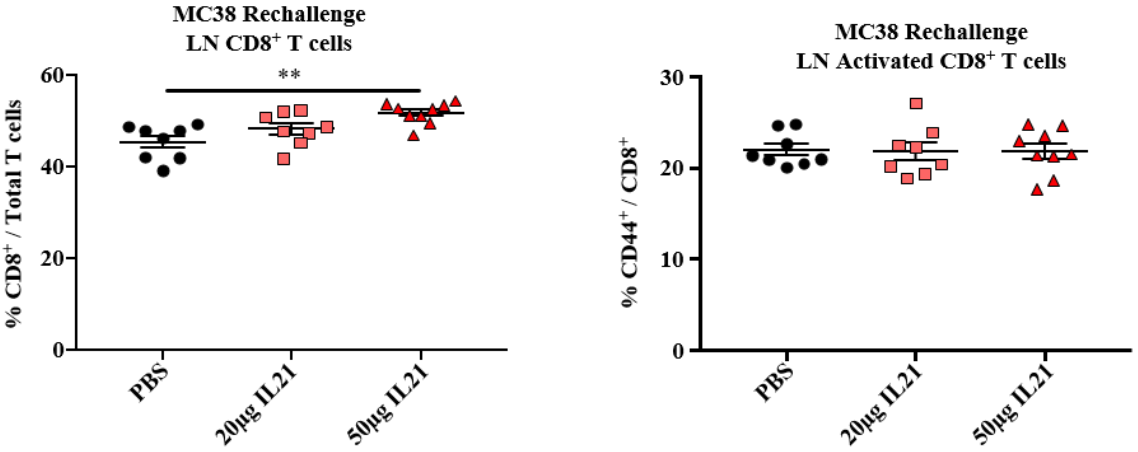
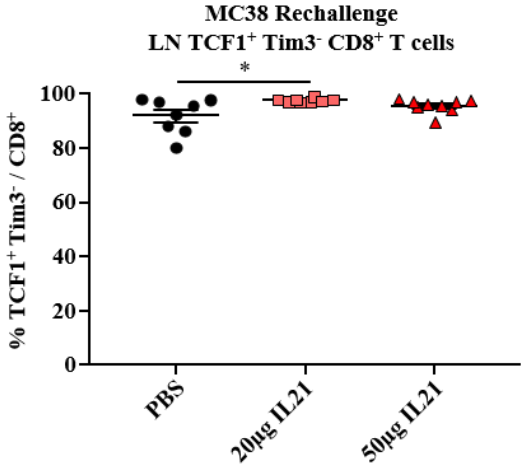


Figure 4.4. IL-21 treatment induces a faster rate of tumour regression. (a-c) Mice were subcutaneously inoculated with tumour cells (1 x 10^6 MC38), 100 µg anti-CD4 and anti-PDL1 intraperitoneal injection every other day commenced after solid tumours were observed on day 5. Mice were intraperitoneally injected with 20 or 50 µg IL21 every other day from day 28 to day 42. Mice were then rechallenged subcutaneously with 10 x 10^6 MC38 cells on day 42 **(b, c)** Tumour size was measured and calculated by using the formula 0.5 x length x breadth x breadth in mm^3. Data were analysed by Two-way ANOVA followed by Tukey's multiple comparison test. Small horizontal lines indicate the mean (± s.e.m.). N = 8 (biological replicate). *$P \leq 0.05$, **$P \leq 0.01$.

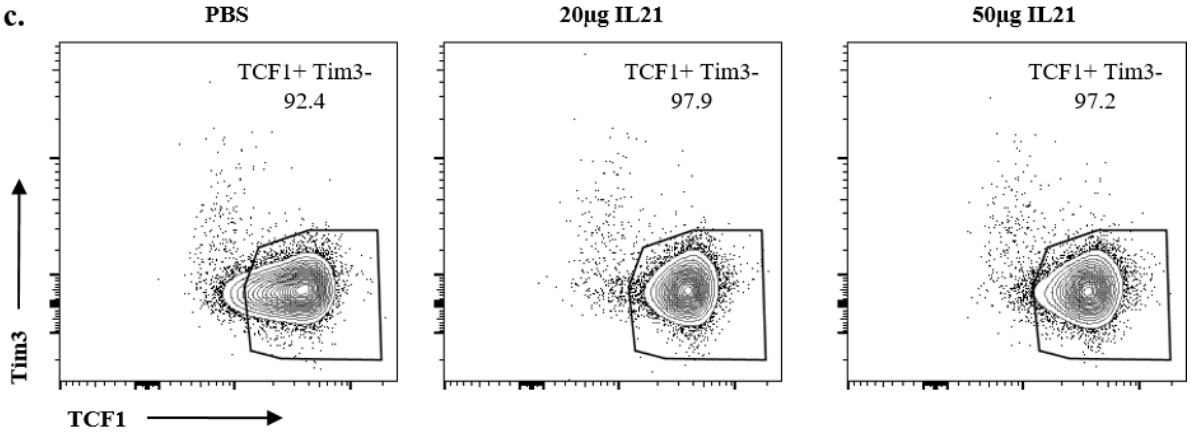
a.


MC38 Rechallenge
LN CD8+ T cells
**
% CD8+ / Total T cells
60
40
20
0
PBS
20µg IL21
50µg IL21
MC38 Rechallenge
LN Activated CD8+ T cells
% CD44+ / CD8+
30
20
10
0
PBS
20µg IL21
50µg IL21

b.


MC38 Rechallenge
LN TCF1+ Tim3- CD8+ T cells
*
% TCF1+ Tim3- / CD8+
100
80
60
40
20
0
PBS
20µg IL21
50µg IL21

c.


PBS
20µg IL21
50µg IL21
TCF1+ Tim3-
92.4
TCF1+ Tim3-
97.9
TCF1+ Tim3-
97.2
Tim3
TCF1

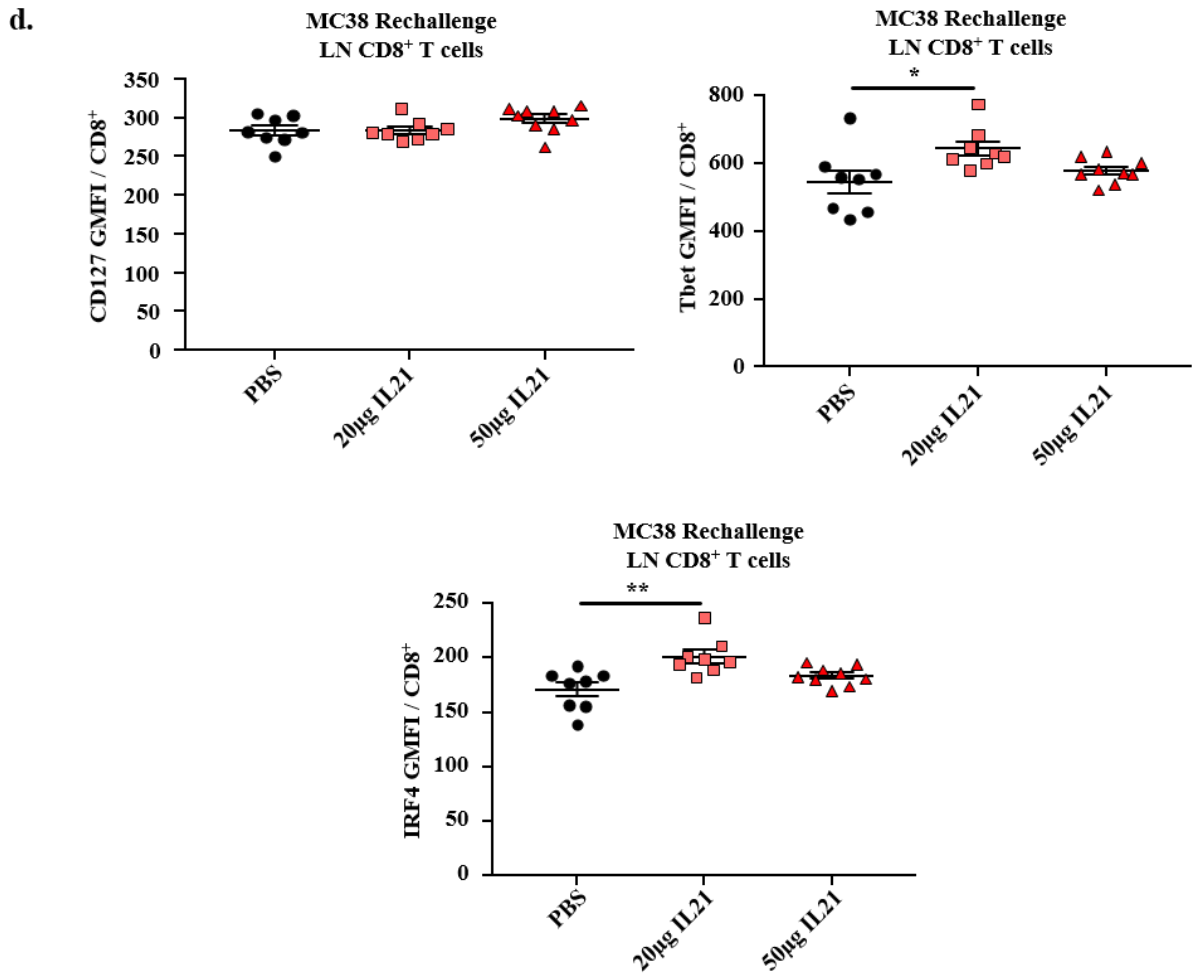


Figure 4.5. IL-21 treatment induces formation of stem cell-like CD8$^+$ T cells with effector phenotype. Mice were treated as described in Figure 4.4a, and were sacrificed on day 14 post tumour rechallenge. Draining lymph nodes at the site of tumour rechallenge were harvested, and lymphocytes were isolated for FACS analysis. **(a)** Percentage of CD8$^+$ cells within total T cells between different treatment groups. Percentage CD44$^+$ CD8$^+$ T cells between different treatment groups. **(b)** Percentage of TCF1$^+$ Tim3$^-$ cells within CD8$^+$ T cells between different treatment groups. **(c)** Representative flow cytometry plots of TCF1$^+$ Tim3$^-$ within CD8$^+$ T cells between different treatment groups. **(d)** GMFI of CD127, Tbet and IRF4 expressions of CD8$^+$ T cells between different treatment groups. Each symbol represents an individual mouse. Data were analysed by One-way ANOVA followed by Tukey's multiple comparison test. Small horizontal lines indicate the mean (± s.e.m.). N = 8 (biological replicate). *$P \leq 0.05$, **$P \leq 0.01$.

4.3 Discussion

Cytokines such as IL-2 and IFN-α have been approved as single agent cancer immunotherapy for patients, albeit the outcome of the treatment might not always be optimal. As demonstrated in the previous chapter, the phase of $CD8^+$ T cell anti-tumour response during which the cytokines were applied will have an impact on the treatment results. Here, I examined the use of IL-21 treatment in different phases of $CD8^+$ T cell anti-tumour response.

In immunogenic tumours AT3 and MC38, IL-21 treatment during the $CD8^+$ T cell priming phase increased $CD8^+$ T cell infiltration and the ratio of $CD8^+$ T cell to Treg ratio. However, these did not translate into an increase tumour inhibition. Further investigation revealed an upregulation of checkpoint inhibitor PD1 which can bind to its ligands expressed on tumour cells and result in the inhibition of $CD8^+$ T cell responses. These observations further highlight that, in the priming phase, IL-21 does not enhance $CD8^+$ T cell killing function when cells are already receiving strong TCR signal.

Conversely, in a poorly immunogenic tumour B16F10, IL-21 more drastically increased the population of activated $CD8^+$ T cells as compared to in the AT3 and MC38 tumours. As expected, treatment of IL-21 also upregulated PD1 expression suggesting its potential use to sensitise non-responding patients towards the PD1 blockade treatment. Indeed, a combination of IL-21 and anti-PD-L1 further augmented the $CD8^+$ T cell to Treg ratio.

Although changes in the $CD8^+$ T cells population have been observed, it did not correspond to significant increase in tumour inhibition. Starting the treatments earlier might have been a solution to this. As treatments only began when tumours had already grown to a substantial size, the solid tumour microenvironment might have already been well-established. The tumour microenvironment is highly immunosuppressive and, once established, will strongly inhibit $CD8^+$ T cells activity [231]. Furthermore, established tumour vasculature can physically impede the penetration of $CD8^+$ T cells into the core of the tumour [232]. Moreover, other exhaustion markers, in addition to PD1, have been reported to inhibit $CD8^+$ T cell function. Tim3, Lag3 and 2B4 are similarly upregulated in activated $CD8^+$ T cells, which inhibits TCR and costimulatory signalling [44, 233].

By contrast, IL-21 treatment in the post-priming phase of $CD8^+$ T cell anti-tumour response has achieved significant results. This could potentially be an outcome of the increase in the $TCF1^+$ $Tim3^-$ $CD8^+$ T cells which reportedly have stem cell-like characteristics [228-230]. This phenomenon was not observed when IL-21 was administered in the priming phase (data not shown), which was potentially why the tumours were not controlled. Especially in chronic diseases, continuous supply of effector $CD8^+$ T cells is critical to a positive immune outcome [228-230].

Notably, a study in 2016 outlined a subset of $CD8^+$ T cells that is crucial in sustaining the $CD8^+$ effector T cells needed to control persistent *Toxoplasma gondii* infection [234]. Broadly marked by the expression of CXCR3 and KLRG1, this group of $CD8^+$ T cell exhibited a memory-effector hybrid phenotype with expression of memory-associated markers such as CD122 and Eomes, and effector-associated markers such as Blimp1 and Tbet. Though certain markers do not overlap in my study, the $CD8^+$ T cells from mice treated with IL-21 post-priming expressed high TCF1, Tbet and IRF4, which also suggest a memory-effector hybrid phenotype.

As observed in the PBS control group, background memory response was already strong and, as a result, diluted the effect of IL-21. One improvement to this model would be to prime $CD8^+$ T cells using OVA-expressing tumours and to rechallenge mice with parental non-OVA-expressing tumours. By doing so, a majority of primed $CD8^+$ T cells will be targeting the foreign protein OVA. With less primed $CD8^+$ T cells generated targeting other tumour peptides, the magnitude of memory response against non-OVA tumour antigens will be largely reduced.

Altogether, IL-21 treatment showed efficacy in both the priming and post-priming phases of $CD8^+$ T cell response. Its effect in post-priming phase appeared to be more significant potentially due to the formation of a stem cell-like $CD8^+$ T cell population. Thus, a comparison of IL-21 treatment with known effector-inducing cytokine IL-2, and memory-inducing cytokine IL-15 will further delineate the function of IL-21 in shaping $CD8^+$ T cells at a cellular, transcriptional and metabolic level.

Chapter 5

The comparison of CD8$^+$ T cell differentiation features induced by IL-2, IL-15 and IL-21

5.1 Introduction

In Chapters 3 and 4, IL-21 was found to be more effective in enhancing CD8$^+$ T cell killing function when administered in the post priming phase of CD8$^+$ T cells. Indeed, the environment where the CD8$^+$ T cells are rested in and the cytokines they are exposed to have been shown to have effects on post-priming development of CD8$^+$ T cells. Thus, further verification using *in vitro* cultures to identify changes in expression of key markers is required. An *in vitro* culture system will allow for an artificially controlled environment to test out function of individual cytokine on CD8$^+$ T cells by limiting the influence of other additional stimulus. Such method has been used to delineate the function of IL-2 in promoting formation of effector CD8$^+$ T cell and IL-15 in promoting formation of memory CD8$^+$ T cell [128, 182, 235]. Therefore, by comparing the effect of IL-21 on CD8$^+$ T cell with the known effector-inducing IL-2 and memory-inducing IL-15, it is then possible to identify the mechanism underlying the increased CD8$^+$ T cell killing function when IL-21 is used post priming.

Cytokines, especially those in the γ_c family, have long been known to play dramatic roles in shaping CD8$^+$ T cell phenotype and response. IL-2 is largely involved in CD8$^+$ T cell effector response [182, 236]. IL-2 signals primarily through the STAT5 pathway [237, 238], which in turn upregulates effector function through changes in expression of the transcription factors – Eomes and Tbet. When CD8$^+$ T cells were activated *in vitro* for two days and subsequently re-cultured in media containing IL-2, an increase in the expression of Eomes but not Tbet was observed with increasing concentration of IL-2 [182]. This correlated with Perforin expression [182]. A similar observation was made when pmel-1 CD8$^+$ T cells were activated using its cognate peptide in the presence of IL-2 [128]. On the other hand, *in vitro* addition of IL-2 when stimulating of *T. gondii*-CD8$^+$ T cell hybridomas with its cognate antigen resulted in an upregulation of Tbet which correlated with enhanced IFNγ [183]. IL-2 signalling induces a feed forward loop by inducing CD25 (IL-2Ra) expression [239], thereby further increasing the responsiveness of activated CD8$^+$ T cells to IL-2. Deficiency in CD25 has been found to reduce CD8$^+$ T cell cytotoxic activity *in vivo* [182]. Correspondingly, intense IL-2 signalling favoured effector CD8$^+$ T cell differentiation at the expense of memory CD8$^+$ T cell formation [182, 240].

Another γ_c cytokine IL-15, produced mainly by dendritic cells and macrophages [241-243], is important for CD8$^+$ T cell homeostasis and promotes memory formation [244-246]. Binding of IL-15 to its receptor induces phosphorylation of STAT3 and STAT5 which then form heterodimers and result in the upregulation of anti-apoptotic protein BCL2 [185, 186], and proto-oncogenes such as c-myc [247]. This ultimately leads to long-term survival of CD8$^+$ T cells. Indeed, *in vitro* culture of antigen-experienced CD8$^+$ T cells with IL-15 resulted in an increase in number of live cells [235]. Other observations from similar *in vitro* culture studies also revealed a decrease in expression of activation markers – CD25 and CD69, an increase in expression of memory marker – CD62L – and an increase in expression of the IL-15 receptor subunit – CD122 [248-250]. Studies done on IL-15-deficient mice also showed the importance of this cytokine in maintaining the memory CD8$^+$ T cell pool post Listeria and viral infections [251, 252].

As part of the same cytokine family, IL-21 has known effects on multiple processes. IL-21 signals mainly through STAT3, and weakly by STAT5 [123, 124]. IL-21 has a role in inducing CD8$^+$ T cell cytotoxicity via the induction of Tbet [108]. Controversially, IL-21 can also inhibit Eomes expression and, at the same time, promote *Tcf7* and *Lef1* which induce memory CD8$^+$ T cell formation [184]. CD62L was also noted to be upregulated in IL-21-induced CD8$^+$ T cells which suggests the induction of the memory phenotype as opposed to an effector phenotype mentioned in the previous study [184]. These mixed observations have potentially been a result of different source of CD8$^+$ T cells – transgenic or polyclonal, and the type of activation method – TCR-peptide or CD3 stimulation. Thus, it remains ambiguous if IL-21 actually promotes the effector or memory CD8$^+$ T cell response.

Furthermore, studies in chronic LCMV model have shown that IL-21 is a cytokine released by the CD4 T cells only in late T cell response [188, 189]. Consistent with that, my findings from the previous chapters have also identified IL-21 as having a more prominent role in the post priming phase of CD8$^+$ T cell response. Indeed, IL-21R knockout was found to have no effect on virus titre when mice were acutely infected with vaccinia virus [151]. Furthermore, the previous effects of IL-21 described *in vitro* has largely been in the priming phase which is early in the T cell response. The effects of IL-21 on the post priming phase of CD8$^+$ T cell response, therefore, remain to be elucidated.

Using flow cytometry, a direct comparison of the changes in expression of different markers – activation, memory, proliferation – in IL-21-induced CD8$^+$ T cells with the known IL-2-induced effector CD8$^+$ T cells and the IL-15-induced memory CD8$^+$ T cells can be made. This will facilitate better understanding of the effect of IL-21 on CD8$^+$ T cells in the post priming phase and provide critical information on the CD8$^+$ T cell subset that IL-21 is promoting.

5.2 Results

5.2.1 IL-21 treatment promotes the upregulation of effector markers as compared to IL-2 and IL-15

To understand the effect of IL-21 on the CD8$^+$ T cell post priming phase, a culture system to create *in vitro* primed CD8$^+$ T cells was used. Adapted from van der Windt et. al. [248], this culture system mimics the development of effector CD8$^+$ T cells and memory CD8$^+$ T cells after an anti-viral response *in vivo*. During infection, CD8$^+$ T cells undergo priming and proliferation in the presence of IL-2. As the infection is being cleared, CD8$^+$ T cells begin to undergo contraction and memory formation which can be broadly categorised under the post-priming phase. With the gradual loss of IL-2 dominance, the effects of other cytokines begin to emerge. Hence, transgenic OT-I CD8$^+$ T cells were first primed with their cognate antigen SIINFEKL peptide and human IL-2 (hIL-2), and then rested in different cytokines to examine changes in expression of different markers **(Figure 5.1)**.

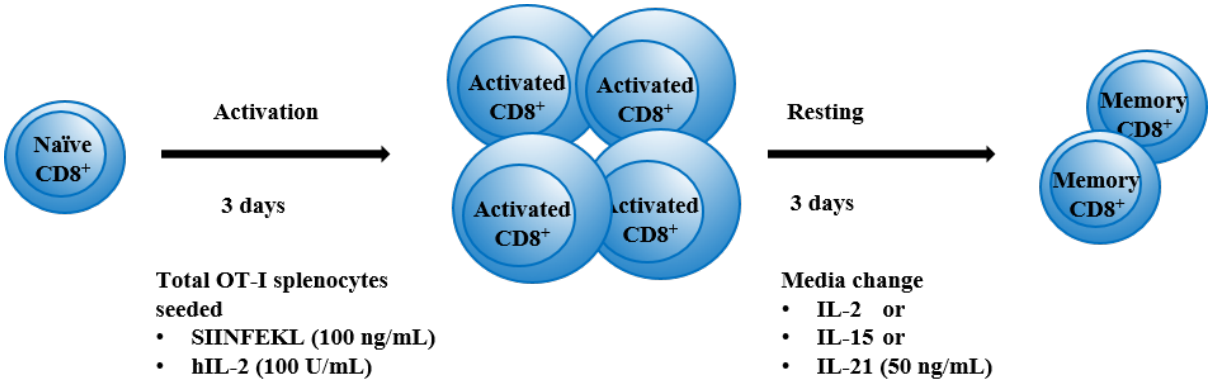
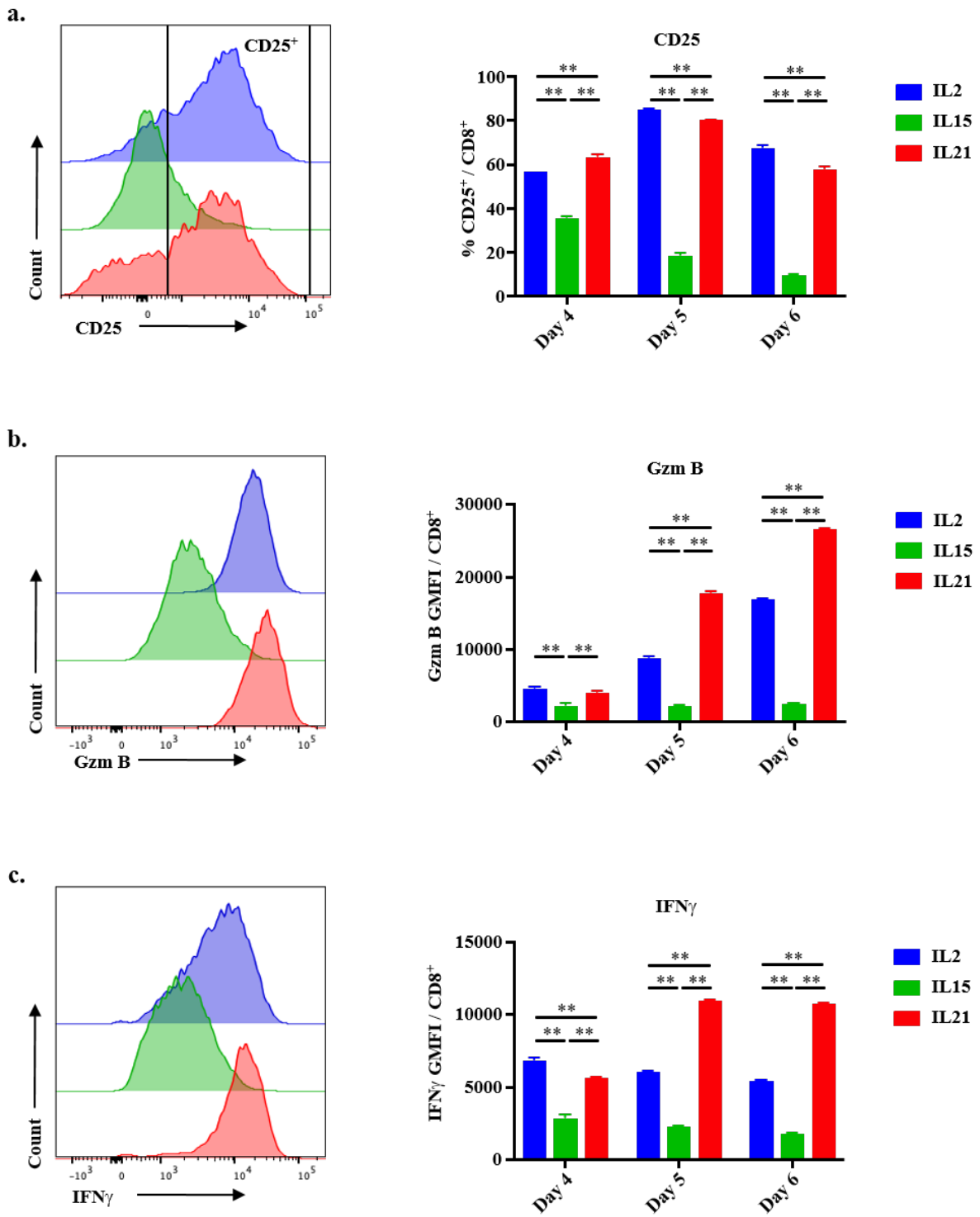


Figure 5.1. Schematic overview of the *in vitro* CD8⁺ T cell cytokine treatment post priming experiment. Total OT-I splenocytes were isolated and primed with 100 ng/mL SIINFEKL peptide and 100 U/mL human IL-2 for three days. Cells were then washed and rested in media containing 50 ng/mL murine IL-2, IL-15, or IL-21 for another three days and changes in expression of different markers were measured by FACS.

CD8⁺ T cells were gated as shown in **Supplementary Figure S4** before further analyses of other markers were done. One of the effector marker upregulated by IL-2 signalling is CD25 [239]. Expectedly, on day 6, IL-2 induced 67% of cells to express CD25 **(Figure 5.2a)**. The percentage of CD25⁺ cells was decreased in IL-15 treatment to 10%, a seven-fold decrease compared to IL-2, suggesting a lower requirement of IL-2 signalling in IL-15-induced memory CD8⁺ T cells **(Figure 5.2a)**. Treatment with IL-21 remarkably increased the percentage of CD25⁺ cells to about 58%, which was significantly lower than the 67% in IL-2 but higher than the 10% in IL-15 **(Figure 5.2a)**.

