The Influence of Environmental Cues on $CD8^+$ T Cell Function and Metabolism

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To John, Lenny, Elo and Lydia.

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Abstract

$CD8^+$ T lymphocytes play a critical role in our protection against pathogens and tumours. $CD8^+$ T cell function is shaped by a myriad of environmental cues that include TCR-stimulation, co-stimuli, pro- and anti-inflammatory cytokines and nutrient availability. Understanding how $CD8^+$ T cells integrate these signals and how they influence T cell output is crucial to better comprehend immune regulation and to find novel targets for immunotherapy.

During the last decade, it has become more evident that T cell metabolism tightly controls T cell fate. This principle has provided a novel signalling network that can be manipulated in order to modulate T cell responses but that still needs to be fully explored. In this thesis, I determine the impact of several environmental cues on $CD8^+$ T cell activation and metabolism. First, the study of signal 1 (TCR-triggering), signal 2 (CD28) and signal 3 (IL-12 or IFNα) revealed that these cooperate to induce a full T cell activation state, contributing separately to T cell functionality and metabolism. Second, the study of the anti-inflammatory cytokine TGFβ established that its effect on the suppression of $CD8^+$ T cell activation is predominantly mediated by the repression of Myc, ultimately impeding TCR-driven metabolic reprogramming. Finally, the study of asparagine (Asn) deprivation showed that $CD8^+$ T cells require the supplementation of this amino acid during early stages of T cell activation, but lose this requirement upon upregulation of the enzyme asparagine synthetase (ASNS). These findings demonstrate that modulation of T cell responses by environmental cues are linked to the regulation of metabolic pathways and shed light into new mechanisms that might be exploited to finely tune T cell function.

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Abbreviations

γc	γ-chain
2-DG	2-deoxyglucose
3PG	3-phosphoglycerate
4E-BPs	4E-binding proteins
AA	Amino acid
ACC1	Acetyl-CoA carboxylase
ACL	ATP citrate lyase
ACT	Adoptive cell transfer
ADAP	Adhesion- and degranulation-promoting adaptor protein
ALL	Acute lymphoblastic leukaemia
AP-1	Activator-protein 1
APCs	Antigen presenting cells
AQP9	Aquaporin 9
ASCT2	Alanine serine cysteine transporter 2
Asn	Asparagine
ASNase	Asparaginase
ASNS	Asparagine synthetase
ATF1	Activating transcription factor 1
ATF4	Activating transcription factor 4
ATP	Adenosine triphosphate
BCR	B cell receptor
BM	Bone marrow
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
CamKK2	Calmodulin-dependent protein kinase kinase-2
CARMA-1	Caspase recruitment domain-containing membrane-associated guanylate kinase protein-1
CAT	Cationic AA transporter
ChEA	ChIP Enrichment Analysis
ChIP	Chromatin immunoprecipitation
CHX	Cycloheximide

CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CPT1	Carnitine palmitoyltransferase 1
CRAC	Calcium-release activated calcium
Csk	C-terminal Src kinase
CTLA-4	Cytotoxic T lymphocyte antigen 4
CTLs	Cytotoxic T lymphocytes
DAG	Diacylglycerol
DCs	Dendritic cells
DHAP	Dihydroxyacetone-phosphate
DMEM	Dulbecco's modified eagle's medium
DN	Double negative
dNTP	Deoxynucleotide
DP	Double positive
EAA	Essential amino acid
EAE	Experimental autoimmune encephalomyelitis
ECAR	Extracellular consumption rate
ELISA	Enzyme-linked immunosorbent assay
Eomes	Eomesodermin
ER	Endoplasmic reticulum
ERRα	Estrogen-related receptor α
ETC	Electron transport chain
FACS	Fluorescence-activated cell sorting
FAD	Flavin adenine dinucleotide
FAO	Fatty acid β-oxidation
FAS	Fatty acid synthesis
FasL	Fas ligand
FASN	Fatty acid synthase
FBS	Fetal bovine serum
FC	Fold-change
FOXO1	Forkhead box protein O1
FoxP3	Forkhead box P3

FSC	Forward scatter
G6P	Glucose-6-phosphate
GADS	GRB2-related adaptor protein
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCN2	General control nonderepressible 2
GEF	Guanine-nucleotide exchange factor
Gln	Glutamine
GrA	Granzyme A
GrB	Granzyme B
GRB2	Growth factor receptor-bound protein 2
GSH	Glutathione
GSK3	Glycogen synthase kinase 3
HBSS	Hank's balanced salt solution
HIF1α	Hypoxia-inducible factor 1 α
HK	Hexokinase
HLA	Human leukocyte antigen
HMGCR	Hydroxymethylglutaryl-CoA reductase
HRP	Horseradish-peroxidase
HSCs	Hematopoietic stem cells
ICAM-1	Intracellular adhesion molecule 1
ICB	Immune checkpoint blockade
IFNγ	Interferon γ
IFNAR	Interferon-alpha receptor
Ig	Immunoglobulin
Ig-SF	Ig-superfamily
IKK	IκB kinase
IL	Interleukin
IL-7Rα	IL-7 receptor α chain
ILCs	Innate lymphoid cells
IP_3	Inositol 1,4,5-triphosphate
IRF4	Interferon regulatory factor 4
IS	Immunological synapse

ITAM	Immunoreceptor tyrosine-based activation motif
ITK	Interleukin-2-inducible T cell kinase
JNK	c-Jun N-terminal kinase
KEGG	Kyoto Encyclopedia of Genes and Genomes
Kyn	Kynurenine
LAG3	Lymphocyte activation gene 3
LAP	Latency-associated peptide
LAT	Linker for activated T cells
LDH	Lactate dehydrogenase
LFA-1	Lymphocyte function-associated antigen 1
LKB1	Liver kinase B1
LPS	Lipopolysaccharides
LTα	Lymphotoxin α
Lyp	Lymphoid tyrosine phosphatase
MALT	Mucosa-associated lymphoid tissue
MAPK	Mitogen-activated protein kinase
MDSCs	Myeloid-derived suppressor cells
MEK1	MAPK/ERK Kinase 1
MHC-I	Major histocompatibility complex class I
MHC-II	Major histocompatibility complex class II
MPECs	Memory-precursor effector cells
MTFA	Mitochondrial transcription factor A
mTOR	Mechanistic Target of Rapamycin
mTORC	mTOR complex
NAD	Nicotinamide adenine dinucleotide
NCK1	Non-catalytic region of tyrosine kinase adaptor protein 1
NEMO	NF-κB essential modulator
NF-κB	Nuclear factor-κB
NF90	Nuclear factor 90
NFAT	Nuclear factor of activated T cells
NK	Natural killers
NP	4-Hydroxy-3-nitrophenylaacetyl

OCR	Oxygen consumption rate
OPP	O-propargyl- puromycin
OVA	Ovalbumin
OXPHOS	Oxidative phosphorylation
PAMPs	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PD-1	Programmed cell death 1
PDH	Pyruvate dehydrogenase
PDK1	Phosphoinositide-dependent kinase 1
PEP	Phosphoenolpyruvate
PGC1α	PPARγ co-activator 1-α
PKC-θ	Protein kinase C θ
PKM2	Pyruvate kinase isozyme M2
PLCγ1	Phospholipase Cγ1
pMHC	Peptide-MHC
PPP	Pentose phosphate pathway
PPARγ	Peroxisome proliferator-activated receptor γ
PRRs	Pattern recognition receptors
pTα	Pre-TCR chain α
PTPN	Non-receptor type protein tyrosine phosphatase
PTPR	Receptor-type protein tyrosine phosphatase
RA	Rheumatoid arthritis
RAGs	Recombination-activating genes
RasGRP	Ras guanyl-releasing protein
RORγt	Receptor-related orphan receptor-γt
RT	Room temperature
S6K	Ribosomal protein S6 kinases
SAM	S-adenosylmethionine
SD	Standard deviation
SEM	Standard error of mean
SFK	SRC family tyrosine kinase
SGOC	Serine glycine one-carbon

SH2	Src homologue 2
SH3	Src homologue 3
SHP	SH2 domain-containing phosphatase
Slc	Solute carrier
SLE	Systemic lupus erythematosus
SLECs	Short-lived effector cells
SLO	Secondary lymphoid organ
SLP-76	SH2 domain-containing leukocyte protein of 76 kDa
SMAD	Small mothers against decapentaplegic
SMURF1	SMAD ubiquitination regulatory factor 1
SNAT	sodium-coupled AA transporters
SNP	Single nucleotide polymorphism
Sos	Son of sevenless
SRC	Spare respiratory capacity
SREBPs	Sterol regulatory element binding-proteins
SSC	Side scatter
STAT	Signal transducer and activator of transcription
STIM1	Stromal interaction molecule 1
TAA	Tumour-associated antigen
Tbet	T-box expressed in T cells
TCA	Tricarboxylic acid
Tcf1	Transcription factor 1
TCR	T cell receptor
T_{FH}	T follicular helper cell
TGFβ	Transforming growth factor β
T_h	T helper cells
TIM3	T cell immunoglobulin and mucin-domain containing 3
TLR	Toll-like receptor
TMB	Tetramethylmenzidine
TME	Tumour microenvironment
TNF	Tumour necrosis factor
TRAF6	TNF receptor associated factor 6

TRAF3	TNF receptor associated factor 3
T_{reg}	Regulatory T cell
TSA	Tumour-specific antigen
UDP-GlnNAc	Uridine diphosphate N-acetylglucosamine
UTR	Untranslated region
VAV1	Vav guanine nucleotide exchange factor 1
ZAP70	ζ-chain-associated protein of 70kDa

Chapter 1 Introduction

1.1 An overview of the immune system.

In the 19th century, Robert Koch described a causative relationship between microorganisms and infectious disease. We are constantly surrounded by pathogens including bacteria, viruses, fungi and parasites but, despite persistent exposure to microorganisms, we rarely become seriously ill. This is a consequence of our immune system, a complex network of organs, cells and molecules that cooperatively protect us against harmful agents.

Upon exposure to an infectious organism, epithelial barriers (skin and mucosal membranes) build the first line of defence of the body. However, fissures are common and further protective mechanisms are needed. Within minutes-hours after exposure, granulocytes (neutrophils, eosinophils and basophils), macrophages, dendritic cells (DCs), natural killer (NK) cells and the complement system get activated. All these components, including the epithelium, comprise the first pillar of immunity: the innate system. Innate cells provide responses characterised by their rapid speed of action and their lack of specificity as they identify, through pattern recognition receptors (PRRs), simple structures that have been conserved during evolution and are common to many pathogens. These structures are known as pathogen-associated molecular patterns (PAMPs) and include lipopolysaccharides (LPS), mannose-rich oligosaccharides, peptidoglycans and unmethylated CpG DNA (Akira et al., 2006; Kawai and Akira, 2010; Brubaker et al., 2015). The activation of innate cells promotes a proinflammatory response that restrains the initial infection and induces the stimulation of the second pillar of immunity: the adaptive system. Adaptive responses are mediated by B and T lymphocytes and are characterised

by their specificity against antigens and their memory capacity. Their specificity relies on the generation of B cell receptors (BCRs) and T cell receptors (TCRs) by B and T cells, respectively. While the BCR recognises non-processed antigens, the TCR recognises processed peptide antigens presented by major histocompatibility (MHC) molecules. Two main types of MHC molecules can be distinguished: MHC class I (MHC-I), which are expressed by virtually all cell types, and MHC class II (MHC-II), which are only expressed by specialised antigen presenting cells (APCs) such as DCs, macrophages and activated B cells. T lymphocytes that recognise antigens in association with MHC-I molecules are known as $CD8^+$ T cells, whilst those that recognise antigens in association with MHC-II molecules are $CD4^+$ T cells (Andersen et al., 2006; Yang, Q. et al., 2010).

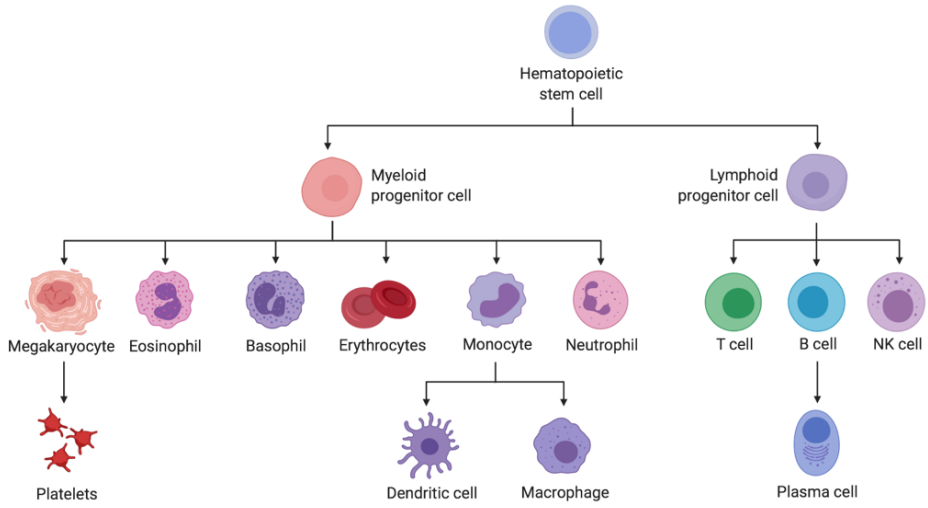
In this thesis, I focus on the study of T lymphocytes and, therefore, I aim to describe in the following sections the development, function and regulation of these cells in the mediation of immune responses.

1.2 T cell development – giving rise to a diverse TCR repertoire.

Postnatally, all immune cells derive from pluripotent haematopoietic stem cells (HSCs) in the bone marrow (BM). HSCs differentiate into common myeloid progenitor (CMP) cells or common lymphoid progenitor (CLP) cells which, in turn, give rise to the myeloid and lymphoid lineages, respectively. The majority of the innate cells, in addition to erythrocytes and platelets, are descended from CMPs. On the other hand, innate lymphoid cells (ILCs), such as NK cells, and B and T lymphocytes arise from CLPs (Kondo, 2010; Yang, Q. et al., 2010) (Fig. 1.1A). Once differentiated, immune cells leave the bone marrow and circulate through the blood and lymphatics to travel to primary and secondary lymphoid organs

(SLOs) and tissues. Nonetheless, an additional step is required for T lymphocytes in order to become functional. Before commitment to the T cell lineage, CLPs exit the BM and traffic to the thymus, where they complete their development after acquiring an specific antigen receptor, i.e., a TCR.

A



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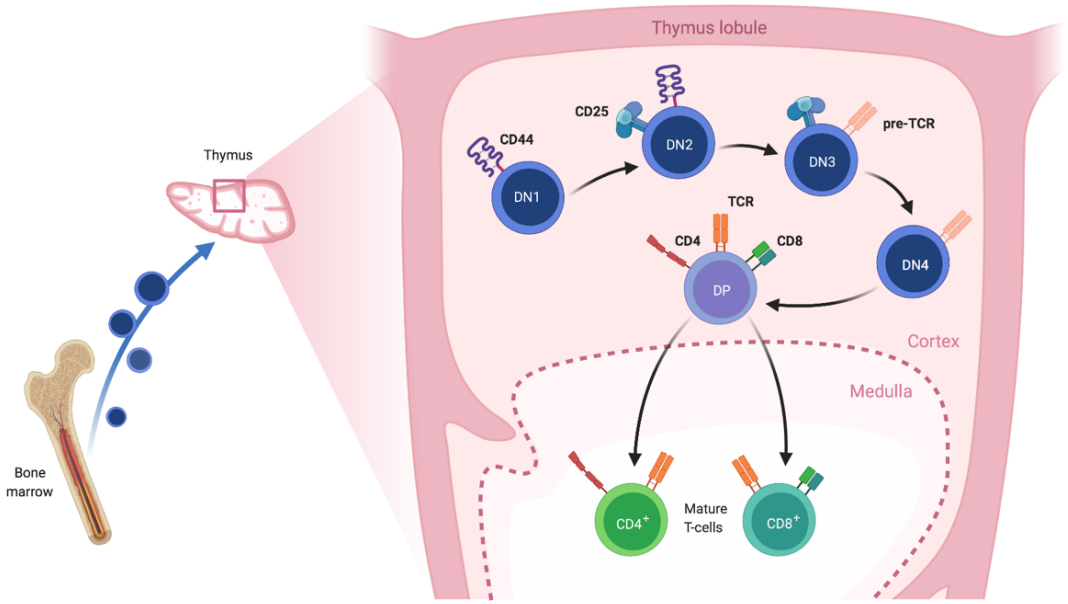


Figure 1.1: Development of T lymphocytes.

(A) HSCs originated in the BM develop into CMPs or CLPs which, in turn, will give rise to the myeloid or lymphoid lineages, respectively. (B) CLPs migrate to the thymus where they acquire a functional TCR. Specifically, T cell early progenitors undergo three different stages, based on the expression of the co-receptors CD4 and CD8: DN (includes DN1, DN2, DN3 and DN4), DP and SP. In the transit from DN2 to DN3, cells express RAG1 and RAG2, which promote the rearrangement of the TCRβ chain locus and the formation of a pre-TCR. The rearrangement of the TCRα chain locus takes place during the DP stage leading to the acquisition of the TCR which functionality is tested through self-pMHC presentation by thymic non-lymphoid cells. Failure to bind self-pMHC or high self-reactivity induces apoptosis, whilst only those T cells that bind to pMHCs with low affinity survive and leave the thymus in search of antigens. *Adapted from "Stem Cell Differentiation from Bone Marrow" and "T-cell Development in Thymus", by Biorender.com (2021). Retrieved from https://app.biorender.com/biorender-templates.*

The development of a diverse TCR repertoire is essential to provide appropriate T cell responses against any exogenous antigen. Multipotent immature lymphocytes that arrive from the BM localise within the thymic cortex. At this stage, T lineage cells are described as double negative (DN) thymocytes as they lack expression of CD4 and CD8 co-receptors (Fig. 1.1B). In mice, DN cells are divided into 4 subtypes: DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^{low}CD25^+$), DN4 ($CD44^-CD25^-$). Commitment to the αβ T cell lineage (95% of T lymphocytes in human circulation) starts during DN2 and continues in DN3 with the rearrangement of the TCRβ chain locus. The recombination-activating genes (RAGs) RAG1 and RAG2 encode for the key enzymes that orchestrate the recombination of antigen receptor variable (V), diversity (D) and joining (J) gene segments (Schatz and Ji, 2011). This recombination allows the production of a diverse TCR repertoire from a limited number of genes and induces the synthesis of a successfully rearranged TCRβ chain. Then, the β chain pairs with an invariant pre-TCR α chain (pTα) forming the so-called pre-TCR (Fig. 1.1B). The surface

expression of the pre-TCR triggers signals that lead to a burst of proliferation and the repression of RAG1 and RAG2 to ensure allelic exclusion at the β locus.

During the DN4 stage, proliferation ceases and CD4 and CD8 molecules start to be expressed giving rise to the double positive (DP) phenotype (Fig. 1.1B). DP cells restore RAG1 and RAG2 expression which induces the rearrangement of the TCRα chain locus and the assembly of a functional TCR:CD3 complex. Upon migration to the medulla, the TCR functionality of the thymocytes is tested through exposure to MHC molecules present in thymic non-lymphoid cells. Here, the majority of DP cells die as a result of a failed productive interaction with self-peptide-MHC (pMHC) complexes in a process known as 'death by neglect'. On the other hand, thymocytes capable of recognising self-pMHC complexes will undergo apoptosis if they are bound with high affinity (negative selection) whereas those recognised with low-moderate affinity (positive selection) will continue the maturation process (Bommhardt et al., 2004). Thus, besides generating a high T cell clonality, the thymus is also responsible for central tolerance. Finally, thymocytes become mature single positive T lymphocytes, $CD4^+CD8^-$ or $CD4^-CD8^+$, depending on the class of MHC molecule that binds the TCR during positive selection (Bommhardt et al., 2004; Palmer and Naeher, 2009; Vicente et al., 2010; Shah and Zúñiga-Pflücker, 2014; Yui and Rothenberg, 2014).

The aforementioned description is a brief summary of the complex process of T cell lineage commitment and differentiation but it is important to note that thymocytes integrate other signals that are also required in this process. For example, early precursors that arrive to the thymic cortex interact with Delta-like ligands that trigger Notch signalling and the subsequent expression of

transcription factors that are essential to establish the T cell identity of precursors. Another key signal integrated by thymocytes is IL-7, which promotes the survival of thymocytes expressing a competent TCR (Hong et al., 2012; Hosokawa and Rothenberg, 2018).

1.3 T cell subpopulations and their role in immune responses.

T cell responses are developed in an antigen-specific manner. I have already introduced the two main T cell populations: $CD4^+$ and $CD8^+$ T lymphocytes; but, what is their role in the diverse immune responses to different pathogens?

Effector $CD8^+$ T cells, also known as cytotoxic T lymphocytes (CTLs), mediate responses against intracellular pathogens, such as viruses, or tumour cells. CTLs are recruited into the site of infection or within the tumour microenvironment (TME) and mediate the lysis of target cells upon TCR-ligation via the exocytosis of cytolytic granules at the immunological synapse (IS) (Andersen et al., 2006). These granules contain perforins and granzymes. Amongst the several types of granzymes (10 in mice and 5 in humans), granzyme A (GrA) and granzyme B (GrB) are the most abundant ones. Mechanistically, perforins form pores in the plasma membranes of target cells to mediate the entry of granzymes in the cell and trigger apoptosis through mitochondrial damage or activation of caspase 3 (Zhang, N. and Bevan, 2011; Voskoboinik et al., 2015). Recent investigations from Bálint et al. (2020) have revealed that perforins and granzymes can also be delivered in supramolecular attack particles (SMAPs), multiprotein complexes that contain trombospondin-1 (TSP-1) in its shell to mediate cytotoxicity. Nonetheless, secretion of cytolytic granules is not the only mechanism by which CTLs can eliminate target cells. For instance, CTLs release cytokines including interferon γ (IFNγ), tumour necrosis factor (TNF) and lymphotoxin α (LTα), which

activate other immune cells such as macrophages, inhibit viral replication, induce MHC-I expression and even kill target cells directly via TNFR-I (Zhang, N. and Bevan, 2011). CTLs also express ligands for the death receptor Fas (FasL) enabling the recruitment and activation of caspases 8 and 10 and, therefore, promoting the extrinsic pathway of apoptosis in target cells (Andersen et al., 2006; Zhang, N. and Bevan, 2011; Halle et al., 2017) (Fig. 1.2).

Conversely, $CD4^+$ T cells are known as 'helper' T (T_h) cells as they produce cytokines that recruit, activate and coordinate other effector cell types and mechanisms. Depending on the cytokine milieu, $CD4^+$ T cells can be differentiated into at least 4 different subtypes: T_h1, T_h2, T_h17 and T_h9 (Murphy and Reiner, 2002; Zhu, J. et al., 2010; Luckheeram et al., 2012). Each subtype is characterized by a distinct cytokine repertoire that mediates pathogen-selective responses. T_h1 cells are involved in type 1 responses - i.e., cell-mediated immunity to intracellular pathogens. T_h1 cells secrete IFN-γ and express CD40L to activate macrophages and induce the inflammatory M1-phenotype. Among other mechanisms, they also produce interleukin-2 (IL-2) to support CTL expansion and secrete other cytokines such as TNF or LTα to promote macrophage recruitment at the site of infection (Zhu, J. et al., 2010). T_h2 cells mediate type 2 immunity including responses against extracellular parasites. To do so, they secrete IL-4, IL-5 and IL-13 to induce epithelial cells repair, muscle contraction for helminth expulsion, activation of M2-macrophages, recruitment of eosinophils and IgE antibody production (Zhu, J. et al., 2010). T_h17 cells coordinate responses against extracellular bacteria and fungi. Their function is mediated by production of IL-17A and IL-17F which mobilise neutrophil responses and promote the generation of antibacterial peptides by epithelial cells (Zhu, J. et al., 2010). Finally, T_h9 cells have been more recently characterised as

crucial sources of IL-9. However, due to the pleiotropic functions of IL-9 (mainly promotes the survival of $CD4^+$ T cells and other immune cells) it is thought that T_h9 cells play a broader role amongst the different types of immunity (Kaplan, 2013). Importantly, T_h cells do not always play a protective role and, instead, have been associated with pathologies such as allergies (T_h2, T_h9) or autoimmune diseases including type 1 diabetes (T_h1), rheumatoid arthritis and multiple sclerosis (T_h17) (Zhu, J. et al., 2010; Hirahara et al., 2013; Zhu, J., 2018).

Additional $CD4^+$ T cell subsets are involved in the regulation of immune responses. T follicular helper cells (T_{FH}) develop in concert with the other subsets and provide help to B cells to promote germinal centre responses and generate class-switched immunoglobulins (Ig) of different isotypes (Luckheeram et al., 2012). Furthermore, naïve $CD4^+$ T cells can also differentiate into peripheral T_{regs} (pT_{regs}), which provide immunosuppressive responses rather than stimulatory. pT_{regs} are characterised by the expression of forkhead box P3 (FoxP3) and CD25 and secrete inhibitory cytokines such as IL-10 and transforming growth factor β (TGFβ) to restore and limit immune responses. Moreover, they prevent autoreactivity against self-peptides providing an important mechanism of peripheral tolerance (Murphy and Reiner, 2002; Sakaguchi et al., 2010; Luckheeram et al., 2012). The majority of FoxP3-expressing T_{regs} are generated in the thymus; these are referred to as natural T_{regs} (nT_{regs}) (Dhamne et al., 2013).

1.4 $CD8^+$ T cell responses – from the thymus to memory formation.

In this thesis, that aims to determine how T cells integrate signals and modulate their metabolism, I focus on the study of $CD8^+$ T lymphocytes.

Naïve $CD8^+$ T cells recirculate throughout the bloodstream and lymphatics into lymph nodes, spleen and mucosa-associated lymphoid tissues (MALT) in search of specific antigen. Until then, naïve T cell peripheral homeostasis is regulated via IL-7 and self-peptide TCR signals which promote survival through the expression of antiapoptotic factors and maintain cells in quiescence (Surh and Sprent, 2008). The accumulation of APCs and T lymphocytes within SLOs facilitates T cell priming. Here, initial T cell binding to APCs is mediated by low-affinity interactions of the cell adhesion molecules lymphocyte function-associated antigen 1 (LFA-1) with the intracellular adhesion molecule 1 (ICAM-1) (Walling and Kim, 2018). If the antigen is successfully recognised by the TCR while receiving appropriate stimulation from co-receptors and cytokines, the T cell will grow, clonally expand and acquire cytotoxic functions differentiating, therefore, into an effector CTL (Fig. 1.2). Activated T cells migrate via the action of chemokines, integrins and selectins to the site of infection or tumour microenvironment (TME), where the CTL recognises target cells and delivers cytolytic granules and cytokines to carry out its effector functions, as described in Section 1.3 (Zhang, N. and Bevan, 2011) (Fig. 1.2). Finally, upon clearance of antigen and cytokine withdrawal most of the lymphocytes will die (contraction phase) but some will form a pool of memory cells to provide long-term responses, which will survive in the periphery due to the action of the homeostatic cytokines IL-7 and IL-15 (Kaech and Cui, 2012; Mueller et al., 2013). When CTLs are not able to efficiently clear antigen, such as during chronic infections or cancer, the memory T cell population will not form and might instead result in an exhausted phenotype (McLane et al., 2019; Franco et al., 2020).

Thus, the life of a $CD8^+$ T cell can be differentiated into several stages that include (1) CLP development within BM, (2) T cell development within the thymus, (3)

naïve T cell homeostasis in the periphery, (4) T cell priming in SLOs, (5) T cell growth proliferation and differentiation, (6) migration to site of infection or TME, (7) delivery of cytotoxic functions and, after pathogen removal, (8) effector cell death and survival of a memory population. In the following sections, I explain the mechanisms by which T cell priming is regulated.

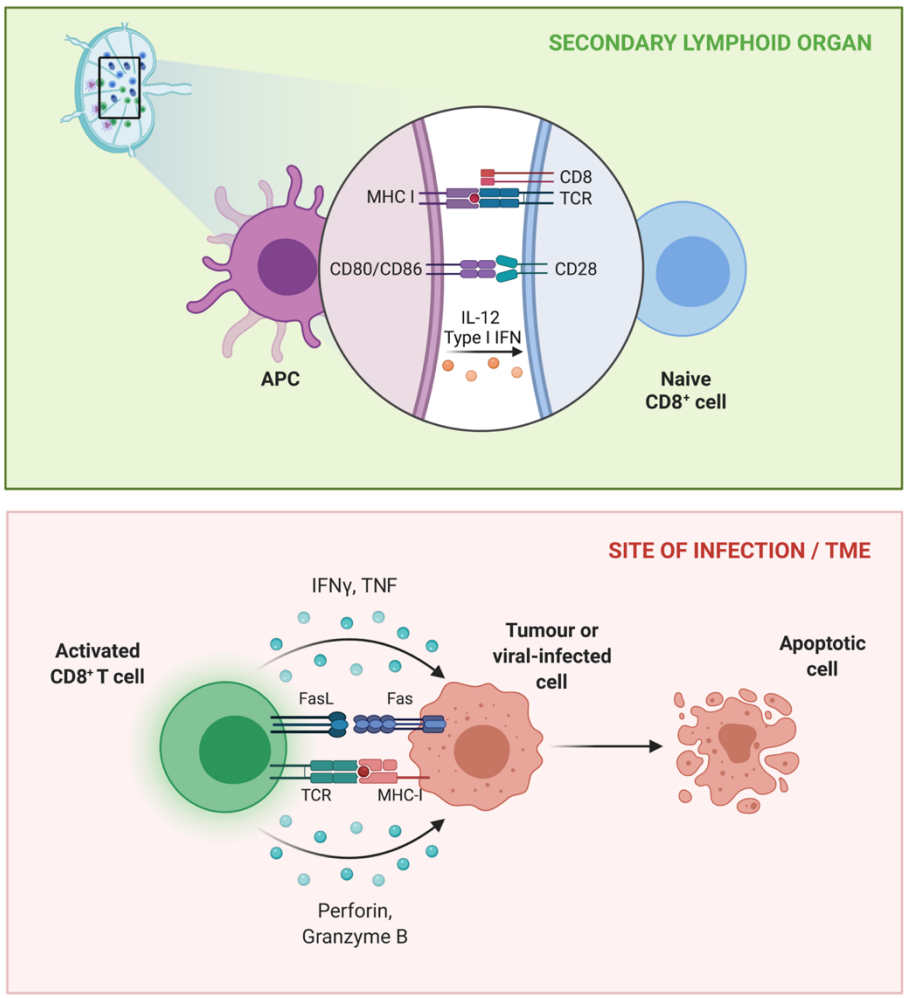


Figure 1.2: CD8⁺ T cell activation and cytotoxic functions.

Naïve CD8⁺ T cells are primed within SLOs, where APCs present antigen. After appropriate pMHC:TCR interaction (signal 1), accompanied by co-stimulation (signal 2) and signal 3 cytokines IL-12 and type I IFNs, activation, clonal expansion and differentiation is triggered. CTLs migrate to the site of infection or the TME, where target cells are detected and killed through the delivery of cytotoxic granules, Fas/FasL interactions or the secretion of cytokines such as IFNγ and TNF.

1.5 T cell priming: signal integration and modulation of CD8$^+$ T cell responses.

CD8$^+$ T cell priming is triggered by antigen presentation via pMHC binding to the TCR. Activation of TCR signalling alone allows for limited proliferation and differentiation of naïve CD8$^+$ T cells (Shahinian et al., 1993; Wang, B. et al., 2000). However, for the development of optimal T cell responses, the 'three signals model' has been established in which the signalling provided by the TCR, co-stimulatory molecules and cytokines synergise to modulate T cell activation, expansion and fate (Mescher et al., 2006; Marchingo et al., 2014) (Fig. 1.2). Further detail of each signal and its role in T cell function are provided in Sections 1.5.1, 1.5.2 and 1.5.3, respectively.

1.5.1 Signal 1: TCR signalling.

1.5.1.1 Structure of MHC-I and TCR complexes.

Antigen recognition by the TCR of CD8$^+$ T lymphocytes is only carried out in the context of MHC-I molecules. MHC-I molecules – HLA-A/B/C in humans and H-2$^{K/D/L}$ in mice - are formed by two polypeptide chains: the α chain and the β_2-microglobulin. The former consists of three domains (α_1, α_2, α_3) and is anchored to the membrane through the α_3 domain, whereas the α_1 and α_2 domains fold together into a single structure where the peptide binds. This structure is known as the peptide-binding cleft and is highly polymorphic. β_2-microglobulin does not span the membrane and binds noncovalently to the α chain (Garcia and Adams, 2005; Rudolph et al., 2006). TCRs are formed by the TCRα and TCRβ chains which are linked by a disulphide bond. Each chain consists of an Ig-like constant domain (C), a variable domain (V) and a transmembrane region that chains the receptor to the T cell membrane. V_α and V_β, similarly to the α_1 and α_2 domains of

the MHC-I molecule, constitute the antigen-binding site (Wucherpfennig et al., 2010).

The TCR alone recognises peptide antigen but cannot directly trigger signalling as it has no intrinsic enzymatic activity. Thus, the TCR associates with the CD3 complex (CD3γ, CD3δ and CD3ε chains) and the homodimer of ζ chains generating the so-called TCR complex (Dong et al., 2019; Hwang et al., 2020). The CD3 complex is composed by two dimers: CD3ε:CD3δ and CD3ε:CD3γ. Each chain contains an extracellular Ig-like domain, a transmembrane region and a cytoplasmic region that contains one immunoreceptor tyrosine-based activation motif (ITAM). On the other hand, ζ chains have a short extracellular region and a long intracellular region that contains three ITAMs. Additionally, the CD8 co-receptor is recruited to the immunological synapse. CD8 is a dimer whose chains (α and β) are constituted by an extended polypeptide with an Ig-like domain in its extracellular region. CD8 binds to the cytoplasmic SRC family tyrosine kinase (SFK) Lck (Veillette et al., 1988; Mørch et al., 2020). Together, the TCR complex in association with the CD8 co-receptor enables TCR-mediated antigen recognition to be translated into an intracellular cascade initiated by tyrosine phosphorylation of the ITAMs that results in activation of a transcriptional programme necessary for T cell activation (Fig. 1.3).

1.5.1.2 Initiation of TCR signalling: regulation of Lck activity.

The extent of Lck-mediated phosphorylation of the CD3 and ζ chain ITAMs determines whether or not the signal received is sufficient to surpass the TCR affinity threshold and initiate the downstream signalling cascade (Stepanek et al., 2014). Lck structure consists of a Src homology 3 (SH3) and an SH2 domain linked to the C-terminal kinase domain in addition to an N-terminal unique domain

that binds the CD8 cytoplasmic tail. Lck activity is regulated via phosphorylation of critical tyrosine sites: the inhibitory Tyr505, situated in the SH2 domain, and the activating Tyr394, located in the kinase domain. Whereas Lck inactivity is commonly associated with Tyr505 phosphorylation and a closed conformation, activated Lck presents an open conformation and Tyr394 phosphorylation. The proteins involved in the phosphorylation/dephosphorylation of those motifs are the receptor-type protein tyrosine phosphatase (PTPR) CD45 and the C-terminal Src kinase (Csk) whose balancing forces constitute a key regulatory axis of Lck activity (Smith-Garvin et al., 2009). Non-stimulated lymphocytes present a basally active Lck characterised by Tyr394 phosphorylation (Nika et al., 2010). After TCR-stimulation, a series of events including CD45 segregation from the TCR complex (Courtney et al., 2019) or diminishment of Csk recruitment (Torgersen et al., 2001) modulate Lck-mediated phosphorylation of ITAMs that subsequently trigger TCR signalling. Other cytosolic phosphatases, such as the protein tyrosine phosphatases non-receptor type 6 (PTPN6; also known as SH2 domain-containing phosphatase (SHP)1) and PTPN22, are also involved in the negative regulation of Lck through direct dephosphorylation of Tyr394 or, in the case of PTPN22, also indirectly via interaction with Csk (Bottini and Peterson, 2014; Gaud et al., 2018). Thus, intricate regulation of early TCR events set a TCR threshold that determines discrimination of exogenous and self-peptides (Acuto et al., 2008; Salmond et al., 2014; Manz et al., 2015; Courtney et al., 2019). In section 1.5.4, the role of PTPN22 in this process will be further described (Fig. 1.3).

1.5.1.3 Proximal TCR signalling.

Lck-mediated phosphorylation of tyrosine residues within ITAMs facilitates the recruitment of proteins containing tandem SH2 domains. In T cells, the protein

recruited is the Syk family kinase ζ-chain-associated protein of 70kDa (ZAP70) (Chan et al., 1991) which, once bound, is activated via Lck-mediated phosphorylation. Activated ZAP70 initiates phosphorylation of the scaffold protein linker for activated T cells (LAT) at multiple sites that are utilised as docking points for the recruitment of several proteins and the formation of the LAT signalosome (Finco et al., 1998; Zhang, W. et al., 1998; Balagopalan et al., 2010). Among the proteins that comprise the LAT signalosome are SH2 domain-containing leukocyte protein of 76 kDa (SLP-76), phospholipase Cγ1 (PLCγ1), growth factor receptor-bound protein 2 (GRB2), vav guanine nucleotide exchange factor 1 (VAV1), GRB2-related adaptor protein (GADS), interleukin-2-inducible T cell kinase (ITK), adhesion- and degranulation-promoting adaptor protein (ADAP) and non-catalytic region of tyrosine kinase adaptor protein 1 (NCK1). The LAT signalosome propagates and branches the TCR signalling into at least three major signalling modules: Ca^{2+}, nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) signalling pathways, leading to the activation of transcription factors that mediate gene expression for T cell activation (Cantrell, D., 1996; Smith-Garvin et al., 2009; Brownlie and Zamoyska, 2013; Navarro and Cantrell, 2014; Gaud et al., 2018; Hwang et al., 2020) (Fig. 1.3).

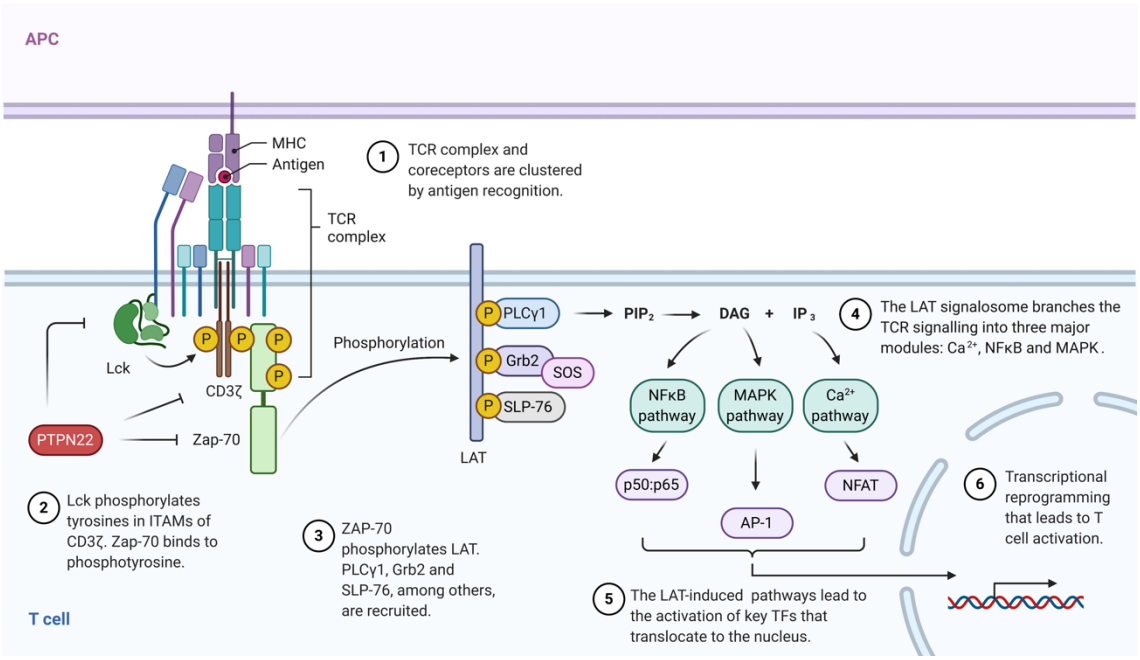


Figure 1.3: T cell receptor signalling.

After TCR-binding, Lck phosphorylates ITAMs within the TCR complex. Consequently, ZAP-70 is recruited and activated, which leads to the phosphorylation of LAT. LAT phosphorylation results in the recruitment of multiple proteins such as PLCγ1, Grb2 and SLP-76 that trigger a myriad of signalling pathways (e.g. NFκB, MAPK, Ca^{2+}). The engagement of these pathways ultimately results in the activation of TFs that induce transcriptional reprogramming and T cell activation. The initiation of the TCR signalling is regulated by the action of tyrosine phosphatases, such as PTPN22. *Adapted from "TCR Downstream Signaling", by Biorender.com (2021). Retrieved from https://app.biorender.com/biorender-templates.*

1.5.1.4 Branching of TCR signalling: Ca^{2+}, NF-κB and MAPK signalling pathways.

How are these modules engaged and how do they result in the activation of transcription factors? In brief, formation of the LAT signalosome induces PLCγ1 recruitment to the inner face of the membrane and its subsequent activation (Sieh et al., 1994). Active PLCγ1 catalyses the conversion of PIP_2 (phosphatidylinositol (4,5)-bisphosphate) into the second messengers diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3) (Brownlie and Zamoyska, 2013; Cantrell, D., 2015; Hwang et al., 2020).

IP_3 binds on the endoplasmic reticulum (ER) to Ca^{2+} channels which releases Ca^{2+} to the cytosol. Depletion of Ca^{2+} within the ER causes clustering of the transmembrane protein stromal interaction molecule 1 (STIM1). Then, STIM1 oligomers interact with the calcium-release activated calcium (CRAC) channels on the plasma membrane triggering extracellular Ca^{2+} uptake. Accumulation of cytosolic Ca^{2+} provokes a conformational change of calmodulin which, in turn, activates calcineurin. Calcineurin is a serine/threonine phosphatase that regulates the localisation of the TF nuclear factor of activated T cells (NFAT) (Oh-hora and Rao, 2008; Hogan et al., 2010). In resting T cells, NFAT is sequestered in the cytosol mainly by the glycogen synthase kinase 3 (GSK3) (Beals et al., 1997). GSK3-mediated phosphorylation dampens NFAT recognition of nuclear transporters thereby preventing entry of NFAT into the nucleus. Upon TCR-stimulation and calcineurin activation, cytosolic NFAT is dephosphorylated allowing transporter recognition, entrance to the nucleus and transcriptional activation of crucial genes, such as IL-2 (Feske, 2007; Oh-hora and Rao, 2008; Hogan et al., 2010; Cantrell, D., 2015).

In contrast, DAG production contributes to the other two signalling modules. First, DAG recruits and activates Ras guanyl-releasing protein (RasGRP), a guanine-nucleotide exchange factor (GEF) that specifically activates the small GTPase Ras (Ebinu et al., 2000; Dower et al., 2000; Roose et al., 2005). Ras, which can also be activated by the Grb-2-recruited GEF Son of sevenless (Sos) (Buday et al., 1994), triggers the three-tiered MAPK cascade: Ras activates Raf, which activates MAPK/ERK Kinase 1 (MEK1), which activates extracellular signal-regulated kinases ERK1 and ERK2 (Genot and Cantrell, 2000). ERKs enter the nucleus and phosphorylates the transcription factor Elk-1 which, consequently, induces c-Fos expression. At the same time, activation of the MAP kinase c-Jun

N-terminal kinase (JNK) by protein kinase C θ (PKC-θ) leads its mobilisation to the nucleus where phosphorylates c-Jun. Afterwards, c-Jun/Fos dimerisation generates and activates activator-protein 1 (AP-1) transcription factor that turns on transcription of important genes for T cell activation (Cantrell, D.A., 2003; Brownlie and Zamoyska, 2013; Cantrell, D., 2015; Hwang et al., 2020).

Moreover, DAG also activates the already mentioned PKC-θ which is also involved in initiation of NF-κB signalling. NF-κB transcription factors are dimers formed by combinations of the NFκB/Rel family proteins p50, p52, RelA/p65, RelB and c-Rel. In resting T lymphocytes, the canonical p50:p65 heterodimer is kept inactive in the cytosol via interaction with the inhibitory protein IκB. During T cell activation, PKC-θ phosphorylates the scaffold protein caspase recruitment domain-containing membrane-associated guanylate kinase protein-1 (CARMA-1) (Matsumoto et al., 2005). CARMA1 associates and forms a multisubunit complex with BCL10 and MALT1 implicated in the recruitment of the E3 ubiquitin ligase TNF receptor associated factor 6 (TRAF-6) (Che et al., 2004; Wang, D. et al., 2004). Next, TRAF-6 makes a polyubiquitin scaffold on itself that recruits TAB1/2 and activates TAK1 (Sun et al., 2004). TAK1 phosphorylates IKK-β, a subunit of the IκB kinase (IKK) complex which is constituted by the serine kinases IKK-α, IKK-β and IKK-γ (also known as NEMO; NF-κB essential modulator). Subsequently, the IKK complex phosphorylates and stimulates IκB ubiquitination which is then degraded by the proteasome. The p50:p65 dimer is then released from IκB and able to access the nucleus and commence transcription (Spitaler and Cantrell, 2004; Matthews and Cantrell, 2009; Brownlie and Zamoyska, 2013; Cantrell, D., 2015; Hwang et al., 2020).

Together, TCR signalling results in the activation of a network of transcription factors that leads to T cell growth, expansion and differentiation. TCR-stimulation results in naïve T cells exiting G0 and entering the cell cycle upon activation and expression of cyclins. Furthermore, TCR signalling stimulates T cell activation pathways and rapid expression of IL-2 and CD25 (α chain of the IL-2 receptor) that promotes T cell growth, proliferation and differentiation (Smith-Garvin et al., 2009). At the same time, transcription factors such as Eomesodermin (Eomes) and T-box expressed in T cells (Tbet) are expressed to mediate $CD8^+$ T cell differentiation and effector function (Kaech and Cui, 2012). Importantly, T cell activation is also accompanied by upregulation of metabolic transcription factors such as Myc that induce a metabolic reprogramming to support energetic and biosynthetic demands (Wang, R. et al., 2011; Buck et al., 2017; Chapman et al., 2019). The metabolism of activated T cells is further described in Section 1.6.

1.5.2 Signal 2: Co-stimulatory signals

Not all self-peptides are presented in the thymus and, therefore, central tolerance alone cannot fully explain non-responsiveness against all self-antigens in peripheral tissues (Bretscher and Cohn, 1970; Lafferty and Cunningham, 1975; König, 2010). This idea gave rise to the hypothesis that an additional signal to TCR-ligation is required for T cell activation. Today, we know that engagement of the TCR alone often induces T cell anergy (Harding et al., 1992; Schwartz, 2003) and that co-stimulatory molecules, predominantly CD28, provide a fully activated state when combined with TCR- and cytokine- stimulation (Marchingo et al., 2014).

1.5.2.1 CD28 signalling and its role in T cell activation

CD28 is an Ig-superfamily (Ig-SF) transmembrane homodimer constitutively expressed in naïve and activated $CD4^+$ and $CD8^+$ T lymphocytes. CD28 binds to CD80 and CD86 (also known as B7.1 and B7.2, respectively) which are expressed by activated, but not resting, APCs. Following TCR-stimulation, CD28 co-localises with the TCR complex within the immunological synapse and mediates a myriad of functions resulting in the amplification of the TCR signalling, increased IL-2 secretion and the induction of bigger and prolonged T cell responses to antigen (Rudd and Schneider, 2003; Miller et al., 2009; Rudd et al., 2009; Boomer and Green, 2010; Esensten et al., 2016). Specifically, TCR-driven phosphorylation of CD28 in its cytoplasmic YMNM and PYAP motifs, mediated predominantly by Lck and Fyn, allows interaction with multiple proximal TCR proteins (Hutchcroft and Bierer, 1994; Raab et al., 1995). In particular, interaction with the p85 subunit of phosphatidylinositol-3 kinase (PI3K) triggers activation of the PI3K-Akt pathway (Rudd and Schneider, 2003; Boomer and Green, 2010). Mechanistically, PI3K induces the conversion of PIP_2 to PIP_3 which, in turn, binds and retains Akt at the plasma membrane facilitating its activation by phosphoinositide-dependent kinase 1 (PDK1). Then, activation of the Akt kinase leads to the phosphorylation and subsequent activation of an array of downstream signals including GSK3, NFκB, nuclear factor 90 (NF90), mechanistic Target of Rapamycin (mTOR) and enhanced expression of pro-survival Bcl-x_L. These pathways synergise with TCR-signalling and promote transcription and mRNA stabilisation of IL-2, cell cycle progression, metabolic reprogramming and survival (Fraser et al., 1991; Boise et al., 1995; Frauwirth, K. A. et al., 2002; Pei et al., 2008; Boomer and Green, 2010). In addition, TCR signalling is further amplified independently of PI3K signalling via Grb2 and Lck

recruitment which induces activation of PLCγ1, PKCθ and JNK, among others (Boomer and Green, 2010).

1.5.2.2 Co-signalling: beyond CD28

Our current understanding of 'signal 2' has evolved to incorporate a more complex regulatory system. Many additional receptors and ligands, both stimulatory and inhibitory, are expressed by T cells and APCs that contribute to determining T cell fate and survival, as well as the degree of T cell responses and their termination (Chen, L. and Flies, 2013). For instance, the co-inhibitory receptor cytotoxic T lymphocyte antigen 4 (CTLA-4) has been established as a crucial signal for self-tolerance (Perez et al., 1997; Alegre et al., 2001; Greenwald et al., 2001). CTLA-4, which is constitutively expressed by T_{regs} and upregulated in conventional T lymphocytes upon TCR-stimulation, binds CD80/CD86 with high affinity and thereby outcompetes CD28. The CD28/CTLA-4/CD80/CD86 regulatory network modulates self-reactivity within the thymus and periphery, impairing T cell priming and highlighting the importance of additional co-signals in the tight regulation of T cell responses (Rudd et al., 2009; Chen, L. and Flies, 2013; Verhagen et al., 2013; Paterson et al., 2015). Other co-signals include the co-stimulatory CD137 (also known as 4-IBB), inducible T cell co-stimulator (ICOS), CD134 (also known as OX40), CD27 and CD244 (also known as 2B4) and the co-inhibitory programmed cell death 1 (PD-1), T cell immunoglobulin and mucin-domain containing 3 (TIM3) and lymphocyte activation gene 3 protein (LAG3), among others (Chen, L. and Flies, 2013).