To further investigate the effector functions, expression levels of cytotoxic markers GzmB, IFNγ and TNFα were explored. To do so, cells were stimulated with PMA, ionomycin, brefeldin A and monensin for four hours prior to FACS. By comparing the GMFIs on day 6, the strong induction of IL-21 on cytotoxic marker expressions can be observed. GzmB (GMFI 26660) in IL-21-induced cells were significantly higher than those of IL-2-induced cells (GMFI 17000) and ten-fold higher than IL-15-induced cells (GMFI 2600) **(Figure 5.2b)**. A similar observation was made in the expression of IFNγ with IL-21-induced cells (GMFI 10755) having significantly higher expression as compared to IL-2- (GMFI 5420) and IL-15-induced cells (GMFI 1830) **(Figure 5.2c)**. TNFα was also higher in IL-21-induced cells (GMFI 59000) as compared to IL-2- (GMFI 41000) and IL-15-induced cells (GMFI 23700) **(Figure 5.2d)**. A comparison across the days of treatment showed that the effect of IL-21 in inducing cytotoxic

markers was subtle on the first day of treatment (day 4), but more prominent on the second and third day (day 5 and 6) of treatment with even stronger expressions than the effector-inducing IL-2 **(Figure 5.2b – d)**.



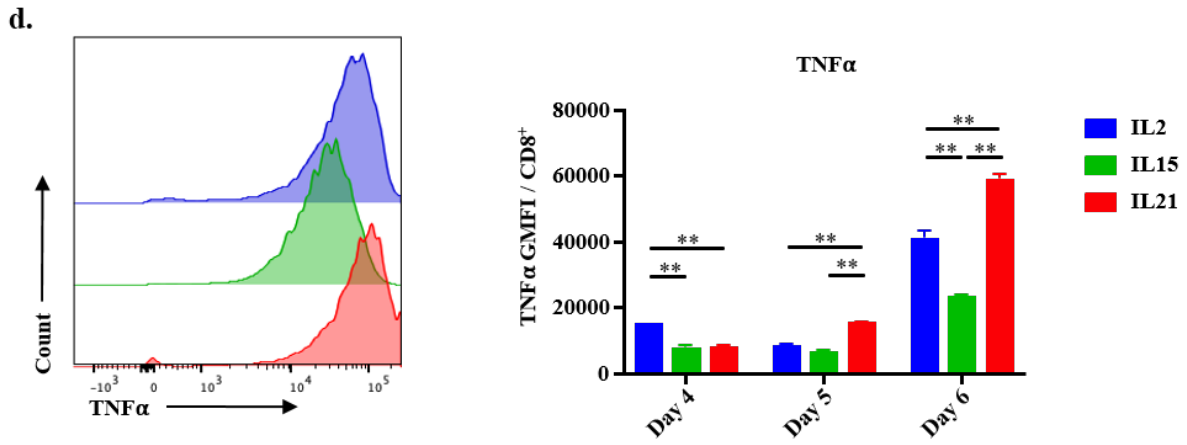


Figure 5.2. IL-21 increases effector marker expression on CD8⁺ T cells. *In vitro* culture was performed as described in Figure 5.1. FACS analyses were performed on cytokine-treated CD8⁺ T cells after four, five and six days of culture. **(a)** Left: Flow cytometry plot displays surface CD25 expression on cytokine-treated CD8⁺ T cells on day 6. Right: Mean percentage of CD25⁺ cytokine-treated CD8⁺ T cells. **(b, c, d)** Intracellular marker analyses of IFNγ, Gzm B and TNFα were performed after cells were stimulated with PMA, ionomycin, brefeldin A and monensin for four hours. **(b)** Left: Flow cytometry plot displays intracellular Gzm B expression of cytokine-treated CD8⁺ T cells on day 6. Right: Mean GMFI of Gzm B of cytokine-treated CD8⁺ T cells. **(c)** Left: Flow cytometry plot displays intracellular IFNγ expression of cytokine-treated CD8⁺ T cells on day 6. Right: Mean GMFI of IFNγ of cytokine-treated CD8⁺ T cells. **(d)** Left: Flow cytometry plot displays intracellular TNFα expression of cytokine-treated CD8⁺ T cells on day 6. Right: Mean GMFI of TNFα of cytokine-treated CD8⁺ T cells. Data were analysed by Two-way ANOVA followed by Tukey's multiple comparison test. Small horizontal lines indicate the mean (± s.e.m.). N = 3 (experimental replicate). *$P \leq 0.05$, **$P \leq 0.01$. Data are representative of two independent experiments.

5.2.2 IL-21 treatment has mixed effects on the expression of memory markers

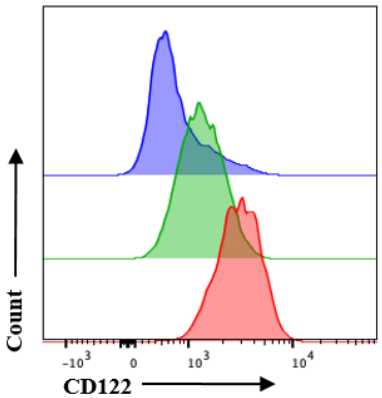
Despite the abovementioned evidences of increased effector characteristics, memory-related markers were also found to be increased in IL-21-induced cells. On day 6, memory markers critical for CD8⁺ T cell survival such as CD122 were at least two-fold higher in IL-21-induced cells (GMFI 2750) compared to IL-2- (GMFI 720) and IL-15-induced cells (GMFI 1300) **(Figure 5.3a)**. A similar observation was made for another pro-survival memory marker CD127, with IL-21-induced cells (GMFI 290) again showing two-fold higher expression than IL-2- (GMFI 140) and IL-15-induced cells (GMFI 136) **(Figure 5.3b)**.

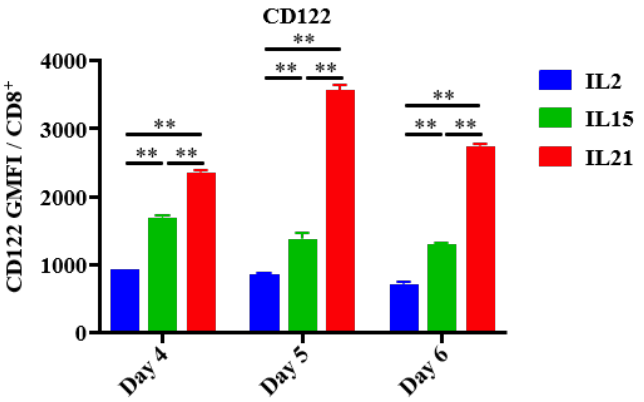
Apart from the ability of memory CD8$^+$ T cells to survive, the ability to home into secondary lymphoid organs allows further stratification of memory CD8$^+$ T cells into more defined subsets [253]. When comparing the homing receptor CCR7 on day 6, IL-21 treatment induced a significantly higher expression of CCR7 (GMFI 1020) as compared to IL-2- (GMFI 640) and IL-15-induced cells (GMFI 400) **(Figure 5.3c)**. However, the comparison of another homing receptor CD62L showed a different expression pattern to CCR7. IL-21 treatment induced a lower expression of CD62L (GMFI 810) as compared to IL-2 (GMFI 1190) and IL-15 (GMFI 1750) **(Figure 5.3d)**.

In an opposite trend to the surface memory markers, the transcription factor TCF1, which is known to drive CD8$^+$ memory T cell differentiation [254], was significantly reduced in IL-21-induced cells (GMFI 1140) by two- to four-fold when compared to IL-2- (GMFI 2420) and IL-15-induced cells (GMFI 4590) respectively **(Figure 5.3e)**.

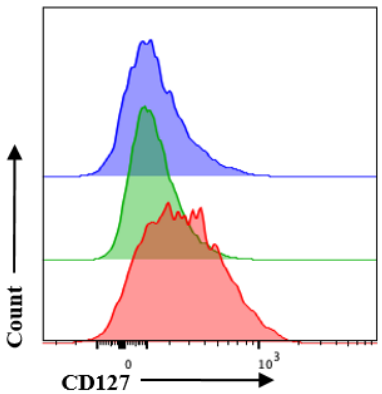
Altogether, mixed effects by IL-21 on the expression of memory markers have been observed. IL-21 has clear stimulatory effect in enhancing CD8$^+$ T cell longevity as suggested by the increase in CD122 and CD127. The expressions of homing receptors and memory-driving transcription factor TCF1, however, were more ambiguous.

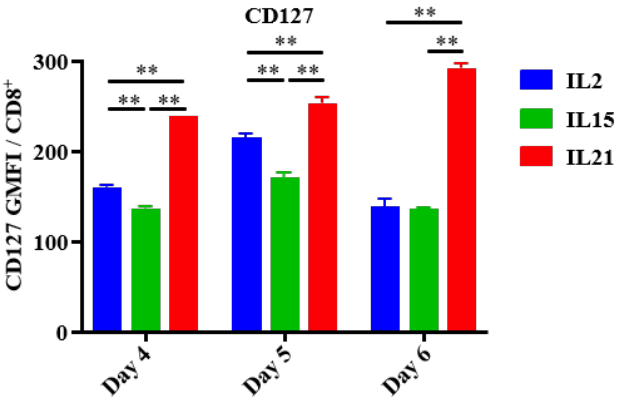
a.


Count
CD122

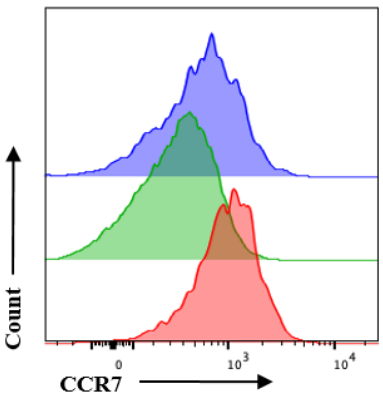

CD122
CD122 GMFI / CD8⁺
4000
3000
2000
1000
0
Day 4
Day 5
Day 6
IL2
IL15
IL21

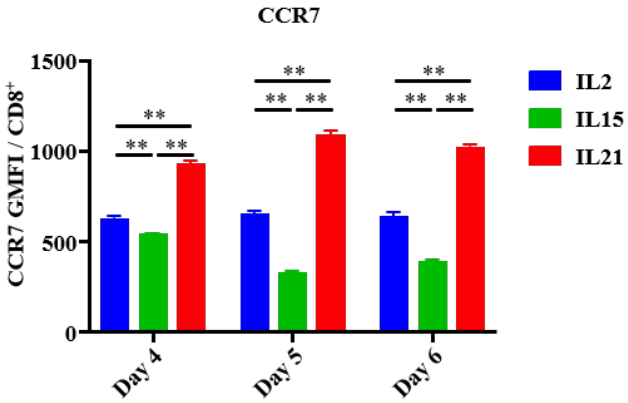
b.


Count
CD127


CD127
CD127 GMFI / CD8⁺
300
200
100
0
Day 4
Day 5
Day 6
IL2
IL15
IL21

c.


Count
CCR7


CCR7
CCR7 GMFI / CD8⁺
1500
1000
500
0
Day 4
Day 5
Day 6
IL2
IL15
IL21

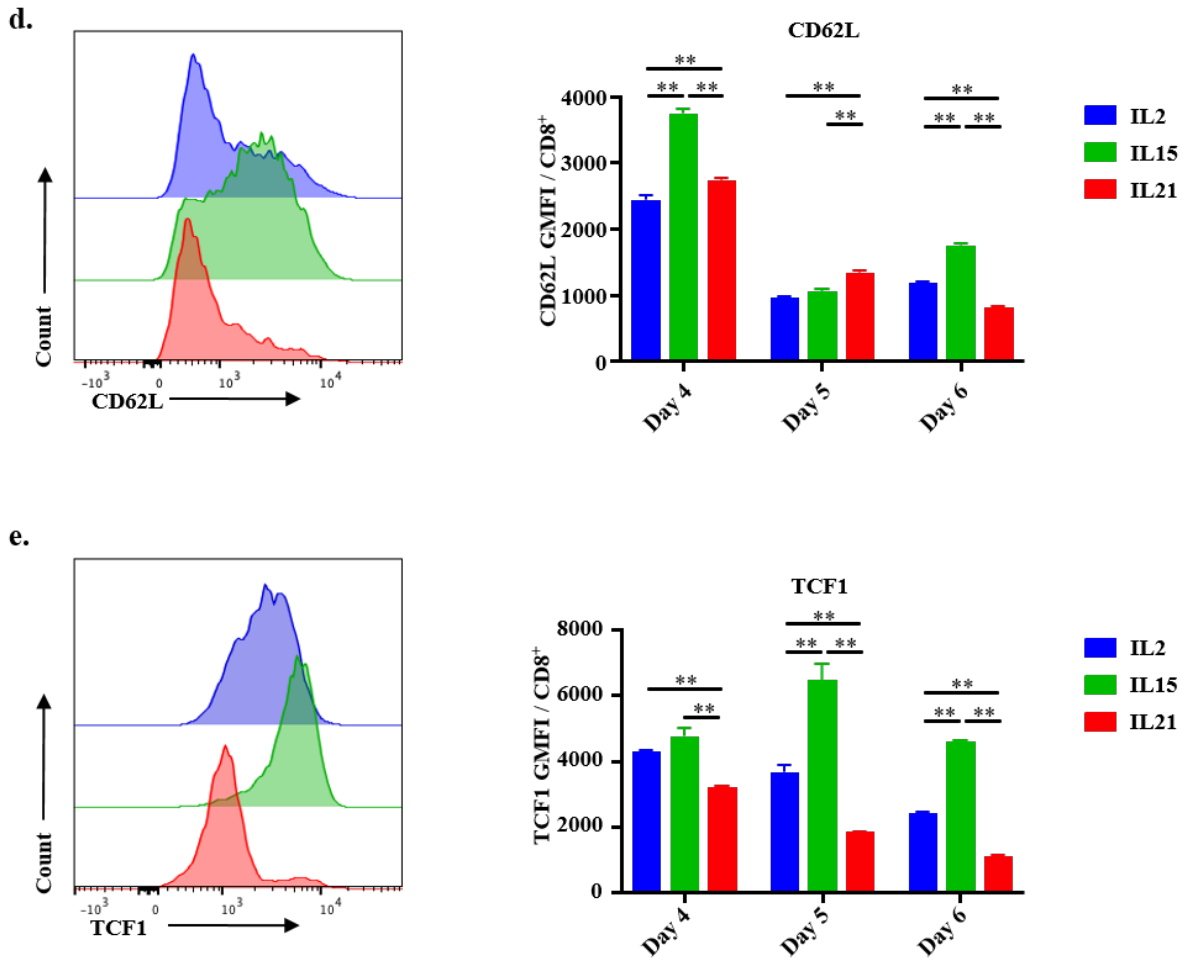


Figure 5.3. IL-21 has mixed effects on memory marker expressions on CD8⁺ T cells. *In vitro* culture was performed as described in Figure 5.1. FACS analyses were performed on cytokine-treated CD8⁺ T cells after four, five and six days of culture. **(a)** Left: Flow cytometry plot displays surface CD122 expression of cytokine-treated CD8⁺ T cells on day 6. Right: Mean GMFI of CD122 expression of cytokine-treated CD8⁺ T cells. **(b)** Left: Flow cytometry plot displays surface CD127 expression of cytokine-treated CD8⁺ T cells on day 6. Right: Mean GMFI of CD127 expression of cytokine-treated CD8⁺ T cells. **(c)** Left: Flow cytometry plot displays surface CCR7 expression of cytokine-treated CD8⁺ T cells on day 6. Right: Mean GMFI of CCR7 expression of cytokine-treated CD8⁺ T cells. **(d)** Left: Flow cytometry plot displays intracellular CD62L expression of cytokine-treated CD8⁺ T cells on day 6. Right: Mean GMFI of CD62L expression of cytokine-treated CD8⁺ T cells. **(e)** Left: Flow cytometry plot displays intracellular TCF1 expression of cytokine-treated CD8⁺ T cells on day 6. Right: Mean GMFI of TCF1 expression of cytokine-treated CD8⁺ T cells. Data were analysed by Two-way ANOVA followed by Tukey's multiple comparison test. Small horizontal lines indicate the mean (± s.e.m.). *$P \leq 0.05$, **$P \leq 0.01$. N = 3 (experimental replicate). Data are representative of two independent experiments.

5.2.3 IL-21 treatment induces the formation of Tbet^{hi} Eomes^{lo} CD8$^+$ T cells

To better understand the changes underlying the transcription factors that control CD8$^+$ T cell effector and memory fates [255-258], in addition to TCF1, the expression levels of Tbet and Eomes were investigated.

During priming, IL-21 has been shown to directly upregulate or maintain Tbet expression by STAT1 signalling [108], and to inhibit Eomes expression [184]. A similar observation was made when IL-21 was added to CD8$^+$ T cells in the post priming phase – a strong induction of Tbet and but not Eomes **(Figure 5.4a)**. Comparing across cytokine treatments, the expressions of Tbet and Eomes were clearly different in IL-21-induced cells as compared to IL-2- and IL-15-induced cells **(Figure 5.4a)**. On day 6, about 17% of IL-21-induced CD8$^+$ T cells became Tbet^{hi} Eomes^{lo}, while IL-2 and IL-15 only induced 1.2% and 0.44% of Tbet^{hi} Eomes^{lo} cells respectively **(Figure 5.4b)**. Inversely, IL-2 and IL-15 induced a higher population of Tbet^{lo} Eomes^{hi} cells at 50% and 30% respectively **(Figure 5.4c)**. Interestingly, the Tbet^{lo} Eomes^{hi} CD8$^+$ T cells have been described as the progenitor cell population needed to sustain Tbet^{lo} Eomes^{hi} terminal effector CD8$^+$ T cells during chronic viral infection [259].

Together with the changes in TCF1 expression, this further suggests that the differences between the cytokine-induced CD8$^+$ T cells occur at a transcriptional level. However, more experiments like RNA-sequencing will be needed to confirm this.

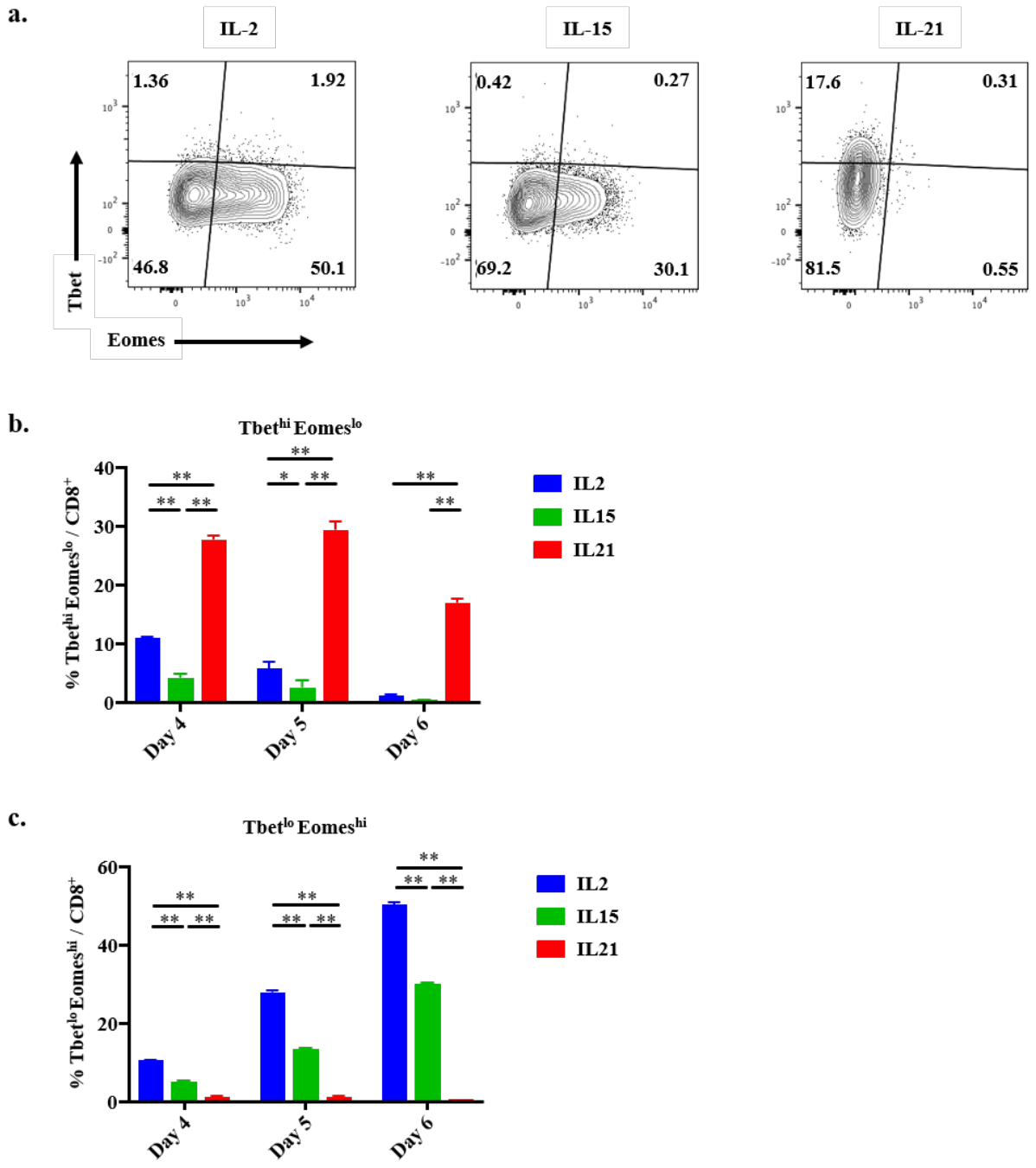


Figure 5.4. IL-21 induces the formation of Tbet^{hi} Eomes^{lo} CD8$^+$ T cells *in vitro*. *In vitro* culture was performed as described in Figure 5.1. Intracellular marker analyses of Tbet and Eomes expressions of cytokine-treated CD8$^+$ T cells after four, five and six days of culture. **(a)** Representative flow cytometry plots of Tbet and Eomes expressions of CD8$^+$ T cells on day 6. **(b)** Mean percentage of Tbet^{hi} Eomes^{lo} cytokine-treated CD8$^+$ T cells. **(c)** Mean percentage of Tbet^{lo} Eomes^{hi} cytokine-treated CD8$^+$ T cells. Data were analysed by Two-way ANOVA followed by Tukey's multiple comparison test. Small horizontal lines indicate the mean (± s.e.m.). *$P \leq 0.05$, **$P \leq 0.01$. N = 3 (experimental replicate). Data are representative of two independent experiments.

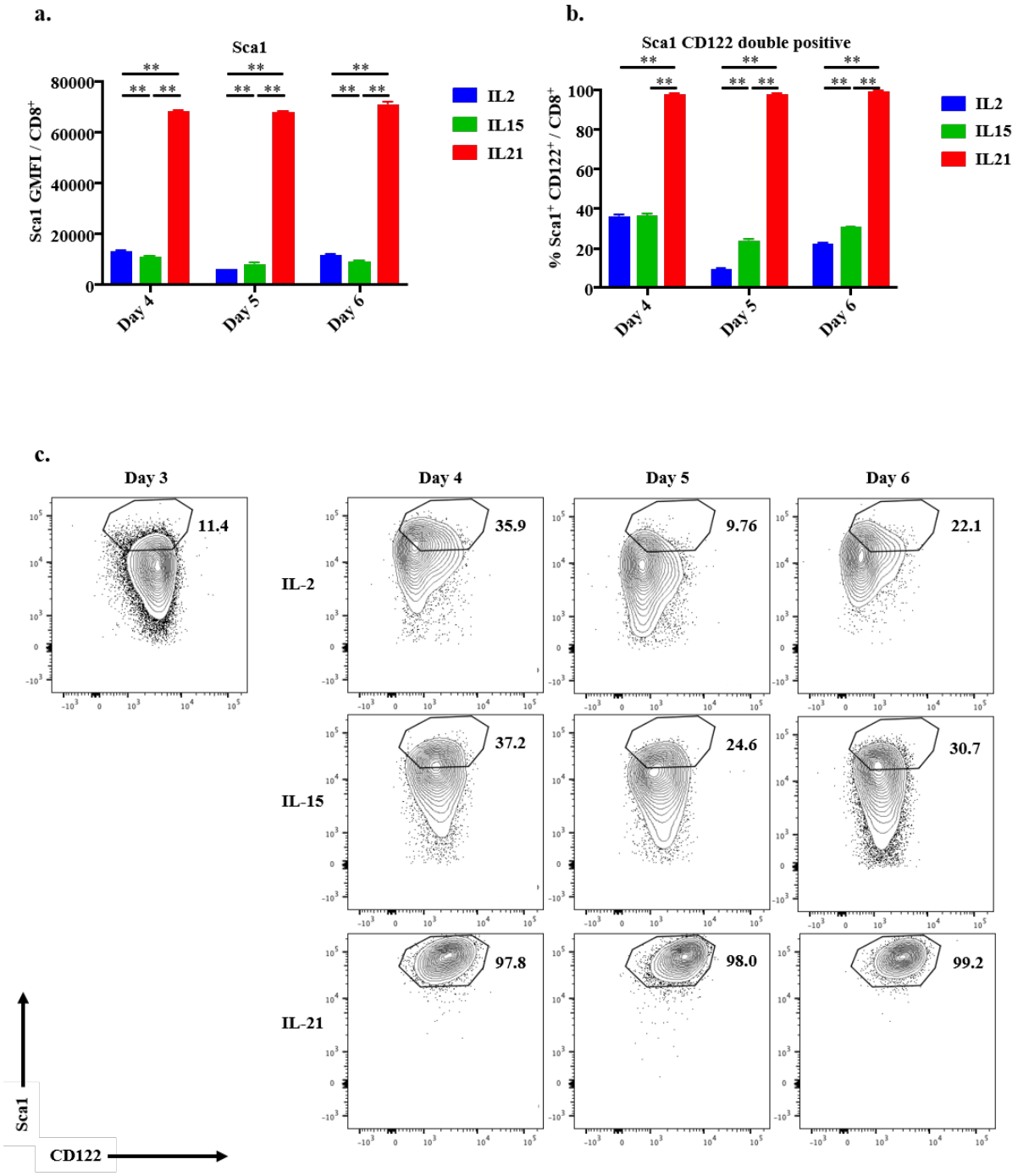
5.2.4 IL-21 treatment induces a stem cell-like CD8$^+$ T cell population that differs from Tscm

A subset of memory CD8$^+$ T cells, known as Tscm, with superior self-renewal, multipotency and anti-tumour response has been recently established [260-263]. Of important note, this subset of cells expresses high Sca1, high CD122, high CD62L and low CD44 in mice [262, 263]. While IL-7 and IL-15 have been proposed to result in the formation of Tscm in humans [261], it is not known if IL-21 can also induce these cells. Furthermore, formation of Tscm has only been shown to be achieved by inhibiting CD8$^+$ T cell differentiation into effector cells early in the priming phase [262]. The theory of directly inducing Tscm from effector CD8$^+$ T cells potentially via de-differentiation still remains unexplored.

On day 6, IL-21 strongly induced Sca1 expression on the CD8$^+$ T cells (GMFI 70800) by up to seven-fold as compared to IL-2 (GMFI 11700) and IL-15 (GMFI 9150) **(Figure 5.5a)**. Together with CD122, a clear double positive population can be identified **(Figure 5.5c)**. In fact, almost all CD8$^+$ T cells treated with IL-21 after one day were Sca1$^+$ CD122$^+$. This expression pattern persisted throughout the duration of the assay (day 4 to 6) **(Figure 5.5b, c)**. This phenomenon could neither be observed in IL-2 nor IL-15 treatments with only 20 to 30% of cells being Sca1$^+$ CD122$^+$ **(Figure 5.5b, c)**.

Further validation of the IL-21-induced cells showed that 66.9% of the cells were CD44^{hi} CD62L^{lo}, commonly recognised as the effector memory CD8$^+$ T cell (Tem) phenotype **(Figure 5.5d)**. IL-2-induced cells also had 49.1% of cells maintained as CD44^{hi} CD62L^{lo} **(Figure 5.5d)**, whereas, most IL-15-induced cells (69.8%) were maintained as the CD44^{hi} CD62L^{hi} central memory CD8$^+$ T cell (Tcm) **(Figure 5.5d)**.

Taken together, IL-21 appears to have induced the formation of a stem cell-like subset with high expressions of Sca1 and CD122. However, this subset differs from the recently discovered Tscm based on their high CD44 and low CD62L expressions which more closely resemble the Tem.


a.
Sca1
Sca1 GMFI / CD8⁺
80000
60000
40000
20000
0
Day 4
Day 5
Day 6
IL2
IL15
IL21
b.
Sca1 CD122 double positive
% Sca1⁺ CD122⁺ / CD8⁺
100
80
60
40
20
0
c.
Day 3
Day 4
Day 5
Day 6
IL-2
IL-15
IL-21
11.4
35.9
9.76
22.1
37.2
24.6
30.7
97.8
98.0
99.2
Sca1
CD122

d.

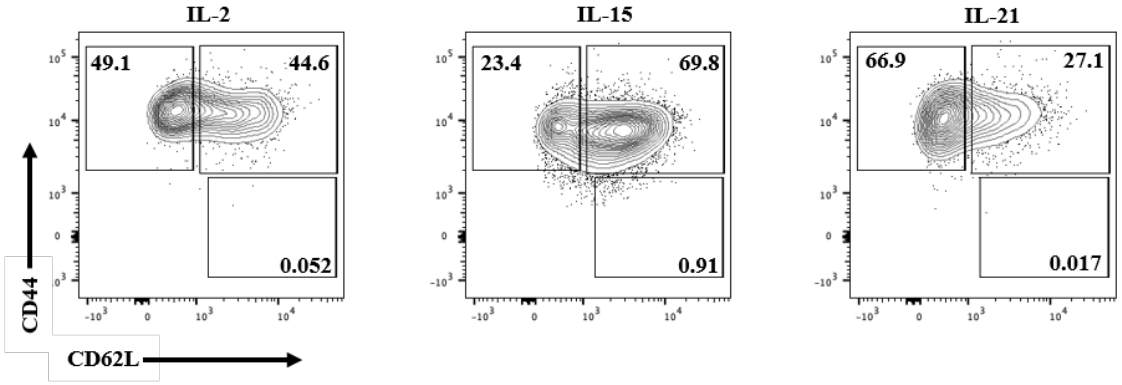


Figure 5.5. IL-21 induces stem cell-like CD8⁺ T cell population that differs from Tscm. *In vitro* culture was performed as described in Figure 5.1. **(a)** Mean GMFI of Sca1 expression of cytokine-treated CD8⁺ T cells. **(b)** Mean percentage of Sca1⁺ CD122⁺ cytokine-treated CD8⁺ T cells. **(c)** Representative flow cytometry plots of Sca1 and CD122 expressions of cytokine-treated CD8⁺ T cells on day 6. **(d)** Representative flow cytometry plots of CD44 and CD62L expressions of cytokine-treated CD8⁺ T cells on day 6. Data were analysed by Two-way ANOVA followed by Tukey's multiple comparison test. Small horizontal lines indicate the mean (± s.e.m.). *$P \leq 0.05$, **$P \leq 0.01$. N = 3 (experimental replicate). Data are representative of two independent experiments.

5.2.5 IL-21 treatment inhibits CD8⁺ T cell proliferation

With partial resemblance to the Tscm, further investigation was prompted to examine its rate of proliferation. It is noted that Tscm has low homeostatic proliferation but high proliferative capacity when stimulated [260, 262].

To study homeostatic proliferation, the expression of Ki67, which is associated to the active phases of the cell cycle [264], was examined. More than 90% of IL-2- and IL-15-induced cells had high expression levels of Ki67 suggesting that cells are still actively proliferating **(Figure 5.6a)**. Whereas for IL-21-induced cells, only 70% of the cells were actively proliferating suggesting that 30% of these cells were quiescent **(Figure 5.6a)**. On further investigation, by comparing GMFI of those Ki67⁺ IL-21-induced cells, it was found that these cells had a reduced expression of Ki67 as compared to IL-2 and IL-15-induced counterparts **(Figure 5.6b)**. This suggests that IL-21 treatment induces the formation of a quiescent subset of cells, and those that are actively proliferating have a reduced rate of proliferation.

Overall, the data on Ki67 show that IL-21 was less effective in promoting CD8⁺ T cell proliferation, whereas IL-2 and IL-15 promoted CD8⁺ T cell proliferation. This, again, offers more evidence of the phenotypic similarities between IL-21-induced cells and Tscm.

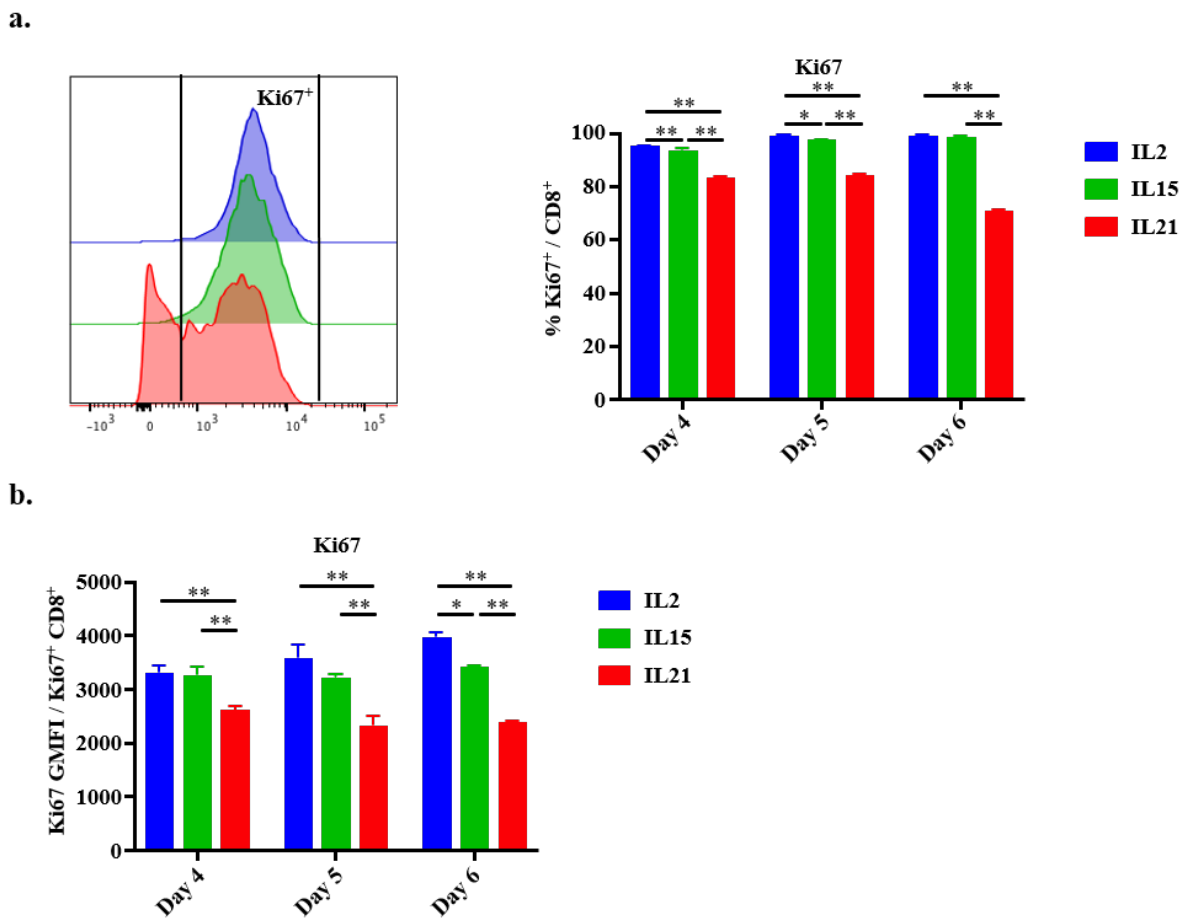


Figure 5.6. IL-21 reduces CD8⁺ T cell proliferation. *In vitro* culture was performed as described in Figure 5.1. **(a)** Left: Flow cytometry plot displays intracellular Ki67 expression of cytokine-treated CD8⁺ T cells on day 6. Right: Mean percentage of Ki67⁺ cytokine-treated CD8⁺ T cells. **(b)** Mean GMFI of Ki67 expression of Ki67⁺ cytokine-treated CD8⁺ T cells from (a). Data were analysed by Two-way ANOVA followed by Tukey's multiple comparison test. Small horizontal lines indicate the mean (± s.e.m.). *$P \leq 0.05$, **$P \leq 0.01$. N = 3 (experimental replicate). Data are representative of two independent experiments.

5.3 Discussion

Though IL-2, IL-15 and IL-21 share overlaps in their signalling pathways such as the JAK-STAT pathway, they perform largely different function on CD8$^+$ T cell post priming. IL-2 maintains effector CD8$^+$ T cells while IL-15 leads to the formation of memory CD8$^+$ T cells. The effect of IL-21 on the post priming phase of CD8$^+$ T cell which involves the crucial memory development process remains largely unknown. Therefore, by using IL-2 and IL-15 as positive controls, I compared the functions of all three cytokines to better understand their distinct roles in this process.

Here, I found that IL-21 treatment induces expressions of effector, memory and proliferative markers that are distinct from IL-2 and IL-15 treatments. These differences were a result of transcriptional changes, which had potentially led to the formation of a novel inducible stem cell-like subset.

The IL-21-induced cells upregulate GzmB, IFNγ and TNFα secretions, hallmarks of effector function. However, it might also suggest a form of cytotoxic or stem capacity, as studies have shown that undifferentiated cells can elicit stronger effector response [262]. A direct comparison of cells before and after re-stimulation through the TCR might serve as a better way to understand their cytotoxic capacity. In addition to that, receptors such as CD122 and CD127 were also highly expressed in IL-21-induced cells, suggesting longevity of these cells though survival assays will need to be performed to validate this.

Unexpectedly, the induction of two homing receptors was found to be different. IL-21 strongly upregulated CCR7 expression, which is important for the homing of CD8$^+$ T cells to an IL-7-rich environment and thus promotes CD8$^+$ T cell homeostasis [265]. Another homing receptor CD62L, however, was found to be downregulated. Though co-expression of both CCR7 and CD62L is commonly found on the central memory CD8$^+$ T cells, their overlapping expressions have been observed to be partial in an intermediate form of memory CD8$^+$ T cells [266]. It is unknown if differential expressions of CCR7 and CD62L have any role to play with regards to the localisation and distribution of CD8$^+$ T cells within secondary lymphoid organs. The difference in localisation pattern may shed light on its role in anti-viral response.

At a transcriptional level, IL-21 clearly induced the formation of a Tbet^{hi} Eomes^{lo} population, which is in marked contrast to the Tbet^{lo} Eomes^{hi} cells induced by IL-2 and IL-15. Indeed, the ratio of Tbet and Eomes has also been reported to play a role in determining CD8$^+$ T cell function and phenotype [259, 267, 268]; with high Tbet/Eomes CD8$^+$ T cells having a stronger capacity to control viral infection [267], and representing a less exhausted CD8$^+$ T cell phenotype [268]. This indicates that IL-21 induced an effector and unexhausted phenotype in the CD8$^+$ T cells.

More notably, in persistent viral infection, the expression pattern of Tbet and Eomes has been shown to represent different population of CD8$^+$ T cells [259]. Tbet^{hi} Eomes^{lo} CD8$^+$ T cells are progenitor cells that give rise to Tbet^{lo} Eomes^{hi} terminal effector subset. The Tbet^{hi} Eomes^{lo} progenitor cells are less proliferative and have lower expression of exhaustion markers as compared to the Tbet^{lo} Eomes^{hi} terminal effector cells [259]. Thus, it is conceivable that IL-21 is involved in the formation of the multipotent progenitor CD8$^+$ T cells.

Another striking observation was the significant upregulation of Sca1and the maintenance of the Sca1$^+$ CD122$^+$ population by IL-21. Sca1 is a marker that positively defines multipotent stem cells in mice [269-271]; Sca1 appears to be associated with c-kit signalling in HSC and progenitor cells [272]. Furthermore, the co-expression of CD122 with Sca1 found on the IL-21-induced cells resembles the recently discovered Tscm. This subset of cells have been shown to be multipotent and have the ability to self-renew [262].

Furthermore, the reduction of proliferative marker Ki67 in IL-21-induced cells potentially suggests a stem cell phenotype. In general, stem cells are largely quiescent and only proliferate to renew itself. This can also be observed in the recently discovered Tscm [260, 262]. The clear segregation of one actively proliferating population and one quiescent population in IL-21-induced cells may indicate that these cells undergo asymmetric division. The quiescent pool may be the stem cell-like cells that propagate to renew itself and, at the same time, produce actively proliferating effector cells. Though commonly observed in stem cells, lymphocytes have been reported to undergo such method of division [273].

However, further investigation of other markers revealed phenotypic differences of the IL-21-induced cells with the Tscm. Firstly, the IL-21-induced cells were mainly of the CD44^{hi} CD62L^{lo} Tem phenotype, unlike the CD44^{lo} CD62L^{hi} Tscm [262]. Secondly, the decreased

TCF1 expression is also different from the high TCF1 expression in Tscm [262]. These might be the limitations of an *in vitro* culture system – IL-21 when used alone *in vitro* might be insufficient to drive complete expression of all Tscm markers. The increase in CD122 and CD127 implies that additional signals from IL-15 or IL-7 might be needed for the IL-21-induced cells. Moreover, IL-21, when studied *in vivo*, has been shown to increase TCF1 expression (Chapter 4) [274]. Thus, this prompts the need for additional stimulation that are missing in an *in vitro* culture system.

Functionally, it remains to be investigated if the IL-21-induced cells possess any stem cell-like capability particularly *in vivo*. Tscm hold great value in controlling chronic diseases [275, 276], therefore by transferring the *in vitro* IL-21-induced cells back into cancer or chronic LCMV infection mice models, their stem cell-like capability can be examined. Repeated rechallenge can also be performed using the chronic LCMV infection model to observe if these cells can persist in mice. *In vivo* induction of this subset by IL-21 should also be studied, for example through the use of the tumour rechallenge model described in Chapter 4.

In Chapter 3 and 4, the importance of IL-21 in the post priming phase of CD8$^+$ T cells over the priming phase was highlighted. This was supported by the increased killing ability and anti-tumour response in the post priming phase but not during the priming phase. Evidence from this Chapter complement those findings and suggest the induction of a distinct subset of CD8$^+$ T cells. With high cytotoxic capacity, high expression of memory markers, and low proliferation, IL-21-induced cells are distinct from cells treated with IL-2 and IL-15. It was also discovered, for the first time, that IL-21 can directly induce the expression of Sca1 on CD8$^+$ T cells. Taken together, it can be speculated that these cells might be an inducible stem cell-like CD8$^+$ T cell subset, and this warrants further investigation in terms of their metabolic profile as metabolic profiles have been shown to be closely related to the function of a cell.

Chapter 6

The metabolic regulation of $CD8^+$ T cells in IL-21 treatment

6.1 Introduction

Metabolism of a cell largely limits the amount of energy it will have and therefore the type of function it can perform [277]. It is not surprising that while cytokine changes the function of CD8$^+$ T cells, it can also dramatically affect its metabolic processes especially during the post priming phase of CD8$^+$ T cells. where they undergo massive changes in their function depending on exposure to different cytokines [164, 278]. Cellular metabolism is also modified in order to support the newly acquired function.

T cells utilise multiple metabolic pathways – glycolysis, oxidative phosphorylation (OXPHOS), fatty acid oxidation (FAO) and more – to maintain their survival and function **(Figure 6.1)**. In particular, naïve T cells mainly undergo catabolic metabolism characterised by the use of glucose breakdown via glycolysis, the tricarboxylic acid (TCA) cycle and eventually OXPHOS via the electron transport chain (ETC) to fuel cell survival [279, 280]. Upon activation, T cells switch to anabolic metabolism where glycolysis predominates to produce ATP and generate precursors such as nucleotides and lipids for proliferation and effector functions [281, 282]. Following removal of antigen, a population of T cells will revert back to catabolic metabolism with OXPHOS primarily dependent on FAO [248, 283].

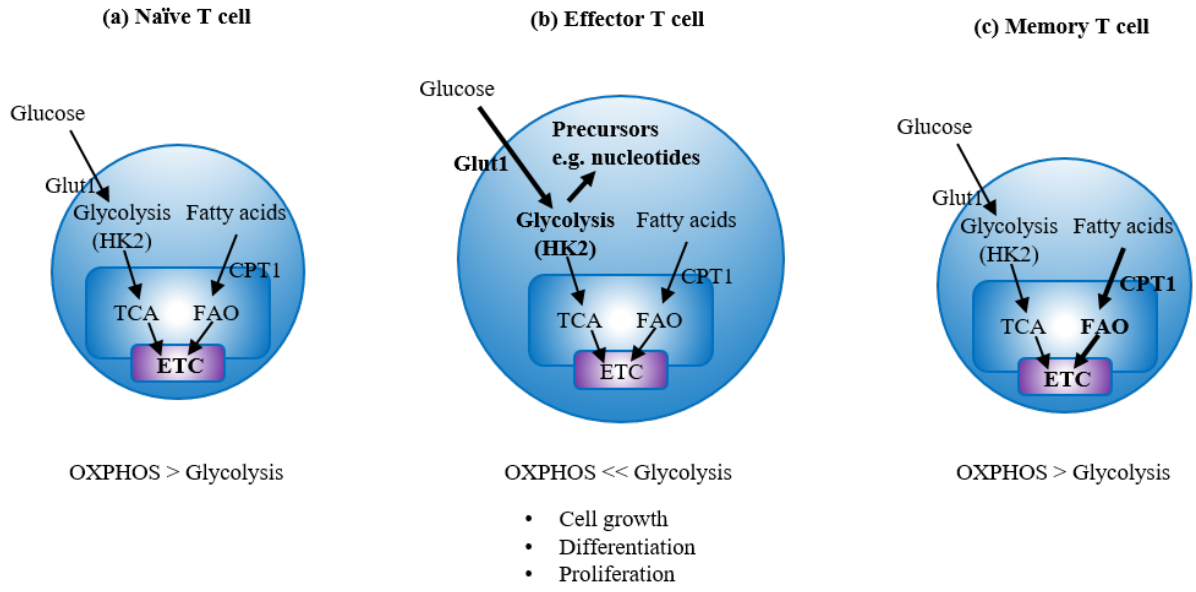


Figure 6.1. Metabolic pathways in different subsets of T cells. (a) Naïve T cells utilise oxidative respiration of the substrate from glycolysis, TCA and FAO through the ETC. **(b)** Upon activation, T cells increase glycolytic activity – increase in GLUT1 transporter for glucose uptake, increase in glycolytic enzyme such as HK2. This, in turn, increases production of precursors such as nucleotides which are needed for cell growth, differentiation and proliferation. **(c)** Following clearance of antigen, a population of T cells forms memory T cells. They are characterised by an increase in FAO, via the increase in CPT1, to supply energy for OXPHOS in the ETC in the event when the pathogen is re-encountered. Figure adapted from [284].

Cytokines play a critical role in shaping T cell metabolic responses. When CD8⁺ T cells are exposed to IL-2 post priming, they continue to maintain effector phenotype as shown by other studies [182, 248], and validated in Chapter 5. These cells are high in basal glycolysis and moderately low in basal oxidative phosphorylation [248]. This supports their high energy requirement to produce large amounts of cytotoxic molecules for their killing function [285].

On the other hand, when primed CD8⁺ T cells are exposed to IL-15, they acquire a memory phenotype based on data from other studies and from Chapter 5 [235, 248]. These cells have low basal glycolysis and high basal OXPHOS, and their level of spare respiratory capacity (SRC) is also higher [248]. SRC is explained as the maximum oxygen consumption rate which in turn represents the maximum energy that can be generated by the cells. Energy production is especially critical for memory CD8⁺ T cells to undergo morphological, functional and proliferative changes when they respond to re-exposure of the same antigen [286]. It has been

discovered that the level of SRC is determined by the level of FAO in cells [248]. This was validated when researchers upregulated FAO in antigen-specific CD8$^+$ T cells and found an increase in the frequency of CD8$^+$ T cell after pathogen challenge [248].

Since mitochondria is one of the most important organelle involved in regulating metabolic activities for example in OXPHOS and FAO [287], changes in mitochondrial mass and morphology would be expected. Indeed, it has been shown that IL-2-induced effector cells have lower mitochondrial mass and higher mitochondrial fission, whereas IL-15-induced memory cells have higher mitochondrial mass and higher mitochondrial fusion [248]. A higher mitochondrial mass would be needed to support higher level of OXPHOS and FAO that is needed for memory CD8$^+$ T cells to rapidly respond to pathogen when re-countered [248]. FAO occurs in mitochondria, hence more mitochondrial mass will allow greater use of fatty acids for energy via OXPHOS, thereby facilitating cell survival in the absence of pro-glycolysis signals. Similarly, fusion in mitochondria in memory CD8$^+$ T cell favours the associations of ETC thereby improving OXPHOS and FAO [288].

To carry out changes in cellular metabolism, cytokines induce specific signalling pathways that directly regulate metabolic pathways. For example, IL-2 promotes glycolysis through the induction of several transcription factors – c-myc, mTOR and AKT [199, 289-292]. T cells isolated from c-myc-deficient mice has been shown to impair activation-induced glycolysis, and this led to compromised T cell growth and proliferation *in vitro* [279]. In a separate study, CD8$^+$ T cells that have higher level of mTOR Complex 1 have higher level of glycolysis [293]. Another signalling pathway through AKT has been found to promote Glucose transporter 1 (GLUT1) trafficking to cell surface, and promote the localisation of the glycolytic enzyme Hexokinase 2 (HK2) to the mitochondria and augmented its activity [294].

With overlapping receptor subunits, IL-15 can signal through similar transcription factors but at different intensity as compared to IL-2. It is believed that heterogeneity in similar signalling pathways as IL-2 is one of the main factors driving differences in CD8$^+$ T cell fate [199, 295, 296]. For example, when activated CD8$^+$ T cells were cultured in IL-2, the AKT signalling was sustained at high levels and CD62L expression was low which is characteristic of effector CD8$^+$ T cells [199, 296]. However, when the same culture was performed with IL-15, only low level of AKT signalling was sustained and it eventually resulted in high level of CD62L [199,

296]. Indeed, dampening of key glycolytic regulators such as mTOR has been shown to favour memory CD8$^+$ T cell formation [297, 298].

Hypoxia-inducible factor 1 alpha (HIF-1α) is another master regulatory transcription factor mediated by cytokines to control metabolism in multiple cell types. Addition of IL-1β to A549 (human lung adenocarcinoma) cells in culture resulted in an increase in the level of HIF-1α protein, which was due to increased HIF-1α protein stability [299]. In CD4$^+$ T cells, a similar observation has been made where addition of IL-6 and TGFβ to culture can induce HIF-1α at both the mRNA and protein levels [300]. When naive CD4$^+$ T cells were cultured in Th17 conditions – anti–IL-2, anti–IL-4, anti–IFNγ, TGF-β1 and IL-23, RNA and protein levels of HIF-1α were found to be increased [301]. This further led to a corresponding upregulation of glycolytic activity such as GLUT1 and HK2 expressions [301].

As shown in Chapter 5, IL-21 can also induce phenotypic changes in CD8$^+$ T cell post priming that are distinct from IL-2 and IL-15. This includes the expressions of Sca1 and CD122, and the reduction in homeostatic cell proliferation, which closely resemble the recently discovered Tscm [262]. Thus, a thorough understanding of the metabolic changes in the IL-21-induced CD8$^+$ T cell will allow us to better interpret its function and its relevance to Tscm, and to predict its role in controlling chronic diseases.

The Seahorse XF Cell Mito Stress Test Kit and Seahorse analyser will be used to study selected metabolic pathways – basal glycolysis, basal OXPHOS, and SRC. This kit chemically disrupts the activity of the electron transport chain by the sequential addition of chemical compounds – Oligomycin, FCCP, Rotenone and Antimycin A **(Figure 6.2a, b)**. Oligomycin is first introduced to inhibit Complex V, this prevents movement of proton into the mitochondria matrix. As a result, Complex IV activity decreases due to limited proton, and oxygen consumption reduces. Next, addition of FCCP disrupts the mitochondria membrane thereby allowing proton to flow across freely. With increased proton concentration, Complex IV is now at its maximum capacity and oxygen consumption is at its maximum too. Finally, Rotenone and Antimycin A inhibits Complex I and III respectively, which effectively shuts down mitochondria respiration. The Seahorse analyser takes measurements of the oxygen consumption rate (OCR) as the readout for OXPHOS, and the rate of proton release from cells, also known as the extracellular acidification rate (ECAR) as the readout for glycolysis. By

comparing metabolic differences of IL-21-induced cells with IL-2-induced effector cells and IL-15-induced memory cells, this study will shed light on the possible function of these cells whether they are more effector- or memory-like, or completely distinct.

Specific pathways within glycolysis and FAO will also be investigated in greater details by looking at expression levels of related genes such as GLUT1, HK2 and carnitine palmitoyltransferase I (CPT1). GLUT1 plays a major role as an important transporter known to mediate glucose uptake into cells through facilitated diffusion [302, 303], while HK2 is the enzyme involved in the first committed step in glycolysis converting Glucose into Glucose-6-phosphate (G6P) [304]. CPT1, which is known as the rate-limiting step in FAO, is involved in the conversion of fatty acids into acyl carnitines for subsequent transfer from cytosol into the mitochondria [305, 306]. Total mitochondrial mass will also be examined as it reveals the ability of cells to generate energy.

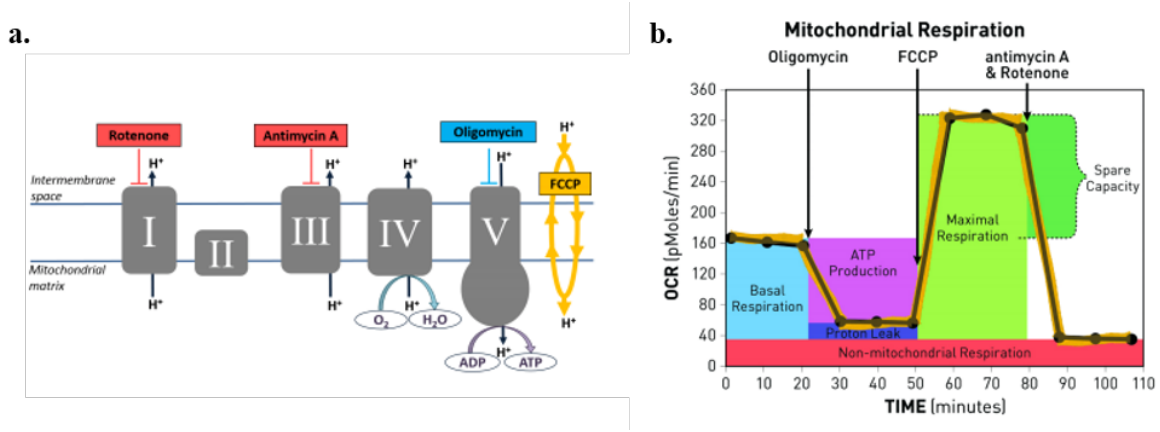


Figure 6.2. Mechanisms of the mitochondrial inhibitors found in the Seahorse mitochondrial stress kit. (a) This kit chemically disrupts the activity of the electron transport chain by the sequential addition of chemical compounds – Oligomycin, FCCP, Rotenone and Antimycin A Oligomycin is first introduced to inhibit Complex V, this prevents movement of proton into the mitochondria matrix. As a result, Complex IV activity decreases due to limited proton, and oxygen consumption reduces. Next, addition of FCCP disrupts the mitochondria membrane thereby allowing proton to flow across freely. With increased proton concentration, Complex IV is now at its maximum capacity and oxygen consumption is at its maximum too. Finally, Rotenone and Antimycin A inhibits Complex I and III respectively, which effectively shuts down mitochondria respiration. **(b)** An example of oxygen consumption rate measured by the Seahorse machine at specific time of chemical compound addition. Figures from Agilent Seahorse XF Cell Mito Stress Kit manual guide.