1.5.3 Signal 3: Cytokines

Once T cells have encountered cognate antigen and co-signals, lymphocytes can proliferate and expand but require additional signals in order to acquire full

effector functions and promote survival and memory formation (Mescher et al., 2006). IL-2, a common γ-chain (γc) multifunctional cytokine, promotes effector responses in both $CD4^+$ and $CD8^+$ T cell subsets (T_h1, T_h2, CTLs) but suppresses the inflammatory T_h17 responses and inhibits T_{FH} cells. Furthermore, IL-2 induces development and maintenance of T_{regs} suggesting that IL-2 fine-tunes the balance of stimulatory and suppressive signals to provide adequate regulation of T cell responses (Fontenot et al., 2005; Ross and Cantrell, 2018). In the context of $CD8^+$ T lymphocytes, IL-2 triggers a metabolic and transcriptional program, characterised by the upregulation of the STAT5-dependent Blimp1 and the engagement of glycolysis, that induces survival, growth and clonal expansion following TCR-stimulation. Moreover, IL-2 signalling determines $CD8^+$ T cell fate: whereas strong and prolonged IL-2 signals drive differentiation into short-lived effector cells (SLECs), shorter IL-2 signals have been associated with the generation of memory-precursor effector cells (MPECs) (Kalia and Sarkar, 2018; Ross and Cantrell, 2018).

In addition to the key role of IL-2 differentiation is determined, in $CD4^+$ T lymphocytes, by cell exposure to cytokines such as IL-12 (T_h1), IL-4 (T_h2) or IL-6, TGFβ and IL-23 (T_h17) (Zhu, J. et al., 2010; Luckheeram et al., 2012). In $CD8^+$ lymphocytes, the major cytokines that support differentiation into CTLs are IL-12 and type I IFNs (IFNα/β) (Curtsinger and Mescher, 2010). IL-12 is a heterodimeric cytokine (p35 and p40 subunits) secreted by macrophages, DCs and B lymphocytes upon intracellular pathogen infection. On the other hand, type I IFNs, known for their potent antiviral function, are secreted by almost all cell types upon infection. Whilst IL-12 signals are sensed by T cells through the IL-12 receptor (formed by IL-12Rβ1 and IL-12Rβ2), IFNα/β signals are integrated via the IFN-

alpha receptor (IFNAR). Both receptors trigger JAK/STAT signalling pathways and regulate T cell effector functions mainly in a STAT4 (IL-12) or a STAT1/2 (IFNα/β) dependent manner (Liu et al., 2005; Decker et al., 2005; Curtsinger et al., 2005). Lack of both IL-12 and IFNα/β has been associated with an inability to promote cytolytic activity, IFNγ production and long-term responses in $CD8^+$ T cells while engaging an anergic state (Curtsinger, J. M. et al., 2003; Curtsinger et al., 2005; Curtsinger and Mescher, 2010). Mechanistically, IL-12 and IFNα/β stimulation regulates expression of effector proteins and differentiation transcription factors (such as GrB, IFNγ and Eomes) through, at least in part, histone acetylation modifications that lead to chromatin remodelling of critical loci and, therefore, maintain effector function and support memory formation (Agarwal et al., 2009; Xiao et al., 2009; Curtsinger and Mescher, 2010).

1.5.4 Modulators of TCR signalling: PTPN22

1.5.4.1 Structure, targets and role of PTPN22 in T lymphocytes

PTPN22 is a cytoplasmic class I protein tyrosine phosphatase predominantly expressed in cells of haematopoietic origin. Human PTPN22 is formed by 807 AAs and its structure is comprised of three major domains: an N-terminal canonical PTP domain, an interdomain and a C-terminal domain containing 4 proline (P), glutamate (E), serine (S), threonine (T) (PEST)-enriched C-terminal motifs, termed P1-P4. The phosphatase activity lies in the PTP domain, in which the amino acid sequence is >90% conserved between the human and mouse proteins. Catalytic activity is critically dependent on amino acid residues C227 and D195. The P1 motif mediates interaction with Csk whilst the interdomain regulates PTPN22 activity inhibiting the catalytic domain through intramolecular interactions (Bottini and Peterson, 2014).

In T lymphocytes, PTPN22, also known as lymphoid tyrosine phosphatase (Lyp) in humans and PEST-enriched protein tyrosine phosphatase (Pep) in mice, targets several TCR downstream signalling proteins. Thus, PTPN22 has been reported to dephosphorylate tyrosine residues within ZAP70, Lck (Tyr394), Fyn or TCRζ limiting proximal TCR signalling (Hasegawa et al., 2004; Wu, J. et al., 2006; Bottini and Peterson, 2014) (Fig. 1.3). PTPN22 control of proximal TCR signalling is tightly linked to Csk as 25-50% of PTPN22 is in complex with Csk in resting T lymphocytes (Cloutier and Veillette, 1999; Vang et al., 2012). Some studies suggest that PTPN22-Csk interaction enables synergistic inhibition of Lck activity (Gjörloff-Wingren et al., 1999; Bottini and Peterson, 2014). However, other reports have established that association with Csk is indeed counter-productive. For example, Fiorillo et al. (2010) described that Lck suppressed PTPN22 through phosphorylation of tyrosine residues, a mechanisms that is reduced when the binding to Csk decreases, suggesting, therefore, that the PTPN22-Csk interaction negatively affects PTPN22 activity. Moreover, Vang et al. (2012) showed that the PTPN22-Csk complex disassociates upon TCR-binding facilitating PTPN22 recruitment into lipid rafts and dephosphorylation of its TCR signalling targets. Another study by Wallis et al. (2017) revealed that a third component, the adaptor protein TNF receptor associated factor (TRAF) 3, is involved in the regulation of PTPN22. Experiments performed using a T cell-specific *Traf3*$^{-/-}$ model suggest that TRAF3 promotes T cell activation inducing a tri-partite complex that regulates localisation and function of both Csk and PTPN22 (Wallis et al., 2017). Thus, the mechanism by which PTPN22 function is regulated upon TCR-stimulation are complex and there is still a lack of complete understanding in this field.

The discrimination between high affinity antigens and low affinity self-peptides strongly relies on early TCR signalling events. Salmond et al. (2014) described, using a *Ptpn22*$^{-/-}$ OT-I transgenic model, which expresses an MHC-I restricted ovalbumin (OVA)-specific TCR, that PTPN22 restricts T cell responses against weak affinity peptides while not impeding T cell activation against strong agonists. *Ptpn22*-deficient T lymphocytes display enhanced T cell responses when stimulated with low-affinity peptides as shown by increased expression of activation markers, IL-2 and effector proteins upon TCR-binding (Salmond et al., 2014; Brownlie et al., 2017; Bray et al., 2018). Thus, PTPN22 is important in determining TCR-signalling strength and setting an affinity threshold for T cell activation in order to avoid hyperreactivity against low affinity self-peptides. These data place PTPN22 in a complex network formed by phosphatases and kinases such as CD45, Csk and other PTPs that allow accurate antigen discrimination and maintain T cell homeostasis through control of proximal TCR signalling (Gaud et al., 2018).

In TCR-transgenic *Ptpn22*$^{-/-}$ mice, cell number and phenotype of both CD4^{+} and CD8^{+} T lymphocytes within the thymus and lymph nodes is comparable to its wild-type counterparts (Salmond et al., 2014; Wu, D.J. et al., 2014), suggesting that the lack of PTPN22 does not significantly affect thymic development of conventional T cells. Nonetheless, naïve *Ptpn22*$^{-/-}$ T cells display enhanced basal activation state and proliferation under lymphopenic conditions (Salmond et al., 2014), highlighting the key role of PTPN22 in maintenance of T cell homeostasis. Despite having a lower TCR activation threshold, total absence of PTPN22 in knockout mice does not result in spontaneous autoimmune defects (Hasegawa et al., 2004). Maine et al. (2012) reported that T_{reg} development was affected upon loss of PTPN22 resulting in an increased number of thymic and peripheral

T_{regs}. Additionally, *Ptpn22*$^{-/-}$ T_{regs} are more immunosuppressive as shown by increased IL-10 secretion and LFA-1-mediated adhesion properties (Brownlie et al., 2012) suggesting that increased T_{reg} numbers and improved function might maintain T cell homeostasis in mice lacking PTPN22.

The lack of PTPN22 also enhances LFA-dependent adhesion resulting in increased stabilisation of APC:T cell conjugates (Brownlie et al., 2012; Salmond et al., 2014; Sanchez-Blanco et al., 2018). Also, PTPN22 is involved in integrin 'outside-in' signalling regulating activation of Lck, ZAP70 and VAV1 following LFA-1:ICAM-1 engagement in migrating T cells (Burn et al., 2016). Furthermore, PTPN22 determines effector function of CTL responses. PTPN22-deficient mice display expanded effector/memory T cell populations and augmented Lck activity in re-stimulated T lymphocytes (Hasegawa et al., 2004). CTLs lacking PTPN22 are reactive against very low-affinity antigens showing higher secretion of inflammatory cytokines (e.g. IFNγ and TNF) and enhanced cytolytic effector responses both *in vitro* and *in vivo* when compared to wild-type CTLs (Salmond et al., 2014; Brownlie et al., 2017; Brownlie et al., 2019).

Additionally, PTPN22 is also significant in the context of other immune cells and T cell subsets, such as T_{FH} cells, limiting formation of germinal centres and Ab production, or myeloid cells, promoting PRR signalling primarily in Toll-like receptor (TLR)-induced IFNα/β responses (Wang, Y. et al., 2013; Maine et al., 2014; Bottini and Peterson, 2014).

1.5.4.2 Role of PTPN22 in autoimmune diseases

PTPN22 function has been strongly linked to autoimmunity. A common single nucleotide polymorphism (SNP) in the *PTPN22* gene, C1858T, results in an amino acid substitution (arginine to tryptophan) at position R620 (PTPN22^{R620W})

and has been associated with an increased risk of developing several autoimmune diseases including type I diabetes, rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) (Begovich et al., 2004; Bottini et al., 2004; Bottini and Peterson, 2014). PTPN22^{R620W} localises in the Csk-interacting P1 motif, greatly reducing PTPN22-Csk association (Bottini et al., 2004; Bottini and Peterson, 2014). Similarly to the lack of consensus in regards to whether or not PTPN22 activity is increased or decreased in association with Csk, discrepancies are shown in mouse studies describing the consequences of PTPN22^{R619W} (equivalent to R620W) expression. Some investigations imply that the polymorphism partially mimics the phenotype of *Ptpn22*$^{-/-}$ T cells, i.e. enhanced activation profiles and effector functions, suggesting a PTPN22 loss-of-function upon Csk dissociation (Zhang, J. et al., 2011; Dai et al., 2013; Burn et al., 2016). Conversely, studies describing PTPN22^{R260W} significance in human T cells have reported a PTPN22 gain-of-function acting as a more potent inhibitor of TCR signalling (Vang et al., 2005; Rieck et al., 2007; Vang et al., 2012).

1.5.4.3 Role of PTPN22 in T cell anti-tumour responses

T cells in autoimmunity are frequently characterised by increased sensitivity to weak TCR agonists and resistance to immune-regulatory mechanisms. By contrast, one of the mechanisms by which tumour cells evade immune responses is by the expression of low-affinity tumour-associated antigens (TAAs) and the manifestation of a harsh TME defined by the presence of immunosuppressive signals such as transforming growth factor β (TGFβ), among many others (Hanahan and Coussens, 2012; Becker et al., 2013; Maueröder et al., 2014). Thus, engineering T cells with autoimmunity-associated risk alleles provide a rational approach in adoptive cell transfer (ACT) therapies. For instance, *Ptpn22*$^{-/-}$ OT-I CTLs mediate enhanced tumour rejection in mice bearing ID8

ovarian carcinoma cells expressing the low-affinity OVA-peptide SIITFEKL (T4) when compared to control, whilst no significant differences were shown against the high-affinity OVA-peptide SIINFEKL (N4) (Brownlie et al., 2017; Brownlie et al., 2019). Importantly, *Ptpn22*$^{-/-}$ CD8^{+} memory T cells retain their enhanced protective capacity during long-term *in vivo* responses (Brownlie et al., 2019). Furthermore, Brownlie et al. (2017) described that *Ptpn22*$^{-/-}$ T lymphocytes are highly resistant to TGFβ-mediated suppression of proliferation as an indirect effect of enhanced TCR signalling and IL-2 secretion. Altogether, these data propose PTPN22 as a potential target for cancer immunotherapy to improve TAA-recognition and anti-tumour responses in ACT therapy (Maueröder et al., 2014; Brownlie et al., 2018).

1.6 Transforming growth factor β (TGFβ)

1.6.1 Classification, activation and signalling

The TGFβ superfamily is a pleiotropic group of cytokines involved in embryonic development, immune regulation and tissue regeneration. It is composed of 32 members divided into the TGFβ and bone morphogenetic protein (BMP) subfamilies. TGFβ1 in particular, as well as TGFβ2 and TGFβ3, members of the TGFβ subfamily, are strongly associated with immune regulation (Batlle and Massagué, 2019).

TGFβ1 (referred as TGFβ from now on) is synthesised as pro-TGFβ, an inactive form that includes a N-terminal portion known as the latency-associated peptide (LAP) forming the so-called small latent complex. LAP encircles the active TGFβ preventing contact with its cognate receptor. After secretion, the complex releases active TGFβ by cleavage of the LAP domain by extracellular proteases

or interaction with αβ integrins (Travis and Sheppard, 2014; Batlle and Massagué, 2019). Then, cells sense TGFβ signals through binding and subsequent assembly of a hetero-tetrameric receptor complex consisting of two type I and two type II receptor components known as TGFβR1 and TGFβR2, respectively. These contain a cytoplasmic domain with both serine/threonine and tyrosine kinase activity (Shi, Y. and Massagué, 2003; Massagué, 2012; David and Massagué, 2018). Following ligand binding, TGFβR2 phosphorylates and activates TGFβR1 which, in turn, phosphorylates the transcription factors small mothers against decapentaplegic homolog 2 (SMAD2) and SMAD3, referred as receptor regulated SMADs, or R-SMADs. SMAD2/3 phosphorylation leads to the formation of heterotrimeric complexes with SMAD4 which then translocate to the nucleus (Massagué, 2012; David and Massagué, 2018). In the nucleus, SMAD complexes interact with cell- and context-dependent co-activators and co-repressors that determine transcriptional outcomes (David and Massagué, 2018). Termination of TGFβ signalling is driven by induction of the inhibitory SMAD6 and SMAD7 that promote degradation of the TGFβ receptor. Additionally, CDK8 and CDK9 induce recognition of SMAD complexes by GSK3β, which marks SMADs for SMAD ubiquitination regulatory factor 1 (SMURF1)-mediated polyubiquitination and subsequent degradation. Importantly, TGFβ also signals via SMAD-independent pathways, including MAPK and PI3K pathways (Batlle and Massagué, 2019).

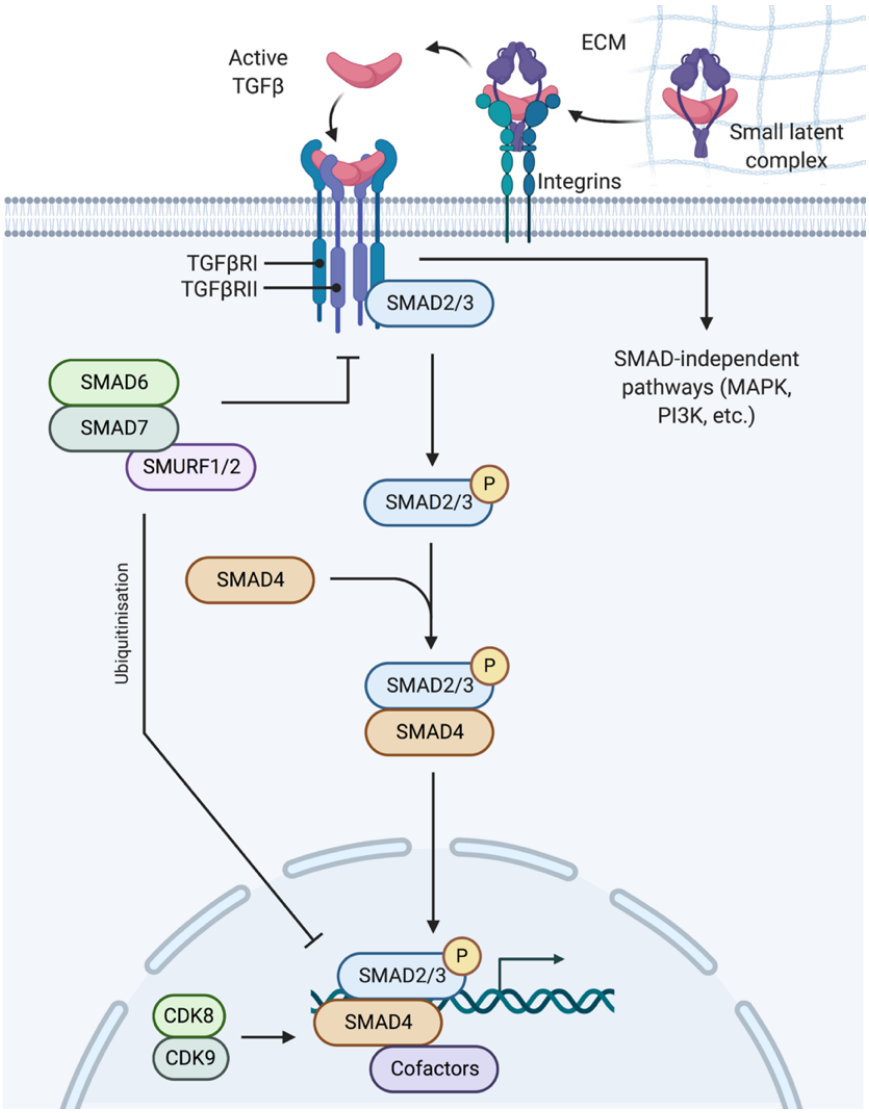


Figure 1.4: TGFβ signalling.

The interaction of the small latent complex with the extracellular matrix (ECM) and integrins releases the active form of TGFβ. TGFβ is then recognised by the TGFβR, which leads to the phosphorylation of TGFβRI by TGFβRII. Consequently, TGFβRI phosphorylates the TFs SMAD2/3, which consequently interact with SMAD4. The SMAD complex translocates to the nucleus and regulates transcription through association with other cofactors. SMAD6 and SMAD7 mediate the termination of TGFβ signalling through SMURF-mediated ubiquitination of SMADs and the TGFβR. Termination of TGFβ signalling can also be mediated by CDK8/9. *Adapted from "TGF-Beta Signaling Pathway", by Biorender.com (2021). Retrieved from https://app.biorender.com/biorender-templates.*

1.6.2 TGFβ in immune regulation

TGFβ has a pivotal role in immune regulation modulating both adaptive and innate lineages. TGFβ is particularly relevant in the development of several T cell lineages within the thymus as well as maintaining peripheral tolerance against self and benign antigens (Sanjabi et al., 2017). For instance, TGFβ induces development of T_{regs} antagonising negative selection and promoting survival of its precursors. Also, TGFβ induces expression of the IL-7 receptor α chain (IL-7Rα) in thymocytes, key for the differentiation of conventional $CD8^+$ T cells (Sanjabi et al., 2017). High IL-7R levels contribute to IL-7 sensing by low-affinity T lymphocytes promoting survival and homeostasis of the peripheral naïve T cell pool (Johnson and Jameson, 2012). The importance of TGFβ as a regulator of peripheral tolerance is shown in transgenic mouse models with abrogated TGFβ signalling that display aberrant lymphoproliferative phenotypes (Gorelik and Flavell, 2000; Lucas et al., 2000; Marie et al., 2006). Furthermore, TGFβ dampens T cell activation by inhibiting IL-2 transcription and specifically suppresses CTL, T_h1 and T_h2 differentiation while promoting development of pTreg, T_h17, T_h9 and T_{FH} cells (Sanjabi et al., 2017). In the context of CTLs, TGFβ represses cytotoxic function by directly inhibiting transcription of key effector proteins and cytokines upon association of SMADs with the co-activator activating transcription factor 1 (ATF1) (Thomas and Massagué, 2005).

1.6.3 TGFβ within the tumour microenvironment

Immune responses are suppressed within the TME through multiple mechanisms including the recruitment and expansion of immunosuppressive cells such as T_{regs} and myeloid-derived suppressor cells (MDSCs), the lack of nutrients and oxygen, poor antigen presentation, the acquisition of an exhausted T cell

phenotype or the accumulation of inhibitory cytokines such as IL-10 and TGFβ (Anderson et al., 2017; Zhang, Z. et al., 2020).

TGFβ within the TME affects several cell types and displays context-dependent effects. TGFβ acts as a tumour suppressor at early stages of cancer progression via cytostatic and proapoptotic mechanisms and as shown by favoured development of premalignant cells into tumours in mice containing genetic ablation of *TGFBRII* or *SMAD4* (Takaku et al., 1998; Muñoz et al., 2006; Bardeesy et al., 2006; Massagué, 2008). However, malignant cells switch off TGFβ-derived suppressive mechanisms and convert TGFβ into a pro-tumorigenic signal. Malignant cells are able to develop resistance to TGFβ as a consequence of mutations in core TGFβ signalling components and its downstream signals promoting survival and proliferation in TGFβ-rich environments. Additionally, tumour cells are also able to use TGFβ to their own advantage and rewire TGFβ programs to instruct metastasis and produce autocrine mitogens (Massagué, 2008).

Besides modulating tumour growth and invasion, TGFβ is key to drive immune evasion as it promotes the differentiation and recruitment of pro-tumour immune cells while suppressing the activity of anti-tumour cells (Flavell et al., 2010; Gigante et al., 2012; Tu et al., 2014). TGFβ impairs activation of immature tumour infiltrating DCs downregulating expression of MHC molecules and co-stimulatory receptors as well as becoming tolerogenic TGFβ-secreting cells that promote differentiation of tumour-specific T_{regs} (Li, M.O. et al., 2006; Flavell et al., 2010). Furthermore, TGFβ induces differentiation of the pro-tumorigenic M2 macrophages (Allavena et al., 2008) and N2 neutrophils (Fridlender et al., 2009). In terms of effector cells, TGFβ inhibits activation of NK cells via attenuation of

IFNγ secretion and downregulation of activation receptors such as NKG2D (Castriconi et al., 2003; Laouar et al., 2005). TGFβ also disrupts $CD8^+$ T lymphocytes killing capacity through inhibition of the effector proteins GrB, GrA, Fas-L, and IFNγ (Thomas and Massagué, 2005). In the context of $CD4^+$ T cells, TGFβ induces FoxP3 and receptor-related orphan receptor-γt (RORγt) expression promoting T_{reg} and T_h17 differentiation, respectively (Li, M.O. and Flavell, 2008).

1.7 Immunometabolism

All cellular processes are sustained by metabolism, an interconnected network of catabolic or anabolic chemical reactions. Catabolism facilitates the breakdown of molecules, ultimately resulting in energy production via adenosine triphosphate (ATP) synthesis. Key energy-producing catabolic pathways include glycolysis, the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS). In the presence of oxygen, a molecule of glucose undergoes glycolysis and is oxidised into two pyruvate molecules that translocate to the mitochondria. Here, pyruvate is converted by pyruvate dehydrogenase (PDH) into acetyl-CoA which, in turn, fuels the TCA cycle. The engagement of the TCA cycle yields free electrons carried by nicotinamide adenine dinucleotide (NAD/NADH) and flavin adenine dinucleotide (FAD/$FADH_2$) and transferred to the electron transport chain (ETC) in order to pump protons (H^+) into the mitochondrial intermembrane space. The membrane potential generated by the H^+ gradient is used by the ATP synthase to generate energy. Furthermore, substrates apart from glucose can be utilised to support ATP production. For instance, fatty acid β-oxidation (FAO) generates acetyl-CoA whilst several amino acids (AAs), such as glutamine, can incorporate at different steps of the TCA cycle. The glycolytic pathway *per se* also

participates in the generation of the membrane potential through the synthesis of NADH, which is then translocated to the mitochondria via the aspartate-malate shuttle. Finally, the generated ATP pool is used to fuel anabolic pathways, e.g. synthesis of fatty acids (FAS), proteins and nucleic acids (O'Neill et al., 2016).

During the last decade, it has become evident that metabolism shapes immunity. T lymphocytes in particular require metabolic modifications to match the energetic and biosynthetic demands needed for activation, growth, clonal expansion, differentiation and effector functions. Moreover, T cell metabolism has also been associated with other non-bioenergetic functions including epigenetic, post-transcriptional and post-translational modifications as well as the utilisation of metabolites as signalling molecules (Gerriets and Rathmell, 2012; Pearce et al., 2013; Pearce and Pearce, 2013; MacIver et al., 2013; Maciolek et al., 2014; Buck et al., 2015; Geltink et al., 2018; Chapman et al., 2019; Shyer et al., 2020). This new understanding has provided a novel and valuable tool to manipulate T cell function in immunotherapy and, therefore, emphasises the importance to further comprehend how T cell metabolism is regulated (O'Sullivan and Pearce, 2015; Chang and Pearce, 2016; Patel and Powell, 2017; Bettencourt and Powell, 2017; Li, X. et al., 2019; Hope and Salmond, 2019; Pålsson-McDermott and O'Neill, 2020). Below, I describe the crosstalk between metabolism and function during the development of T cell responses followed by some examples on how this knowledge can been exploited to manipulate immunity in disease.

1.7.1 Metabolic reprogramming in activated T lymphocytes

Quiescent naive T cells take up low levels of nutrients and display a basal metabolic activity characterised by the engagement of catabolic pathways such as OXPHOS and FAO to maintain housekeeping functions. Upon antigen

recognition, T lymphocytes undergo a metabolic shift that drives the cell into an anabolic programme that supports growth and rapid clonal proliferation. Specifically, activated T cells upregulate the expression of nutrient transporters and, subsequently, significantly increase nutrient uptake in order to provide sufficient building blocks for the synthesis of new macromolecules. Furthermore, activated T cells mainly rely on aerobic glycolysis, glutaminolysis, FAS and serine glycine one-carbon (SGOC) metabolism (MacIver et al., 2013; Buck et al., 2015; Geltink et al., 2018; Chapman et al., 2019; Shyer et al., 2020).