6.2 Results

6.2.1 IL-21 treatment promotes glycolysis

The culture system used was similar to that in Chapter 5 **(Figure 5.1)**, where OT-I CD8$^+$ T cells were first primed with their cognate antigen SIINFEKL peptide and hIL-2, and then rested in different cytokines. Cells were then purified and used for downstream analysis.

To begin, the most fundamental metabolic pathway of all – glycolysis was examined. It is an important step of aerobic respiration and provides the substrate needed for OXPHOS to occur.

GLUT1 is an important transporter known to mediate glucose uptake into cells through facilitated diffusion [302, 303]. It is produced intracellularly and gets trafficked to the cell surface under certain signalling cues in times of need to increase glucose uptake [307]. Based on surface staining, IL-21 can be observed to induce higher expression of GLUT1 (GMFI 590). This was two-fold higher than IL-15-induced cells (GMFI 360) and was also significantly higher than IL-2-induced cells (GMFI 510) **(Figure 6.3a)**. Following that, intracellular GLUT1 was also studied. The level of intracellular GLUT1 expression in IL-21-induced cells is higher (GMFI 2000) than IL-15-induced cells (GMFI 1210), albeit being lower than IL-2-induced cells (GMFI 2500) **(Figure 6.3b)**.

Turning now to study the glycolytic process, HK2, the enzyme involved in the first committed step in glycolysis converting Glucose into G6P [304], was investigated via real-time PCR. In particular, increased level of HK2 has been shown to be characteristic of effector CD8$^+$ T cells [308]. Since IL-2-induced cells exhibit effector CD8$^+$ T cell phenotype as shown in Chapter 5 and by previous publications [248, 286], relative expression of HK2 in IL-15- and IL-21-induced cells were made against IL-2-induced cells. The level of HK2 was observed to be almost similar in IL-15-induced cells and 1.5-fold higher in IL-21-induced cells **(Figure 6.3c)**.

Next, using the Seahorse analyser, basal glycolysis rate was measured in the form of proton release from cells, also known as the ECAR. IL-21 induced a glycolytic rate that is similar to that of IL-2-induced cells, and significantly higher than that of IL-15-induced cells **(Figure 6.3d)**.

Overall, these data suggest that IL-21-induced cells have increased ability to uptake glucose which is a basic requirement in order to increase glycolysis. In addition, IL-21-induced cells have likely an increased conversion of glucose to G6P, which is the first rate-limiting step of glycolysis. Upon direct measurement of the glycolytic rates, it was found that IL-21-induced cells have higher rate of glycolysis than IL-2- and IL-15-induced cells. Hence, these confirm that IL-21-induced cells have increased glycolysis and have upregulated critical parts of the pathway like glucose uptake and G6P conversion to achieve it.

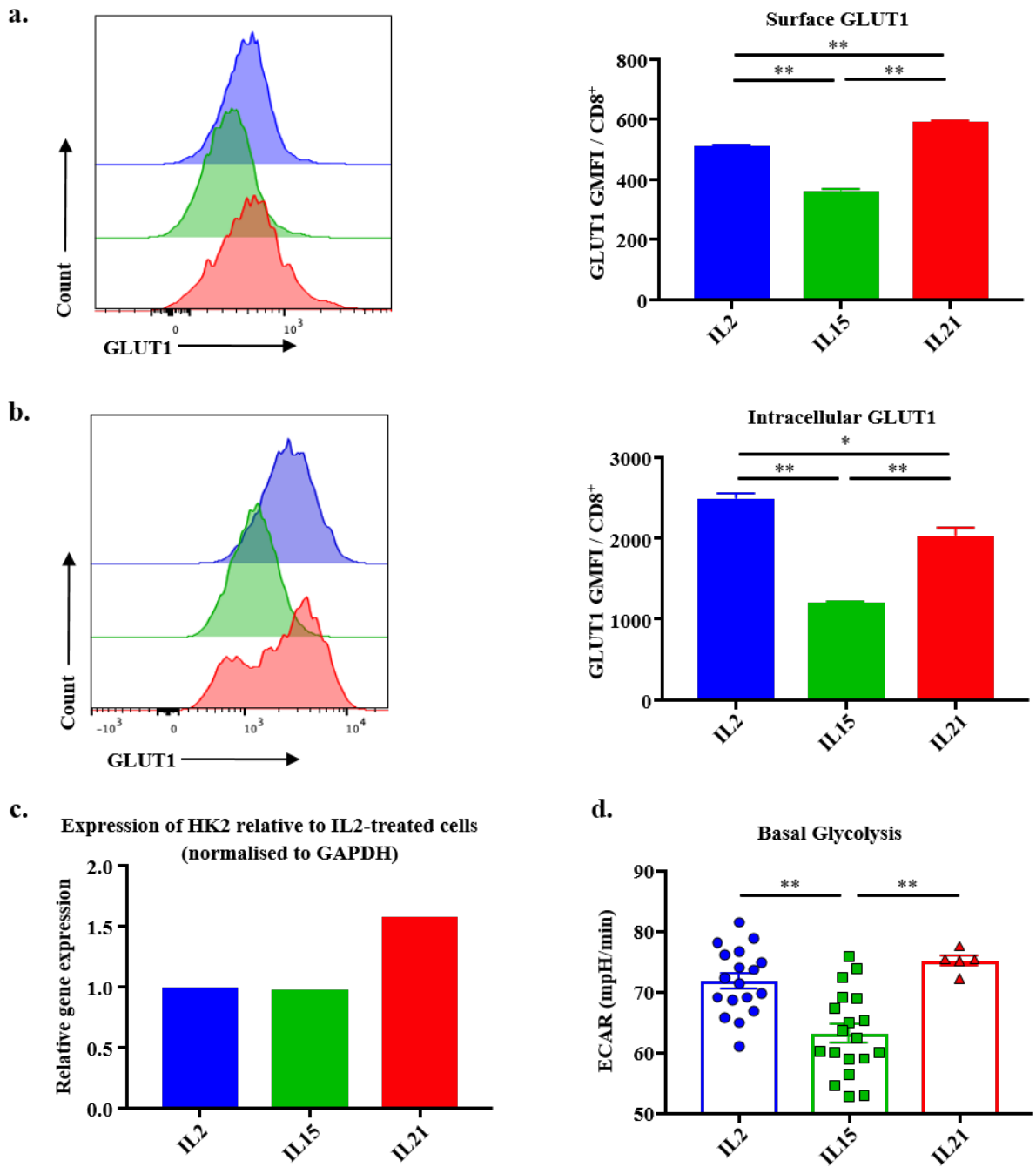


Figure 6.3. IL-21 increases CD8⁺ T cell glycolysis. *In vitro* culture was performed as described in Figure 5.1. FACS, real-time PCR and seahorse analyses were performed on cytokine-treated CD8⁺ T cells after six days of culture. **(a)** Left: Flow cytometry plot displays surface GLUT1 expression on cytokine-treated CD8⁺ T cells. Right: Mean GMFI of surface GLUT1 expression on cytokine-treated CD8⁺ T cells. **(b)** Left: Flow cytometry plot displays intracellular GLUT1 expression in cytokine-treated CD8⁺ T cells. Right: Mean GMFI of intracellular GLUT1 expression in cytokine-treated CD8⁺ T cells. **(c)** Relative HK2 mRNA expression in cytokine-treated CD8⁺ T cells. **(d)** ECAR measurement of cytokine-treated CD8⁺ T cells under basal conditions. Data were analysed by One-way ANOVA. Small horizontal lines indicate the mean (± s.e.m.). **P* ≤0.05, ***P* ≤0.01. **(a – c)** N = 3 (experimental replicate).

(d) N = 5 to 18 (experimental replicate). Data are representative of two independent experiments.

6.2.2 IL-21 treatment induces higher mitochondrial mass but inhibits oxidative phosphorylation and fatty acid oxidation

Mitochondria is a type of organelle that is highly involved in cellular metabolism and overall energy production [309]. Therefore, with different energy requirements, it can be expected that mitochondrial mass will vary in different subsets of CD8⁺ T cells [248, 286, 310]. To measure mitochondrial mass in cells, the MitoTracker Green, a fluorescent label that accumulates in the mitochondrial matrix and binds to mitochondrial proteins, was used. What stood out from the analysis was the higher mitochondrial mass (GMFI 6000) in IL-21-induced cells. This was approximately two-fold higher when compared to IL-2-induced cells (GMFI 3550), and three-fold higher when compared to IL-15-induced cells (GMFI 2600) **(Figure 6.4)**.

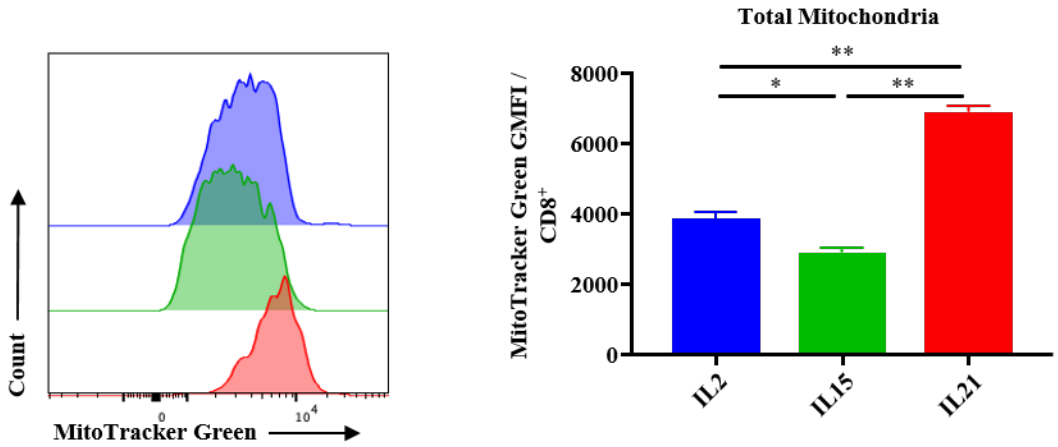


Figure 6.4. IL-21 increases total mitochondrial mass. *In vitro* culture was performed as described in Figure 5.1. FACS analysis was performed on cytokine-treated CD8⁺ T cells after six days of culture. Left: Flow cytometry plot displays MitoTracker Green staining in cytokine-treated CD8⁺ T cells. Right: Mean GMFI of MitoTracker Green in cytokine-treated CD8⁺ T cells. Data were analysed by One-way ANOVA. Small horizontal lines indicate the mean (± s.e.m.). *$P \leq 0.05$, **$P \leq 0.01$. N = 3 (experimental replicate). Data are representative of two independent experiments.

To understand key metabolic processes that happen in the mitochondria, I turned to look at basal OXPHOS and SRC. This was done by studying the level of oxygen uptake by cells using

the Seahorse XF Cell Mito Stress Test Kit and Seahorse analyser. This kit chemically disrupts the activity of the electron transport chain by the sequential addition of chemical compounds – Oligomycin, FCCP, Rotenone and Antimycin A **(Figure 6.2a, b)**. It is clear in **Figure 6.5a** that IL-2-, IL-15- and IL-21-induced cells have different level of response to each compound.

When comparing basal OXPHOS, IL-15-induced cells had the highest level at about 102 pmol/min as compared to 99 pmol/min and 76 pmol/min in IL-2- and IL-21-induced cells respectively **(Figure 6.5b)**. Together with data from Figure 6.3d, IL-21-induced cells have a tendency to use glycolysis over OXPHOS.

SRC is the ability of mitochondria to rapidly produce energy when cells are exposed to increased stress or work [311-313]. The difference between maximal OCR and basal OCR will be the SRC of cells. Memory CD8$^+$ T cells are known to respond well to stress by having higher SRC as compared to effector CD8$^+$ T cells [248]. Similar to what was published, IL-15-induced cells had the highest level of SRC at 102 pmol/min, while IL-2-induced cells had a low level of SRC at 82 pmol/min. Interestingly, while IL-21-induced cells display memory-like phenotype as shown in Chapter 5, it had a low level of SRC 83 pmol/min similar to IL-2-induced cells **(Figure 6.5c)**.

Another function of the mitochondria in metabolism is in the regulation of FAO [314]. Cells can turn to its FA reserve to upregulate metabolism and energy production [315], this includes the memory CD8$^+$ T cells which have been shown to utilise FAO to support its high SRC [248]. Thus, it is important to determine if IL-21-induced cells also have lower level of FAO.

Widely known as the rate-limiting step of FAO, CPT1 is involved in the conversion of fatty acids into acyl carnitines for subsequent transfer from cytosol into the mitochondria [305, 306] . Three isoforms of CPT1 namely CPT1a, CPT1b and CPT1c exist and were investigated in this study. As expected, IL-15-induced cells upregulated all three CPT1 expressions by 1.1- to 1.3-fold higher than IL-2-induced cells to support its SRC. IL-21-induced cells, on the contrary, downregulated CPT1 by close to two-fold when compared to IL-2-induced cells **(Figure 6.5d)**.

Altogether, IL-21-induced cells have been shown to have higher mitochondria content as compared to IL-2- and IL-15-induced cells. However, this observation did not correlate with

higher level mitochondrial-related processes. Counter-intuitively, IL-21-induced cells had lower basal OXPHOS, lower SRC and lower FAO.

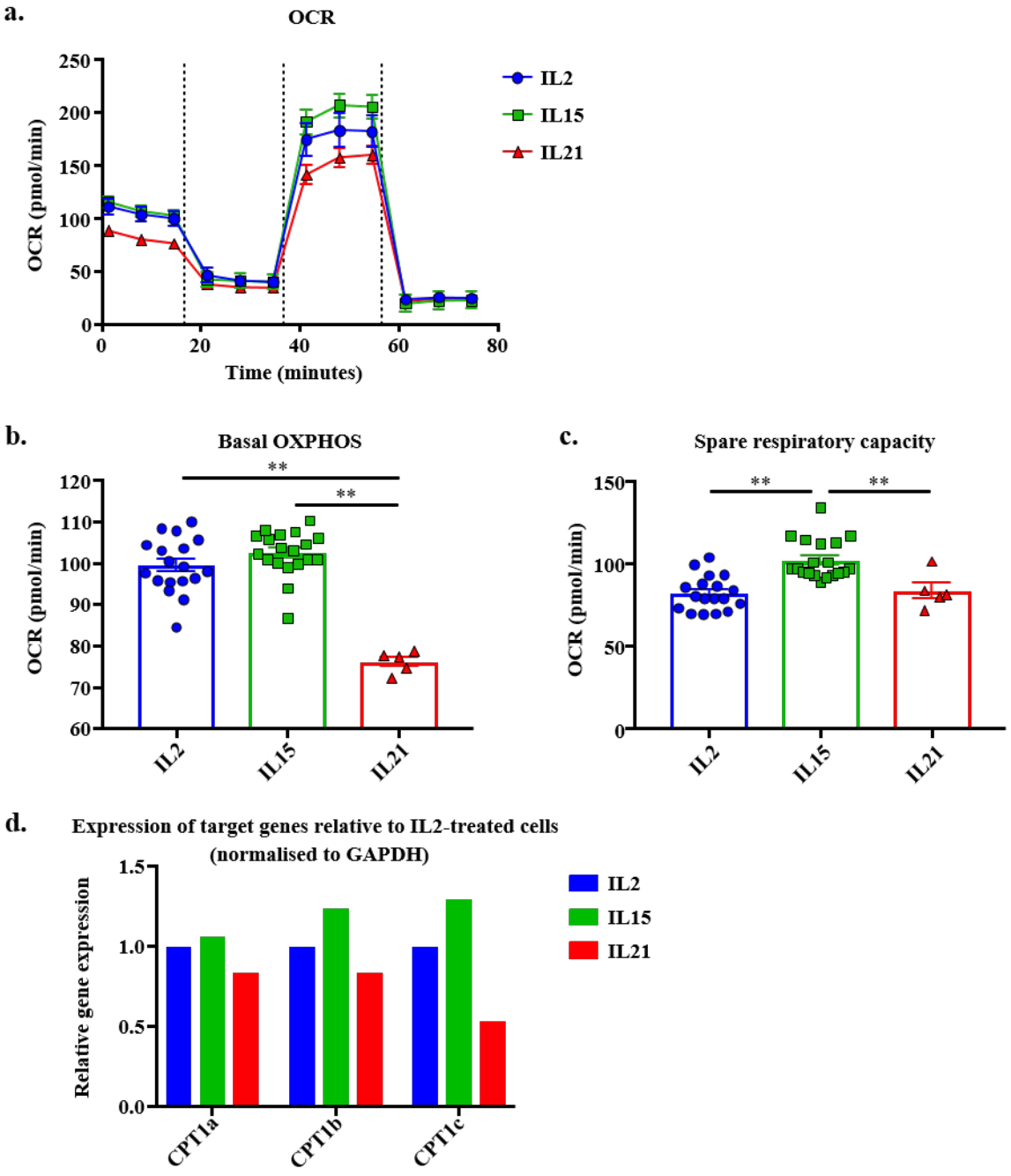


Figure 6.5. IL-21 decreases CD8⁺ T cell OXPHOS, SRC and FAO. *In vitro* culture was performed as described in Figure 5.1. FACS, real-time PCR and seahorse analyses were performed on cytokine-treated CD8⁺ T cells after six days of culture. **(a)** OCRs of cytokine-treated CD8⁺ T cells in real-time under basal and in response to mitochondrial inhibitors in Figure 6.2. **(b)** OCR of cytokine-treated CD8⁺ T cells under basal conditions. **(c)** SCR calculated from the difference between maximal OCR and basal OCR cytokine-treated CD8⁺ T cells. **(d)** Relative CPT1a, CPT1b and CPT1c mRNA expression in cytokine-treated CD8⁺

T cells. Data were analysed by One-way ANOVA. Small horizontal lines indicate the mean (± s.e.m.). $^{**}P \leq 0.01$. **(a – c)** N = 5 to 18 (experimental replicate). (d) N = 3 (experimental replicate). Data are representative of two independent experiments.

6.2.3 IL-21 treatment induces HIF-1α that drives the metabolic switch

Changes in metabolic profiles are brought about by important modifications in intrinsic cellular signalling pathways [50, 316, 317]. Accordingly, it can be postulated that transcription factors are heavily involved in modulating metabolic changes in CD8$^+$ T cells after IL-21 treatment.

One candidate transcription factor is HIF-1α. The shift in CD8$^+$ T cell metabolism towards high glycolysis [318], low OXPHOS [319, 320], and low FAO [321] observed in IL-21-induced cells resembles that of HIF-1α-induced metabolic changes. HIF-1α has been found to regulate CD8$^+$ T cell metabolism thereby influencing their function, differentiation and migration [322]. To compare the changes in HIF-1α expression level, HIF-1α protein level was examined in CD8$^+$ T cells after different cytokine treatment using FACS. IL-21 was found to significantly upregulate HIF-1α level (GMFI 6160) as opposed to IL-2 (GMFI 4560) and IL-15 (GMFI 4550) **(Figure 6.6a)**.

However, it can be argued that the increase in HIF-1α protein level was achieved either through preventing the degradation in HIF-1α protein or through increasing the transcription of HIF-1α. Thus, to test whether the effect was due to a transcriptional upregulation, the mRNA expression was verified using real-time PCR. When normalised to IL-2-induced cells, IL-21-induced cells had a two-fold increase in HIF-1α expression, while IL-15-induced cells had a two-fold decrease in HIF-1α expression **(Figure 6.6b)**.

Taken together, these results suggest that IL-21 treatment resulted in higher expression of HIF-1α which could potentially have direct implication on the metabolic changes of high glycolysis, low OXPHOS, and low FAO.

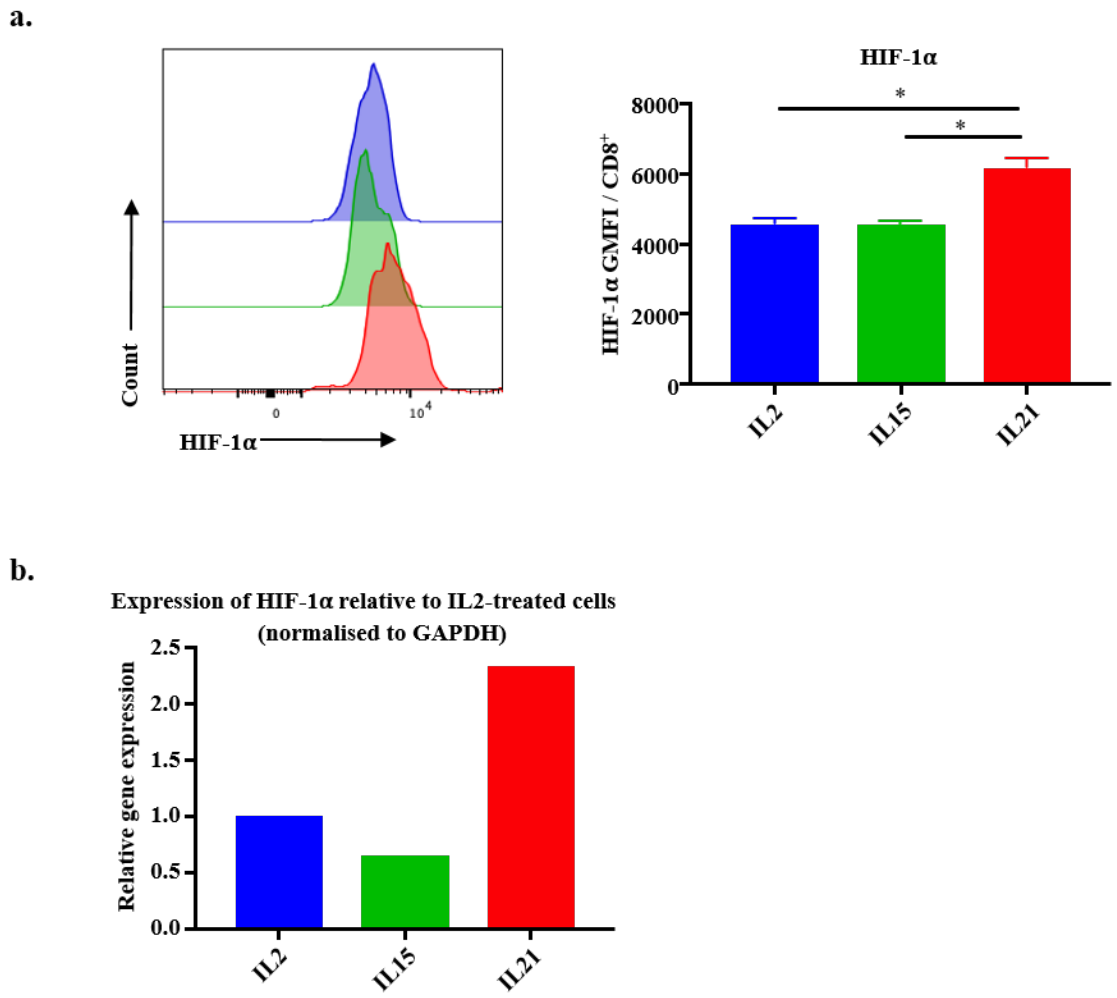


Figure 6.6. IL-21 increases CD8⁺ T cell HIF-1α expression. *In vitro* culture was performed as described in Figure 5.1. FACS, real-time PCR and seahorse analyses were performed on cytokine-treated CD8⁺ T cells after six days of culture. **(a)** Left: Flow cytometry plot displays intracellular HIF-1α expression in cytokine-treated CD8⁺ T cells. Right: Mean GMFI of intracellular HIF-1α expression in cytokine-treated CD8⁺ T cells. **(b)** Relative HIF-1α mRNA expression in cytokine-treated CD8⁺ T cells. Data were analysed by One-way ANOVA. Small horizontal lines indicate the mean (± s.e.m.). *$P \leq 0.05$. N = 3 (experimental replicate). Data are representative of two independent experiments.

6.3 Discussion

As shown in published literature, cytokines induce critical changes in CD8$^+$ T cells metabolic profiles which are directly related to their functional performance [248, 283, 323]. Also as supported by the findings in previous chapters, IL-21 induces distinct phenotypic changes in CD8$^+$ T cells that are different from IL-2 and IL-15. The IL-21-induced cells show high effector capacity, high memory marker expression, high stem cell marker expression, and low proliferation, all of which are characteristics of a stem cell-like CD8$^+$ T cell subset. However, the underlying metabolic changes driving these phenotypic changes remain unknown. Better understanding of their metabolic changes can also shed light on the function of these cells by then comparing the metabolic profiles with known cell subset that have been already published.

The effect of IL-21 on T cell metabolism has been explored by Loschinski et al. [324]. In that study, they first cultured human T cells with either IL-2 or IL-21 over five days in the presence of activation beads, then tested the cytokine-induced T cells for various metabolic processes. They found that IL-21 enhanced the memory phenotype in human T cells. As compared to IL-2, IL-21 decreased GLUT1 expression and glucose uptake in the form of 6-NBDG. Correspondingly, IL-21 also lowered glycolysis, while concomitantly increasing basal OXPHOS and SRC. Though providing interesting insights into IL-21-induced T cell metabolism, their findings lacked a clear distinction between CD4$^+$ and CD8$^+$ T cells. Furthermore, there was also no clear segregation of the different subset of T cells – naïve, effector and memory. If the intention of the study was to understand the kinetics of IL-21 on naïve T cell priming, then the Loschinski et al study, along with several others investigating the effect of IL-21 on CD8$^+$ T cells, might have overlooked the prominent role of IL-21 in the post-priming phase of CD8$^+$ T cell.

Here, I demonstrated that at 50 ng/mL, the effect of IL-21 on metabolic changes of the CD8$^+$ T cells in the post-priming phase are distinct from IL-2 and IL-15. Contrary to the findings of Loschinski et al. [324], the IL-21-induced CD8$^+$ T cells have high basal glycolysis, low basal OXPHOS, low SRC, and low FAO. On further analysis, its unique metabolic profile was found to be potentially a result of an increased HIF-1α transcription induced by IL-21.

IL-21 appears to have induced the reliance of CD8$^+$ T cells towards glycolysis. In order to support the increase in glycolysis, cells upregulated GLUT1 expression on the surface to ensure that there is an increase in ability to uptake substrate for glycolysis. However, this might not represent an increased in glycose uptake. Ideally, a glucose uptake assay using 2-NBDG would be more effective to confirm this hypothesis. Together with an upregulation in HK2, the key enzyme in catalysing the conversion of the first step in glycolysis, it can be speculated that overall glycolysis was increased in IL-21-induced cells.

Indeed, basal rate of glycolysis was found to be increased in IL-21-induced cells to a similar level as IL-2-induced effector cells, and significantly higher than IL-15-induced cells. This result might explain the similar level of cytotoxic ability of IL-21-induced cells described in Chapter 5.