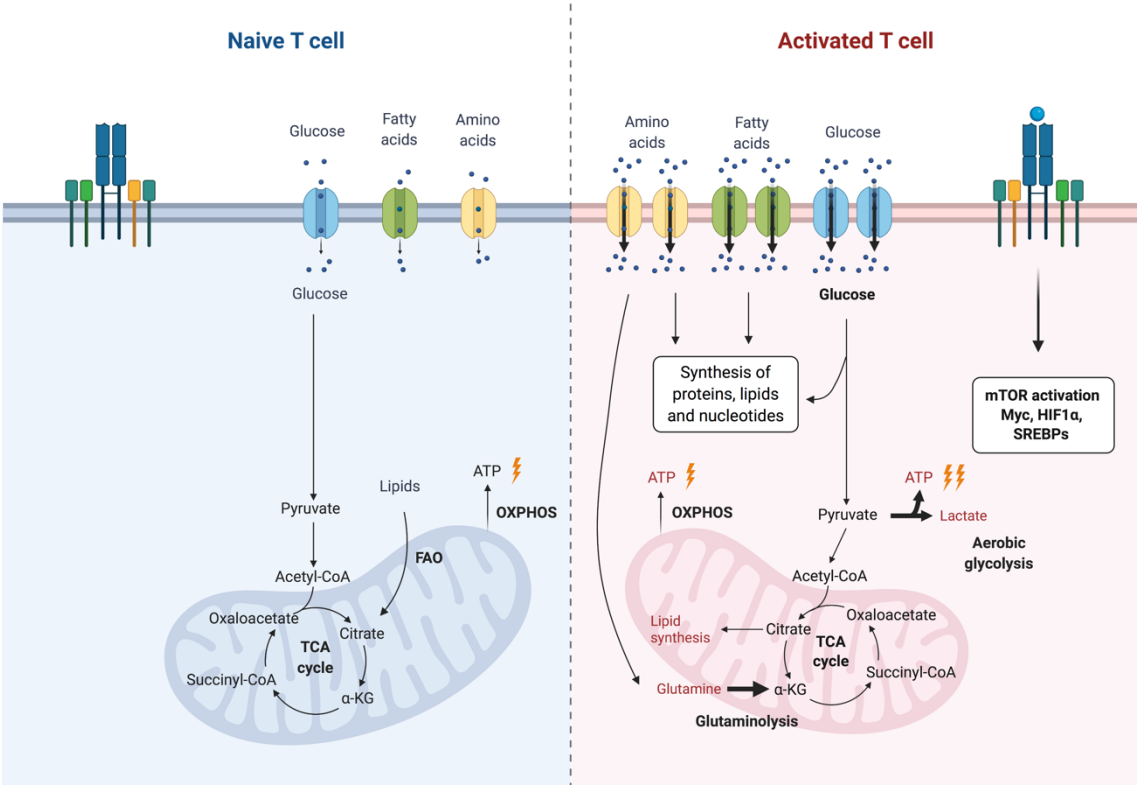


Figure 1.5: Metabolism of activated T lymphocytes.

Naïve T cells display a low metabolic activity, characterised by low nutrient uptake and the engagement of OXPHOS and FAO. Upon TCR-stimulation, the activation of mTOR and the expression of metabolic TFs (e.g. Myc, HIF1α, SREBPs) leads to a metabolic reprogramming that benefits the synthesis of new macromolecules through the preferential engagement of aerobic glycolysis and glutaminolysis.

How does antigen recognition by the TCR lead to the metabolic reprogramming of activated T cells? Transcription factors such as Myc, hypoxia-inducible factor 1 alpha (HIF1α) and sterol regulatory element binding-proteins (SREBPs) family members have been strongly associated with the upregulation of nutrient transporters and metabolic-related genes (Hough et al., 2015). Additionally, the mTOR/AMPK axis modulates T cell metabolism while sensing energy status and nutrient availability triggering a context-dependent response and, therefore, providing metabolic plasticity during T cell function (Ma et al., 2017b; Myers et al., 2019; Saravia et al., 2020; Shyer et al., 2020).

1.7.1.1 Transcriptional regulation of the T cell metabolic reprogramming

The proto-oncogenic transcription factor c-Myc (referred as Myc from now on) is involved in a wide range of human cancers and contributes to the regulation of cell cycle and metabolism. In T lymphocytes, Myc expression is rapidly upregulated in response to TCR-stimulation and sustained by the action of costimulatory receptors, IL-2 and nutrient availability (Preston et al., 2015; Swamy et al., 2016). Wang, R. et al. (2011) identified Myc as a key transcription factor for the metabolic reprogramming of activated T cells and, particularly, for the induction of glycolysis and glutaminolysis. A more recent study by Marchingo et al. (2020) described that Myc null T cells show no disruption in the upregulation of glucose transporter GLUT1 and little impact in the expression of glycolytic enzymes. Instead, Myc null T cells display strong inhibition of the lactate transporter Slc16a1 and several amino acid transporters including Slc1a5, Slc7a1 and, particularly, Slc7a5. In the same study, Slc7a5-deficient T cells largely mimicked the phenotype of Myc null T cells suggesting that, mechanistically, Myc might trigger T cell metabolic reprogramming via the induction of amino acid transporters. Importantly, Myc also induces expression of

AP4, a transcription factor that sustains the Myc-regulated metabolic program after Myc downregulation during TCR signalling withdrawal. (Chou et al., 2014). Nonetheless, TCR-induced Myc expression alone is not sufficient to trigger T cell activation and sustain T cell function and differentiation. HIF1α is usually stabilised under hypoxic conditions and induces gene expression of all glycolytic enzymes, including lactate dehydrogenase (LDH) and GLUT1, to minimise oxygen consumption. However, HIF1α can also be induced by the TCR and despite being dispensable for early T cell activation and proliferation is, similarly to AP4, required to sustain glycolysis and glutaminolysis throughout differentiation and expansion of $CD8^+$ T cells (Wang, R. et al., 2011; Finlay et al., 2012).

In the context of lipid metabolism, activated T cells induce the mTOR-dependent SREBP1 and SREBP2 which bind to promoter regions of genes encoding key enzymes for fatty acid and cholesterol synthesis, such as fatty acid synthase (FASN), acetyl-CoA carboxylase (ACC1) and hydroxymethylglutaryl-CoA reductase (HMGCR). $CD8^+$ T lymphocytes lacking SREBP signalling strikingly display an altered lipid anabolic program in response to mitogen resulting in an impaired capacity to blast, proliferate and survive whilst indirectly affecting glycolysis and glutaminolysis due to a compromised bioenergetic status (Kidani et al., 2013; Lochner et al., 2015).

The transcription factors estrogen-related receptor α (ERRα) and mitochondrial transcription factor A (MTFA) are also required for T cell activation and have been particularly associated with the regulation of mitochondrial function (Michalek et al., 2011b; Baixauli et al., 2015). The TCR-induced increase of mitochondrial function is accompanied by heightened mitochondrial mass and dynamics (Ron-

Harel et al., 2016; Fischer et al., 2018; Rambold and Pearce, 2018). How and when mitochondrial biogenesis is regulated is largely unknown but it has been strongly linked with the peroxisome proliferator-activated receptor γ (PPARγ) co-activator 1-α (PGC1α) (Scharping et al., 2016; Dumauthioz et al., 2020).

1.7.1.2 mTOR/AMPK

mTOR is a constitutively expressed serine/threonine kinase that performs as a central node integrating environmental immune and metabolic cues in order to regulate T cell function. mTOR signals through two main complexes, the rapamycin-sensitive mTOR complex 1 (mTORC1) and rapamycin-insensitive mTOR complex 2 (mTORC2). mTORC1 is a key metabolic regulator during T cell activation and differentiation. mTORC1 is rapidly activated in T cells upon TCR-stimulation by a PI3K-Akt-independent mechanism involving PDK1 (Finlay et al., 2012). Furthermore, mTORC1 activity is modulated by intracellular levels of nutrients: amino acids can be directly sensed via Rag GTPases whilst low glucose availability reduces the ATP:AMP ratio promoting AMPK activation and, subsequently, mTORC1 suppression. mTORC1 downstream targets include the translational regulators 4E-binding proteins (4E-BPs) and ribosomal protein S6 kinases (S6Ks), thereby promoting protein synthesis (Chapman and Chi, 2015; Saxton and Sabatini, 2017; Zeng and Chi, 2017; Myers et al., 2019). However, a recent proteomic analysis by Hukelmann et al. (2016) established that, in T cells, mTOR selectively influences specific subsets of proteins rather than having a global effect on protein abundance. For instance, rapamycin-treated CTLs significantly downregulate the expression of glucose transporters and enzymes involved in glycolysis and cholesterol metabolism but not those involved in glutaminolysis and OXPHOS (Hukelmann et al., 2016; Howden et al., 2019). Consistently, naïve T cells stimulated *in vitro* in the presence of rapamycin are

unable to undergo T cell metabolic reprogramming and enter an anergic state (Araki et al., 2010; Shi, L.Z. et al., 2011). The mechanisms underlying the control of T cell metabolism by mTORC1 are suggested to be dependent on activation of transcription factors such as HIF1α (Finlay et al., 2012) and SREBPs (Kidani et al., 2013), but not Myc (Finlay et al., 2012; Howden et al., 2019; Marchingo et al., 2020).

By contrast, AMPK counterbalances mTOR activity in the regulation of T cell metabolism. AMPK, formed by 3 subunits (α, β, γ), senses AMP/ADP accumulation and maintains the ATP/ADP ratio promoting energy-producing processes while suppressing anabolic pathways. Furthermore, AMPKα1 can be activated directly by liver kinase B1 (LKB1) and calmodulin-dependent protein kinase kinase-2 (CamKK2) in response to energetic stress and Ca^{2+} release after TCR-ligation, respectively. One of the best characterised AMPK targets is ACC1, an enzyme involved in the generation of malonyl-CoA which serves as a substrate for FAS while blocking FAO through allosteric inhibition of CPT1 (Ma et al., 2017b; Saravia et al., 2020). Furthermore, as mentioned above, AMPK is known to directly inhibit mTOR via activation of the TSC1-TSC2 complex or phosphorylation of the mTOR binding partner Raptor. In CTLs, AMPK selectively senses glucose deprivation initiating a metabolic adaptive response based on a glycolysis-to-glutaminolysis switch derived from mTORC1 inactivation (Blagih et al., 2015). AMPKα1-deficient T lymphocytes, despite being able to proliferate and differentiate into effector T cells, provide deficient primary responses due to an impaired accumulation of both $CD4^+$ and $CD8^+$ T cells in infection sites (Rolf et al., 2013). Is speculated that T lymphocytes lacking AMPKα1 fail to adapt and survive within the nutrient-compromised inflammatory microenvironment which

might explain their poor capacity in the generation of memory T cells (Rolf et al., 2013).

1.7.1.3 Aerobic glycolysis in activated T lymphocytes

Conversion of pyruvate, the final glycolytic product, into lactate via lactate dehydrogenase (LDH) is a transition seen in hypoxic environments to minimise oxygen consumption. However, it is likely that all proliferating cells use aerobic glycolysis as a central metabolic pathway – i.e. lactate production regardless of oxygen levels, a phenomenon known as 'the Warburg effect'. Within minutes after TCR-stimulation, T lymphocytes impair mitochondrial pyruvate import and facilitate breakdown into lactate (Menk et al., 2018). Furthermore, rapid mTOR activation as well as Myc and HIF1α upregulation promote expression of glycolytic enzymes and glucose transporters that sustain glucose metabolism during T cell activation and differentiation (Wang, R. et al., 2011; Salmond, 2018). As shown by analysis of the impact of glucose deprivation or treatment with 2-deoxyglucose (2-DG), an inhibitor of hexokinase (HK), or studies of transgenic mice lacking GLUT1, glycolytic inhibition abolishes activation-induced expansion of effector T cells while significantly affecting survival (Macintyre et al., 2014). In effector T cells, limiting glucose availability is sensed by AMPK which, in turns, inhibits mTOR activation resulting in slowed proliferation and reduced IFNγ secretion (Blagih et al., 2015).

Aerobic glycolysis is a relatively inefficient bioenergetic pathway as only 2 ATPs are generated per glucose molecule. However, glycolytic flux provides accumulation of intermediaries required for additional functions. For instance, glucose-6-phosphate (G6P) is diverted into the pentose phosphate pathway (PPP) which provides pentoses for nucleotide synthesis and maintains the

NADPH/$NADP^+$ ratio. G6P also fuels the hexosamine pathway driving the generation of uridine diphosphate N-acetylglucosamine (UDP-GlcNAc), a substrate used for O-GlcNAcylation of key T cell signalling molecules such as Myc, NFAT or c-Rel. 3-phosphoglycerate (3PG) is used as a substrate for serine generation contributing to SGOC metabolism, whilst dihydroxyacetone-phosphate (DHAP) produces glycerol for the synthesis of complex lipids (Seki and Gaultier, 2017; Dimeloe et al., 2017; Shyer et al., 2020). Moreover, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) act as a posttranscriptional regulator by directly binding to AU-rich elements within the 3' untranslated region (UTR) of IFNγ mRNA and preventing translation (Chang et al., 2013). After TCR-stimulation, GAPDH is diverted into the glycolytic pathway, disengaging from the 3'-UTR and initiating IFNγ translation. Importantly, the enolase-derived product phosphoenolpyruvate (PEP) performs as a metabolic checkpoint modulating cytosolic Ca^{2+} concentrations and NFAT activation (Ho et al., 2015). Therefore, aerobic glycolysis is essential during T cell activation not only to support ATP and biomass production, but also contributing to the generation of signalling metabolites that directly implicate glycolysis on T cell effector function.

1.7.1.4 Mitochondrial metabolism in activated T lymphocytes

The Warburg effect, first identified in tumour cells, was initially described as the engagement of aerobic glycolysis in parallel to the inhibition of OXPHOS (Warburg, 1956; Liberti and Locasale, 2016). Nonetheless, T lymphocytes also require mitochondrial metabolism to support activation (Sena et al., 2013; Tan et al., 2017). Despite the shift of pyruvate metabolism towards lactate, pyruvate levels still augment and fuel the TCA cycle upon TCR-stimulation. Furthermore,

the TCA cycle is predominantly replenished by glutamine (Gln)-derived α-ketoglutarate in a process known as glutaminolysis. Fatty acids are also utilised as an additional source of TCA intermediaries as FAO-derived acyl-CoA enters the mitochondria via carnitine palmitoyltransferase 1 (CPT1) and induces acetyl-CoA synthesis (Lochner et al., 2015). Importantly, the relevance of mitochondrial metabolism goes beyond coupling the TCA cycle and the ETC to produce ATP. ETC-derived ROS has been described as regulator of mTOR and NFAT activation and has been described as an additional 'signal 3' during T cell activation (Sena et al., 2013; Mak et al., 2017; Chamoto et al., 2017). Moreover, Krebs intermediaries mediate alternative T cell functions including its utilisation as posttranslational regulators or as substrates for biosynthesis. For example, citrate can be used to produce lipids via ATP citrate lyase (ACL) and oxalacetate to produce nucleotides, whilst malate and succinate can be used to modify proteins (Mehta et al., 2017). Besides supporting the Krebs cycle and OXPHOS, mitochondria sustain SGOC metabolism, a complex network that comprises the folate and methionine cycle and that contributes to glutathione synthesis, redox balance and epigenetic remodelling via accumulation of the methyl donor S-adenosylmethionine (SAM) (Ron-Harel et al., 2016; Ma et al., 2017a).

1.7.1.5 Amino acid uptake in activated T lymphocytes

Amino acids represent, in addition to glucose and lipids, the third pillar that sustain T cell metabolism. The expression of AA transporters, such as Slc7a5 (large neutral AA transporter 1; LAT1), Slc3a2 (CD98) and Slc1a5 (alanine serine cysteine transporter 2; ASCT2), is highly upregulated upon TCR-stimulation in a Myc-dependent manner (Marchingo et al., 2020; Wang, W. and Zou, 2020). As shown by Sinclair et al. (2013), Slc7a5-deficient T lymphocytes are unable to clonally expand and differentiate into effector T cells indicating that Slc7a5-

mediated AA transport is essential to undergo T cell metabolic reprogramming after antigen recognition. On the other hand, ASCT2, a major glutamine transporter, despite being dispensable for T cell proliferation and IL-2 production, is associated with optimal mTORC1 activation resulting in defective Myc expression and effector differentiation in activated T cells lacking ASCT2 (Nakaya et al., 2014). Importantly, activated T lymphocytes require extracellular uptake of both essential and several non-essential AAs emphasising the high demand on AAs and its importance in the modulation of T cell responses (Kelly and Pearce, 2020).

The mechanisms underlying T cell suppression in the absence of AAs are mainly associated with impaired protein synthesis and/or mTORC1 inactivation (Kelly and Pearce, 2020). However, T cells are able to induce an adaptive response in this scenario. Mechanistically, T cells can sense AA deprivation upon interaction of uncharged tRNAs with general control nonderepressible 2 (GCN2). GCN2 activates ATF4, an stress-induced transcription factor that upregulates the expression of genes involved in metabolic reprogramming that leads to the synthesis of AA (Yang et al., 2018). In addition to protein synthesis and the regulation of mTORC1 activity, recent investigations have linked several AAs to other T cell functions. For example, (1) glutamine is used as a substrate for anaplerosis, glutathione generation (Mak et al., 2017), O-GlcNAcylation (Swamy et al., 2016) and asparagine (Asn) synthesis via asparagine synthetase (ASNS) (Hope et al., 2020); (2) serine incorporates into SGOC metabolism, as mentioned in section 1.7.1.3; (3) methionine uptake is a rate limiting step for SAM generation in activated T cells, as shown by impaired RNA and histone methylation upon deprivation of external methionine supply (Sinclair et al., 2019; Klein Geltink and Pearce, 2019); (4) intracellular arginine accumulation directly modulates T cell

survival while promoting a transition from glycolysis to OXPHOS (Geiger et al., 2016). Therefore, AAs are essential pleiotropic metabolites for T cell function involved in protein synthesis, mTORC1 activation, signalling and regulation of gene expression.

1.7.2 Metabolism determines T cell fate

Changes in metabolism remarkably determine T cell differentiation into an effector vs memory phenotype. Resting memory T lymphocytes, as they do not proliferate and produce little or no cytokines, mainly rely on catabolic pathways such as FAO or OXPHOS. Unlike naïve T cells, memory T cells are characterised by an increased spare respiratory capacity (SRC) that promotes longevity and rapid recall responses (van der Windt et al., 2012). Enhanced mitochondrial capacity, driven by the common γ chain cytokines IL-7 and IL-15 as well as CD28 co-stimulation (Cui et al., 2015; Klein Geltink et al., 2017), has been associated with increased FAO since CPT1 overexpression increases SRC and generation of memory T cell populations *in vivo* (Pearce et al., 2009). Interestingly, FAO is preferentially fuelled by *de novo* FAS as shown by impaired memory formation in T cells lacking aquaporin 9 (AQP9), a mediator of glycerol import (O'Sullivan et al., 2014). Furthermore, memory T cells present increased mitochondrial mass driven by high expression of PGC1α (Scharping et al., 2016; Chamoto et al., 2017). In contrast, the metabolism of short-lived effector T cells is characterised by high glycolytic and mTORC1 activity. Attenuation of the glycolytic flux, either by 2-DG or rapamycin supply in low doses, has been associated with the acquisition of memory-like phenotypes and long-term responses *in vivo* (Araki et al., 2009; Sukumar et al., 2013). Similarly, enforcement of mitochondrial function through PGC1α or FAO stimulation also drives memory formation sustaining

persistent and durable responses (Chamoto et al., 2017; Li, W. and Zhang, 2020; Dumauthioz et al., 2020).

In the context of $CD4^+$ T lymphocytes, each subset also has a unique metabolic profile. In general terms, effector CD4 T_h cells (T_h1, T_h2 and T_h17) preferentially rely on glycolysis whilst T_{regs} depend on FAO and OXPHOS (Michalek et al., 2011a; Gerriets and Rathmell, 2012; MacIver et al., 2013; Buck et al., 2015). Thus, the glycolytic/mitochondrial dichotomy comprises a key regulatory axis that determines T cell fate and, as discussed below, provides a therapeutic tool to manipulate T cell immunity.

1.7.3 Immunometabolism as an emerging target in immunotherapy

The understanding of immunometabolism and its regulation has provisioned a new mechanism to attenuate or boost T cell responses in autoimmune diseases and cancer, respectively. Particular interest is arising in the field of anti-tumour responses as the TME imposes metabolic barriers, either by nutrient and oxygen-depletion or the secretion of immunomodulatory metabolites, that dampen effective T cell responses (Ho and Kaech, 2017; Sugiura and Rathmell, 2018; Hope and Salmond, 2019; Lim et al., 2020).

Investigations revealing the direct implications of glycolysis in T cell effector function have been used to improve cytotoxicity of T lymphocytes in ACT therapies by enforcing accumulation of glycolytic intermediaries, such as PEP (Ho et al., 2015). Nonetheless, constitutive glycolytic flux is associated with terminal differentiation of CTLs (Kishton et al., 2017). Instead, new studies have been focused on the modulation of mitochondrial metabolism to reinforce memory-like phenotypes providing long-term T cell responses and survival

(Geiger et al., 2016; Klein Geltink et al., 2017; Chamoto et al., 2017; Li, W. and Zhang, 2020).

Unravelling how pro-tumour cells such as $T_{regs,}$ MDSCs or M2 macrophages are favoured within the TME has also been of interest in cancer therapy. For instance, recent research by Wang, H. et al. (2020) revealed that intratumoral T_{regs} adapt to lactate-rich environments switching to a lipid metabolism mediated by the upregulation of CD36, a fatty acid translocase, that sustains mitochondrial fitness and suppressive function selectively within the TME. Consequently, tumour rejection substantially improves in T_{reg}-specific CD36-ablated mice models while not affecting to peripheral homeostasis proposing CD36 as a potential target for cancer therapy. Co-inhibitory molecules also influence T cell metabolism *per se* (Patsoukis et al., 2015). Therapies combining immune checkpoint blockade (ICB) and metabolism-directed strategies have provided enhanced therapeutic efficiency suggesting that targeting metabolism is also promising in this context (Hope and Salmond, 2019; Li, X. et al., 2019). Another attractive strategy is to metabolically compromise tumour cells. Tumour cells are strongly dependent on nutrient availability to sustain aberrant proliferation and, therefore, nutrient deprivation limits tumour progression. A good example of this is the utilisation of asparaginases, which deprives tumour cells from the AA Asn, as a conventional chemotherapeutic adjuvant in acute lymphoblastic leukaemia (ALL) (Chiu et al., 2020).

On the other hand, immune cell metabolism is also aberrant in autoimmunity and, therefore, the manipulation of autoreactive T cell metabolism is increasingly becoming a field of interest (Teng et al., 2019). Particularly, limiting glucose metabolism successfully reduces T_h1, T_h17 and T_{FH} hyperreactivity resulting in disease attenuation (Yin et al., 2015; Choi et al., 2018; Abboud et al., 2018;

Kornberg et al., 2018). For example, a recent study by Angiari et al. (2020) showed that the utilisation of TEPP-46, a small molecule that acts on pyruvate kinase isozyme M2 (PKM2), the enzyme that converts PEP into pyruvate, inhibited the development of experimental autoimmune encephalomyelitis (EAE). PKM2 is mostly present as a monomer/dimer in activated T cells, which has been associated with moonlighting activities beyond canonical glycolytic control (Luo et al., 2011). Instead, monomer/dimer PKM2 translocates to the nucleus and regulates gene expression of HIF1α which, in turn, promotes expression of glycolytic enzymes. Mechanistically, TEPP-46 enforces PKM2 tetramerization and exit from the nucleus resulting in glycolysis attenuation, decreased T_h1 and T_h17 polarization and inhibited EAE development.

Altogether, these studies indicate that the role of metabolism in immunity goes beyond sustaining immune function bioenergetically and, rather than being only a consequence of T cell signalling, metabolism *per se* belongs to the complex signalling machinery that regulates T cell responses. This finding has provisioned researchers with a whole new spectrum of possibilities to manipulate immune responses and has positioned the study of immunometabolism as a state-of-the-art field.

1.8 Thesis aims

As described above, emerging investigations have provided evidence that metabolic modulation of T lymphocytes is a strategy that can be successfully exploited for immunotherapy. The outcome of T cell responses is defined by the integration of a wide array of environmental signals that include TCR signalling, co-stimulation, cytokines or nutrient availability (Ramsay and Cantrell, 2015) (Fig. 1.6). Adequate control of these signals leads to optimal immune responses whilst

its deregulation is associated with the development of autoimmunity and cancer progression (Bantug et al., 2018). Further understanding how metabolism is shaped by these signals during the development of CD8$^+$ T cell responses will shed light into novel strategies to precisely manipulate T cell function in disease and acquire better clinical outcomes.

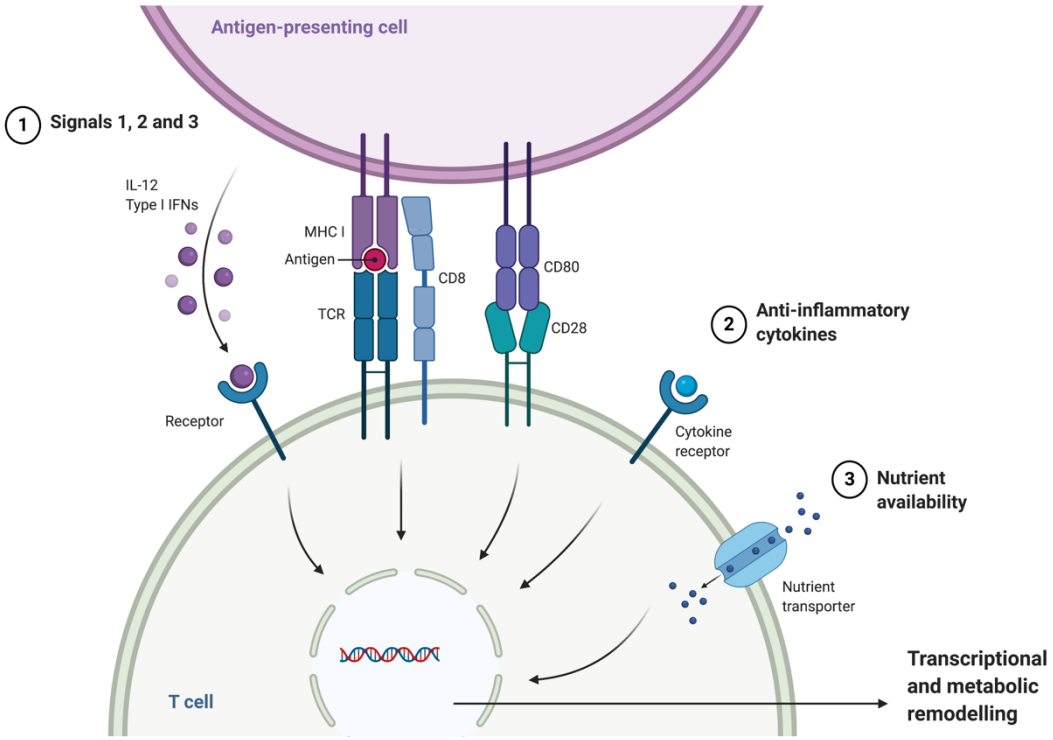


Figure 1.6: Environmental cues are integrated to dictate CD8$^+$ T cell fate.
CD8$^+$ T cell function is influenced by the presence of environmental signals that modify their gene expression and metabolic profile. Amongst the main signals that shape CD8$^+$ T cells we find: (1) 'Signals 1, 2 and 3', which include TCR signalling, co-stimulation (e.g. CD28) and inflammatory cytokines (e.g. IL-12 and type I IFNs); (2) anti-inflammatory cytokines and (3) the availability of nutrients.

Here, in order to gain further understanding of the fundamental mechanisms that regulate CD8$^+$ T cell responses with the long-term goal of identifying new targets for immunotherapy, I aim to investigate how some of these environmental cues are integrated by CD8$^+$ T cells and how these differentially influence their function and metabolism. Specifically, in this thesis I have aimed to:

1. Define how signals 1, 2 and 3 are integrated to mediate with $CD8^+$ T cell activation and the TCR-induced metabolic reprogramming. The study of 'Signals 1, 2 and 3' and its influence on T cell function and metabolism is included in Chapter 3.
2. Determine the effect of TGFβ during $CD8^+$ T cell priming and subsequent metabolic reprogramming. The study of TGFβ and its influence on T cell function and metabolism is included in Chapter 4 and Chapter 5.
3. Interrogate the requirements for the amino acid asparagine to sustain $CD8^+$ T cell function and metabolism. The study of asparagine availability and its influence on T cell function and metabolism is included in Chapter 6.

Chapter 2 Materials and methods

2.1 Transgenic mice

All mice were maintained at the St. James Biomedical Services (SBS) animal facility (University of Leeds, UK). Experiments were performed in accordance with UK Home Office project licence PDAD2D507. Age-matched (7-12 weeks) mice were used in the majority of the experiments. However, some repeated experiments were performed using older mice (28-32 weeks) due to the difficulties caused by the COVID-19 situation between March and August 2020.

2.1.1 *Rag1*$^{-/-}$ *CD45.1*$^{+}$ OT-I mice

The OT-I mouse strain expresses a transgenic TCR that specifically recognises an H-2K^{b}-restricted ovalbumin peptide ($OVA_{257-264}$). Single amino acid substitutions in this peptide alter the affinity for the OT-I TCR (Hogquist et al., 1994). Additionally, this OT-I strain lacks mature B cells and T cells expressing non-transgenic TCRs (*Rag1*$^{-/-}$ genotype).

2.1.2 *Ptpn22*$^{-/-}$ *Rag1*$^{-/-}$ *CD45.2*$^{+}$ OT-I mice

OT-I mice with global deletion of *Ptpn22* were generated as described previously (Salmond et al., 2014). This mouse strain is also RAG1-deficient. Furthermore, *Ptpn22*$^{-/-}$ T cells express a CD45.2 allele, whilst *Ptpn22*$^{+/+}$ T cells (Section 2.1.1) express CD45.1. This is used to distinguish both cell types in *in vitro* and *in vivo* experiments where these cells are co-cultured or co-transferred, respectively.

2.1.3 *Asns*$^{Tm1a(EUCOMM)/Wtsi}$ mice

The genetically-modified mouse strain *Asns*$^{Tm1a(EUCOMM)/Wtsi}$, referred from now on as *Asns*^{Tm1a}, was generated by the International Mouse Phenotyping Consortium (IMPC). When imported to the University of Leeds SBS animal facility, *Asns*^{Tm1a}

mice, originally on a C57BL/6N background, were back-crossed with C57BL/6J mice. *Asns*^{Tm1a} mice contain a gene-trap insertion in intron 2 of the *Asns* locus that results in an hypomorphic *Asns* allele (Ruzzo et al., 2013) which, when expressed homozygously, reduces ASNS protein to <10% of control levels (Hope et al., 2020). Polymerase chain reaction (PCR), as described in Section 2.2, was performed to determine the genotype of *Asns*^{Tm1a} mice. When performing experiments with *Asns*^{Tm1a} mice, WT littermates or in-house C57BL/6 mice were used as controls.

2.2 *Asns*^{Tm1a} genotyping

2.2.1 DNA extraction

First, DNA from ear samples was extracted adding lysis buffer [100mM Tris (pH8.5), 5mM EDTA, 0.2% SDS, 200mM NaCl] and proteinase K (100μg/ml; Roche) overnight at 56°C. The following day, samples were centrifuged (13000rpm, 10min) and supernatants were transferred to 1.5mL tubes containing isopropanol. Samples were centrifuged (13000rpm, 10min), the supernatant was discarded and pellets were washed carefully in 70% EtOH and dried for at least 30 min. TE buffer (10mM Tris, 1mM EDTA) was added and samples were kept overnight at 56°C.

2.2.2 Polymerase chain reaction (PCR)

PCR master mix containing deoxynucleotides (dNTPs; 10mM, Invitrogen), $MgCl_2$ (50mM, Thermo Scientific), KAPA2G Fast HotStart DNA Polymerase (Kapa Biosystems) and dH_20 was mixed in PCR tubes with the forward (ASNS-5arm-WTF: 5' GCATTTAAGTGCACAGGAGGA 3'; 20μM, Sigma) and reverse primers (ASNS-Crit-WTR: 5' ACAAGGGTCAGGCATCAGAG 3'; 5mut-R1: 5'GAACTTCGGAATAGGAACTTCG 3'; 20μM, Sigma) plus the indicated sample.

PCR was performed using a 96-well thermocycler (Applied Biosystems) following the next protocol:

- Stage 1: 95°C, 1 minute.
- Stage 2 (x29 cycles): 95°C, 10 seconds → 60°C, 10 seconds → 72°C, 1 second.
- Stage 3: 72°C, 30 seconds. Then, samples were kept at 16°C until further analysis.

2.2.3 Electrophoresis

PCR products, mixed with BlueJuice™ gel loading buffer (Invitrogen), were analysed through electrophoresis (Bio-Rad) in a 2% agarose gel containing ethidium bromide (Alfa Aesar). The amplified Asns-WT (223pb) or Asns-Tm1a (123pb) fragments were detected using ChemiDoc imagers (Bio-Rad). Asns-WT and Asns-Tm1a bands were distinguished according to a 100bp DNA ladder (Invitrogen).

2.3 Cell culture

2.3.1 Reagents

For all experiments included in Results Chapters 3, 4 and 5 T cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with penicillin-streptomycin, 50μM β-mercaptoethanol and 5% heat-inactivated Fetal Bovine Serum (FBS) (Table 2.1). All experiments included in Chapter 6, unless otherwise stated, were performed using Dulbecco's Modified Eagle's Medium (DMEM) supplemented with penicillin-streptomycin, 50μM β-mercaptoethanol, 5% dialysed FBS, ± 2mM L-glutamine ± 300μM anhydrous L-Asparagine (Table 2.1).

Reagent	Final concentration	Manufacturer
β-mercaptoethanol	50μM	Gibco #21985-023
Dialysed FBS	NA	Gibco #1050064
DMEM	NA	Gibco #11880-036
FBS	NA	Gibco #26140087
IMDM	NA	Gibco #12440-053
PBS	NA	Gibco #10010-015
L-Asparagine	300μM	Sigma #A4159
L-Glutamine	2mM	Gibco #25030-081
Penicillin/Streptomycin	100IU/mL penicillin 100μg/mL streptomycin	Gibco #15140-122
anti-CD25 (cl 3C7)	5μg/ml	BioLegend #101906
anti-CD28 (cl 37.51)	1μg/mL	BioLegend #102116
hIL-2	1ng/mL or 20ng/mL	PeproTech #200-02
TGFβ	5ng/mL	PeproTech #100-21
IFNα	20ng/ml	BioLegend #752802
IL-12	2ng/ml	PeproTech #210-12
IL-7	10ng/mL	PeproTech #217-17
SIINFEKL (N4)	10^{-6}M-10^{-8}M	Cambridge Peptides
SIITFEKL (T4)	10^{-6}M-10^{-8}M	Cambridge Peptides
SIIGFEKL (G4)	10^{-6}M-10^{-8}M	Cambridge Peptides

Table 2.1: List of reagents for cell culture.

NA – Not applicable; IU – international units.

2.3.2 T lymphocyte stimulation

Naïve T lymphocytes were obtained from lymph nodes and/or spleens of OT-I (*Ptpn22*$^{+/+}$ or *Ptpn22*$^{-/-}$). Single cell suspensions were obtained by filtering the cells through a 70 μm strainer (Sigma Aldrich). No additional cell purification was needed for lymph nodes from OT-I mice (>90% CD8$^+$ T cells, of which >80% are naïve CD8$^+$ T cells). Spleen samples were treated with Ammonium-Chloride-Potassium (ACK) lysing buffer to remove red blood cells and utilised without any further purification. In all experiments, viable T cell concentration was determined by trypan exclusion, counted with a haemocytometer and adjusted to a final concentration of 1-2x10^6 cells/ml.

For activation of OT-I T lymphocytes, cells were stimulated with the ovalbumin-derived peptides SIINFEKL, SIITFEKL or SIIGFEKL (hereafter referred as OVA-N4, OVA-T4 or OVA-G4 respectively) (Table 2.1). OVA-N4, OVA-T4 and OVA-G4 differ in their fourth amino acid (asparagine in OVA-N4, threonine in OVA-T4, glycine in OVA-G4), which results in a modification in their TCR affinity [OVA-N4 K_d = 54μM; OVA-T4 K_d = 444μM; OVA-G4 K_d = >1000μM] (Stepanek et al., 2014). Where indicated, T lymphocytes were also stimulated with combinations of recombinant human IL-2 (hIL-2), recombinant human TGFβ, murine IL-12, murine IFNα and/or soluble anti-CD28. Moreover, T cells were cultured with IL-7 as a control for non-TCR activated cells (Table 2.1). Cells were incubated at 37°C and 5% CO_2 for the indicated time at figure legends.

After appropriate incubation, the density separation medium Lympholyte®-M (Cedarlane) was occasionally utilised to remove dead cells prior to assay initiation.

2.3.3 Expansion of CTLs

OT-I T cells from lymph nodes (2x10^6 cells/ml) or spleens (2.5x10^6 cells/ml) were stimulated with N4 peptide (10^{-9}M) for 48h. Then, cells were washed twice in T cell medium to remove excess N4. Cell density was adjusted to 2.5x10^5 cells/ml and maintained with hIL-2 (20ng/ml) for 4 days further.

2.3.4 Cell culture – ID8 cell line

ID8 is a mouse ovarian carcinoma cell line (Roby et al., 2000). Three OVA-expressing variants have been used: ID8-N4, ID8-T4 and ID8-V4 (originally a gift from D. Zehn, Technical University Munich). In addition, these cell lines stably express Firefly luciferase. Cells were maintained in IMDM and split when they

reached a 70% confluence. Cells were washed in PBS to remove excess media and FBS, then were treated with trypsin for 2 minutes to allow detachment.

2.3.5 Killing assays

Target cells (ID8-N4, ID8-T4, ID8-V4) were cultured for 5h in 48-well plates at a density of $2x10^4$ cells/well. After cells adhered, wild-type and *Ptpn22*$^{-/-}$ OT-I CTLs were added to the wells at effector:target (E:T) ratios of 2:1, 10:1 and 20:1. ID8 cells with no CTLs was used as a negative control. Cells were incubated overnight at 37°C, 5% CO2. Then, supernatant was discarded and each well was washed in PBS (37°C) to remove cell debris before fresh medium was added to the wells. Finally, target cell viability was quantified by measuring luciferase activity, following addition of luciferin (Regis Technologies), with bioluminescence imaging using IVIS Spectrum and Living Image software (Perkin Elmer) or Cytation 5 Imaging Plate Reader (BioTek).

2.3.6 ID8 and OT-I co-culture experiments

Target cells (ID8-N4, ID8-T4, ID8-V4) were cultured for 5h in 48-well plates at a density of $2x10^4$ cells/well. After cells adhered, OT-I naïve T cells were added to the wells at a density of $2x10^5$ cells/well. Where indicated, the TGFβRI selective inhibitor SB431542 (5μM, Sigma Aldrich) was added to the wells. Cells were incubated for 48h at 37°C, 5% CO2. Then, cells were trypsinised and analysed by flow cytometry.

2.4 Flow cytometry

All Fluorescence-Activated Cell Sorting (FACS)-based experiments were performed using LSRII (BD Biosciences) or LX Cytoflex (Beckman Coulter) flow cytometers. Afterwards, data was analysed using FlowJo® v10.3 software. All

experiments were performed with unstained and single colour staining samples used as negative and positive controls for each fluorophore. Compensation was manually adjusted in the flow cytometer prior to acquisition of experimental samples.

A minimum of 20000 events were collected per sample and stored ungated. Before measurement of the indicated marker, all T lymphocytes were gated using the following procedure (Fig. 2.1):

- Lymphocyte gate using FSC-A/SSC-A.
- Removal of doublets using FSC-A/FSC-H.
- Removal of dead cells using live-dead Aqua dye (1:700, Molecular Probes) or zombie NIR™ (1:1000, BioLegend), as indicated.
- In some experiments, CD8β$^+$ gating was performed to optimise population purification.
- In some experiments, mixed *Ptpn22*$^{+/+}$ and *Ptpn22*$^{-/-}$ T cells were distinguished by CD45.1/CD45.2 expression, respectively.

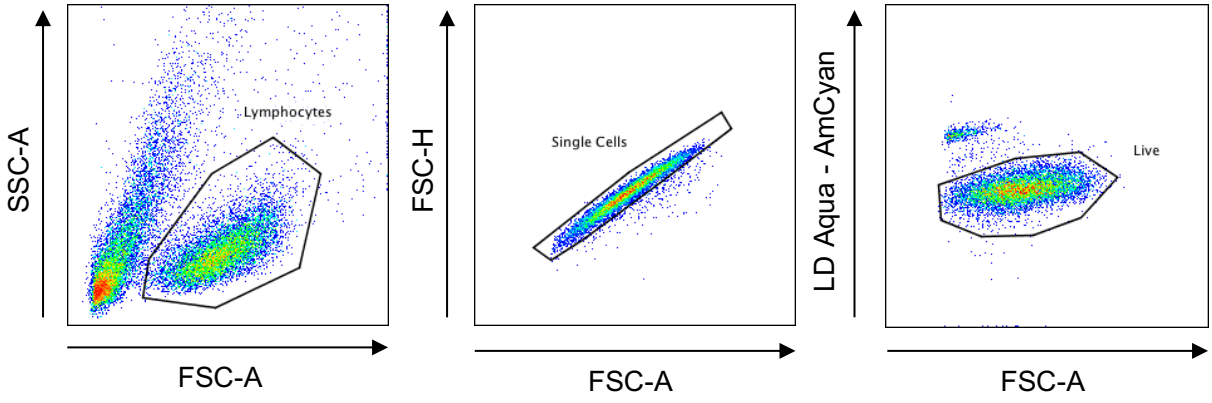


Figure 2.1: Gating strategy in flow cytometry experiments.

Dot plots are representative of the strategy used to assess activated T cells gated as shown above (Lymphocytes > Single cells > Live cells). FSC-A – Forward-scatter area; FSC-H – Forward-scatter height; SSC-A – Side-scatter area.

To be noted, in all histograms shown in this thesis, the Y axis is normalised and represents the percentage of events relative to the maximum value.

2.4.1 Surface staining

After appropriate stimulation, cells were transferred to FACS tubes, washed in PBS and stained with the indicated conjugated antibodies and cell viability dye at 4°C in the dark in PBS (Table 2.2). After 15 minutes, cells were washed and re-suspended in PBS prior to analysis with the flow cytometer. Occasionally, cell fixation was performed using 2% formaldehyde in PBS.

2.4.2 Intracellular staining

Typically, cells were fixed and permeabilized using FoxP3 fix/permeabilisation buffer (eBioscience) followed by staining with the indicated conjugated antibodies in the dark for 20 minutes at 4°C in permeabilisation buffer (eBioscience) (Table 2.2). Afterwards, cells were washed in permeabilisation buffer, re-suspended in PBS and analysed with the flow cytometer. For TNF and IFNγ staining, cells were previously treated in culture with 2.5μg/ml Brefeldin A (Sigma Aldrich), an inhibitor of protein transport from the ER to the Golgi, to retain cytokines intracellularly.

For phospho-rpS6 (pS6) Ser240/244 and phospho-SMAD2 Ser465/467 detection, cells were fixed in 4% formaldehyde followed by permeabilization in ice-cold 90% methanol. Cells were stained for 1h at RT with the indicated antibody diluted in 0.5% bovine serum albumin (BSA, Tocris) in PBS (Table 2.2). As the utilised pS6 (Ser240/244) antibody was unconjugated, an additional staining, performed with the PB conjugated secondary goat Anti-Rabbit IgG (1:1000, LifeTechonologies, #P10994), also diluted in 0.5% BSA in PBS, was required.

		Fluorophore	Dilution factors	Company	Catalogue number	Clone
Surface antibodies	CD8β	PE-Cy7	1:400	BioLegend	126616	YTS156.7.7
	CD25	FITC	1:200		102006	PC61
	CD25	PE			101904	3C7
	CD36	PE			102606	HM36
	CD44	APC-Cy7			101916	IM7
	CD45.1	AF647			103028	A20
	CD69	PercP-Cy5.5			110720	H1.2F3
	CD71	FITC			113806	RI7217
	CD98	PercP-Cy5.5			128218	RL388
Intracellular antibodies	Eomes	AF488	1:100	Invitrogen	4336342	Dan11mag
	Tbet	PE			644810	4B10
	IFNγ	AF488			505813	XMG1.2
	GrB	BV421			515408	GB11
	TNF	PercP-Cy5.5			506322	MP6-XT22
	c-Myc	APC	1:50	Cell Signalling Technologies	13871S	D84C12
	GLUT1	AF488	1:400	Abcam	ab195359	EPR3915
	ASCT2	Unconjugated	1:50	Cell Signalling Technologies	5345S	V501
	pS6	Unconjugated	1:200	Cell Signalling Technologies	5363S	D68F8
	pSMAD2	AF647	1:50	Cell Signalling Technologies	68550S	E8F3R

Table 2.2: List of antibodies utilised for FACS analysis.

AF488 – Alexa Fluor 488; AF647 – Alexa Fluor 647; APC – Allophycocyanin; BV421 – Brilliant Violet 421; Cy – Cyanine; FITC – Fluorescein isothiocyanate; PE – Phycoerythrin; PercP – Peridinin-Chlorophyll-protein; PB – Pacific Blue.

2.4.3 Proliferation assays

Prior to stimulation, naïve T lymphocytes were stained in PBS with CellTrace™ Violet Cell Proliferation Dye (5μM; Invitrogen) for 20 minutes at 37°C in the dark. Cells were stimulated as indicated in Figure legends and then analysed by flow cytometry (~405/450 nm). To calculate the division index, FlowJo® v10.3 was

utilised. This software automatically counted the number of cells included in each generation and calculated the average of divisions.

2.4.4 Protein synthesis assays

For protein synthesis assays, cells were treated with 20μM O-propargyl-puromycin (OPP; Jena Bioscience), an alkyne puromycin analogue that incorporates into newly synthesised proteins, for 10min. As negative control, 100μg/ml cyclohexamide (CHX; Sigma Aldrich) was added 15min prior to OPP labelling to stop protein synthesis. OPP incorporation was analysed using Click-iT™ Plus Alexa Fluor™ 488 Picolyl Azide Toolkit (Invitrogen) followed by flow cytometry detection.

2.4.5 Nutrient uptake assays

2.4.5.1 Glucose uptake assays

To measure glucose uptake, the fluorescent glucose analogue 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG; Abcam) was added to the cultures at a concentration of 50μM for 1h. Then, cells were washed in PBS twice and stained with cell viability dye for 5 minutes. Cells were analysed by flow cytometry (~465/540 nm).

2.4.5.2 Fatty acid uptake assays

To measure lipid uptake, the fluorescent fatty acid analogue 4,4-Difluoro-5,7-Dimethyl-4-Bora-3a,4a-Diaza-s-Indacene-3-Hexadecanoic Acid (BODIPY™ FL C_{16}; Thermo Fisher) was added to the cultures at a concentration of 2μM for 30 min. Then, cells were washed in PBS twice and stained with cell viability dye for 5 minutes. Cells were analysed by flow cytometry (~505/510 nm).

2.4.5.3 Amino acid uptake assays

To measure AA uptake, kynurenine (Kyn), a tryptophan metabolite with self-fluorescent properties that utilises the system-L AA transporters to enter the cell, was used (Sinclair et al., 2018). T cells were cultured with Kyn in Hank's Balanced Salt Solution (HBSS, Sigma-Aldrich) and fixed with 2% paraformaldehyde after 4min. Then, cells were washed in PBS, stained with cell viability dye for 5min and analysed by flow cytometry (~380/480 nm).

2.5 Metabolic assays

2.5.1 Seahorse XFe96 Analyser

Extracellular consumption rate (ECAR) and O_2 consumption rate (OCR) values, indicators of glycolysis and OXPHOS, respectively, were acquired using a Seahorse XFe96 Analyzer. The Seahorse XFe96 Analyser utilises a specialised 96-well plate with two compartments: the Seahorse XFe96 cell culture microplate (bottom) and a sensor cartridge (top) that, when ensembled together, form a microchamber that allows real-time ECAR and OCR detection. Furthermore, the sensor cartridge contains four ports per well to enable injection of compounds during the assay.

2.5.1.1 General Seahorse XFe96 Analyser protocol

First, the sensor cartridge was hydrated overnight at 37°C in a non-CO_2 incubator using XF calibrant solution. On the day of the assay, the 96-well microplate was coated with 25μL/well of a 22.4μg/ml Cell-Tak (Corning), 1M NaOH, 1M $NaHCO_3$ solution. After 20min incubation at room temperature (RT), the microplate was washed twice with sterile H_20 and air dried for 30min. Then, T lymphocytes (10^5 cells/well) were transferred to the microplate in XF base media supplemented with glucose (10mM, omitted for Glycolytic Test, see below), glutamine (2mM)

and pyruvate (1mM). The plate was centrifuged (300rpm for 1min), left 30min to allow cells to adhere and immediately analysed following system calibration. Where indicated, MitoStress Test or Glycolytic Test were performed by adding specific compounds diluted in the supplemented XF media into the cartridge ports. The compounds were sequentially added in the same order as shown in Table 2.3. Three measurements were taken of baseline ECAR/OCR levels and after each compound injection. Each measurement took 3min followed by 3min of mixing and 2min of waiting. At least five replicates per sample were analysed. Data was acquired using Wave software.

		Compound(s)	Final concentration
MitoStress Test	1	Oligomycin	1μM
	2	FCCP	1.5μM
	3	Rotenone / antimycin A	0.5μM
Glycolytic Test	1	Glucose	10mM

Table 2.3: List of compounds utilised for metabolic assessment with Seahorse XFe96.

FCCP - 2-[2-[4-(trifluoromethoxy)phenyl]hydrazinylidene]-propanedinitrile

To calculate basal ECAR/OCR, all values acquired prior to injection of the first compound, including the three measurements of all technical replicates, were used. When performing a MitoStress Test, maximal OCR was calculated with all values acquired after FCCP injection and prior to rotenone / antimycin A injection, including the three measurements of all technical replicates. Spare respiratory capacity (SRC) was calculated as the difference between maximal and basal OCR (Fig. 2.2). When performing a Glycolytic Test, glycolytic capacity was

calculated using all values acquired after glucose injection, including the three measurements of all technical replicates.

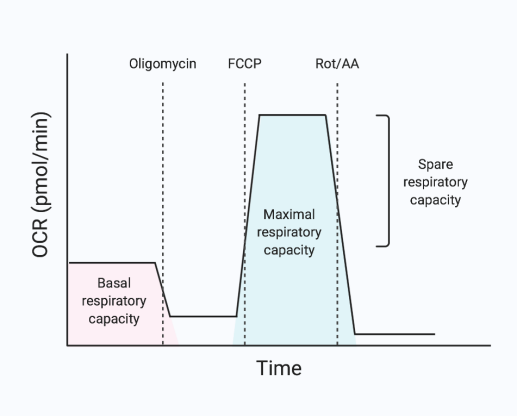


Figure 2.2: Schematic representation of MitoStress Test data.