When comparing other metabolic processes, IL-21 was discovered to reduce OXPHOS, SRC, and FAO which are key energy-producing mechanisms especially for memory CD8$^+$ T cells. Ironically, mitochondrial mass was increased despite the reduction in abovementioned mitochondrial-regulated processes. Unexpectedly, the IL-15-induced cells in my experiment did not have a higher level of mitochondria as reported in a previous publication [248]. A more accurate way to measure mitochondrial mass could be the direct measurement of mitochondria DNA.

The level of mitochondrial mass in IL-21-induced cells, if confirmed by mitochondria DNA to be higher than IL-2- and IL-15-induced cells, will suggest that mitochondria in these cells have functions other than cellular respiration. It has been reported that mitochondria are also needed in apoptosis [325], other biosynthetic pathways [326], or calcium homeostasis [327]. It would be interesting to test the predominant role of mitochondria in IL-21-induced cells.

As observed from Chapter 5, IL-21-induced cells share overlapping phenotypic traits with Tscm. A similar metabolic profile can be expected, but this is not the case. Tscm have been shown to have high OXPHOS, low glycolysis, high SRC, high FAO [328]. Hence, this suggests that IL-21-induced cells belong to a different subset of cells despite sharing common expressions of certain surface markers such as CD122 and Sca1.

Surprisingly, a comparison with other cell types reveals that the IL-21-induced metabolic phenotype is similar to that of HSC. HSCs have high glycolysis, low OXPHOS, low SRC and high mitochondrial mass [329, 330]. As HSCs reside in hypoxic niches of the bone marrow cavity, they mainly rely on anaerobic glycolysis as their source of ATP production [329]. Furthermore, the use of glycolysis over OXPHOS will greatly reduce the production of reactive oxygen species which is detrimental to cell stemness [331, 332]. Upon receiving cues to differentiate, HSCs undergo metabolic switch from glycolysis to OXPHOS in order to meet the robust energy demand [333]. As such, IL-21-induced cells, with a similar metabolic profile as HSCs, may have a similar cellular function.

Knockouts of IL-21 and its receptor have been shown to interfere with the ability to control chronic infection [150-152]. Interestingly, IL21R-deficient CD8$^+$ T cells were found to be more exhausted with higher expression of PD1 [151, 152]. Therefore, the role of IL-21 could potentially be either to reverse exhaustion and reinvigorate CD8$^+$ T cells to become active again, or to promote a constant supply of fresh effector CD8$^+$ T cells. Evidences from Chapter 4 show that IL-21 treatment drives CD8$^+$ T cell exhaustion instead of inhibiting it. With observations of the phenotypic changes in cells driven by IL-21, it is highly conceivable that the latter scenario is true – IL-21 drives the formation of a stem cell-like subset of cells which undergo asymmetric cell division to maintain itself and to replenish new effector CD8$^+$ T cells to fight pathogen.

The possibility of IL-21-induced cells to undergo self-renewal is supported by the enhanced expression of HIF-1α. Originally discovered in cancer cells, HIF-1α, as its name suggests, is only induced under hypoxic environment and drives cancer progression [334, 335]. It does so by promoting survival of cancer cells through the upregulation of key metabolic pathways such as glycolysis to compensate for the low oxygen availability [334, 335]. Interestingly, HIF-1α also has a crucial role in maintaining stem cell self-renewal ability [336, 337] The HSCs in HIF-1α-deficient mice were found to decrease in numbers due to the loss of their ability to maintain cell cycle quiescence [336, 337], while overexpression of HIF-1α in mesenchymal stem cells increased cell survival *in vitro* [338, 339]. Like cancer cells, stem cells are also known to thrive in hypoxic environment [329]. Stem cells require high HIF-1α to increase glycolysis and to reduce OXPHOS to maintain quiescence [336].

Oncogenic signals have been shown to induce STAT3 signalling which directly promotes the transcription of HIF-1α [340, 341], while HIF-1α in turn upregulates glycolytic processes such as induction of GLUT1 [342]. Since STAT3 is the most prominent signalling molecule of the IL-21 receptor [124], IL-21 can potentially signal through STAT3 to mediate the direct transcription of HIF-1α.

Current studies are underway in our laboratory to investigate the role of HIF-1α in IL-21-mediated metabolic changes. For example, can we overcome the metabolic defects in IL21 receptor knockout cells by overexpressing HIF-1α? As mentioned in Chapter 5, validation of the stem cell-like properties is required by showing persistence of cells in repeated rechallenge of pathogen. It could also be helpful to identify if the IL-21-induced upregulation of HIF-1α is due to STAT3. This can be achieved by using knockout or knockdown of STAT3 followed by exposure of cells to IL-21. In addition, further investigation into the mitochondrial biology may also shed light into the impact of IL-21 as STAT3 has been shown to directly affect mitochondrial gene expression and regulate mitochondrial respiration.

Collectively, with the findings from Chapters 5 and 6, it is plausible that IL-21 shapes CD8$^+$ T cell post priming into a new subset of stem cell-like population defined by Sca1$^+$ CD122$^+$ CD127$^+$ CCR7$^+$ Tbet$^+$ Ki67^{lo}. These cells resemble the HSCs metabolically with high glycolysis, low OXPHOS, low SRC, low FAO, and high mitochondrial mass. This is further substantiated by high expression of HIF-1α which is a key transcription factor mediating stem cell metabolism.

Chapter 7

General Discussion

7.1 $CD8^+$ T cell – phases of antigen response, memory diversity and their therapeutic applications in cancer

$CD8^+$ T cell responses exist in different phases depending on when the $CD8^+$ T cell is exposed to the antigen. When a naïve $CD8^+$ T cell is exposed to its cognate antigen, it undergoes a priming phase which involves the sensing and interpreting of three separate signals – TCR, CD28 costimulation and cytokine [343]. Depending on the intensity of each signal, they are summed up linearly which then determine the eventual division fate of the $CD8^+$ T cells [137]. Cytokine concentration and the type of cytokine during priming have been shown to affect $CD8^+$ T cell division to a large extent [137].

After the antigen and the prominent effector cytokine IL-2 retract, $CD8^+$ T cells begin to undergo a post-priming phase. This is also widely known as the contraction and maintenance phases of $CD8^+$ T cells where the majority of short-lived effector cells undergo apoptosis, and those that survive undergo memory formation and thereafter long-term maintenance [344]. At this point, cues in the form of cytokine can influence $CD8^+$ T cell survival, proliferation and memory formation. Cytokines known to play important roles in this phase include IL-7 and IL-15. IL-7 can promote $CD8^+$ T cell survival [187], while IL-15 can promote memory $CD8^+$ T cell development [244, 246, 345].

$CD8^+$ T cell memory exists in many distinct subsets namely but not limited to Tem, Tcm, Tscm and Trm. Primarily classified by their respective expression markers, the differences between them go far beyond that with each subset being functionally, metabolically, and transcriptionally distinct [260, 328, 346, 347]. As $CD8^+$ T cells undergo the maturation process, they gradually acquire more effector functions and simultaneously lose their self-renewal capacity and long-term therapeutic value. Furthermore, the "stemness" of transferred cells in ACT has been shown to affect *in vivo* survival and anti-tumour capacity [348-350], supporting the approach of using more undifferentiated $CD8^+$ T cells to improve anti-tumour response.

Since more undifferentiated subsets of $CD8^+$ T cell memory – Tcm and the recently characterised Tscm – demonstrate far superior anti-tumour response and better persistence *in vivo*, the generation of these $CD8^+$ T cell subsets could therefore be the key to long-lasting immune protection of cancer immunotherapy. Multiple factors have been shown to contribute

to the lineage formation of $CD8^+$ T cell memory – the magnitude of antigen and costimulation [351-353], the cytokine environment [70, 202, 354-356], and the initial naïve T cell precursor frequency [357]. As such, thorough understanding and precise manipulation of the abovementioned factors may tweak $CD8^+$ T cell memory formation in favour of Tcm and Tscm lineages.

7.2 IL-21 is a candidate cytokine to generate long-term $CD8^+$ T cell memory post priming

IL-21, in a similar sense, can function in both phases of $CD8^+$ T cell response. As shown by Marchingo et al [137], TCR signalling, costimulation and cytokine have a summative effect to determine the eventual division fate of $CD8^+$ T cell. My results further this field of knowledge by showing that IL-21 stimulation is unable to adequately compensate TCR signal to improve activation of $CD8^+$ T cells. This is also true when killing ability of $CD8^+$ T cells were viewed as an output of $CD8^+$ T cell priming. IL-21 could only promote $CD8^+$ T cell killing against weak affinity antigen by two-fold in the priming phase *in vivo*. This increase in magnitude of $CD8^+$ T cell killing could be the result of enhanced proliferation and effector function and not an increase in $CD8^+$ T cell antigen sensitivity.

In the post priming phase, IL-21 was found to greatly enhance the magnitude of $CD8^+$ T cell killing. However, there was no change in the EC_{50} of the killing of target cells. These suggest that IL-21 does not enhance $CD8^+$ T cell antigen sensitivity, but instead achieves higher magnitude of killing potentially through the induction of $CD8^+$ T cell proliferation or the formation of a memory subset that can elicit stronger recall response.

Similar observations were made when IL-21 was tested in different tumour models. In a primary tumour model, we observed an increase in the population of activated $CD8^+$ T cells. This was likely due to the division of those already activated cells and not an increase $CD8^+$ T cell activation. In addition, there was an increase in $CD8^+$ T cell/Treg ratio which is a prognostic indication of cancer treatment [210, 211]. There was, however, no significant increase in killing of the tumour cells as evident in the tumour sizes.

When IL-21 was administered in the post-priming phase of $CD8^+$ T cell *in vivo*, there was a significant control of tumour growth. This was potentially a result of an increase in the stem cell-like $CD8^+$ T cells which are $TCF1^+$ $Tim3^-$. These cells have been shown to have outstanding persistence in chronic viral control and are required to supply effector $CD8^+$ T cells [228, 230, 358]. In PD1 blockade, these cells were found to respond by rapidly increasing proliferation [229]. Together with the increase in Tbet, it provides strong evidence that this subset of cells possibly represents the progenitor cells needed to generate $CD8^+$ T effector cells [359]. It can be speculated that IL-21 promotes the formation of such cells during the $CD8^+$ T cell contraction or maintenance phase.

Compelling evidences from previous publications further support this hypothesis. Comparison of cytokine production by $CD4^+$ T cells showed that IL-21 production predominated in chronic viral infection (day 9) and was further enhanced in the later stage of infection (day 30) [188]. On the contrary, IL-2 production by $CD4^+$ T cells and IL-15 level in spleen were quickly upregulated in early stages of acute viral infection (day 9 and day 2 respectively) [188, 360]. Consistently, the knockouts of IL-21 and its receptor have been shown to dramatically reduce the ability to form viral-specific cells in chronic LCMV-infected mice [150-152]. This corresponded to the inability of mice to control virus. However, removal of IL-21 signalling did not affect the viral-specific cells in mice infected with acute LCMV.

By comparing the effect of IL-21 in different phases of $CD8^+$ T cell response in a FTA killing assay and tumour model, I have, for the first time, confirmed that IL-21 plays a more important role in $CD8^+$ T cell post-priming phase. This further supports the physiological observations that IL-21 production is only increased late in chronic viral response.

It is not difficult to speculate an evolutionary advantage for this physiological response. Since Tfh response is increased in the later phase of chronic infection [361], the level of IL-21 in the body will also peak later [150]. Interestingly, in the later phase response of $CD8^+$ T cell against chronic infection, $CD8^+$ T cells have been shown to undergo T cell exhaustion marked by decreased proliferation and effector function [362-364]. Therefore, the role of IL-21 might be to maintain a healthy pool of antigen-experienced $CD8^+$ T cells with high differentiation and can rapidly replace the exhausted $CD8^+$ T cells.

7.3 IL-21 supports the generation of $CD8^+$ T cells with progenitor-like and stem cell-like phenotypes

When primed $CD8^+$ T cells were rested in IL-21, $CD8^+$ T cells were found to upregulate memory markers like CD122, CD127 and CCR7. The level of expression of these markers on IL-21-induced cells was revealed to be significantly higher than IL-2-induced effector and IL-15-induced memory cells. As these markers are crucial in maintaining the survival and homing of $CD8^+$ T cells, higher expression might indicate that these cells are longer-lasting and have higher tendency to stay in the secondary lymphoid organs [187, 235, 365].

With high Tbet to Eomes ratio, the IL-21-induced cells differ completely from the IL-2-induced effector and IL-15-induced memory $CD8^+$ T cells. The expression of these markers closely resembled a unique population of progenitor $CD8^+$ T cells that are $Tbet^{hi}$ $Eomes^{lo}$. In viral infection, this population of cells is needed to generate continuous supply of $Tbet^{lo}$ $Eomes^{hi}$ effector cells [259].

Furthermore, the IL-21-induced cells resembled the recently discovered Tscm which are $Sca1^+$ $CD122^+$ $CD44^-$ $CD62L^+$ [262]. The phenotype of $Sca1^+$ $CD122^+$ $CD44^+$ $CD62L^-$ suggests partial overlap of the IL-21-induced cells with the Tscm. Previous publication has shown that Wnt signaling is needed to arrest $CD8^+$ T cell differentiation and through this generates Tscm [262]. However, in my experimental setup, $CD8^+$ T cells were treated only after they had been completely primed which explains why they were $CD44^+$ $CD62L^-$. It is conceivable that the IL-21-induced cells might represent an inducible subset from fully differentiated or primed $CD8^+$ T cells as opposed to the Tscm which arise from naïve $CD8^+$ T cells.

Consistently, the IL-21-induced cells were found to have a low homeostatic proliferation rate. This suggests that IL-21-induced cells are highly quiescent. Once again, this is a similar to Tscm. It is plausible that the IL-21-induced cells undergo asymmetric proliferation *in vitro* to maintain a healthy pool of stem cell-like cells while also generating effector cells.

Despite *in vitro* results proposing a stem cell-like and progenitor-like phenotype, the role of IL-21 in the formation and maintenance of this subset *in vivo* remains unknown. Can IL-21 administration lead to increased formation and maintenance of such cells? Will the knockout

of IL-21 or IL-21 receptor lead to dissolution of this subset? Also, functional assays will be needed to determine the stem cell-like ability of the IL-21-induced cells. It will be important to understand if these cells are truly quiescent or are they just apoptotic and not proliferating as well.

If the abovementioned questions could be addressed, then IL-21 could be the critical cytokine involved in generating the progenitor stem cell-like $CD8^+$ T cell subset. Administration of IL-21 could be a form of treatment for chronic infections or diseases which depend heavily on the regenerative capacity of $CD8^+$ T cells.

7.4 IL-21 reduces OXPHOS and increases glycolysis of $CD8^+$ T cells

Examination of the metabolic profile of IL-21-induced cells revealed that these cells have high basal glycolysis, low basal OXPHOS, low SRC and low FAO. This differs largely from the IL-15-induced memory cells which have low basal glycolysis, high basal OXPHOS, high SRC and high FAO. Though exhibiting a similar metabolic profile, IL-2-induced effector cells have slightly lower basal glycolysis, lower expression of GLUT1 and lower glycolytic enzyme HK2 expression, while also having higher basal OXPHOS. In addition to that, IL-21-induced cells also express higher mitochondria mass as compared to IL-2-induced effector and IL-15-induced memory $CD8^+$ T cells. Hence, these findings propose that IL-21-induced cells are neither effector nor memory $CD8^+$ T cells.

Cellular metabolism has been shown to reflect the function of each type of cell [277]. Therefore, the comparison of the metabolic profile of IL-21-induced cells with that of other cell types in the hematopoietic lineage will shed light on its potential function. Intriguingly, the metabolic profile of the HSCs appear to be similar to that of IL-21-induced cells [329, 330]. Thus, it is possible to postulate a certain degree of functional overlap.

Notably, future experiments should include RNA sequencing of the different cytokine-induced cells to better characterise the effect of each cytokine on the $CD8^+$ T cells and elucidate the differences in their transcriptomics. More importantly, as IL-21 has been shown here to induce

a stem cell-like population and, at the same time, increase effector function, single-cell RNA sequencing will be needed to identify if two distinct populations of cells were induced.

7.5 IL-21 induction of stem cell-like phenotype could be through HIF-1α

While changes in the transcriptional landscape are fundamental to long-lasting cell fate and function, only a selected few have been noted to drastically impact metabolism. One such is HIF-1α. This present study demonstrates that IL-21 can upregulate HIF-1α at both the transcriptional and the protein level.

Though it still remains to be tested, IL-21 can potentially upregulate HIF-1α through STAT3 signalling pathway. This STAT3-HIF-1α axis has been observed in cancer cells where the induction of STAT3 signalling by oncogenic signals directly promoted the transcription of HIF-1α [340, 341]. This is reminiscent of Wnt signalling being the master regulator of Tscm [262], and BCL6 signalling of Tfh [117]. It is therefore possible that HIF-1α can be the key to orchestrate the formation of this progenitor stem cell-like $CD8^+$ T cells. Studies to further delineate the role of HIF-1α can be performed. For example, overexpression or knockdown of HIF-1α can be done to observe for changes in the formation of this cell subset.

The hypoxic environment in solid tumours has been proven to be a challenging obstacle to overcome in cancer immunotherapy [366, 367]. This is partly due to the ability of hypoxia to induce $CD8^+$ T cells apoptosis [368]. Since HIF-1α has been shown to promote the survival of HSCs in hypoxic environments [329], will an elevated level of HIF-1α expression in $CD8^+$ T cells, therefore, allow the cells survive better in the solid tumour microenvironment?

Furthermore, tertiary lymphoid structures have been observed to be formed inside solid tumours and have been shown to correlate with prognosis of the cancer in patients [369, 370]. It is, however, not known if the $CD8^+$ T cells in those tertiary lymphoid structures have developed special mechanisms to survive in the hypoxic environment. It is likely that this subset of $CD8^+$ T cells can form these tertiary niches inside the tumour microenvironment and

act as the source of progenitor $CD8^+$ T cells that constantly replenishes the anti-tumour effector $CD8^+$ T cells.

7.6 The understanding of IL-21 role can help to improve checkpoint blockade therapy and offer better therapeutic outcome when used together

PD1 blockade is a form of immunotherapy that reinvigorates exhausted $CD8^+$ T cells in human patients. However, not all patients respond to this treatment. Resistance to checkpoint blockade therapy can be broadly categorised into three major factors [371]. First, the inability to generate sufficient anti-tumour $CD8^+$ T cells which typically arises from the lack of neoantigen and impaired processes to generate them [101, 372]. Second, the inability for $CD8^+$ T cells to exert their effector functions which can be due to the inhibitory tumour microenvironment or intrinsic defects in the effector signalling pathways [373, 374]. Third, the dysfunctional formation of $CD8^+$ T cell memory which is a result of epigenetic modification during terminal T cell exhaustion [375-377].

The use of IL-21 can improve generation of anti-tumour $CD8^+$ T cells as observed by its increased antigen sensitivity towards weak affinity antigen and increased magnitude of effector response in the FTA killing assay. Furthermore, increased $CD8^+$ T cell activation was also observed in the poorly immunogenic B16F10 tumour model. In other words, this can enhance $CD8^+$ T cell recognition of TAA which tend to have a weaker affinity as compared to neoantigen. Therefore, IL-21 treatment might be a short-term solution to increase overall anti-tumour response by increasing $CD8^+$ T cell activation and sensitising patients to PD1 blockade treatment. However, prudence needs to be taken as target of healthy non-malignant cells might occur.

In addition, IL-21 enhances the formation of a progenitor-like stem cell-like $CD8^+$ T cell population. A recent study by Ribas et al. showed that the presence and expansion of intratumoural $CD8^+$ Tem are critical to a positive PD1 blockade response [378]. It can be speculated that the presence of memory cells which are less differentiated than Tem may play a more important role in checkpoint blockade responses. As such, the stem cell-like $CD8^+$ T

cells induced by IL-21, which share overlapping phenotypic and metabolic traits with Tscm and HSC, might prove to be an asset in checkpoint blockade.

7.7 Concluding remarks

In summary, my research reveals that the temporal exposure of $CD8^+$ T cell to cytokine has a major impact in determining its response. In particular for IL-21, it has a more prominent role in the post-priming phase of $CD8^+$ T cell by shaping the memory development towards a more stem cell-like phenotype.

A hypothetical model of cytokine production and phases of $CD8^+$ T cell response can be derived from my findings **(Figure 7.1)**. During acute virus infection, differentiation of Th1 cells, which predominantly produce IL-2, is promoted. This induces formation and maintenance of effector $CD8^+$ T cells that have high cytotoxic ability to remove viral-infected cells. While $CD8^+$ T cells exposed to IL-15-secreting cells such as DCs and macrophages or monocytes will form memory $CD8^+$ T cells.

On the other hand, during late stage of chronic virus infection, differentiation of Tfh is promoted resulting in high IL-21 secretion **(Figure 7.2)**. This induces formation of the progenitor stem cell-like $CD8^+$ T cells marked by $Sca1^+$ $CD122^+$ $CD127^+$ $CCR7^+$ $Tbet^{hi}$ $Eomes^{lo}$ $Ki67^-$. This group of $CD8^+$ T cells could potentially remain quiescent and persist long-term. As such, they could functionally be similar to the HSCs which are multipotent and mainly exist to support the production of more differentiated cells. They may divide via asymmetric cell division giving rise to $Ki67^+$ cells which subsequently go on to become other $CD8^+$ T cell subsets, or to $Ki67^-$ cells which continue to remain quiescent and regenerate themselves.

To end off, my research has shed light on an inducible form of progenitor stem cell-like $CD8^+$ T cells which forms the basis of a treatment regimen for cancer patients. Patients who have successful primary cancer remission from the first cancer therapy can be placed on IL-21 supplements in the form of weekly injection to boost the formation of the stem cell-like $CD8^+$ T cells. This will be a preventative measure to completely remove the possibility of cancer relapses.

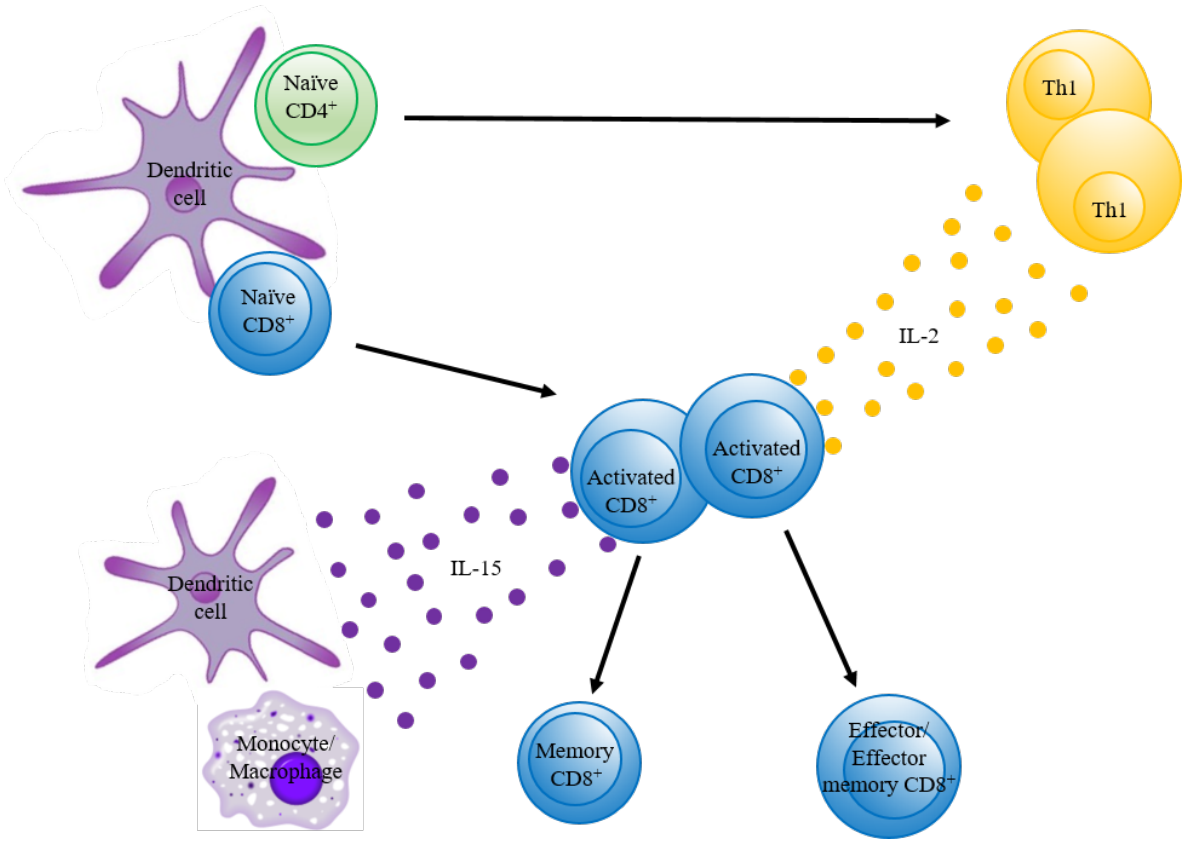


Figure 7.1. Hypothetical model of CD8$^+$ T cell response in acute infection. CD4$^+$ T cells predominantly differentiates into the Th1 subtype which produces IL-2, and this induces formation and maintenance of effector CD8$^+$ T cells. Presence of IL-15-secreting cells such as DCs and macrophages or monocytes will drive memory CD8$^+$ T cell formation.

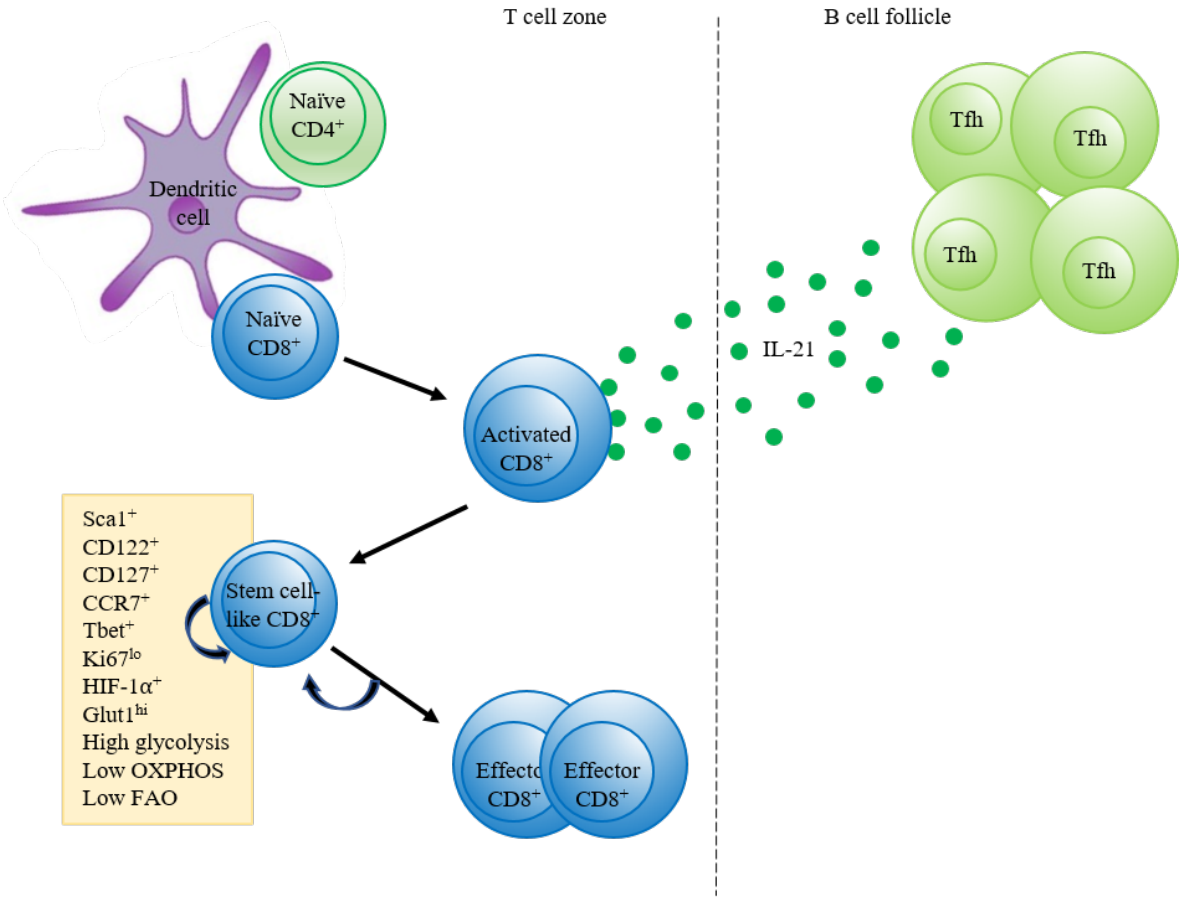


Figure 7.2. Hypothetical model of CD8$^+$ T cell response in chronic infection. CD4$^+$ T cells predominantly differentiates into the Tfh subtype which produces IL-21, and this induces formation the stem cell-like CD8$^+$ T cells. This subset is capable of renewing itself and replenishing the effector CD8$^+$ T cell pool.