2.5.2 ATP assay

Levels of ATP were determined using Luminescent ATP Detection Assay Kit (Abcam, ab113849). Briefly, T cells (1 x 10^5 cells/well with at least three replicates per sample) were lysed adding a detergent solution for 5min. Then, substrate solution, which contains luciferase enzyme and luciferin, was added to the wells and the plate was stored in the dark for 10min. As the luciferase reaction requires ATP, the emitted light correlates with ATP levels. Luminescence was measured using a Cytation 5 Imaging Plate Reader (BioTek). ATP levels were quantified generating an ATP standard curve.

2.6 Enzyme-Linked ImmunoSorbent Assay (ELISA)

2.6.1 Reagents

Item	Manufacturer
Mouse IL-2 DuoSet ELISA	R&D Systems #DY402-05
Mouse IFNγ DuoSet ELISA	R&D Systems #DY485-05
DuoSet Ancilliary Reagent Kit 2	R&D Systems #DY008
ELISA MAX™ Standard Set Mouse IL-2	BioLegend #431001
ELISA MAX™ Standard Set Mouse IFNγ	BioLegend #430801
Biotin Rat Anti-Mouse IgM	BD BioSciences #553406
Biotin Rat Anti-Mouse IgG1	BD BioSciences #550331
eBioscience™ Avidin-HRP	Invitrogen #18-4100-94
TMB substrate solution	LifeTechnologes #7335

Table 2.4: List of reagents utilised for ELISA.

For IL-2 and IFNγ detection, capture and detection antibodies, as well as horseradish-peroxidase (HRP)-conjugated streptavidin were provided in Mouse IL-2 DuoSet ELISA, Mouse IFNγ DuoSet ELISA, ELISA MAX™ Standard Set Mouse IL-2 or ELISA MAX™ Standard Set Mouse IFNγ.

The utilised solutions and buffers were included in DuoSet Ancilliary Reagent Kit 2 or prepared as followed:

- Wash buffer: 0.05% Tween 20 in PBS, pH 7.2 - 7.4
- Block buffer: 1% BSA in PBS, pH 7.2 – 7.4, 0.2 μm filtered
- Reagent diluent: 0.1% BSA, 0.05% Tween 20 in Tris-buffered Saline (20mM Trizma base, 150mM NaCl), pH 7.2 – 7.4, 0.2 μm filtered
- Substrate solution: 1:1 mixture of Color Reagent A (H_20_2) and Color Reagent 2 (Tetramethylmenzidine; TMB)
- Stop solution: 2N H_2SO_4

For IgG and IgM detection, biotinylated anti-mouse IgM and IgG antibodies and other standard reagents were purchased individually, as shown in Table 2.4. Buffers and solutions were prepared, unless otherwise specified in Section 2.6.3, as shown above.

2.6.2 General ELISA protocol for IL-2 and IFNγ

To measure IL-2 and IFNγ levels, 1 x 10^5 T lymphocytes per well were cultured in 96-well plates. When measuring IL-2 levels, T cells were cultured in the presence of anti-CD25 blocking antibody to prevent IL-2 consumption. At least three replicates per sample were analysed. After incubation under appropriate stimulation, supernatants were collected and stored at -20°C until further analysis. Cytokine levels in supernatants were quantified following manufacturer's instructions (see section 2.5.1). Briefly, the following assay procedure was performed: (1) 96-well plates were coated overnight at RT with plate-bound anti-mouse capture antibody. (2) The plate was blocked with block buffer for at least 1h at RT. (3) Diluted samples or standards were added to the wells for 2h at RT. (4) Detection antibody was added to the wells for 2h at RT. (5) Working dilution of streptavidin-HRP was added to each well for 20min at RT in the dark. (6) Substrate solution was added to each well at RT in the dark until colour reaction was completed and stopped with stop buffer. (7) Optical density was determined using a microplate reader (Thermo Fisher) set to 450nm. Data analysis was performed in Microsoft Excel by generating a standard curve. Between each step, reagents were aspirated and wells were washed at least three times with wash buffer. During incubation times, the plate was sealed and covered with an adhesive strip.

2.6.3 General ELISA protocol for IgG and IgM

4-Hydroxy-3-nitrophenylaacetyl (NP)-specific IgG and IgM levels in serum were acquired using the following procedure: (1) 96-well plates were coated with NP-BSA (Ratio > 20; 10μg/mL; Biosearch Technologies) in carbonate buffer (0.05M, pH9.5) overnight at 4°C. (2) Plate was blocked for 1h at RT. (3) Samples diluted in 0.1% BSA in PBS were added with five 4-fold serial dilutions (1:200, 1:800, 1:3200, 1:12800, 1:51200) and incubated overnight at 4°C. (4) Detection antibody (biotinylated anti-mouse IgM or anti-mouse IgG1; 1:1500 in 0.1% BSA in PBS) was added to the wells for 1h at RT. (5) Avidin-HRP (1:1000 in PBS) was added to each well for 1h at RT in the dark. (6) TMB was added to each well at RT in the dark until colour reaction was completed and stopped with stop buffer. (7) Optical density was determined using a microplate reader (Thermo Fisher) set to 450nm. Between each step, reagents were aspirated and wells were washed at least three times with wash buffer. During incubation times, the plate was sealed and covered with an adhesive strip.

2.7 RNA-seq

2.7.1 Sample preparation

OT-I T lymphocytes (5 x 10^6 cells per condition) were stimulated *in vitro* with OVA-T4 (10^{-8}M) ± TGFβ (5ng/ml). After 24h, cells were centrifuged (15000 x g for 1min) at RT and resuspended in TRI Reagent® (Zymo Research, R2050-1-50), a solution that lyses and deproteinises the sample.

2.7.2 RNA extraction

RNA was isolated using Direct-zol™ RNA Miniprep Plus (Zymo Research, R2070), a spin-column based kit that purifies total RNA directly from TRI Reagent®. Briefly, samples were mixed in a 1:1 ratio with ethanol and transferred

into a Zymo-Spin™ IIICG Column in a collection tube. Columns were centrifuged and transferred into new collection tubes. After DNase I treatment, columns were washed twice in Direct-zol™ RNA PreWash solution and once in RNA Wash Buffer for 1min. Finally, columns were transferred into RNase-free tubes and RNA was eluted by adding 100μL of RNase-free water. Upon centrifugation, flow-through was collected and RNA purity was checked using NanoDrop™ spectrophotometer (A_{260}/A_{280} and A_{260}/A_{230} > 1.8). All steps were performed at RT and centrifugation was set at 15000 x *g* for 30sec, unless specified. Samples were stored at -70°C until further analysis.

2.7.3 RNA-seq data analysis

RNA sequencing (RNA-seq) procedure and data analysis was performed by Novogene Bioinformatic Technology Co. (Hong Kong). Briefly, upon arrival, samples were quantified and quality tested: (1) RNA degradation and contamination was monitored on 1% agarose gels, (2) RNA purity was assessed by the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA) and (3) RNA integrity and quantitation was determined using the RNA Nao 6000 Assay Kit of the Bioanalyser 2100 system (Agilent Technologies, CA, USA). After validation, mRNA was purified from total RNA. Fragmentation was carried out and cDNA was synthesised. Then, PCR was performed according to a sequencing library generated using NEBNext® Ultra RNA Library Prep Kit for Illumina. PCR products were purified and library quality was assessed. Finally, after cluster generation, the library preparations were sequenced on an Illumina platform.

For data analysis, raw data was first processed and low quality reads were removed. Paired-end clean reads were mapped to the reference genome (NCBI/UCSC/Ensembl). Read numbers of each gene were counted and Reads

Per Kilobase of exon model per Million (RPKM) was calculated based on the length of the gene. Differentially expressed genes (DEGs) were identified after statistical analysis, as indicated in Section 1.8.

For Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis or chromatin immunoprecipitation (ChIP) Enrichment Analysis (ChEA), RNA-seq data was processed using Enrichr. Only DEGs with a fold-change (FC) > 1.5 or FC > 2, as determined in Figure legends, were analysed. FC was calculated as the ratio of the two compared conditions [i.e. “condition 1” (C1)/ “condition 2”(C2)] using the average RPKM values of the biological replicates.

For the generation of heatmaps, the RPKM ratio of the two compared conditions in each biological replicate, both C1/C2 and C2/C1, was performed. Values in heatmaps represent the logarithm with base 2 of the ratios.

2.8 Statistical analysis

All data was processed using GraphPad Prism v7.0d software. Statistical analysis was performed applying paired or unpaired two-tailed Student’s *t*-test, Mann-Whitney test and one- or two- way ANOVA with Tukey’s or Sidak’s multiple comparison tests, as appropriate. Differences were considered significant for *p*-value<0.05. Number of experiments and technical replicates are indicated in Figure legends.

For RNA-seq data, differential expression analysis was determined using DESeq2 R package and *p*-values were adjusted with the Benjamini and Hochberg’s approach, as assessed by Novogene Co. Differences were considered significant when adjusted *p*-value < 0.05.

2.9 Generation of figures

All figures were created with GraphPad Prism v7.0d or Biorender.com.

Chapter 3 The integration of signals 1, 2 and 3 and its impact on CD8$^+$ T cell activation and metabolism

3.1 Introduction

The signals required to induce a CD8$^+$ T cell response include: (1) TCR triggering, (2) co-stimuli and (3) inflammatory cytokines. Whilst TCR-triggering promotes T cell activation, co-stimulatory receptors, mainly CD28, support population expansion and survival while inflammatory cytokines drive differentiation. Nonetheless, it is unlikely that these processes are uniquely regulated by one of these signals, but rather, that they synergise with the ultimate outcome of promoting an optimal T cell response. Furthermore, it is well-established that the quantitative and qualitative balance of signals 1, 2 and 3 received during TCR-priming imprints T cell fate (Kaech and Cui, 2012). Thus, investigating how these signals are integrated, their predominant roles and how they cooperate is key to understand the regulation CD8$^+$ T cell responses and, therefore, find new mechanisms for its manipulation (Etxeberria et al., 2020).

Although the effect of these signals on T cell function has been extensively studied (Mescher et al., 2006; Tian et al., 2007; Zehn, D. et al., 2009; Corse et al., 2011; Esensten et al., 2016; Richard et al., 2018), it is less known how they influence T cell activity through metabolic modulation. The purpose of this chapter is to define how signals 1, 2 and 3 distinctively shape CD8$^+$ T lymphocytes, both functionally and metabolically. Specifically, here I aim to:

1. Define how TCR signal strength controls early events of CD8$^+$ T cell activation and metabolic reprogramming.
2. Determine how TCR signal strength influences CTL function.

3. Establish how signals 2 and 3 regulate $CD8^+$ T cell activation and metabolism.
4. Unravel how distinct signal 3 cytokines, either IL-12 or IFNα, modulate $CD8^+$ T cell activation and metabolism.

3.2 Results

3.2.1 TCR signalling strength determines early $CD8^+$ T cell activation

The first keystone of $CD8^+$ T cell activation is TCR signalling, which can be influenced by the quantity or quality of pMHC:TCR interactions. In order to study how TCR signal strength affects $CD8^+$ T cell activation, I used T lymphocytes from a *Rag1*$^{-/-}$ OT-I mouse strain. OT-I T cells express a transgenic TCR that specifically recognises an ovalbumin (OVA)-peptide in the context of MHC-I molecules (Hogquist et al., 1994). Furthermore, modifying the sequence of the OVA-peptide (SIINFEKL or OVA-N4) generates peptides with different affinities for the OT-I TCR. Here, the following peptides, from high to low affinity, were used: OVA-N4 (K_d = 54μM), OVA-T4 (SIITFEKL, K_d = 444μM) and OVA-G4 (SIIGFEKL, K_d = >1000μM) (Stepanek et al., 2014). As an additional tool to assess TCR signal strength, I also used lymphocytes from *Ptpn22*$^{-/-}$ OT-I mice. As described in Section 1.5.4, PTPN22 is a protein tyrosine phosphatase that inhibits early TCR signalling and, therefore, its deficiency increases TCR signal strength, particularly in response to low affinity peptides. Thus, in the following experiments, TCR signal strength was evaluated by modulating cell-extrinsic (antigen affinity) and cell-intrinsic (lack of PTPN22) mechanisms.

First, *Ptpn22*$^{+/+}$ or *Ptpn22*$^{-/-}$ OT-I naïve T cells were stimulated *in vitro* with either OVA-N4, OVA-T4 or OVA-G4 peptides (10^{-8}M). Moreover, T cells were cultured with IL-7 (10ng/ml), a homeostatic cytokine that prevents cell death in the absence of TCR-binding, as an unstimulated or negative control (indicated as 'No TCR' in figures). One key indicator of TCR-induced activation is an increase in cell size or blastogenesis. After 48h of incubation and as assessed by FSC-A/SSC-A plots during FACS analysis, T cells grew upon stimulation with OVA-N4

and OVA-T4. However, OVA-N4 stimulation resulted in a small, but consistent, increase in T cell size when compared to OVA-T4 stimulated cells. By contrast, T cells stimulated with the very low affinity peptide OVA-G4 were only able to engage a moderate growth (Fig. 3.1A).

Furthermore, T cell viability was determined by exclusion of Live/Dead Aqua dyes and FACS analysis. As expected, OVA-G4 stimulated T cells displayed very high rates of cell death, with <5% of the cells surviving after 48h in the absence of homeostatic signals (Fig. 3.1B). On the other hand, ~80% of the OT-I cells were alive in the presence of the high affinity peptide OVA-N4, whilst the proportion decreased to ~50% in the presence of OVA-T4. The absence of PTPN22 did not impact on levels of cell death (Fig. 3.1B).

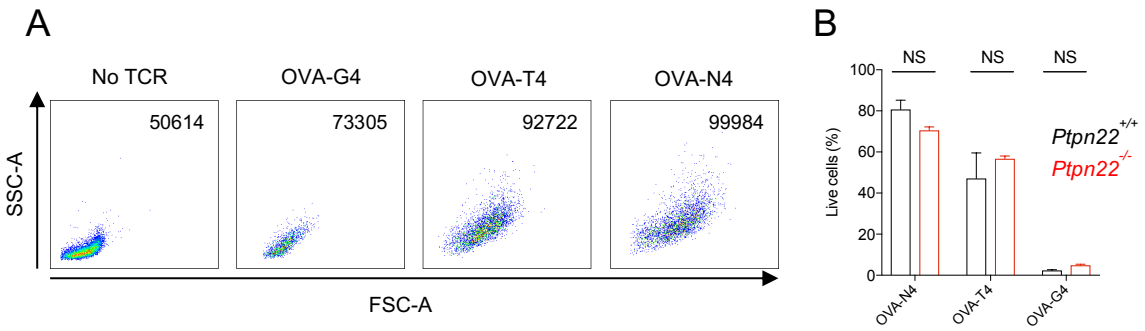


Figure 3.1: TCR-signalling strength determines CD8⁺ T cell growth and survival.

Ptpn22$^{+/+}$ and *Ptpn22*$^{-/-}$ OT-I T lymphocytes were stimulated *in vitro* with OVA-N4, OVA-T4 or OVA-G4 (10^{-8}M) for 48h. T cells cultured in the presence of IL-7 (10ng/ml) were utilised as unstimulated/negative controls. (A) T cell growth was determined by FACS analysis. Representative FSC-A/SSC-A dot plots of wild-type OT-I T cells after exclusion of dead cells and doublets is shown. Values indicate FSC MFI. (B) Cell death was assessed by FACS analysis using the viability dye LD Aqua. Data is from one of at least three experiments. Data in graphs represents mean and SD of technical triplicates. NS – Not significant, as assessed by two-way ANOVA with Sidak's multiple comparisons test. Differences in the effect of OVA-N4 vs OVA-T4 vs OVA-G4 were shown statistically significant as determined by row factor variation with $p < 0.0001$.

Next, in order to further assess the activation state of the cells, I measured the levels of the cell surface molecule CD44 and the effector protein granzyme B (GrB), known to be upregulated in activated $CD8^+$ T cells. Almost all T cells (~90%) were $CD44^+$ after stimulation with OVA-N4 or OVA-T4 whereas only ~60% of the cells were $CD44^+$ when primed with OVA-G4 (Fig. 3.2). Although *Ptpn22*$^{-/-}$ T cells did not show any increase in the proportion of cells expressing CD44 in any condition, the amounts of CD44, determined by mean fluorescence intensity (MFI) in $CD44^+$ cells, were higher in both OVA-N4- and OVA-T4-stimulated T cells, but not in OVA-G4-stimulated T cells (Fig. 3.2). T cells stimulated with OVA-G4 did not upregulate GrB whilst almost all T cells primed with the high affinity OVA-N4 had high levels of GrB expression. By contrast, the stimulation mediated by OVA-T4 induced an intermediary phenotype where not only the proportion of GrB^+ cells was diminished by ~20%, but also the levels that they expressed were significantly reduced (Fig. 3.2). The loss of PTPN22 resulted in elevated GrB expression in OVA-T4 stimulated T cells, increasing both the proportion of cells and the quantity that they expressed (Fig. 3.2).

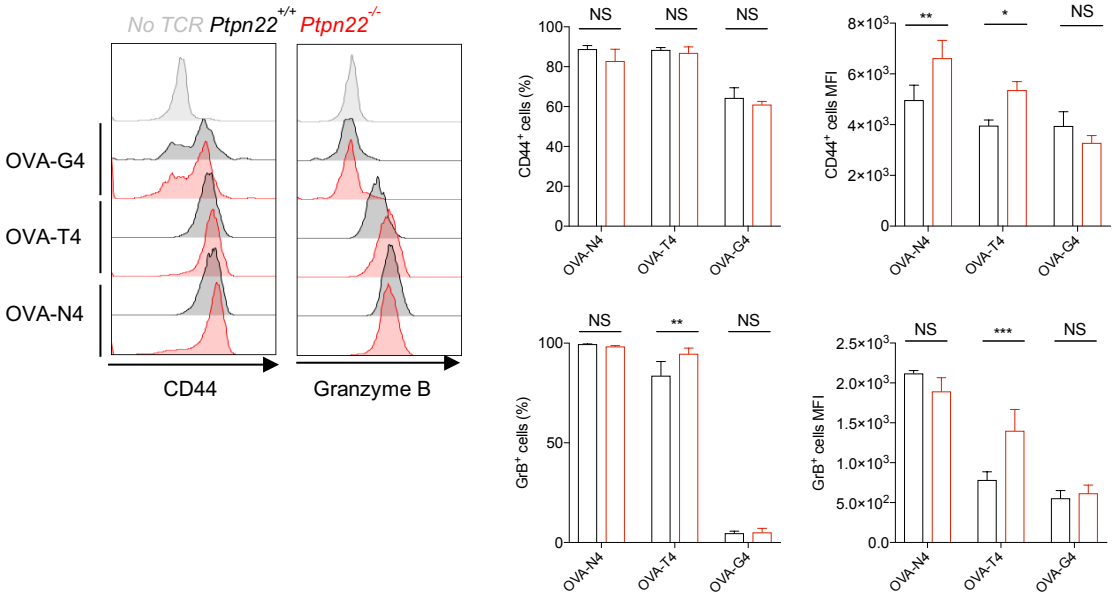


Figure 3.2: TCR-signalling strength determines upregulation of activation markers after antigen recognition.

Ptpn22$^{+/+}$ and *Ptpn22*$^{-/-}$ OT-I T lymphocytes were stimulated *in vitro* with OVA-N4, OVA-T4 or OVA-G4 (10^{-8}M) for 48h. T cells cultured in the presence of IL-7 (10ng/ml) were utilised as unstimulated/negative controls. Upon incubation, CD44 and GrB expression was analysed after staining with conjugated antibodies followed by FACS analysis. Data is from one of at least three experiments. Data in graphs represents mean and SD of technical triplicates. NS – Not significant; * - P<0.05; ** - P<0.005; *** - P<0.0005, as assessed by two-way ANOVA with Sidak's multiple comparisons test. Differences in the effect of OVA-N4 vs OVA-T4 vs OVA-G4 were shown statistically significant as determined by row factor variation with $p < 0.0001$.

Then, I assessed the impact of the strength of TCR signaling on IL-2 production and proliferation. In this experiment and the followings, I decided to exclude the OVA-G4 stimulated T cells due to their poor responsiveness and the low levels of T cell survival experienced during the previous assays. Thus, from now on, only the comparison between OVA-N4 and OVA-T4 stimulated T cells is shown. After 24h of TCR-stimulation *in vitro*, IL-2 levels in culture supernatants were analysed by ELISA. In *Ptpn22*$^{+/+}$ T cells, stimulation with the high affinity peptide OVA-N4 induced a 3-fold increase in IL-2 levels when compared to OVA-T4 stimulated cells (Fig. 3.3A). Moreover, *Ptpn22*$^{-/-}$ T cells showed enhanced IL-2

secretion relative to control cells in both conditions (Fig. 3.3A). To examine the proliferative capacity of T cells, naïve OT-I T cells were stained with Cell Trace Violet (CTV) prior to TCR-stimulation. CTV is used as a label to trace cell generations based on dye dilution. After 72h of TCR activation, consistent with my previous data, OVA-N4 stimulated T cells displayed enhanced proliferative capacity, as shown by an increase proportion of proliferating cells (~100% vs ~60% with OVA-T4) and division index (~3 vs ~1 with OVA-T4) (Fig. 3.3B). PTPN22-deficiency served to boost T cell proliferation, but only against the low affinity peptide OVA-T4 (Fig. 3.3B).

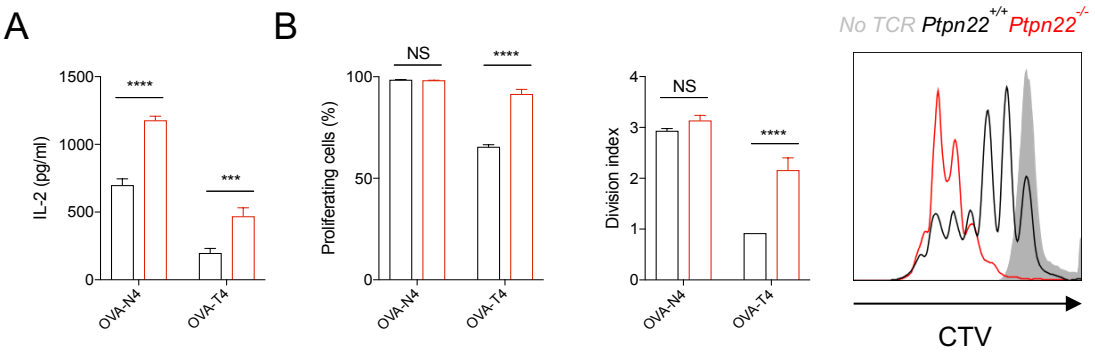


Figure 3.3: IL-2 secretion and proliferative capacity of CD8⁺ T cells is defined by the strength of TCR signal.

Ptpn22$^{+/+}$ and *Ptpn22*$^{-/-}$ OT-I T lymphocytes were stimulated *in vitro* with OVA-N4 or OVA-T4 (10^{-8}M). T cells cultured in the presence of IL-7 (10ng/ml) were utilised as unstimulated/negative controls. (A) Levels of secreted IL-2 in supernatants were measured by ELISA after 24h of TCR-stimulation in the presence of blocking CD25 Ab. (B) T cell proliferative capacity was assessed after 72h of stimulation by Cell Trace Violet (CTV)-labelling and FACS analysis. On the right, a representative histogram of OVA-T4-stimulated T cells is shown. Data is from one of three experiments. Data in graphs represents mean and SD of technical triplicates. NS – Not significant; *** - P<0.0005; **** - P<0.0001, as assessed by two-way ANOVA with Sidak's multiple comparisons test. Differences in the effect of OVA-N4 vs OVA-T4 vs OVA-G4 were shown statistically significant as determined by row factor variation with $p < 0.0001$.

Taken together, these results indicate, in accordance with previous reports (Zehn, Dietmar et al., 2009; Corse et al., 2011; Salmond et al., 2014; Richard et

al., 2018), that TCR signal strength determines T cell growth, survival, activation and proliferation upon TCR-priming.

3.2.2 TCR signalling strength determines CTL function

After differentiation, CTLs migrate to the site of infection or TME, where they recognise antigens to deliver effector functions. So, what is the role of antigen affinity in this scenario? In order to answer this question, I differentiated both *Ptpn22*$^{+/+}$ and *Ptpn22*$^{-/-}$ naïve OT-I T cells into CTLs *in vitro* during 6d (for protocol see Section 2.3.3). CTLs were restimulated with either OVA-N4 or OVA-T4 (10^{-8}M) in the presence of brefeldin A, a repressor of the ER to Golgi protein transport that provokes the intracellular accumulation of cytokines synthesised within the re-stimulation time frame. After 4h of re-stimulation, the levels of IFNγ and TNF were assessed by intracellular FACS analysis. CTLs re-stimulated with the high affinity peptide OVA-N4 displayed a higher proportion of IFNγ^{+} and TNF^{+} cells when compared to OVA-T4 re-stimulated CTLs (80% vs 60%, respectively) (Fig. 3.4A). No differences were detected between *Ptpn22*$^{+/+}$ and *Ptpn22*$^{-/-}$ CTLs (Fig. 3.4A). Moreover, I tested whether TCR signal strength was also affecting IL-2 secretion of CTLs. After 24h of re-stimulation, I measured the levels of IL-2 in supernatants and saw that, consistently, re-challenging CTLs with OVA-N4 doubled the amounts of IL-2 when comparing with OVA-T4 re-stimulated CTLs. Here, the lack of PTPN22 resulted in increased IL-2 levels in both conditions, but the effect was more accentuated when re-stimulating with OVA-T4 (Fig. 3.4B).

Thus, these results further validate that TCR signal strength plays an important role in the magnitude of the response in both naïve and effector CD8^{+} T lymphocytes.