References

References

1. Stewart BW, W.C., *International Agency for Research on Cancer, World Health Organization.* 2014, Lyon: WHO Press: World cancer report 2014
2. Davis, B. and M. Goodwin, *Cancer incidence projections: Australia, 2011 to 2020.* 2012, Cancer Series no. 66. Cat. No. CAN 62. Canberra: AIHW.: Australian Institute of Health and Welfare.
3. Dranoff, G., *Cytokines in cancer pathogenesis and cancer therapy.* Nat Rev Cancer, 2004. **4**(1): p. 11-22.
4. Herrera, V. and J. Parsonnet, *Helicobacter pylori and gastric adenocarcinoma.* Clin Microbiol Infect, 2009. **15**(11): p. 971-6.
5. Sepulveda, A.R., *Helicobacter, Inflammation, and Gastric Cancer.* Curr Pathobiol Rep, 2013. **1**(1): p. 9-18.
6. Di Bisceglie, A.M., *Hepatitis B and hepatocellular carcinoma.* Hepatology (Baltimore, Md.), 2009. **49**(5 Suppl): p. S56-S60.
7. Hussain, S.P., L.J. Hofseth, and C.C. Harris, *Radical causes of cancer.* Nat Rev Cancer, 2003. **3**(4): p. 276-85.
8. Soucek, L., et al., *Mast cells are required for angiogenesis and macroscopic expansion of Myc-induced pancreatic islet tumors.* Nat Med, 2007. **13**(10): p. 1211-8.
9. Sparmann, A. and D. Bar-Sagi, *Ras-induced interleukin-8 expression plays a critical role in tumor growth and angiogenesis.* Cancer Cell, 2004. **6**(5): p. 447-58.
10. Szebeni, G.J., et al., *Pro-Tumoral Inflammatory Myeloid Cells as Emerging Therapeutic Targets.* International journal of molecular sciences, 2016. **17**(11): p. 1958.
11. Hanada, T., et al., *IFNgamma-dependent, spontaneous development of colorectal carcinomas in SOCS1-deficient mice.* J Exp Med, 2006. **203**(6): p. 1391-7.
12. Aspord, C., et al., *Breast cancer instructs dendritic cells to prime interleukin 13-secreting CD4+ T cells that facilitate tumor development.* J Exp Med, 2007. **204**(5): p. 1037-47.
13. DeNardo, D.G., et al., *CD4(+) T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages.* Cancer Cell, 2009. **16**(2): p. 91-102.
14. Langowski, J.L., et al., *IL-23 promotes tumour incidence and growth.* Nature, 2006. **442**(7101): p. 461-5.
15. Wang, L., et al., *IL-17 can promote tumor growth through an IL-6-Stat3 signaling pathway.* J Exp Med, 2009. **206**(7): p. 1457-64.
16. Grivennikov, S., et al., *IL-6 and Stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer.* Cancer Cell, 2009. **15**(2): p. 103-13.
17. Park, E.J., et al., *Dietary and genetic obesity promote liver inflammation and tumorigenesis by enhancing IL-6 and TNF expression.* Cell, 2010. **140**(2): p. 197-208.
18. Wu, Y., et al., *Stabilization of snail by NF-kappaB is required for inflammation-induced cell migration and invasion.* Cancer cell, 2009. **15**(5): p. 416-428.
19. Ehrlich, P., *Ueber den jetzigen stand der karzinomforschung.* Ned Tijdschr Geneeskd. 1909;5:73–290.
20. Old, L.J. and E.A. Boyse, *IMMUNOLOGY OF EXPERIMENTAL TUMORS.* Annu Rev Med, 1964. **15**: p. 167-86.
21. Klein, G., *Tumor antigens.* Annu Rev Microbiol, 1966. **20**: p. 223-52.

22. Shankaran, V., et al., *IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity.* Nature, 2001. **410**(6832): p. 1107-11.
23. Diefenbach, A. and D.H. Raulet, *The innate immune response to tumors and its role in the induction of T-cell immunity.* Immunol Rev, 2002. **188**: p. 9-21.
24. Garrido, F., et al., *Implications for immunosurveillance of altered HLA class I phenotypes in human tumours.* Immunol Today, 1997. **18**(2): p. 89-95.
25. Fauriat, C., et al., *Education of human natural killer cells by activating killer cell immunoglobulin-like receptors.* Blood, 2010. **115**(6): p. 1166-74.
26. Smyth, M.J., N.Y. Crowe, and D.I. Godfrey, *NK cells and NKT cells collaborate in host protection from methylcholanthrene-induced fibrosarcoma.* Int Immunol, 2001. **13**(4): p. 459-63.
27. van der Bruggen, P., et al., *A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma.* Science, 1991. **254**(5038): p. 1643-7.
28. Boon, T., P.G. Coulie, and B. Van den Eynde, *Tumor antigens recognized by T cells.* Immunol Today, 1997. **18**(6): p. 267-8.
29. Echchakir, H., et al., *Evidence for in situ expansion of diverse antitumor-specific cytotoxic T lymphocyte clones in a human large cell carcinoma of the lung.* Int Immunol, 2000. **12**(4): p. 537-46.
30. Karanikas, V., et al., *High frequency of cytolytic T lymphocytes directed against a tumor-specific mutated antigen detectable with HLA tetramers in the blood of a lung carcinoma patient with long survival.* Cancer Res, 2001. **61**(9): p. 3718-24.
31. Zhang, L., et al., *Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer.* N Engl J Med, 2003. **348**(3): p. 203-13.
32. Kondratiev, S., et al., *Intratumoral CD8+ T lymphocytes as a prognostic factor of survival in endometrial carcinoma.* Clin Cancer Res, 2004. **10**(13): p. 4450-6.
33. Chen, Z., et al., *Intratumoral CD8(+) cytotoxic lymphocyte is a favorable prognostic marker in node-negative breast cancer.* PLoS One, 2014. **9**(4): p. e95475.
34. Fridman, W.H., et al., *The immune contexture in human tumours: impact on clinical outcome.* Nat Rev Cancer, 2012. **12**(4): p. 298-306.
35. Van Lint, S., et al., *Priming of Cytotoxic T Lymphocyte Responses by Dendritic Cells: Induction of Potent Anti-tumor Immune Responses*. 2013.
36. Vasaturo, A., et al., *Clinical Implications of Co-Inhibitory Molecule Expression in the Tumor Microenvironment for DC Vaccination: A Game of Stop and Go.* Front Immunol, 2013. **4**: p. 417.
37. Wirth, T.C. and F. Kühnel, *Neoantigen Targeting-Dawn of a New Era in Cancer Immunotherapy?* Frontiers in immunology, 2017. **8**: p. 1848-1848.
38. Gilboa, E., *The makings of a tumor rejection antigen.* Immunity, 1999. **11**(3): p. 263-70.
39. Durgeau, A., et al., *Recent Advances in Targeting CD8 T-Cell Immunity for More Effective Cancer Immunotherapy.* Frontiers in immunology, 2018. **9**: p. 14-14.
40. *The problem with neoantigen prediction.* Nature Biotechnology, 2017. **35**: p. 97.
41. Herbst, R.S., et al., *Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients.* Nature, 2014. **515**(7528): p. 563-7.
42. Karwacz, K., et al., *PD-L1 co-stimulation contributes to ligand-induced T cell receptor down-modulation on CD8+ T cells.* EMBO Mol Med, 2011. **3**(10): p. 581-92.
43. Wang, Y., et al., *Regulation of PD-L1: Emerging Routes for Targeting Tumor Immune Evasion.* Frontiers in pharmacology, 2018. **9**: p. 536-536.

44. Turnis, M.E., L.P. Andrews, and D.A.A. Vignali, *Inhibitory receptors as targets for cancer immunotherapy.* European journal of immunology, 2015. **45**(7): p. 1892-1905.
45. Rosenberg, S.A., et al., *Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. A preliminary report.* N Engl J Med, 1988. **319**(25): p. 1676-80.
46. Rosenberg, S.A. and N.P. Restifo, *Adoptive cell transfer as personalized immunotherapy for human cancer.* Science, 2015. **348**(6230): p. 62-8.
47. Johnson, L.A., et al., *Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen.* Blood, 2009. **114**(3): p. 535-46.
48. Kalos, M., et al., *T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia.* Sci Transl Med, 2011. **3**(95): p. 95ra73.
49. Porter, D.L., et al., *Chimeric antigen receptor T cells persist and induce sustained remissions in relapsed refractory chronic lymphocytic leukemia.* Sci Transl Med, 2015. **7**(303): p. 303ra139.
50. Brentjens, R.J., et al., *Safety and persistence of adoptively transferred autologous CD19-targeted T cells in patients with relapsed or chemotherapy refractory B-cell leukemias.* Blood, 2011. **118**(18): p. 4817-28.
51. Kerkar, S.P., et al., *Tumor-specific CD8+ T cells expressing interleukin-12 eradicate established cancers in lymphodepleted hosts.* Cancer Res, 2010. **70**(17): p. 6725-34.
52. Pegram, H.J., et al., *Tumor-targeted T cells modified to secrete IL-12 eradicate systemic tumors without need for prior conditioning.* Blood, 2012. **119**(18): p. 4133-41.
53. Avanzi, M.P., et al., *IL-18 Secreting CAR T Cells Enhance Cell Persistence, Induce Prolonged B Cell Aplasia and Eradicate CD19+ Tumor Cells without Need for Prior Conditioning.* Blood, 2016. **128**(22): p. 816.
54. Wang, Z., et al., *New development in CAR-T cell therapy.* J Hematol Oncol, 2017. **10**(1): p. 53.
55. Hartmann, J., et al., *Clinical development of CAR T cells-challenges and opportunities in translating innovative treatment concepts.* EMBO molecular medicine, 2017. **9**(9): p. 1183-1197.
56. D'Aloia, M.M., et al., *CAR-T cells: the long and winding road to solid tumors.* Cell Death Dis, 2018. **9**(3): p. 282.
57. Yano, S., et al., *Distribution and function of EGFR in human tissue and the effect of EGFR tyrosine kinase inhibition.* Anticancer Res, 2003. **23**(5a): p. 3639-50.
58. Sasada, T., et al., *Immune Responses to Epidermal Growth Factor Receptor (EGFR) and Their Application for Cancer Treatment.* Front Pharmacol, 2016. **7**: p. 405.
59. Luo, X., et al., *EGFRvIII mediates hepatocellular carcinoma cell invasion by promoting S100 calcium binding protein A11 expression.* PLoS One, 2013. **8**(12): p. e83332.
60. Del Vecchio, C.A., et al., *Epidermal growth factor receptor variant III contributes to cancer stem cell phenotypes in invasive breast carcinoma.* Cancer Res, 2012. **72**(10): p. 2657-71.
61. Harlin, H., et al., *Chemokine expression in melanoma metastases associated with CD8+ T-cell recruitment.* Cancer Res, 2009. **69**(7): p. 3077-85.
62. Bouzin, C. and O. Feron, *Targeting tumor stroma and exploiting mature tumor vasculature to improve anti-cancer drug delivery.* Drug Resist Updat, 2007. **10**(3): p. 109-20.

63. Nelson, J., et al., *The endothelin axis: emerging role in cancer.* Nat Rev Cancer, 2003. **3**(2): p. 110-6.
64. Caruana, I., et al., *Heparanase promotes tumor infiltration and antitumor activity of CAR-redirected T lymphocytes.* Nature medicine, 2015. **21**(5): p. 524-529.
65. Vuillefroy de Silly, R., et al., *Phenotypic switch of CD8(+) T cells reactivated under hypoxia toward IL-10 secreting, poorly proliferative effector cells.* Eur J Immunol, 2015. **45**(8): p. 2263-75.
66. Fischer, K., et al., *Inhibitory effect of tumor cell-derived lactic acid on human T cells.* Blood, 2007. **109**(9): p. 3812-9.
67. Maude, S.L., et al., *Chimeric antigen receptor T cells for sustained remissions in leukemia.* N Engl J Med, 2014. **371**(16): p. 1507-17.
68. Louis, C.U., et al., *Antitumor activity and long-term fate of chimeric antigen receptor-positive T cells in patients with neuroblastoma.* Blood, 2011. **118**(23): p. 6050-6.
69. Xu, Y., et al., *Closely related T-memory stem cells correlate with in vivo expansion of CAR.CD19-T cells and are preserved by IL-7 and IL-15.* Blood, 2014. **123**(24): p. 3750-3759.
70. Hurton, L.V., et al., *Tethered IL-15 augments antitumor activity and promotes a stem-cell memory subset in tumor-specific T cells.* Proc Natl Acad Sci U S A, 2016. **113**(48): p. E7788-e7797.
71. Lenschow, D.J., T.L. Walunas, and J.A. Bluestone, *CD28/B7 system of T cell costimulation.* Annu Rev Immunol, 1996. **14**: p. 233-58.
72. Krummel, M.F. and J.P. Allison, *CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation.* The Journal of experimental medicine, 1995. **182**(2): p. 459-465.
73. Walunas, T.L., et al., *CTLA-4 can function as a negative regulator of T cell activation.* Immunity, 1994. **1**(5): p. 405-13.
74. Lindsten, T., et al., *Characterization of CTLA-4 structure and expression on human T cells.* J Immunol, 1993. **151**(7): p. 3489-99.
75. Fallarino, F., P.E. Fields, and T.F. Gajewski, *B7-1 engagement of cytotoxic T lymphocyte antigen 4 inhibits T cell activation in the absence of CD28.* J Exp Med, 1998. **188**(1): p. 205-10.
76. Lee, K.M., et al., *Molecular basis of T cell inactivation by CTLA-4.* Science, 1998. **282**(5397): p. 2263-6.
77. Schneider, H., et al., *CTLA-4 disrupts ZAP70 microcluster formation with reduced T cell/APC dwell times and calcium mobilization.* Eur J Immunol, 2008. **38**(1): p. 40-7.
78. Tivol, E.A., et al., *Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4.* Immunity, 1995. **3**(5): p. 541-7.
79. Waterhouse, P., et al., *Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4.* Science, 1995. **270**(5238): p. 985-8.
80. Leach, D.R., M.F. Krummel, and J.P. Allison, *Enhancement of antitumor immunity by CTLA-4 blockade.* Science, 1996. **271**(5256): p. 1734-6.
81. Yang, Y.F., et al., *Enhanced induction of antitumor T-cell responses by cytotoxic T lymphocyte-associated molecule-4 blockade: the effect is manifested only at the restricted tumor-bearing stages.* Cancer Res, 1997. **57**(18): p. 4036-41.
82. Hodi, F.S., et al., *Improved survival with ipilimumab in patients with metastatic melanoma.* N Engl J Med, 2010. **363**(8): p. 711-23.
83. Robert, C., et al., *Ipilimumab plus dacarbazine for previously untreated metastatic melanoma.* N Engl J Med, 2011. **364**(26): p. 2517-26.

84. Schadendorf, D., et al., *Pooled Analysis of Long-Term Survival Data From Phase II and Phase III Trials of Ipilimumab in Unresectable or Metastatic Melanoma.* J Clin Oncol, 2015. **33**(17): p. 1889-94.
85. Takahashi, T., et al., *Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4.* J Exp Med, 2000. **192**(2): p. 303-10.
86. Read, S., V. Malmstrom, and F. Powrie, *Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation.* J Exp Med, 2000. **192**(2): p. 295-302.
87. Oderup, C., et al., *Cytotoxic T lymphocyte antigen-4-dependent down-modulation of costimulatory molecules on dendritic cells in CD4+ CD25+ regulatory T-cell-mediated suppression.* Immunology, 2006. **118**(2): p. 240-9.
88. Misra, N., et al., *Cutting edge: human CD4+CD25+ T cells restrain the maturation and antigen-presenting function of dendritic cells.* J Immunol, 2004. **172**(8): p. 4676-80.
89. Wing, K., et al., *CTLA-4 control over Foxp3+ regulatory T cell function.* Science, 2008. **322**(5899): p. 271-5.
90. Qureshi, O.S., et al., *Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4.* Science, 2011. **332**(6029): p. 600-3.
91. Yamazaki, T., et al., *Expression of programmed death 1 ligands by murine T cells and APC.* J Immunol, 2002. **169**(10): p. 5538-45.
92. Baumeister, S.H., et al., *Coinhibitory Pathways in Immunotherapy for Cancer.* Annu Rev Immunol, 2016. **34**: p. 539-73.
93. Hui, E., et al., *T cell costimulatory receptor CD28 is a primary target for PD-1-mediated inhibition.* Science (New York, N.Y.), 2017. **355**(6332): p. 1428-1433.
94. Kamphorst, A.O., et al., *Rescue of exhausted CD8 T cells by PD-1-targeted therapies is CD28-dependent.* Science (New York, N.Y.), 2017. **355**(6332): p. 1423-1427.
95. Topalian, S.L., et al., *Safety, activity, and immune correlates of anti-PD-1 antibody in cancer.* N Engl J Med, 2012. **366**(26): p. 2443-54.
96. Brahmer, J.R., et al., *Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates.* J Clin Oncol, 2010. **28**(19): p. 3167-75.
97. Topalian, S.L., et al., *Survival, durable tumor remission, and long-term safety in patients with advanced melanoma receiving nivolumab.* J Clin Oncol, 2014. **32**(10): p. 1020-30.
98. Robert, C., et al., *Pembrolizumab versus Ipilimumab in Advanced Melanoma.* N Engl J Med, 2015. **372**(26): p. 2521-32.
99. Wolchok, J.D., et al., *Overall Survival with Combined Nivolumab and Ipilimumab in Advanced Melanoma.* N Engl J Med, 2017. **377**(14): p. 1345-1356.
100. Larkin, J., et al., *Combined Nivolumab and Ipilimumab or Monotherapy in Untreated Melanoma.* The New England journal of medicine, 2015. **373**(1): p. 23-34.
101. Schumacher, T.N. and R.D. Schreiber, *Neoantigens in cancer immunotherapy.* Science, 2015. **348**(6230): p. 69-74.
102. Rizvi, N.A., et al., *Cancer immunology. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer.* Science (New York, N.Y.), 2015. **348**(6230): p. 124-128.
103. Van Allen, E.M., et al., *Genomic correlates of response to CTLA-4 blockade in metastatic melanoma.* Science (New York, N.Y.), 2015. **350**(6257): p. 207-211.
104. Le, D.T., et al., *PD-1 Blockade in Tumors with Mismatch-Repair Deficiency.* The New England journal of medicine, 2015. **372**(26): p. 2509-2520.

105. Le, D.T., et al., *Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade.* Science (New York, N.Y.), 2017. **357**(6349): p. 409-413.
106. Spolski, R. and W.J. Leonard, *Interleukin-21: basic biology and implications for cancer and autoimmunity.* Annu Rev Immunol, 2008. **26**: p. 57-79.
107. Zeng, R., et al., *Synergy of IL-21 and IL-15 in regulating CD8+ T cell expansion and function.* J Exp Med, 2005. **201**(1): p. 139-48.
108. Sutherland, A.P., et al., *IL-21 promotes CD8+ CTL activity via the transcription factor T-bet.* J Immunol, 2013. **190**(8): p. 3977-84.
109. Parrish-Novak, J., et al., *Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function.* Nature, 2000. **408**(6808): p. 57-63.
110. Sivori, S., et al., *IL-21 induces both rapid maturation of human CD34+ cell precursors towards NK cells and acquisition of surface killer Ig-like receptors.* Eur J Immunol, 2003. **33**(12): p. 3439-47.
111. Brady, J., et al., *The interactions of multiple cytokines control NK cell maturation.* J Immunol, 2010. **185**(11): p. 6679-88.
112. Kasaian, M.T., et al., *IL-21 limits NK cell responses and promotes antigen-specific T cell activation: a mediator of the transition from innate to adaptive immunity.* Immunity, 2002. **16**(4): p. 559-69.
113. Peluso, I., et al., *IL-21 counteracts the regulatory T cell-mediated suppression of human CD4+ T lymphocytes.* J Immunol, 2007. **178**(2): p. 732-9.
114. Kim-Schulze, S., et al., *Local IL-21 promotes the therapeutic activity of effector T cells by decreasing regulatory T cells within the tumor microenvironment.* Mol Ther, 2009. **17**(2): p. 380-8.
115. Li, Y. and C. Yee, *IL-21 mediated Foxp3 suppression leads to enhanced generation of antigen-specific CD8+ cytotoxic T lymphocytes.* Blood, 2008. **111**(1): p. 229-35.
116. Nurieva, R.I., et al., *Bcl6 mediates the development of T follicular helper cells.* Science, 2009. **325**(5943): p. 1001-5.
117. Yu, D., et al., *The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment.* Immunity, 2009. **31**(3): p. 457-68.
118. Nurieva, R., et al., *Essential autocrine regulation by IL-21 in the generation of inflammatory T cells.* Nature, 2007. **448**(7152): p. 480-3.
119. Ozaki, K., et al., *A critical role for IL-21 in regulating immunoglobulin production.* Science, 2002. **298**(5598): p. 1630-4.
120. Zotos, D., et al., *IL-21 regulates germinal center B cell differentiation and proliferation through a B cell-intrinsic mechanism.* J Exp Med, 2010. **207**(2): p. 365-78.
121. Linterman, M.A., et al., *IL-21 acts directly on B cells to regulate Bcl-6 expression and germinal center responses.* J Exp Med, 2010. **207**(2): p. 353-63.
122. Spolski, R. and W.J. Leonard, *Interleukin-21: a double-edged sword with therapeutic potential.* Nature Reviews Drug Discovery, 2014. **13**: p. 379.
123. Asao, H., et al., *Cutting edge: the common gamma-chain is an indispensable subunit of the IL-21 receptor complex.* J Immunol, 2001. **167**(1): p. 1-5.
124. Zeng, R., et al., *The molecular basis of IL-21-mediated proliferation.* Blood, 2007. **109**(10): p. 4135-42.
125. Wan, C.K., et al., *Opposing roles of STAT1 and STAT3 in IL-21 function in CD4+ T cells.* Proc Natl Acad Sci U S A, 2015. **112**(30): p. 9394-9.
126. Strengell, M., et al., *IL-21 in synergy with IL-15 or IL-18 enhances IFN-gamma production in human NK and T cells.* J Immunol, 2003. **170**(11): p. 5464-9.
127. Cui, W., et al., *An interleukin-21-interleukin-10-STAT3 pathway is critical for functional maturation of memory CD8+ T cells.* Immunity, 2011. **35**(5): p. 792-805.

128. Hinrichs, C.S., et al., *IL-2 and IL-21 confer opposing differentiation programs to CD8+ T cells for adoptive immunotherapy.* Blood, 2008. **111**(11): p. 5326-33.
129. Kwon, H., et al., *Analysis of interleukin-21-induced Prdm1 gene regulation reveals functional cooperation of STAT3 and IRF4 transcription factors.* Immunity, 2009. **31**(6): p. 941-52.
130. Li, P., et al., *BATF-JUN is critical for IRF4-mediated transcription in T cells.* Nature, 2012. **490**(7421): p. 543-6.
131. Ciofani, M., et al., *A validated regulatory network for Th17 cell specification.* Cell, 2012. **151**(2): p. 289-303.
132. Glasmacher, E., et al., *A genomic regulatory element that directs assembly and function of immune-specific AP-1-IRF complexes.* Science, 2012. **338**(6109): p. 975-80.
133. Leonard, W.J. and C.-K. Wan, *IL-21 Signaling in Immunity.* F1000Research, 2016. **5**: p. F1000 Faculty Rev-224.
134. Tian, Y. and A.J. Zajac, *IL-21 and T Cell Differentiation: Consider the Context.* Trends Immunol, 2016. **37**(8): p. 557-568.
135. Curtsinger, J.M. and M.F. Mescher, *Inflammatory cytokines as a third signal for T cell activation.* Current opinion in immunology, 2010. **22**(3): p. 333-340.
136. Corthay, A., *A three-cell model for activation of naive T helper cells.* Scand J Immunol, 2006. **64**(2): p. 93-6.
137. Marchingo, J.M., et al., *T cell signaling. Antigen affinity, costimulation, and cytokine inputs sum linearly to amplify T cell expansion.* Science, 2014. **346**(6213): p. 1123-7.
138. Moroz, A., et al., *IL-21 enhances and sustains CD8+ T cell responses to achieve durable tumor immunity: comparative evaluation of IL-2, IL-15, and IL-21.* J Immunol, 2004. **173**(2): p. 900-9.
139. Davis, I.D., et al., *An open-label, two-arm, phase I trial of recombinant human interleukin-21 in patients with metastatic melanoma.* Clin Cancer Res, 2007. **13**(12): p. 3630-6.
140. Thompson, J.A., et al., *Phase I study of recombinant interleukin-21 in patients with metastatic melanoma and renal cell carcinoma.* J Clin Oncol, 2008. **26**(12): p. 2034-9.
141. Davis, I.D., et al., *Clinical and biological efficacy of recombinant human interleukin-21 in patients with stage IV malignant melanoma without prior treatment: a phase IIa trial.* Clin Cancer Res, 2009. **15**(6): p. 2123-9.
142. Petrella, T.M., et al., *Interleukin-21 has activity in patients with metastatic melanoma: a phase II study.* J Clin Oncol, 2012. **30**(27): p. 3396-401.
143. Frederiksen, K.S., et al., *IL-21 induces in vivo immune activation of NK cells and CD8(+) T cells in patients with metastatic melanoma and renal cell carcinoma.* Cancer immunology, immunotherapy : CII, 2008. **57**(10): p. 1439-1449.
144. Grunwald, V., et al., *A phase I study of recombinant human interleukin-21 (rIL-21) in combination with sunitinib in patients with metastatic renal cell carcinoma (RCC).* Acta Oncol, 2011. **50**(1): p. 121-6.
145. Bhatia, S., et al., *Recombinant interleukin-21 plus sorafenib for metastatic renal cell carcinoma: a phase 1/2 study.* J Immunother Cancer, 2014. **2**: p. 2.
146. Singh, H., et al., *Reprogramming CD19-specific T cells with IL-21 signaling can improve adoptive immunotherapy of B-lineage malignancies.* Cancer research, 2011. **71**(10): p. 3516-3527.
147. Kinter, A.L., et al., *The common gamma-chain cytokines IL-2, IL-7, IL-15, and IL-21 induce the expression of programmed death-1 and its ligands.* J Immunol, 2008. **181**(10): p. 6738-46.