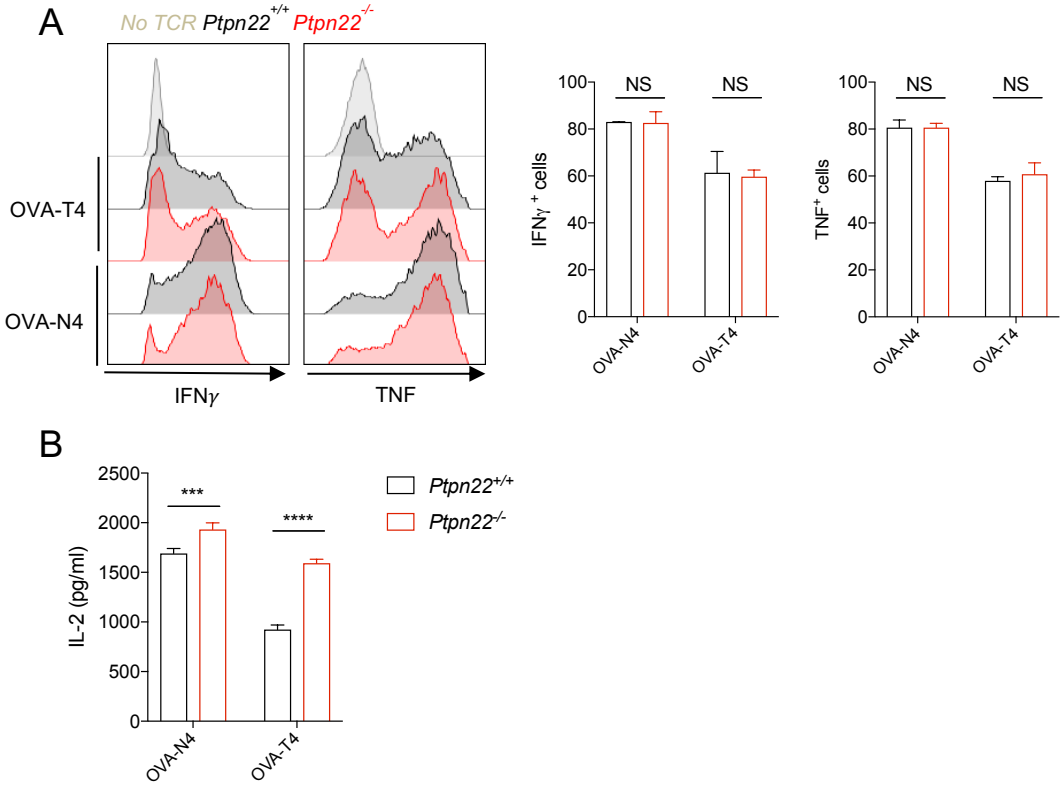


Figure 3.4: Cytokine secretion in CTLs is regulated by TCR affinity.

Ptpn22$^{+/+}$ and *Ptpn22*$^{-/-}$ CTLs were re-stimulated *in vitro* with OVA-N4 or OVA-T4 (10^{-8}M). T cells cultured in the presence of IL-7 (10ng/ml) were utilised as unstimulated/negative controls. (A) IFNγ and TNF levels were determined by FACS analysis after 4h of re-stimulation in the presence of Brefeldin A. (B) Levels of secreted IL-2 in supernatants were measured by ELISA after 24h of TCR-stimulation in the presence of αCD25. Data is from one of at least three experiments. Data in graphs represents mean and SD of technical triplicates. NS – Not significant; **** - P<0.0001, as assessed by two-way ANOVA with Sidak's multiple comparisons test. Differences in the effect of OVA-N4 vs OVA-T4 vs OVA-G4 were shown statistically significant as determined by row factor variation with $p < 0.0001$.

3.2.3 Antigen affinity regulates the TCR-induced metabolic reprogramming

T cell activation is accompanied by a metabolic reprogramming that is predominantly mediated by Myc (Wang, R. et al., 2011). After confirming that higher affinity peptides provide stronger T cell responses (Fig. 3.1-4), I assessed whether peptide affinity also impacted on T cell metabolic reprogramming.

First, the impact of antigen affinity on TCR-induced Myc expression was assessed. Naïve OT-I T cells were stimulated *in vitro* with OVA-N4 or OVA-T4 for 48h and results showed that, in accordance with my data and consistently with previous reports (Preston et al., 2015), Myc expression increased with ligand potency, with OVA-N4 stimulated T cells expressing approximately twice as much Myc as compared with OVA-T4 stimulated T cells (Fig. 3.5).

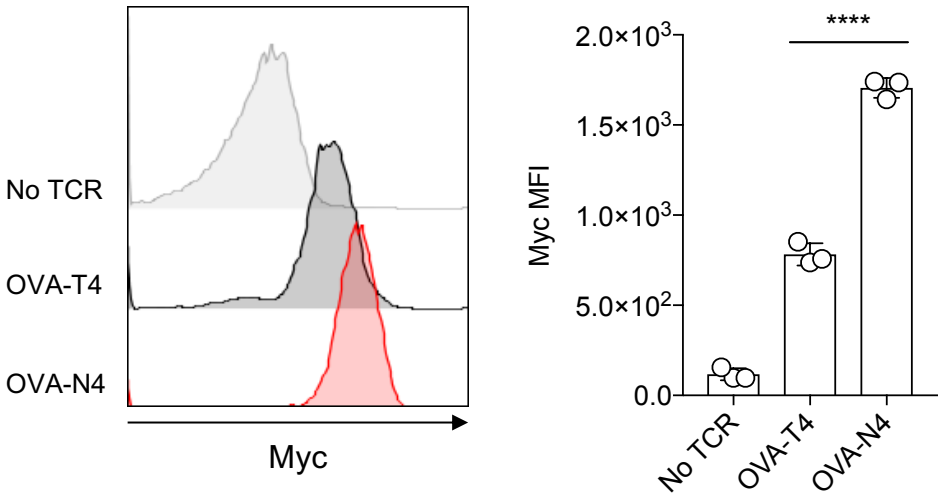


Figure 3.5: Antigen affinity determines Myc expression upon TCR-priming.

OT-I T lymphocytes were stimulated *in vitro* with OVA-N4 or OVA-T4 (10^{-8}M). T cells cultured in the presence of IL-7 (10ng/ml) were utilised as unstimulated/negative controls. After 48h of incubation, Myc expression was assessed by FACS analysis. Data is from one of at least three experiments. Data in graphs represents mean and SD. Dots represent technical triplicates. **** - P<0.0001, as assessed by one-way ANOVA with Tukey's multiple comparisons test.

One of the main roles of Myc during T cell activation is the upregulation of AA transporters, such as Slc7a5 or Slc1a5 (Marchingo et al., 2020), that allow the uptake of AAs to support the synthesis of new proteins required for growth, proliferation and differentiation. Therefore, I hypothesised that the attenuated growth and proliferation presented by OVA-T4 stimulated T cells (Fig. 3.1-3) could be associated with a diminished protein synthesis ability caused by a lower Myc expression. To test this, I utilised O-propargyl puromycin (OPP), a puromycin analogue, that incorporates into newly translated proteins and can be detected by flow cytometry. As a negative control, T cells were treated with cycloheximide (CHX), a blocker of translation, 15min prior to OPP labelling. As expected, T cells primed with the low affinity peptide OVA-T4 had lower levels of protein synthesis as compared with OVA-N4 stimulated cells (Fig. 3.6).

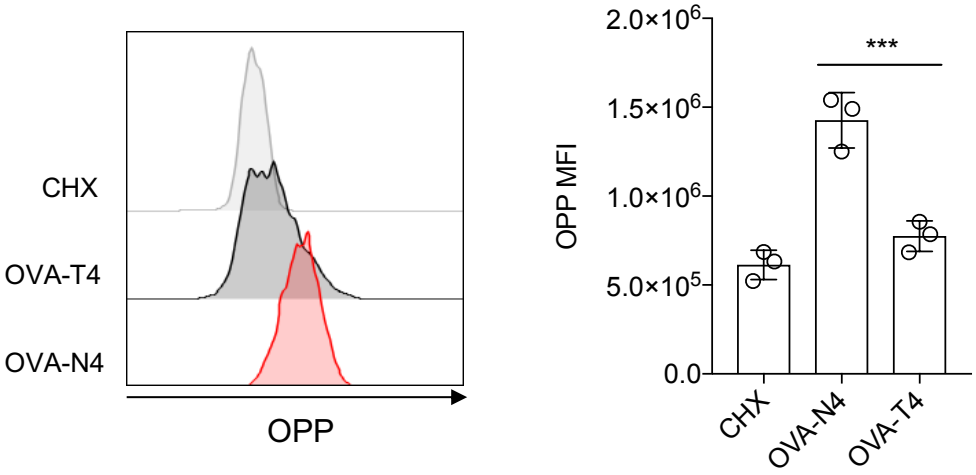


Figure 3.6: Antigen affinity determines protein synthesis upon TCR-priming.

OT-I T lymphocytes were stimulated *in vitro* with OVA-N4 or OVA-T4 (10^{-8}M). After 48h of incubation, Myc expression was assessed by FACS analysis. Data is from one of three experiments. Data in graphs represents mean and SD. Dots represent technical triplicates. *** - P<0.0005, as assessed by one-way ANOVA with Tukey's multiple comparisons test.

Another feature of TCR-induced metabolic reprogramming is the upregulation of nutrient transporters and subsequent nutrient uptake. Following the previous rationale, I speculated that lower affinity peptides would induce a reduced nutrient uptake. OVA-T4 stimulated T cells displayed a trend for diminished uptake of a fluorescent glucose analogue, 2NBDG, when compared to OVA-N4 stimulated T cells, although it did not reach statistical significance (Fig. 3.7A). Similarly, the uptake of BODIPY-C16, a fluorescent fatty acid analogue, was also reduced (Fig. 3.7B).

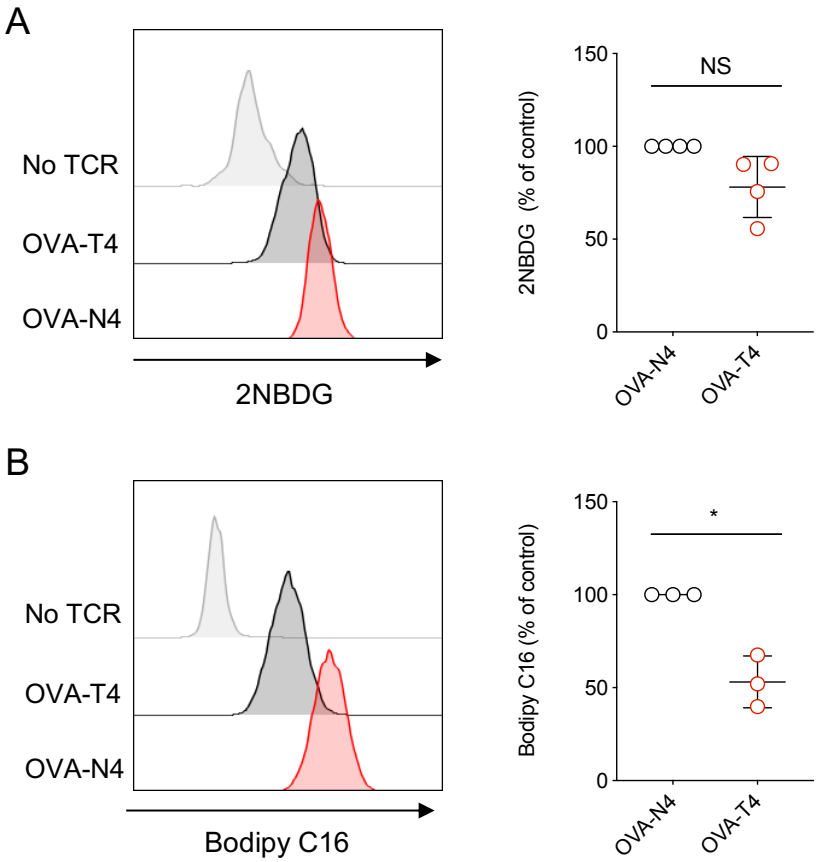


Figure 3.7: Low antigen affinity restricts nutrient uptake upon TCR-stimulation.

OT-I T lymphocytes were stimulated *in vitro* with OVA-N4 or OVA-T4 (10^{-8}M). After 48h of incubation, 2NBDG (A) or Bodipy C16 (B) uptake was determined by FACS analysis. Data is from four (A) or three (B) experiments. Data in graphs represents mean and SD. Dots represent biological replicates. NS – Not significant; * - P<0.05, as assessed by two-tailed paired Student's *t*-test.

To further define how TCR signalling strength affected the metabolic activity of T cells I used the Seahorse XFe96, an analyser that measures in real-time the extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) of cells cultured *in vitro*, serving as readouts for aerobic glycolysis and OXPHOS, respectively. The analysis in real-time allows several metabolic parameters to be determined through the injection of drugs at different timepoints of the assay. Specifically, I performed the 'MitoStress Test'. This assay first measures the basal cell respiration, then, the addition of oligomycin into culture wells inhibits the ATP synthase and shuts down the OCR linked to cellular ATP production. The second injection, FCCP, collapses the proton gradient and increases OCR to its maximum capacity. The difference between the maximal respiration and the basal respiration permits the calculation of the spare respiratory capacity (SRC), known as the ability of the cells to produce extra ATP under situations of sudden high energy demand. Finally, rotenone and antimycin A, inhibitors of complex I and III of the ETC, respectively, are added completely blocking mitochondrial respiration (Fig. 3.8A).

OT-I T cells were stimulated for 24h with either OVA-N4 or OVA-T4. Results of the Mitostress test indicated that antigen affinity impacted upon mitochondrial activity, as shown by diminished basal respiratory capacity and SRC in low affinity OVA-T4 stimulated T cells (Fig. 3.8B and C). Furthermore, although this is a mitochondrial test, the Seahorse XFe96 simultaneously measures ECAR values. Consistently, I found that the basal ECAR, i.e, prior to oligomycin injection, was also decreased in OVA-T4 stimulated cells as compared to OVA-N4 stimulated cells (Fig. 3.7C). As indicated by the ECAR/OCR ratio, OVA-N4 stimulation provokes a general enhancement of metabolic activity, increasing both OXPHOS and aerobic glycolysis rather than benefiting a particular pathway (Fig. 3.8C).

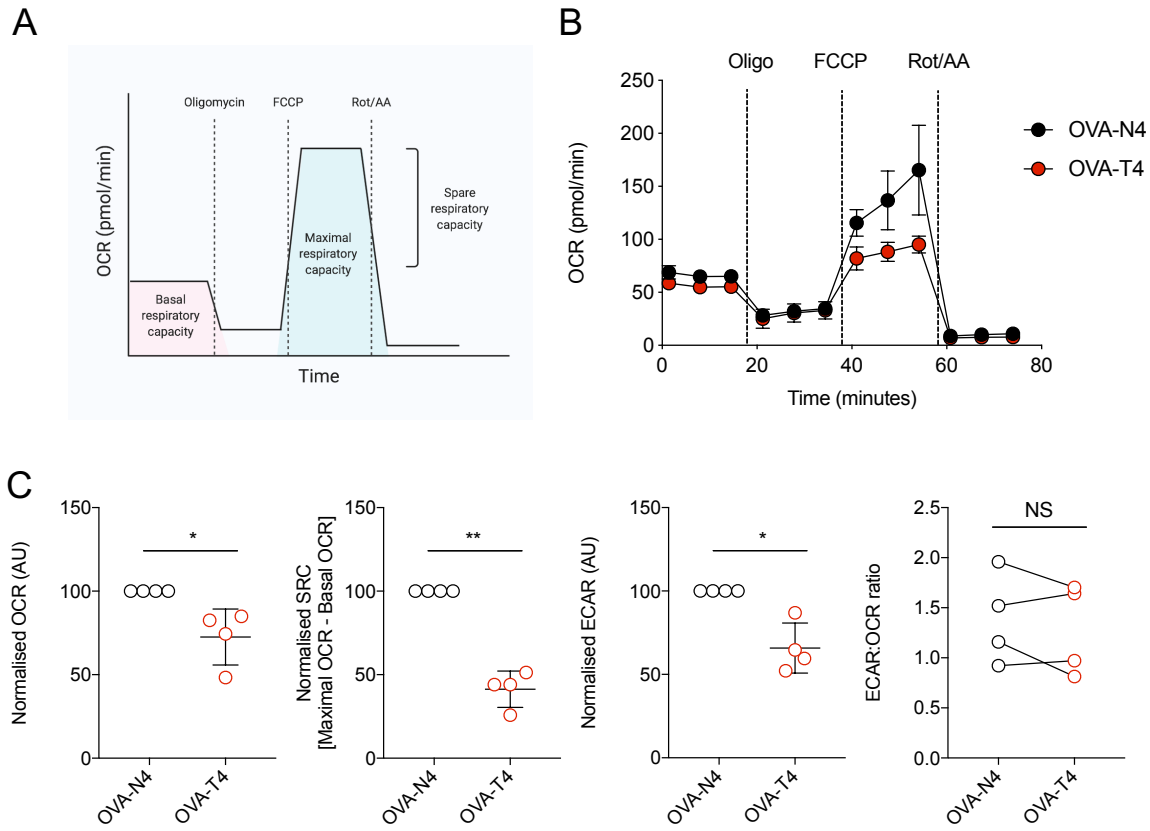


Figure 3.8: The metabolic activity of activated CD8⁺ T cells is shaped by TCR affinity.

OT-I T lymphocytes were stimulated *in vitro* with OVA-N4 or OVA-T4 (10^{-8}M) for 24h. After incubation, a MitoStress Test using the Seahorse XFe96 analyser was performed. (A) Schematic representation of MitoStress Test data, showing the key parameters of mitochondrial function. (B) Representative MitoStress Test profile of OVA-N4 vs OVA-T4 stimulated T cells. (C) Summarised data from four biological replicates. From left to right: basal OCR, SRC, basal ECAR and basal ECAR:OCR ratio. Data in graphs represents mean and SEM (B) or SD (C). Dots (C) represent biological replicates. NS – Not significant; * - P<0.05, ** - P<0.005, as assessed by two-tailed paired Student's *t*-test.

3.2.4 Signals 1, 2 and 3 cooperate to induce optimal CD8⁺ T cell responses

Above, I focused on describing how signal 1 (i.e., TCR signalling) influences CD8⁺ T cell activation and metabolic reprogramming. However, as mentioned in previous sections, T cells require additional inputs to acquire a fully activated phenotype (Mescher et al., 2006). In order to investigate how signals 2 (co-stimulation) and 3 (cytokines) assist signal 1 with this objective, naïve OT-I T cells

were stimulated with 'Signal 1' only, 'Signal 1 + Signal 2' or 'Signal 1 + Signal 2 + Signal 3'. OVA-T4 was utilised as a provider of signal 1, as I reasoned that the effects of signals 2 and 3 were more likely to be observed under suboptimal TCR-stimulations. The predominant co-stimulatory receptor CD28 was used as a provider of signal 2. Finally, either IL-12 or IFNα (type I IFN), key inflammatory cytokines that drive the differentiation of $CD8^+$ T cells (Curtsinger, Julie M. et al., 2003; Curtsinger et al., 2005), were used as providers of signal 3.

After stimulating naïve OT-I T cells as described, I observed that the effects provisioned by the addition of signals 2 and 3 varied depending on the readout, suggesting that they contributed to different aspects of T cell function rather than being simply 'amplifiers' of $CD8^+$ T cell activation. First, results demonstrated that, upon 48h of stimulation, T cells were able to fully blast in the presence of signal 1 alone, whilst the addition of signals 2 and 3 did not further change cell size (Fig. 3.9A). By contrast, and as expected, CD28 supplementation significantly boosted IL-2 production when compared to T cells stimulated with only OVA-T4 (Fig. 3.9B). The addition of IL-12 or IFNα did not further increase IL-2 production (Fig. 3.9B). However, IL-12 and IFNα stimulation did impact upon other parameters of T cell activation. For example, both cytokines, but not CD28, strongly enhanced GrB upregulation (Fig. 3.9C). Despite comparable effects on GrB expression, IL-12 and IFNα differed in their regulation of the transcription factors Tbet and Eomes: whereas IL-12 enhanced Tbet expression, IFNα signalling led to upregulated Eomes upregulation (Fig. 3.9C). Here, the addition of CD28 alone moderately enhanced Tbet expression, but no differences were observed with Eomes (Fig. 3.9C).

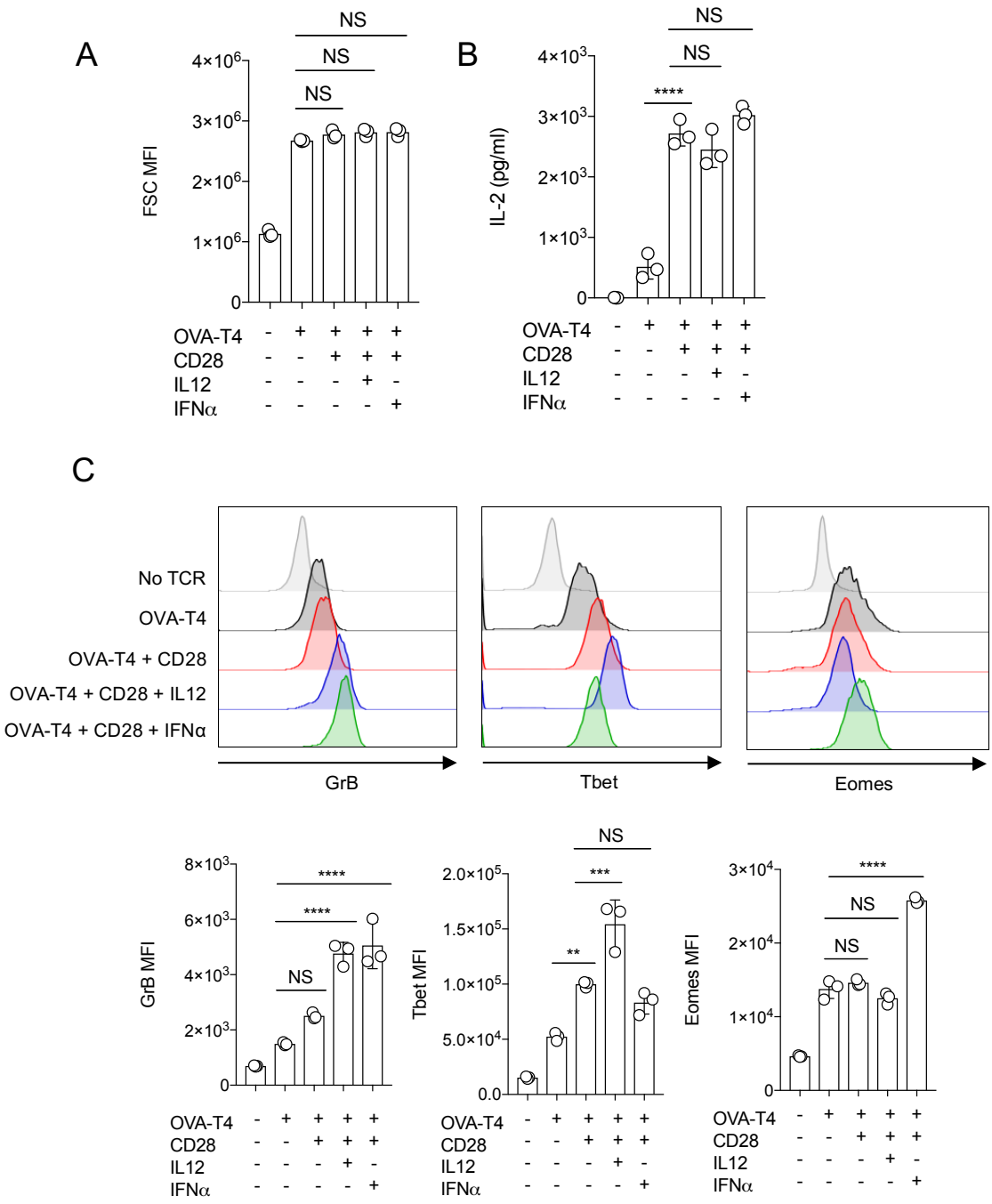


Figure 3.9: Signals 1, 2 and 3 differently regulate CD8⁺ T cell activation.

OT- I naïve T cells were stimulated with OVA-T4 (10^{-8}M) ± CD28 (1μg/ml) ± IL-12 (2ng/ml) or IFNα (20ng/ml) for 24h (B) or 48h (A, C). (A) Cell size was assessed by FACS analysis determining MFI of FSC-A. (B) Levels of IL-2 in supernatants were measured by ELISA. (C) GrB, Tbet and Eomes expression were determined by IC staining with conjugated antibodies followed by FACS analysis. Data is from one of at least four independent experiments. Dots represent technical replicates. Data represents mean and SD. NS – Not significant; ** - P<0.005, *** - 0.0005; **** - P<0.0001, as assessed by one-way ANOVA with Tukey's multiple comparisons test.

3.2.5 Signals 1, 2 and 3 distinctively shape CD8$^+$ T cell metabolism

Above, I have strengthened the idea that signals 1, 2 and 3 distinctively regulate CD8$^+$ T cell function to cooperatively induce full activation. So, how is that mirrored in the metabolism of CD8$^+$ T cells? Several studies have reported that CD28 signalling regulates both glycolytic and mitochondrial metabolism (Frauwirth, Kenneth A. et al., 2002; Klein Geltink et al., 2017), but little is known about the impact of IL-12 and IFNα in this context.

Before describing the results obtained in regards to the study of signals 1, 2 and 3 and its impact on T cell metabolism, it is important to mention that the following data presented a few inconsistencies amongst its repeated experiments. Due to the limitations and lack of time caused by the COVID-19 situation during 2020, these experiments could not be further repeated. However, my findings show a strong trend towards the data described below, but these are not definitive statements and additional assays are required for confirmation.