148. Selby, M. and A. Korman, *Methods of treating cancer using an il-21 polypeptide and an anti-pd-1 antibody*, B.-M. Squibb, Editor. 2013.
149. Lewis, K.E., et al., *Interleukin-21 combined with PD-1 or CTLA-4 blockade enhances antitumor immunity in mouse tumor models.* Oncoimmunology, 2017. **7**(1): p. e1377873-e1377873.
150. Elsaesser, H., K. Sauer, and D.G. Brooks, *IL-21 is required to control chronic viral infection.* Science, 2009. **324**(5934): p. 1569-72.
151. Frohlich, A., et al., *IL-21R on T cells is critical for sustained functionality and control of chronic viral infection.* Science, 2009. **324**(5934): p. 1576-80.
152. Yi, J.S., M. Du, and A.J. Zajac, *A vital role for interleukin-21 in the control of a chronic viral infection.* Science, 2009. **324**(5934): p. 1572-6.
153. Iannello, A., et al., *Dynamics and consequences of IL-21 production in HIV-infected individuals: a longitudinal and cross-sectional study.* J Immunol, 2010. **184**(1): p. 114-26.
154. Chevalier, M.F., et al., *HIV-1-specific interleukin-21+ CD4+ T cell responses contribute to durable viral control through the modulation of HIV-specific CD8+ T cell function.* J Virol, 2011. **85**(2): p. 733-41.
155. Cubas, R.A., et al., *Inadequate T follicular cell help impairs B cell immunity during HIV infection.* Nat Med, 2013. **19**(4): p. 494-9.
156. Curtsinger, J.M., et al., *Inflammatory cytokines provide a third signal for activation of naive CD4+ and CD8+ T cells.* J Immunol, 1999. **162**(6): p. 3256-62.
157. Au-Yeung, B.B., et al., *IL-2 Modulates the TCR Signaling Threshold for CD8 but Not CD4 T Cell Proliferation on a Single-Cell Level.* Journal of immunology (Baltimore, Md. : 1950), 2017. **198**(6): p. 2445-2456.
158. Chen, Z., et al., *Efficient production of recombinant IL-21 proteins for pre-clinical studies by a two-step dilution refolding method.* Int Immunopharmacol, 2013. **16**(3): p. 376-81.
159. Sondergaard, H., et al., *Interleukin 21 therapy increases the density of tumor infiltrating CD8+ T cells and inhibits the growth of syngeneic tumors.* Cancer Immunol Immunother, 2007. **56**(9): p. 1417-28.
160. Li, Y., M. Bleakley, and C. Yee, *IL-21 influences the frequency, phenotype, and affinity of the antigen-specific CD8 T cell response.* J Immunol, 2005. **175**(4): p. 2261-9.
161. White, L., et al., *Differential effects of IL-21 and IL-15 on perforin expression, lysosomal degranulation, and proliferation in CD8 T cells of patients with human immunodeficiency virus-1 (HIV).* Blood, 2007. **109**(9): p. 3873-80.
162. Quah, B.J.C., et al., *The use of fluorescent target arrays for assessment of T cell responses in vivo.* Journal of visualized experiments : JoVE, 2014(88): p. e51627-e51627.
163. Swain, S.L., *T-Cell Subsets: Who does the polarizing?* Current Biology, 1995. **5**(8): p. 849-851.
164. Cox, M.A., L.E. Harrington, and A.J. Zajac, *Cytokines and the Inception of CD8 T Cell Responses.* Trends in immunology, 2011. **32**(4): p. 180-186.
165. Jenkins, M.K. and R.H. Schwartz, *Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo.* The Journal of Experimental Medicine, 1987. **165**(2): p. 302.
166. Schwartz, R.H., *T cell anergy.* Annu Rev Immunol, 2003. **21**: p. 305-34.
167. Hsieh, C.S., et al., *Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages.* Science, 1993. **260**(5107): p. 547-549.

168. Seder, R.A., et al., *Interleukin 12 acts directly on CD4+ T cells to enhance priming for interferon gamma production and diminishes interleukin 4 inhibition of such priming.* Proceedings of the National Academy of Sciences, 1993. **90**(21): p. 10188.
169. Le Gros, G., et al., *Generation of interleukin 4 (IL-4)-producing cells in vivo and in vitro: IL-2 and IL-4 are required for in vitro generation of IL-4-producing cells.* J Exp Med, 1990. **172**(3): p. 921-9.
170. Kopf, M., et al., *Disruption of the murine IL-4 gene blocks Th2 cytokine responses.* Nature, 1993. **362**: p. 245.
171. Bettelli, E., et al., *Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells.* Nature, 2006. **441**(7090): p. 235-8.
172. Mangan, P.R., et al., *Transforming growth factor-beta induces development of the T(H)17 lineage.* Nature, 2006. **441**(7090): p. 231-4.
173. Zheng, S.G., et al., *IL-2 is essential for TGF-beta to convert naive CD4+CD25- cells to CD25+Foxp3+ regulatory T cells and for expansion of these cells.* J Immunol, 2007. **178**(4): p. 2018-27.
174. Nurieva, R.I., et al., *Generation of follicular helper T cells is mediated by IL-21 but independent of TH1, TH2 or TH17 lineages.* Immunity, 2008. **29**(1): p. 138-149.
175. Vogelzang, A., et al., *A fundamental role for interleukin-21 in the generation of T follicular helper cells.* Immunity, 2008. **29**(1): p. 127-37.
176. Agarwal, P., et al., *Gene regulation and chromatin remodeling by IL-12 and type I IFN in programming for CD8 T cell effector function and memory.* J Immunol, 2009. **183**(3): p. 1695-704.
177. Curtsinger, J.M., et al., *Type I IFNs provide a third signal to CD8 T cells to stimulate clonal expansion and differentiation.* J Immunol, 2005. **174**(8): p. 4465-9.
178. Manz, B.N., et al., *T-cell triggering thresholds are modulated by the number of antigen within individual T-cell receptor clusters.* Proceedings of the National Academy of Sciences of the United States of America, 2011. **108**(22): p. 9089-9094.
179. Deshpande, P., et al., *IL-7– and IL-15–mediated TCR sensitization enables T cell responses to self-antigens.* Journal of immunology (Baltimore, Md. : 1950), 2013. **190**(4): p. 1416-1423.
180. Au-Yeung, B., et al., *IL-2 modulates the TCR signaling threshold for CD8 but not CD4 T cell proliferation on a single cell level.* Journal of immunology (Baltimore, Md. : 1950), 2017. **198**(6): p. 2445-2456.
181. Casey, K.A. and M.F. Mescher, *IL-21 promotes differentiation of naive CD8 T cells to a unique effector phenotype.* J Immunol, 2007. **178**(12): p. 7640-8.
182. Pipkin, M.E., et al., *Interleukin-2 and inflammation induce distinct transcriptional programs that promote the differentiation of effector cytolytic T cells.* Immunity, 2010. **32**(1): p. 79-90.
183. Sa, Q., J. Woodward, and Y. Suzuki, *IL-2 produced by CD8+ immune T cells can augment their IFN-γ production independently from their proliferation in the secondary response to an intracellular pathogen.* Journal of immunology (Baltimore, Md. : 1950), 2013. **190**(5): p. 2199-2207.
184. Hinrichs, C.S., et al., *IL-2 and IL-21 confer opposing differentiation programs to CD8+ T cells for adoptive immunotherapy.* Blood, 2008. **111**(11): p. 5326-5333.
185. Shenoy, A.R., S. Kirschnek, and G. Hacker, *IL-15 regulates Bcl-2 family members Bim and Mcl-1 through JAK/STAT and PI3K/AKT pathways in T cells.* Eur J Immunol, 2014. **44**(8): p. 2500-7.
186. Berard, M., et al., *IL-15 promotes the survival of naive and memory phenotype CD8+ T cells.* J Immunol, 2003. **170**(10): p. 5018-26.

187. Bradley, L.M., L. Haynes, and S.L. Swain, *IL-7: maintaining T-cell memory and achieving homeostasis.* Trends Immunol, 2005. **26**(3): p. 172-6.
188. Elsaesser, H., K. Sauer, and D.G. Brooks, *IL-21 is required to control chronic viral infection.* Science (New York, N.Y.), 2009. **324**(5934): p. 1569-1572.
189. Xin, G., et al., *A Critical Role of IL-21-Induced BATF in Sustaining CD8-T-Cell-Mediated Chronic Viral Control.* Cell reports, 2015. **13**(6): p. 1118-1124.
190. Sagerström, C.G., et al., *Activation and differentiation requirements of primary T cells in vitro.* Proceedings of the National Academy of Sciences of the United States of America, 1993. **90**(19): p. 8987-8991.
191. Man, K., et al., *The transcription factor IRF4 is essential for TCR affinity-mediated metabolic programming and clonal expansion of T cells.* Nat Immunol, 2013. **14**(11): p. 1155-65.
192. Yao, S., et al., *Interferon regulatory factor 4 sustains CD8(+) T cell expansion and effector differentiation.* Immunity, 2013. **39**(5): p. 833-45.
193. Nayar, R., et al., *TCR signaling via Tec kinase ITK and interferon regulatory factor 4 (IRF4) regulates CD8+ T-cell differentiation.* Proc Natl Acad Sci U S A, 2012. **109**(41): p. E2794-802.
194. Zehn, D., S.Y. Lee, and M.J. Bevan, *Complete but curtailed T-cell response to very low-affinity antigen.* Nature, 2009. **458**(7235): p. 211-214.
195. Yi, J.S., M. Du, and A.J. Zajac, *A Vital Role For IL-21 in the Control of a Chronic Viral Infection.* Science (New York, N.Y.), 2009. **324**(5934): p. 1572-1576.
196. Mehlhop-Williams, E.R. and M.J. Bevan, *Memory CD8+ T cells exhibit increased antigen threshold requirements for recall proliferation.* J Exp Med, 2014. **211**(2): p. 345-56.
197. Wherry, E.J., et al., *Lineage relationship and protective immunity of memory CD8 T cell subsets.* Nat Immunol, 2003. **4**(3): p. 225-34.
198. Sallusto, F., J. Geginat, and A. Lanzavecchia, *Central memory and effector memory T cell subsets: function, generation, and maintenance.* Annu Rev Immunol, 2004. **22**: p. 745-63.
199. Sinclair, L.V., et al., *Phosphatidylinositol-3-OH kinase and nutrient-sensing mTOR pathways control T lymphocyte trafficking.* Nat Immunol, 2008. **9**(5): p. 513-21.
200. Manjunath, N., et al., *Effector differentiation is not prerequisite for generation of memory cytotoxic T lymphocytes.* J Clin Invest, 2001. **108**(6): p. 871-8.
201. Klebanoff, C.A., et al., *Central memory self/tumor-reactive CD8+ T cells confer superior antitumor immunity compared with effector memory T cells.* Proc Natl Acad Sci U S A, 2005. **102**(27): p. 9571-6.
202. Williams, M.A., A.J. Tyznik, and M.J. Bevan, *Interleukin-2 signals during priming are required for secondary expansion of CD8+ memory T cells.* Nature, 2006. **441**(7095): p. 890-3.
203. Zeng, R., et al., *Synergy of IL-21 and IL-15 in regulating CD8+ T cell expansion and function.* The Journal of experimental medicine, 2005. **201**(1): p. 139-148.
204. Di Carlo, E., et al., *IL-21 induces tumor rejection by specific CTL and IFN-gamma-dependent CXC chemokines in syngeneic mice.* J Immunol, 2004. **172**(3): p. 1540-7.
205. Furukawa, J., et al., *Interleukin-21 gene transfection into mouse bladder cancer cells results in tumor rejection through the cytotoxic T lymphocyte response.* J Urol, 2006. **176**(3): p. 1198-203.
206. Day, C.L., et al., *PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression.* Nature, 2006. **443**(7109): p. 350-4.
207. Wherry, E.J., *T cell exhaustion.* Nat Immunol, 2011. **12**(6): p. 492-9.

208. Efremova, M., et al., *Targeting immune checkpoints potentiates immunoediting and changes the dynamics of tumor evolution.* Nature communications, 2018. **9**(1): p. 32-32.
209. Castle, J.C., et al., *Exploiting the Mutanome for Tumor Vaccination.* Cancer Research, 2012. **72**(5): p. 1081.
210. Preston, C.C., et al., *The ratios of CD8+ T cells to CD4+CD25+ FOXP3+ and FOXP3- T cells correlate with poor clinical outcome in human serous ovarian cancer.* PloS one, 2013. **8**(11): p. e80063-e80063.
211. Twyman-Saint Victor, C., et al., *Radiation and dual checkpoint blockade activate non-redundant immune mechanisms in cancer.* Nature, 2015. **520**(7547): p. 373-7.
212. Juneja, V.R., et al., *PD-L1 on tumor cells is sufficient for immune evasion in immunogenic tumors and inhibits CD8 T cell cytotoxicity.* The Journal of experimental medicine, 2017. **214**(4): p. 895-904.
213. Matsuzaki, J., et al., *Tumor-infiltrating NY-ESO-1-specific CD8+ T cells are negatively regulated by LAG-3 and PD-1 in human ovarian cancer.* Proc Natl Acad Sci U S A, 2010. **107**(17): p. 7875-80.
214. Muenst, S., et al., *The presence of programmed death 1 (PD-1)-positive tumor-infiltrating lymphocytes is associated with poor prognosis in human breast cancer.* Breast Cancer Res Treat, 2013. **139**(3): p. 667-76.
215. Mandai, M., et al., *Dual Faces of IFNgamma in Cancer Progression: A Role of PD-L1 Induction in the Determination of Pro- and Antitumor Immunity.* Clin Cancer Res, 2016. **22**(10): p. 2329-34.
216. Garcia-Diaz, A., et al., *Interferon Receptor Signaling Pathways Regulating PD-L1 and PD-L2 Expression.* Cell Rep, 2017. **19**(6): p. 1189-1201.
217. Sheppard, K.A., et al., *PD-1 inhibits T-cell receptor induced phosphorylation of the ZAP70/CD3zeta signalosome and downstream signaling to PKCtheta.* FEBS Lett, 2004. **574**(1-3): p. 37-41.
218. Parry, R.V., et al., *CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms.* Mol Cell Biol, 2005. **25**(21): p. 9543-53.
219. Hui, E., et al., *T cell costimulatory receptor CD28 is a primary target for PD-1-mediated inhibition.* Science, 2017. **355**(6332): p. 1428-1433.
220. Teng, M.W.L., et al., *Classifying Cancers Based on T-cell Infiltration and PD-L1.* Cancer research, 2015. **75**(11): p. 2139-2145.
221. Snyder, A., et al., *Genetic Basis for Clinical Response to CTLA-4 Blockade in Melanoma.* New England Journal of Medicine, 2014. **371**(23): p. 2189-2199.
222. Hugo, W., et al., *Genomic and Transcriptomic Features of Response to Anti-PD-1 Therapy in Metastatic Melanoma.* Cell, 2016. **165**(1): p. 35-44.
223. Ueha, S., et al., *Robust Antitumor Effects of Combined Anti-CD4-Depleting Antibody and Anti-PD-1/PD-L1 Immune Checkpoint Antibody Treatment in Mice.* Cancer Immunol Res, 2015. **3**(6): p. 631-40.
224. Whiteside, T.L., *Disarming suppressor cells to improve immunotherapy.* Cancer Immunol Immunother, 2012. **61**(2): p. 283-288.
225. Alizadeh, D. and N. Larmonier, *Chemotherapeutic targeting of cancer-induced immunosuppressive cells.* Cancer Res, 2014. **74**(10): p. 2663-8.
226. Ngiow, S.F., et al., *A Threshold Level of Intratumor CD8+ T-cell PD1 Expression Dictates Therapeutic Response to Anti-PD1.* Cancer Res, 2015. **75**(18): p. 3800-11.
227. Juneja, V.R., et al., *PD-L1 on tumor cells is sufficient for immune evasion in immunogenic tumors and inhibits CD8 T cell cytotoxicity.* J Exp Med, 2017. **214**(4): p. 895-904.

228. Wu, T., et al., *The TCF1-Bcl6 axis counteracts type I interferon to repress exhaustion and maintain T cell stemness.* Science immunology, 2016. **1**(6): p. eaai8593.
229. Im, S.J., et al., *Defining CD8+ T cells that provide the proliferative burst after PD-1 therapy.* Nature, 2016. **537**(7620): p. 417-421.
230. Utzschneider, D.T., et al., *T Cell Factor 1-Expressing Memory-like CD8(+) T Cells Sustain the Immune Response to Chronic Viral Infections.* Immunity, 2016. **45**(2): p. 415-27.
231. Gajewski, T.F., H. Schreiber, and Y.-X. Fu, *Innate and adaptive immune cells in the tumor microenvironment.* Nature immunology, 2013. **14**(10): p. 1014-1022.
232. Lanitis, E., M. Irving, and G. Coukos, *Targeting the tumor vasculature to enhance T cell activity.* Curr Opin Immunol, 2015. **33**: p. 55-63.
233. Fuertes Marraco, S.A., et al., *Inhibitory Receptors Beyond T Cell Exhaustion.* Frontiers in immunology, 2015. **6**: p. 310-310.
234. Chu, H.H., et al., *Continuous Effector CD8(+) T Cell Production in a Controlled Persistent Infection Is Sustained by a Proliferative Intermediate Population.* Immunity, 2016. **45**(1): p. 159-171.
235. Zhang, X., et al., *Potent and selective stimulation of memory-phenotype CD8+ T cells in vivo by IL-15.* Immunity, 1998. **8**(5): p. 591-9.
236. Mitchell, D.M., E.V. Ravkov, and M.A. Williams, *Distinct roles for IL-2 and IL-15 in the differentiation and survival of CD8+ effector and memory T cells.* J Immunol, 2010. **184**(12): p. 6719-30.
237. Friedmann, M.C., et al., *Different interleukin 2 receptor beta-chain tyrosines couple to at least two signaling pathways and synergistically mediate interleukin 2-induced proliferation.* Proc Natl Acad Sci U S A, 1996. **93**(5): p. 2077-82.
238. Lin, J.X., et al., *Critical Role of STAT5 transcription factor tetramerization for cytokine responses and normal immune function.* Immunity, 2012. **36**(4): p. 586-99.
239. Depper, J.M., et al., *Interleukin 2 (IL-2) augments transcription of the IL-2 receptor gene.* Proc Natl Acad Sci U S A, 1985. **82**(12): p. 4230-4.
240. Kalia, V., et al., *Prolonged interleukin-2Ralpha expression on virus-specific CD8+ T cells favors terminal-effector differentiation in vivo.* Immunity, 2010. **32**(1): p. 91-103.
241. Anderson, D.M., et al., *Functional characterization of the human interleukin-15 receptor alpha chain and close linkage of IL15RA and IL2RA genes.* J Biol Chem, 1995. **270**(50): p. 29862-9.
242. Stonier, S.W., et al., *Dendritic cells drive memory CD8 T-cell homeostasis via IL-15 transpresentation.* Blood, 2008. **112**(12): p. 4546-4554.
243. Musso, T., et al., *Human monocytes constitutively express membrane-bound, biologically active, and interferon-gamma-upregulated interleukin-15.* Blood, 1999. **93**(10): p. 3531-9.
244. Tan, J.T., et al., *Interleukin (IL)-15 and IL-7 jointly regulate homeostatic proliferation of memory phenotype CD8+ cells but are not required for memory phenotype CD4+ cells.* J Exp Med, 2002. **195**(12): p. 1523-32.
245. Kennedy, M.K., et al., *Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice.* The Journal of experimental medicine, 2000. **191**(5): p. 771-780.
246. Ku, C.C., et al., *Control of homeostasis of CD8+ memory T cells by opposing cytokines.* Science, 2000. **288**(5466): p. 675-8.
247. Bianchi, T., et al., *c-Myc acts downstream of IL-15 in the regulation of memory CD8 T-cell homeostasis.* Blood, 2006. **107**(10): p. 3992-9.

248. van der Windt, G.J.W., et al., *Mitochondrial respiratory capacity is a critical regulator of CD8+ T cell memory development.* Immunity, 2012. **36**(1): p. 68-78.
249. Judge, A.D., et al., *Interleukin 15 controls both proliferation and survival of a subset of memory-phenotype CD8(+) T cells.* The Journal of experimental medicine, 2002. **196**(7): p. 935-946.
250. Rubinstein, M.P., et al., *IL-7 and IL-15 differentially regulate CD8+ T-cell subsets during contraction of the immune response.* Blood, 2008. **112**(9): p. 3704-3712.
251. Sandau, M.M., et al., *IL-15 regulates both quantitative and qualitative features of the memory CD8 T cell pool.* Journal of immunology (Baltimore, Md. : 1950), 2010. **184**(1): p. 35-44.
252. Schluns, K.S., et al., *Cutting edge: requirement for IL-15 in the generation of primary and memory antigen-specific CD8 T cells.* J Immunol, 2002. **168**(10): p. 4827-31.
253. Zhang, Q. and F.G. Lakkis, *Memory T Cell Migration.* Frontiers in immunology, 2015. **6**: p. 504-504.
254. Zhou, X., et al., *Differentiation and persistence of memory CD8(+) T cells depend on T cell factor 1.* Immunity, 2010. **33**(2): p. 229-240.
255. Intlekofer, A.M., et al., *Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin.* Nat Immunol, 2005. **6**(12): p. 1236-44.
256. Szabo, S.J., et al., *Distinct effects of T-bet in TH1 lineage commitment and IFN-gamma production in CD4 and CD8 T cells.* Science, 2002. **295**(5553): p. 338-42.
257. Pearce, E.L., et al., *Control of effector CD8+ T cell function by the transcription factor Eomesodermin.* Science, 2003. **302**(5647): p. 1041-3.
258. Joshi, N.S., et al., *Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor.* Immunity, 2007. **27**(2): p. 281-95.
259. Paley, M.A., et al., *Progenitor and terminal subsets of CD8+ T cells cooperate to contain chronic viral infection.* Science, 2012. **338**(6111): p. 1220-5.
260. Gattinoni, L., et al., *A human memory T cell subset with stem cell-like properties.* Nat Med, 2011. **17**(10): p. 1290-7.
261. Cieri, N., et al., *IL-7 and IL-15 instruct the generation of human memory stem T cells from naive precursors.* Blood, 2013. **121**(4): p. 573-84.
262. Gattinoni, L., et al., *Wnt signaling arrests effector T cell differentiation and generates CD8+ memory stem cells.* Nat Med, 2009. **15**(7): p. 808-13.
263. Zhang, Y., et al., *Host-reactive CD8+ memory stem cells in graft-versus-host disease.* Nat Med, 2005. **11**(12): p. 1299-305.
264. Scholzen, T. and J. Gerdes, *The Ki-67 protein: from the known and the unknown.* J Cell Physiol, 2000. **182**(3): p. 311-22.
265. Jung, Y.W., et al., *CCR7 expression alters memory CD8 T-cell homeostasis by regulating occupancy in IL-7- and IL-15-dependent niches.* Proc Natl Acad Sci U S A, 2016. **113**(29): p. 8278-83.
266. Unsoeld, H. and H. Pircher, *Complex memory T-cell phenotypes revealed by coexpression of CD62L and CCR7.* Journal of virology, 2005. **79**(7): p. 4510-4513.
267. Popescu, I., et al., *T-bet:Eomes balance, effector function, and proliferation of cytomegalovirus-specific CD8+ T cells during primary infection differentiates the capacity for durable immune control.* Journal of immunology (Baltimore, Md. : 1950), 2014. **193**(11): p. 5709-5722.
268. Buggert, M., et al., *T-bet and Eomes are differentially linked to the exhausted phenotype of CD8+ T cells in HIV infection.* PLoS pathogens, 2014. **10**(7): p. e1004251-e1004251.

269. Asakura, A., et al., *Myogenic specification of side population cells in skeletal muscle.* J Cell Biol, 2002. **159**(1): p. 123-34.
270. Tamaki, T., et al., *Identification of myogenic-endothelial progenitor cells in the interstitial spaces of skeletal muscle.* The Journal of cell biology, 2002. **157**(4): p. 571-577.
271. Howell, J.C., M.C. Yoder, and E.F. Srour, *Hematopoietic potential of murine skeletal muscle-derived CD45(-)Sca-1(+)c-kit(-) cells.* Exp Hematol, 2002. **30**(8): p. 915-24.
272. Jahn, T., et al., *Analysing c-kit internalization using a functional c-kit-EGFP chimera containing the fluorochrome within the extracellular domain.* Oncogene, 2002. **21**(29): p. 4508-20.
273. Chang, J.T., et al., *Asymmetric T lymphocyte division in the initiation of adaptive immune responses.* Science, 2007. **315**(5819): p. 1687-91.
274. Snell, L.M., et al., *CD8(+) T Cell Priming in Established Chronic Viral Infection Preferentially Directs Differentiation of Memory-like Cells for Sustained Immunity.* Immunity, 2018. **49**(4): p. 678-694.e5.
275. Flynn, J.K. and P.R. Gorry, *Stem memory T cells (TSCM)-their role in cancer and HIV immunotherapies.* Clinical & translational immunology, 2014. **3**(7): p. e20-e20.
276. Ribeiro, S.P., et al., *The CD8(+) memory stem T cell (T(SCM)) subset is associated with improved prognosis in chronic HIV-1 infection.* J Virol, 2014. **88**(23): p. 13836-44.
277. Metallo, C.M. and M.G. Vander Heiden, *Understanding metabolic regulation and its influence on cell physiology.* Molecular cell, 2013. **49**(3): p. 388-398.
278. Cui, W. and S.M. Kaech, *Generation of effector CD8+ T cells and their conversion to memory T cells.* Immunological reviews, 2010. **236**: p. 151-166.
279. Wang, R., et al., *The transcription factor Myc controls metabolic reprogramming upon T lymphocyte activation.* Immunity, 2011. **35**(6): p. 871-882.
280. Rathmell, J.C., et al., *In the absence of extrinsic signals, nutrient utilization by lymphocytes is insufficient to maintain either cell size or viability.* Mol Cell, 2000. **6**(3): p. 683-92.
281. Bauer, D.E., et al., *Cytokine stimulation of aerobic glycolysis in hematopoietic cells exceeds proliferative demand.* Faseb j, 2004. **18**(11): p. 1303-5.
282. Buck, M.D., D. O'Sullivan, and E.L. Pearce, *T cell metabolism drives immunity.* J Exp Med, 2015. **212**(9): p. 1345-60.
283. O'Sullivan, D., et al., *Memory CD8(+) T cells use cell-intrinsic lipolysis to support the metabolic programming necessary for development.* Immunity, 2014. **41**(1): p. 75-88.
284. Green, W.D. and M.A. Beck, *Obesity altered T cell metabolism and the response to infection.* Current Opinion in Immunology, 2017. **46**: p. 1-7.
285. Chang, C.H., et al., *Posttranscriptional control of T cell effector function by aerobic glycolysis.* Cell, 2013. **153**(6): p. 1239-51.
286. van der Windt, G.J., et al., *CD8 memory T cells have a bioenergetic advantage that underlies their rapid recall ability.* Proc Natl Acad Sci U S A, 2013. **110**(35): p. 14336-41.
287. Vakifahmetoglu-Norberg, H., A.T. Ouchida, and E. Norberg, *The role of mitochondria in metabolism and cell death.* Biochem Biophys Res Commun, 2017. **482**(3): p. 426-431.
288. Buck, M.D., et al., *Mitochondrial Dynamics Controls T Cell Fate through Metabolic Programming.* Cell, 2016. **166**(1): p. 63-76.

289. Preston, G.C., et al., *Single cell tuning of Myc expression by antigen receptor signal strength and interleukin-2 in T lymphocytes.* The EMBO journal, 2015. **34**(15): p. 2008-2024.
290. Finlay, D.K., et al., *PDK1 regulation of mTOR and hypoxia-inducible factor 1 integrate metabolism and migration of CD8+ T cells.* J Exp Med, 2012. **209**(13): p. 2441-53.
291. Hukelmann, J.L., et al., *The cytotoxic T cell proteome and its shaping by the kinase mTOR.* Nat Immunol, 2016. **17**(1): p. 104-12.
292. Cho, J.H., et al., *Unique features of naive CD8+ T cell activation by IL-2.* J Immunol, 2013. **191**(11): p. 5559-73.
293. Pollizzi, K.N., et al., *mTORC1 and mTORC2 selectively regulate CD8$^+$ T cell differentiation.* The Journal of clinical investigation, 2015. **125**(5): p. 2090-2108.
294. MacIver, N.J., R.D. Michalek, and J.C. Rathmell, *Metabolic regulation of T lymphocytes.* Annual review of immunology, 2013. **31**: p. 259-283.
295. Finlay, D. and D.A. Cantrell, *Metabolism, migration and memory in cytotoxic T cells.* Nature reviews. Immunology, 2011. **11**(2): p. 109-117.
296. Cornish, G.H., L.V. Sinclair, and D.A. Cantrell, *Differential regulation of T-cell growth by IL-2 and IL-15.* Blood, 2006. **108**(2): p. 600-8.
297. Araki, K., et al., *mTOR regulates memory CD8 T-cell differentiation.* Nature, 2009. **460**(7251): p. 108-12.
298. Pollizzi, K.N., et al., *Asymmetric inheritance of mTORC1 kinase activity during division dictates CD8(+) T cell differentiation.* Nat Immunol, 2016. **17**(6): p. 704-11.
299. Jung, Y.J., et al., *IL-1beta-mediated up-regulation of HIF-1alpha via an NFkappaB/COX-2 pathway identifies HIF-1 as a critical link between inflammation and oncogenesis.* Faseb j, 2003. **17**(14): p. 2115-7.
300. Dang, E.V., et al., *Control of T(H)17/T(reg) balance by hypoxia-inducible factor 1.* Cell, 2011. **146**(5): p. 772-784.
301. Shi, L.Z., et al., *HIF1alpha-dependent glycolytic pathway orchestrates a metabolic checkpoint for the differentiation of TH17 and Treg cells.* The Journal of experimental medicine, 2011. **208**(7): p. 1367-1376.
302. Mueckler, M., et al., *Sequence and structure of a human glucose transporter.* Science, 1985. **229**(4717): p. 941-5.
303. Thorens, B. and M. Mueckler, *Glucose transporters in the 21st Century.* Am J Physiol Endocrinol Metab, 2010. **298**(2): p. E141-5.
304. Wilson, J.E., *Isozymes of mammalian hexokinase: structure, subcellular localization and metabolic function.* J Exp Biol, 2003. **206**(Pt 12): p. 2049-57.
305. Deberardinis, R.J., J.J. Lum, and C.B. Thompson, *Phosphatidylinositol 3-kinase-dependent modulation of carnitine palmitoyltransferase 1A expression regulates lipid metabolism during hematopoietic cell growth.* J Biol Chem, 2006. **281**(49): p. 37372-80.
306. Ramsay, R.R. and V.A. Zammit, *Carnitine acyltransferases and their influence on CoA pools in health and disease.* Mol Aspects Med, 2004. **25**(5-6): p. 475-93.
307. Maciver, N.J., et al., *Glucose metabolism in lymphocytes is a regulated process with significant effects on immune cell function and survival.* J Leukoc Biol, 2008. **84**(4): p. 949-57.
308. Sukumar, M., et al., *Inhibiting glycolytic metabolism enhances CD8+ T cell memory and antitumor function.* The Journal of clinical investigation, 2013. **123**(10): p. 4479-4488.
309. Tzameli, I., *The evolving role of mitochondria in metabolism.* Trends Endocrinol Metab, 2012. **23**(9): p. 417-9.

310. Masson, J.J.R., et al., *Assessment of metabolic and mitochondrial dynamics in CD4+ and CD8+ T cells in virologically suppressed HIV-positive individuals on combination antiretroviral therapy.* PloS one, 2017. **12**(8): p. e0183931-e0183931.
311. Ferrick, D.A., A. Neilson, and C. Beeson, *Advances in measuring cellular bioenergetics using extracellular flux.* Drug Discov Today, 2008. **13**(5-6): p. 268-74.
312. Nicholls, D.G., *Spare respiratory capacity, oxidative stress and excitotoxicity.* Biochem Soc Trans, 2009. **37**(Pt 6): p. 1385-8.
313. Nicholls, D.G., et al., *Bioenergetic profile experiment using C2C12 myoblast cells.* J Vis Exp, 2010(46).
314. Eaton, S., K. Bartlett, and M. Pourfarzam, *Mammalian mitochondrial beta-oxidation.* The Biochemical journal, 1996. **320 (Pt 2)**(Pt 2): p. 345-357.
315. Houten, S.M. and R.J.A. Wanders, *A general introduction to the biochemistry of mitochondrial fatty acid β-oxidation.* Journal of inherited metabolic disease, 2010. **33**(5): p. 469-477.
316. Man, K., et al., *Transcription Factor IRF4 Promotes CD8(+) T Cell Exhaustion and Limits the Development of Memory-like T Cells during Chronic Infection.* Immunity, 2017. **47**(6): p. 1129-1141.e5.
317. Klein-Hessling, S., et al., *NFATc1 controls the cytotoxicity of CD8(+) T cells.* Nat Commun, 2017. **8**(1): p. 511.
318. Seagroves, T.N., et al., *Transcription factor HIF-1 is a necessary mediator of the pasteur effect in mammalian cells.* Mol Cell Biol, 2001. **21**(10): p. 3436-44.
319. Kim, J.W., et al., *HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia.* Cell Metab, 2006. **3**(3): p. 177-85.
320. Papandreou, I., et al., *HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption.* Cell Metab, 2006. **3**(3): p. 187-97.
321. Huang, et al., *HIF-1-mediated suppression of acyl-CoA dehydrogenases and fatty acid oxidation is critical for cancer progression.* Cell Rep, 2014. **8**(6): p. 1930-1942.
322. Palazon, A., et al., *An HIF-1alpha/VEGF-A Axis in Cytotoxic T Cells Regulates Tumor Progression.* Cancer Cell, 2017. **32**(5): p. 669-683.e5.
323. Cui, G., et al., *IL-7-Induced Glycerol Transport and TAG Synthesis Promotes Memory CD8+ T Cell Longevity.* Cell, 2015. **161**(4): p. 750-761.
324. Loschinski, R., et al., *IL-21 modulates memory and exhaustion phenotype of T-cells in a fatty acid oxidation-dependent manner.* Oncotarget, 2018. **9**(17): p. 13125-13138.
325. Youle, R.J. and A.M. van der Bliek, *Mitochondrial fission, fusion, and stress.* Science, 2012. **337**(6098): p. 1062-5.
326. Vander Heiden, M.G., L.C. Cantley, and C.B. Thompson, *Understanding the Warburg effect: the metabolic requirements of cell proliferation.* Science, 2009. **324**(5930): p. 1029-33.
327. Rizzuto, R., et al., *Mitochondria as sensors and regulators of calcium signalling.* Nat Rev Mol Cell Biol, 2012. **13**(9): p. 566-78.
328. Sukumar, M., et al., *Mitochondrial Membrane Potential Identifies Cells with Enhanced Stemness for Cellular Therapy.* Cell Metab, 2016. **23**(1): p. 63-76.
329. Simsek, T., et al., *The distinct metabolic profile of hematopoietic stem cells reflects their location in a hypoxic niche.* Cell Stem Cell, 2010. **7**(3): p. 380-90.
330. de Almeida, M.J., et al., *Dye-Independent Methods Reveal Elevated Mitochondrial Mass in Hematopoietic Stem Cells.* Cell Stem Cell, 2017. **21**(6): p. 725-729.e4.
331. Ito, K., et al., *Regulation of oxidative stress by ATM is required for self-renewal of haematopoietic stem cells.* Nature, 2004. **431**(7011): p. 997-1002.

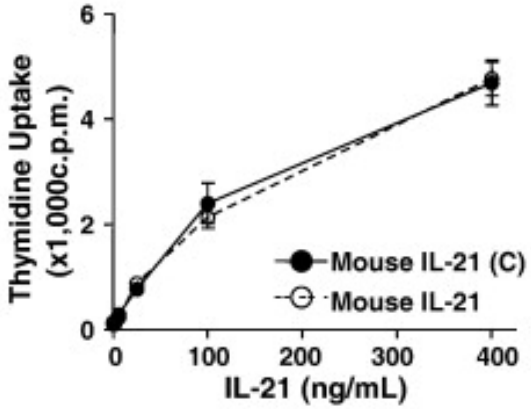
332. Jang, Y.Y. and S.J. Sharkis, *A low level of reactive oxygen species selects for primitive hematopoietic stem cells that may reside in the low-oxygenic niche.* Blood, 2007. **110**(8): p. 3056-63.
333. Yu, W.M., et al., *Metabolic regulation by the mitochondrial phosphatase PTPMT1 is required for hematopoietic stem cell differentiation.* Cell Stem Cell, 2013. **12**(1): p. 62-74.
334. Jun, J.C., et al., *Hypoxia-Inducible Factors and Cancer.* Current sleep medicine reports, 2017. **3**(1): p. 1-10.
335. Masoud, G.N. and W. Li, *HIF-1α pathway: role, regulation and intervention for cancer therapy.* Acta pharmaceutica Sinica. B, 2015. **5**(5): p. 378-389.
336. Takubo, K., et al., *Regulation of the HIF-1alpha level is essential for hematopoietic stem cells.* Cell Stem Cell, 2010. **7**(3): p. 391-402.
337. Li, L., et al., *Hypoxia inducible factor-1alpha (HIF-1alpha) is required for neural stem cell maintenance and vascular stability in the adult mouse SVZ.* J Neurosci, 2014. **34**(50): p. 16713-9.
338. Martinez, V.G., et al., *Overexpression of hypoxia-inducible factor 1 alpha improves immunomodulation by dental mesenchymal stem cells.* Stem Cell Res Ther, 2017. **8**(1): p. 208.
339. Lv, B., et al., *Hypoxia inducible factor 1α promotes survival of mesenchymal stem cells under hypoxia.* American journal of translational research, 2017. **9**(3): p. 1521-1529.
340. Jung, J.E., et al., *STAT3 is a potential modulator of HIF-1-mediated VEGF expression in human renal carcinoma cells.* Faseb j, 2005. **19**(10): p. 1296-8.
341. Xu, Q., et al., *Targeting Stat3 blocks both HIF-1 and VEGF expression induced by multiple oncogenic growth signaling pathways.* Oncogene, 2005. **24**(36): p. 5552-60.
342. Chen, C., et al., *Regulation of glut1 mRNA by hypoxia-inducible factor-1. Interaction between H-ras and hypoxia.* J Biol Chem, 2001. **276**(12): p. 9519-25.
343. Curtsinger, J.M., D.C. Lins, and M.F. Mescher, *Signal 3 determines tolerance versus full activation of naive CD8 T cells: dissociating proliferation and development of effector function.* The Journal of experimental medicine, 2003. **197**(9): p. 1141-1151.
344. Williams, M.A. and M.J. Bevan, *Effector and memory CTL differentiation.* Annu Rev Immunol, 2007. **25**: p. 171-92.
345. Kennedy, M.K., et al., *Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice.* J Exp Med, 2000. **191**(5): p. 771-80.
346. Cui, G., et al., *IL-7-Induced Glycerol Transport and TAG Synthesis Promotes Memory CD8(+) T Cell Longevity.* Cell, 2015. **161**(4): p. 750-761.
347. Crompton, J.G., et al., *Lineage relationship of CD8(+) T cell subsets is revealed by progressive changes in the epigenetic landscape.* Cell Mol Immunol, 2016. **13**(4): p. 502-13.
348. Hinrichs, C.S., et al., *Human effector CD8+ T cells derived from naive rather than memory subsets possess superior traits for adoptive immunotherapy.* Blood, 2011. **117**(3): p. 808-14.
349. Wang, X., et al., *Engraftment of human central memory-derived effector CD8+ T cells in immunodeficient mice.* Blood, 2011. **117**(6): p. 1888-98.
350. Berger, C., et al., *Adoptive transfer of effector CD8+ T cells derived from central memory cells establishes persistent T cell memory in primates.* J Clin Invest, 2008. **118**(1): p. 294-305.
351. van Faassen, H., et al., *Reducing the stimulation of CD8+ T cells during infection with intracellular bacteria promotes differentiation primarily into a central (CD62LhighCD44high) subset.* J Immunol, 2005. **174**(9): p. 5341-50.

352. Bondanza, A., et al., *Suicide gene therapy of graft-versus-host disease induced by central memory human T lymphocytes.* Blood, 2006. **107**(5): p. 1828-36.
353. Gett, A.V., et al., *T cell fitness determined by signal strength.* Nat Immunol, 2003. **4**(4): p. 355-60.
354. Manjunath, N., et al., *Effector differentiation is not prerequisite for generation of memory cytotoxic T lymphocytes.* Journal of Clinical Investigation, 2001. **108**(6): p. 871-878.
355. Weninger, W., et al., *Migratory Properties of Naive, Effector, and Memory Cd8(+) T Cells.* The Journal of Experimental Medicine, 2001. **194**(7): p. 953-966.
356. Cieri, N., et al., *IL-7 and IL-15 instruct the generation of human memory stem T cells from naive precursors.* Blood, 2013. **121**(4): p. 573.
357. Marzo, A.L., et al., *Initial T cell frequency dictates memory CD8(+) T cell lineage commitment.* Nature immunology, 2005. **6**(8): p. 793-799.
358. Lin, W.-H.W., et al., *CD8(+) T Lymphocyte Self-Renewal during Effector Cell Determination.* Cell reports, 2016. **17**(7): p. 1773-1782.
359. Paley, M.A., et al., *Progenitor and terminal subsets of CD8+ T cells cooperate to contain chronic viral infection.* Science (New York, N.Y.), 2012. **338**(6111): p. 1220-1225.
360. Richer, M.J., et al., *Inflammatory IL-15 is required for optimal memory T cell responses.* The Journal of clinical investigation, 2015. **125**(9): p. 3477-3490.
361. Fahey, L.M., et al., *Viral persistence redirects CD4 T cell differentiation toward T follicular helper cells.* J Exp Med, 2011. **208**(5): p. 987-99.
362. Wherry, E.J. and R. Ahmed, *Memory CD8 T-cell differentiation during viral infection.* J Virol, 2004. **78**(11): p. 5535-45.
363. Gallimore, A., et al., *Induction and exhaustion of lymphocytic choriomeningitis virus-specific cytotoxic T lymphocytes visualized using soluble tetrameric major histocompatibility complex class I-peptide complexes.* J Exp Med, 1998. **187**(9): p. 1383-93.
364. Zajac, A.J., et al., *Viral immune evasion due to persistence of activated T cells without effector function.* J Exp Med, 1998. **188**(12): p. 2205-13.
365. Brinkman, C.C., J.D. Peske, and V.H. Engelhard, *Peripheral tissue homing receptor control of naïve, effector, and memory CD8 T cell localization in lymphoid and non-lymphoid tissues.* Frontiers in immunology, 2013. **4**: p. 241-241.
366. Petrova, V., et al., *The hypoxic tumour microenvironment.* Oncogenesis, 2018. **7**(1): p. 10-10.
367. Barsoum, I.B., et al., *Mechanisms of hypoxia-mediated immune escape in cancer.* Cancer Res, 2014. **74**(24): p. 7185-90.
368. Sun, J., et al., *Hypoxia induces T-cell apoptosis by inhibiting chemokine C receptor 7 expression: the role of adenosine receptor A(2).* Cell Mol Immunol, 2010. **7**(1): p. 77-82.
369. Colbeck, E.J., et al., *Tertiary Lymphoid Structures in Cancer: Drivers of Antitumor Immunity, Immunosuppression, or Bystander Sentinels in Disease?* Frontiers in immunology, 2017. **8**: p. 1830-1830.
370. Sautes-Fridman, C., et al., *Tertiary Lymphoid Structures in Cancers: Prognostic Value, Regulation, and Manipulation for Therapeutic Intervention.* Front Immunol, 2016. **7**: p. 407.
371. Jenkins, R.W., D.A. Barbie, and K.T. Flaherty, *Mechanisms of resistance to immune checkpoint inhibitors.* British journal of cancer, 2018. **118**(1): p. 9-16.
372. Pitt, J.M., et al., *Resistance Mechanisms to Immune-Checkpoint Blockade in Cancer: Tumor-Intrinsic and -Extrinsic Factors.* Immunity, 2016. **44**(6): p. 1255-69.

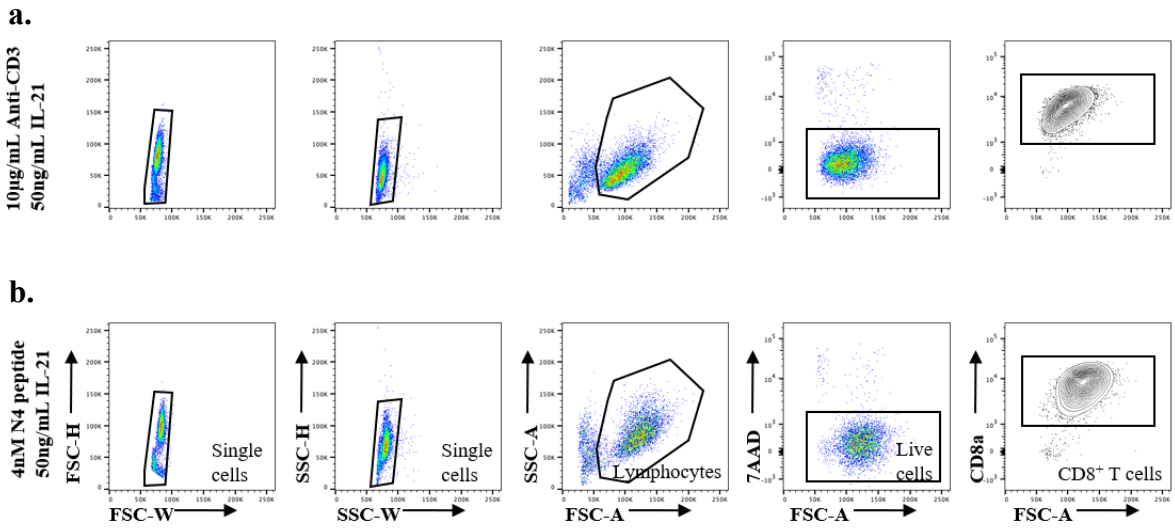
373. Marincola, F.M., et al., *Escape of human solid tumors from T-cell recognition: molecular mechanisms and functional significance.* Adv Immunol, 2000. **74**: p. 181-273.
374. Bronte, V., et al., *Boosting antitumor responses of T lymphocytes infiltrating human prostate cancers.* J Exp Med, 2005. **201**(8): p. 1257-68.
375. O'Donnell, J.S., et al., *Resistance to PD1/PDL1 checkpoint inhibition.* Cancer Treat Rev, 2017. **52**: p. 71-81.
376. Pauken, K.E., et al., *Epigenetic stability of exhausted T cells limits durability of reinvigoration by PD-1 blockade.* Science, 2016. **354**(6316): p. 1160-1165.
377. Sen, D.R., et al., *The epigenetic landscape of T cell exhaustion.* Science, 2016. **354**(6316): p. 1165-1169.
378. Ribas, A., et al., *PD-1 Blockade Expands Intratumoral Memory T Cells.* Cancer Immunol Res, 2016. **4**(3): p. 194-203.

Appendix

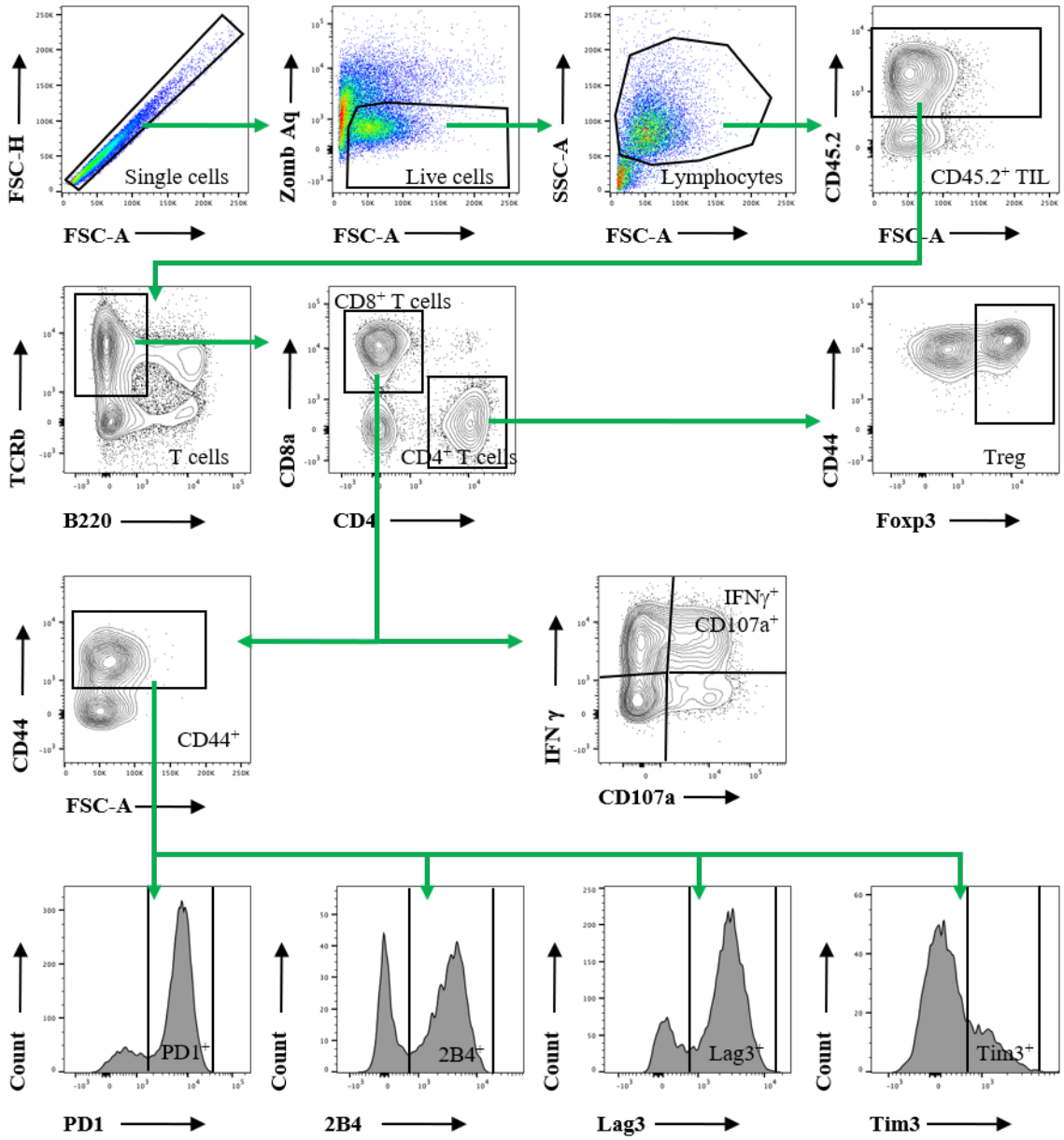
Supplementary Figures



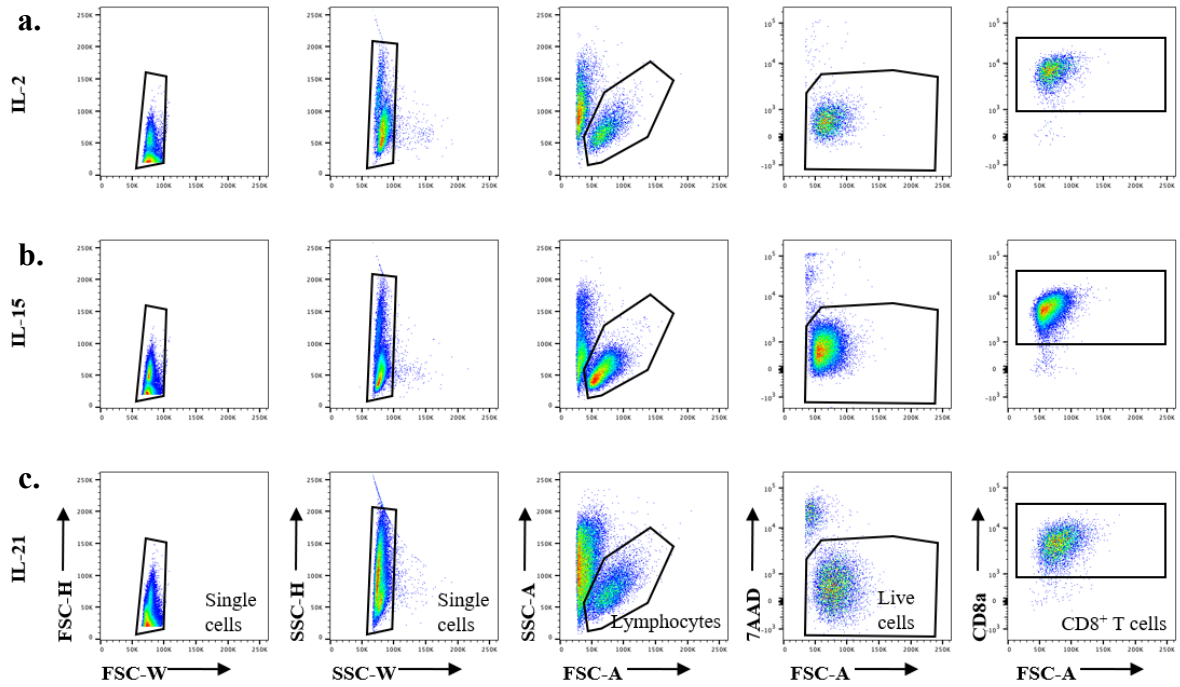
Supplementary Figure S1. In-house produced murine IL-21 has comparable activity to commercial IL-21. Ba/F3-msIL-21R was cultured in the presence of mouse IL-21 [^{3}H]-labelled thymidine was added for the last 18 h of culture. Incorporated radioactivities were measured. Mouse IL-21 (C): proteins from the commercial supply (Peprotech). Figure from [158].



Supplementary Figure S2. Gating strategy for *in vitro* cultured CD8$^+$ T cells. (a) Polyclonal cells and **(b)** transgenic OT-I cells were first gated for singlets, lymphocytes, 7AAD$^-$ and finally CD8a$^+$.



Supplementary Figure S3. Gating strategy for TIL. TIL were first gated for singlets, Zombie Aq$^-$, lymphocytes, CD45.2$^+$, TCRb$^+$ B220$^-$ and then either CD8a$^+$ or CD4$^+$. CD8$^+$ T cells and CD4$^+$ T cells were subsequently used for analyses of other markers.



Supplementary Figure S4. Gating strategy for *in vitro* cultured CD8$^+$ T cells. Activated CD8$^+$ T cells that were rested in **(a)** IL-2, **(b)** IL-15 and **(c)** IL-21 were first gated for singlets, lymphocytes, 7AAD$^-$ and finally CD8a$^+$.