As described in Section 1.7.1.2, the key integrator of environmental cues is mTOR which, in turn, regulates T cell metabolism accordingly (Chapman and Chi, 2015). Hence, in order to examine how signals 1, 2 and 3 were involved in its regulation upon priming, OT-I T cells were stimulated with OVA-T4 $\pm$ CD28 $\pm$ IL-12 or IFNα for 48h. After incubation, I quantified mTOR activation based on the phosphorylation of its downstream target the ribosomal protein S6. My results showed that stimulation with OVA-T4 alone upregulated mTOR activity. Nevertheless, stimulation with CD28 seemed to potently enhance S6 phosphorylation, whilst the addition of signal 3 cytokines did not further impact on S6 phosphorylation (Fig. 3.10).

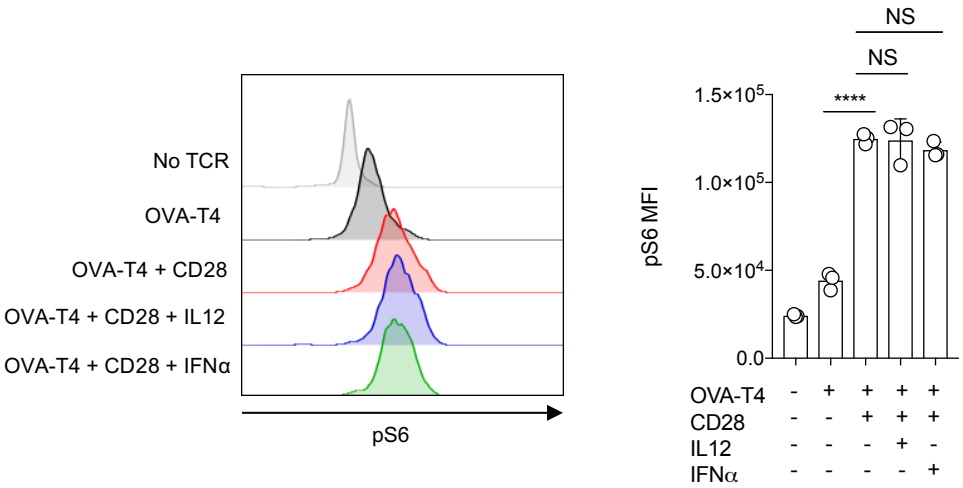


Figure 3.10: Signal 2 boosts mTOR activation upon TCR-priming.

OT- I naïve T cells were stimulated with OVA-T4 (10^{-8}M) ± CD28 (1μg/ml) ± IL-12 (2ng/ml) or IFNα (20ng/ml) for 48h. After incubation, S6 phosphorylation was quantified by IC staining and FACS analysis. Data is from one of two independent experiments. Dots represent technical replicates. Data represents mean and SD. NS – Not significant; **** - P<0.0001, as assessed by one-way ANOVA with Tukey's multiple comparisons test.

Next, I evaluated how signals 1, 2 and 3 affected the upregulation of nutrient uptake during T cell activation. As assessed using 2-NBDG, stimulation with only OVA-T4 promoted high levels of glucose uptake that were not further increased by the addition of signals 2 and 3 (Fig. 3.11A). By contrast, as determined using Bodipy-C16, both IL-12 and IFNα, but not CD28, significantly increased lipid uptake (Fig. 3.11B).

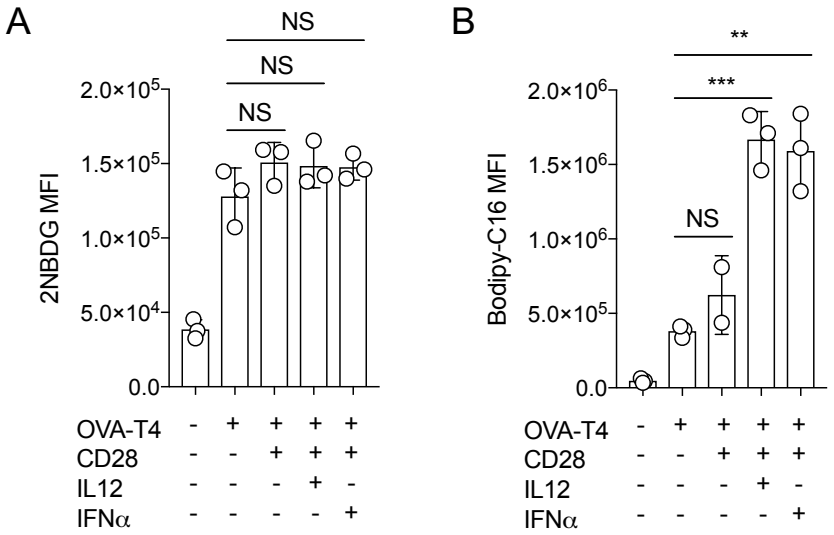


Figure 3.11: Signal 3 enhances lipid uptake upon TCR-stimulation.

Naïve OT- I T cells were stimulated with OVA-T4 (10^{-8}M) ± CD28 (1μg/ml) ± IL-12 (2ng/ml) or IFNα (20ng/ml) for 48h. After incubation, uptake of 2NBDG (A) or Bodipy-C16 (B) was assessed by FACS analysis. Data is from one of at least four independent experiments. Dots represent technical replicates. Data represents mean and SD. NS – Not significant; ** - P<0.005; *** - P<0.0005, as assessed by one-way ANOVA with Tukey's multiple comparisons test.

As lipids are an important fuel for mitochondrial metabolism, it was possible that the signals provided by IL-12 and IFNα shaped mitochondrial activity. To test this hypothesis, a MitoStress Test was performed using the Seahorse XFe96 analyser, as described in Section 1.2.3. First, results suggested that, after 48h of stimulation, CD28 signalling enhanced both OCR and ECAR basal values when compared to T cells stimulated with OVA-T4 alone (Fig. 3.12A-C). However, CD28 did not promote variations in the ECAR:OCR ratio suggesting that Signal 2 amplifies T cell metabolic activity rather than promoting a certain pathway (Fig. 3.12D). By contrast, the addition of IL-12 or IFNα seemed to lead to a change on the type of metabolism that T cells were engaging. On the one hand, IL-12 boosted basal ECAR while preventing the CD28-induced augment in OCR (Fig. 3.12A-C). Consequently, the ECAR:OCR ratio significantly increased in this condition implying that IL-12-treated T cells preferentially engaged aerobic

glycolysis (Fig. 3.12D). On the other hand, T cells stimulated with OVA-T4 + CD28 + IFNα presented an increase in both OCR and ECAR basal levels (Fig. 3.12A-C). Nevertheless, the ECAR:OCR ratio was <1 suggesting that these T cells favored mitochondrial metabolism rather than glycolysis (Fig. 3.12D). Furthermore, T cells stimulated with IFNα displayed a heightened maximal respiration leading to an improved SRC, also observed in the presence of CD28 alone (Fig. 3.12E). Taken together, these findings suggest that, whilst IL-12 promoted the engagement of glycolysis, IFNα-treated T cells shifted their metabolism in the benefit of mitochondrial activity.

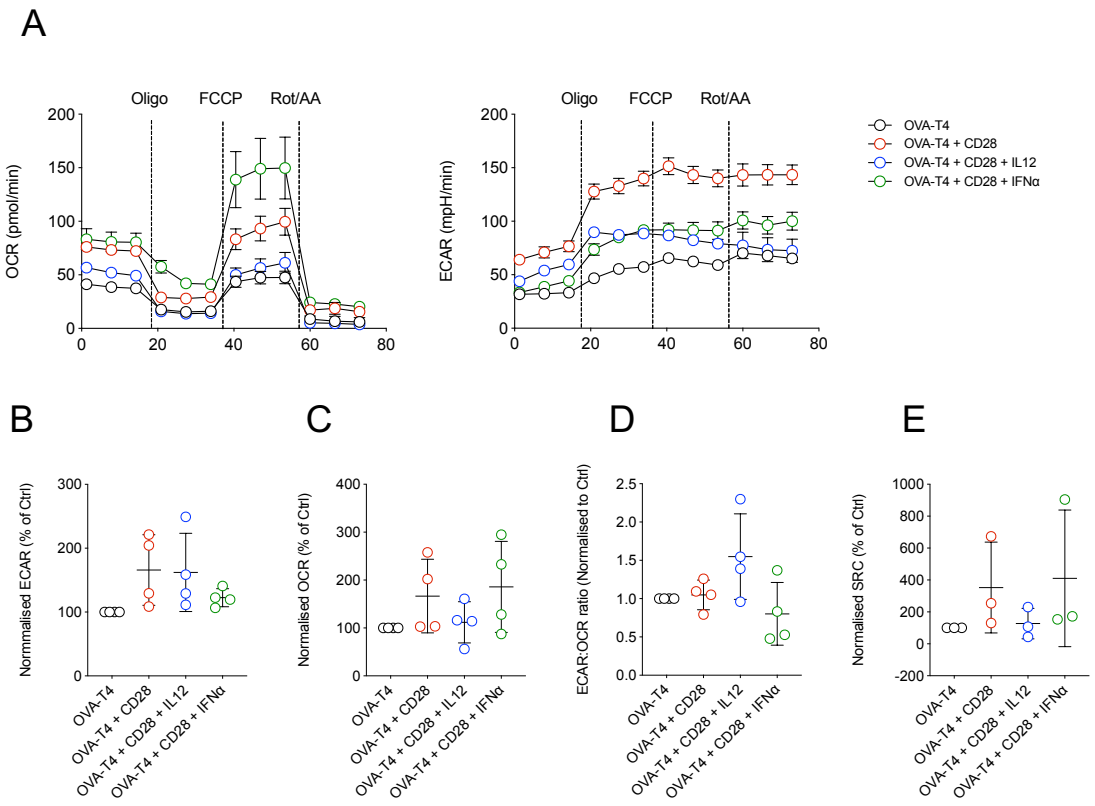


Figure 3.12: CD28, IL-12 and IFNα shape the metabolic activity of activated CD8⁺ T cells.

OT-I T lymphocytes were stimulated *in vitro* with OVA-T4 (10^{-8}M) ± CD28 (1μg/ml) ± IL-12 (2ng/ml) or IFNα (20ng/ml) for 48h. After incubation, a MitoStress Test using the Seahorse XFe96 analyser was performed. (A) Representative MitoStress Test profile (left) and ECAR trace (right) of T cells stimulated under the indicated conditions. (B) Basal OCR values. (C) Basal ECAR values. (D) Basal ECAR:OCR ratio. (E) SRC, i.e., difference between maximal and basal OCR. In (A), data is representative of one of three independent experiments. Data represents mean with SEM. In (B, C, D, E), graphs collect data from three independent experiments. Data is normalised to control (OVA-T4) and represents mean with SD. Dots represent biological replicates (n=4).

3.3 Discussion

The principle that $CD8^+$ T cells require the integration of at least 3 types of signals (TCR signalling, co-stimulation and cytokines) to become fully activated has been extensively studied and well-established in previous studies. However, how these signals cooperated in a metabolic level was less understood. Here, I have reinforced the hypothesis that signals 1, 2 and 3 add together, both functionally and metabolically, to promote optimal $CD8^+$ T cell activation.

Regarding to the influence of TCR signalling strength (signal 1), the experiments described here involving antigens with different affinities concluded that TCR signalling strength is key to determine the extent of $CD8^+$ T cell responses: whereas T cells stimulated with the high-affinity peptide OVA-N4 displayed high rates of T cell growth, activation, proliferation and cytokine secretion upon TCR-binding, these were decreased in T cells stimulated with the low affinity peptide OVA-T4. Consistently, previous investigations have described a correlative strength-response relationship, indicating that T cells stimulated with strong peptides show enhanced outputs (Zehn, D. et al., 2009; King et al., 2012). However, strong TCR signals are not only achieved by strong antigen affinities but also by high antigen doses. Here, for practical reasons, I have only chosen one antigen dose but it is important to highlight that both parameters define the magnitude of T cell responses. In fact, it is well-established that an increased antigen density partially compensates weak pMHC:TCR interactions suggesting, therefore, that increasing OVA-T4 abundance T cells could achieve the same activation state of OVA-N4 activated T cells (Rosette et al., 2001; Salmond et al., 2014; Allison et al., 2016; Mayya and Dustin, 2016).

The results presented here have further demonstrated that the manipulation of TCR signalling through cell-intrinsic mechanisms also regulates the magnitude of

T cell responses. Specifically, PTPN22-deficiency, in accordance with other studies, benefited T cell activation and expansion after TCR-priming (Bottini and Peterson, 2014; Salmond et al., 2014; Brownlie et al., 2017; Bray et al., 2018). Salmond et al. (2014) described that PTPN22 is crucial to discriminate weak self-peptides from exogenous antigens, with PTPN22 acting as a 'brake' of early TCR signalling and unwanted T cell responses upon recognition of self-peptides. These findings suggest, therefore, that the presence or absence of PTPN22 in T cells entails a significant difference particularly in the context of stimulations with low-affinity peptides. This phenomenon has also been observed here with, for example, the T cells' proliferative rates or GrB expression: whilst PTPN22-deficiency induced an improvement of both parameters when recognising the low-affinity OVA-T4, no changes were shown after stimulation with OVA-N4 (Fig. 3.2 and 3.3). In spite of the general accordance of my PTPN22 data with previous findings, some discrepancies can be found when analysing TNF and IFNγ production in CTLs (Fig. 3.4A). In these experiments, by contrast with other studies (Salmond et al., 2014; Brownlie et al., 2019), the lack of PTPN22 did not enhance its expression. However, we should note that CTLs are more responsive than naïve T cells to low affinity antigens (Stone et al., 2009). As mentioned above, in order to see a significant difference in *Ptpn22*$^{-/-}$ T cells, these should be stimulated under sub-optimal conditions, achieved by reducing the affinity and/or amount of antigen. It is therefore plausible that the *in vitro* stimulations performed here have been sufficient to show differences in naïve T cells but not in all parameters of CTLs.

Altogether, it all seems to indicate that an increased TCR signal strength is associated with an improved T cell response, but what does exactly mean that a response is 'improved'? Is that improvement qualitative, quantitative or both?

Richard et al. (2018) recently demonstrated that whilst low affinity peptides provide weaker responses, the observed effects are rather a consequence of a delayed response caused by a limited or slower TCR-binding. However, naïve T cells are ultimately capable of differentiating into CTLs of the same 'quality' irrespective of the strength of the initial stimulation (Richard et al., 2018). Hence, these findings suggest that antigen affinity regulates the speed of action and the magnitude of a T cell response inducing an improvement only on a population level, but not on a single-cell level. Furthermore, antigen affinity not only plays an important role during TCR-priming but also upon re-challenge of CTLs on sites of infection or the TME, dictating the amount of secreted cytokines and also their killing capacity (Fig. 3.4) (Salmond et al., 2014; Brownlie et al., 2019).

Zehn, D. et al. (2009) studied the progression of monoclonal $CD8^+$ T cells *in vivo* in response to a panel of six TCR ligands with different affinities. They concluded that, although lower-affinity peptides can provide a complete response and generate T cell memory, T cells exit lymphoid organs earlier leading to a shorter expansion phase. In terms of efficiency of T cell responses, Zhong et al. (2013) described that TCRs with a higher affinity for a variant of gp100 (gp206), a melanocyte protein, displayed delayed tumour growth and more severe ocular autoimmunity (destruction of melanocytes in the eye) when compared to lower TCR affinities. These findings suggest, therefore, that manipulating TCR or antigen affinity is a promising strategy to enhance T cell responses. On the other hand, another report by Corse et al. (2010) demonstrated that pMHC:TCR interactions sustained for too long impaired T cell proliferation and memory formation. More recently, Zahm et al. (2017) proved that higher affinity epitopes induce higher levels of inhibitory receptors, which ultimately resulted counterproductive for anti-tumor activity. These investigations imply that an

increase in ligand potency does not always lead to an improved CTL response and that is crucial to find appropriate pMHC:TCR interactions in order to maximise the efficacy of $CD8^+$ T cell responses.

OVA-T4 stimulated T cells displayed lower levels of Myc when compared to T cells stimulated with high affinity OVA-N4 (Fig. 3.5). Preston et al. (2015) established, also using the OT-I model, that antigen affinity proportionally correlates with the number of T cells that trigger Myc expression within the first hours upon TCR-priming. However, at later stages, the levels of Myc are maintained through the action of IL-2. The experiments described here were performed at later timepoints, after 48h of stimulation, suggesting that the observed differences are likely to be a consequence of IL-2 diminishment. As described in Section 1.7.1.1, the role of Myc has been strongly associated with its capacity to induce the upregulation of AA transporters, such as Slc7a5, in order to sustain protein synthesis, which was accordingly enhanced in OVA-N4 stimulated T cells (Fig. 3.6). I have also observed that antigen affinity controls nutrient uptake as well as glycolytic and mitochondrial activity (Fig. 3.7-8). King et al. (2012) showed that $CD8^+$ T cells stimulated with high affinity peptides displayed increased differentiation into SLECs when compared to those stimulated with low affinity peptides. As mentioned in Section 1.7.2, the balance between glycolytic and mitochondrial activity is key to determine terminal vs memory differentiation and it has been proposed as a potential modulatory axis to dictate T cell fate. Thus, it is expected that high T cell affinity, glycolysis and SLEC differentiation are associated. Further exploring how pMHC:TCR interactions modulate T cell metabolism will provide new insight into the mechanisms by which signal 1 regulates T cell responses.

In regards to the role of CD28 stimulation (signal 2) during T cell activation, it has been well established that CD28 promotes T cell expansion due to IL-2 secretion, as also described in Fig. 3.9B (Fraser et al., 1991; Appleman et al., 2000; Sanchez-Lockhart et al., 2004). Considering that the induction of IL-2 secretion by CD28 is thought to be mediated by PI3K activation, it was unsurprising that, following the classic PI3K/Akt/mTOR pathway, pS6 phosphorylation was also particularly boosted upon CD28 stimulation (Zheng et al., 2007). In the context of metabolism, early studies confirmed that CD28 stimulation leads to an enhanced glucose uptake and aerobic glycolysis (Frauwirth, Kenneth A. et al., 2002; Jacobs et al., 2008). More recently, Klein Geltink et al. (2017) also proved that T cells primed with CD28 not only provoked an increase in 2NBDG uptake and glycolysis, but also promoted mitochondrial priming, crucial for memory development. Consistently, my data strongly suggested that addition of CD28 favored both glycolytic and mitochondrial metabolism, as shown by increased ECAR and OCR values and SRC (Fig. 3.12). On the other hand, the 2NBDG experiments did not completely corroborate previous findings. However, it is essential to mention again that the experiments performed to investigate the role of signals 1, 2 and 3 on T cell metabolism (Fig. 3.10-12) showed a degree of variability (e.g., few repeats showed a moderate but not consistent upregulation of 2NBDG uptake) and those require to be repeated.

Finally, in regards to the role of IL-12 and IFNα (signal 3), it has been determined that these are essential to allow productive T cell responses and differentiation of $CD8^+$ T cells (Curtsinger, J. M. et al., 2003; Curtsinger et al., 2005; Mescher et al., 2006; Curtsinger and Mescher, 2010). My findings showed, in accordance with these statements, that an added signal 3 during TCR-stimulation boosted the expression of GrB and differentiation transcription factors implying that,

whereas IL-12 and IFNα are not required for T cell priming and expansion, these play a relevant role in T cell differentiation (Fig. 3.9C). Both IL-12 and IFNα have been associated not only with the acquisition of an effector phenotype, but also with a subsequent development of a memory population. However, the underlying mechanisms of this regulation are less understood and whether each cytokine favours a distinct developmental programme or whether IL-12 and IFNα have redundant roles is still under debate (Agarwal et al., 2009; Curtsinger and Mescher, 2010). Differentiation into terminally-differentiated effector T cells is generally associated with a higher Tbet/Eomes ratio and the engagement of glycolysis. By contrast, memory formation is favoured by the maintenance of Eomes expression and an enhanced mitochondrial metabolism (Araki et al., 2009; Kaech and Cui, 2012; Klein Geltink et al., 2017). The findings of this chapter indicate that IL-12 increases Tbet levels at the expenses of Eomes upregulation, whilst IFNα has the opposite effect (Fig. 3.9C) (Takemoto et al., 2006; Rao et al., 2010; Martinet et al., 2015). Furthermore, my results suggest that IL-12 pushes towards a glycolytic phenotype while repressing the mitochondrial priming derived by CD28 stimulation (Fig. 3.12). These results might imply that IL-12 and IFNα regulate T cell development in a different manner, promoting either an effector or a memory phenotype, respectively. Similarly, this hypothesis has been previously proposed in human $CD8^+$ T cells in the context of memory formation, with IL-12 inducing the development of an effector memory T cell population and IFNα/β driving the differentiation of central memory T cells (Ramos et al., 2009; Chowdhury et al., 2011). Thus, albeit having overlapping functions, it is likely that the responsiveness to IL-12 and type I IFNs dictates $CD8^+$ T cell fate. Here, my findings suggest that the modulation of T cell differentiation by inflammatory cytokines is also performed on a metabolic level.

Recent investigations have been focused on the search of different mechanisms that modulate the glycolytic/mitochondrial axis in order to manipulate T cell fates (see Section 1.7.2). How IL-12 and IFNα regulate T cell metabolism and whether these cytokines could be used with this objective will be of interest in future investigations.

Taken together, this chapter has served as a basis to show that the final output of fully activated $CD8^+$ T cells depends on a myriad of signals that differently shape its function and metabolism. In the following chapters, I investigate how other environmental cues intervene during $CD8^+$ T cell activation and metabolic reprogramming.

Chapter 4 The influence of TGFβ during $CD8^+$ T cell activation

4.1 Introduction

In the previous chapter, I explored how $CD8^+$ T lymphocytes integrate signals 1, 2 and 3 to induce T cell activation and metabolic reprogramming. However, T cell responses are not only shaped by these key signals, but also, by the balance of pro-inflammatory and anti-inflammatory cytokines present in the tissue microenvironment.

TGFβ is an essential immunoregulatory cytokine that controls T cell responses both in the thymus and the periphery, playing an essential role in the maintenance of homeostasis and tolerance (Sanjabi et al., 2017). Moreover, TGFβ is recognised as one of the most influential cytokines in cancer and its neutralisation is in the spotlight as a promising cancer therapy (Teixeira et al., 2020; Groeneveldt et al., 2020). Although anti-TGFβ therapies frequently aim to restrict tumour invasion and metastasis, it has been reported that targeting TGFβ also benefits anti-tumour immune responses (Yang, L. et al., 2010; Groeneveldt et al., 2020). In fact, specific blockade of TGFβ signalling in T lymphocytes reinforces rejection of transplanted B16 and EL4 tumours in mice, indicating that TGFβ plays a crucial role in the depletion of T cell responses against tumours (Gorelik and Flavell, 2001). Similarly, the lack of TGFβ signalling in T cells prevents the development of tumours in a mouse model of spontaneous prostate cancer (Donkor et al., 2011). In these studies, TGFβ decreased the number and activity of tumour-specific CTLs, which has been generally associated with a deleterious effect of TGFβ during $CD8^+$ T cell priming within draining LNs (dLNs). However,

these investigations utilised transgenic mice expressing a dominant-negative TGFβRII, a model that blocks TGFβ signalling in both $CD4^+$ and $CD8^+$ T lymphocytes. Due to the pleiotropic effects that TGFβ exerts (Li, M.O. and Flavell, 2008; Batlle and Massagué, 2019), whether other T cell lineages and to what extent these influenced the TGFβ-mediated suppression of $CD8^+$ T cell responses is unclear. A few studies have explored the direct effects of TGFβ during $CD8^+$ T cell priming *in vitro* but there is still a lack of in-depth understanding in this field and its underlying mechanisms (Thomas and Massagué, 2005; Brownlie et al., 2017). Another gap in our current knowledge of the mechanism of action of TGFβ in the suppression of anti-tumour T cell responses is whether TGFβ exclusively inhibits the activity of $CD8^+$ T cells during early stages of activation or whether this also occurs to the fully differentiated CTLs that migrate to the TME.

In this chapter, I aim to:

1. Define in detail the impact of TGFβ treatment during the priming of $CD8^+$ T cells
2. Assess the mechanisms by which TGFβ modulates T cell activation by analysis of the TCR-induced T cell transcriptome using RNA-seq
3. Determine the effects of TGFβ on fully-differentiated CTL effector functions.

4.2 Results

4.2.1 Tumour-derived TGFβ suppresses activation of CD8$^+$ T cells

To assess the effects of TGFβ during early CD8$^+$ T cell activation I used OT-I TCR transgenic T cells stimulated *in vitro* with OVA-peptide ± exogenous TGFβ (5ng/ml). It has been proposed that the effects of TGFβ are dependent on the strength of the TCR-stimulation (Arumugam et al., 2015; Brownlie et al., 2017; Gunderson et al., 2020) and, therefore, I used both the high affinity peptide OVA-N4 and the lower affinity OVA-T4 to stimulate OT-I T cells. T cells cultured in the presence of the homeostatic cytokine IL-7 (10ng/ml) were utilised as a negative control (no TCR stimulation). After 48h of TCR-stimulation, the levels of the activation markers CD25, CD44 and CD71, as well as granzyme B, a well-established target of the SMAD2/3 transcription factors (Thomas and Massagué, 2005), were determined. CD25 is the α subunit of the IL-2 receptor, further constituted by the γ and β subunits (also known as γc and CD122, respectively). Whilst γc and CD122 are present in naïve T cells, CD25, which is the high-affinity receptor of IL-2, is only upregulated in activated T cells. By contrast, CD71 is the transferrin receptor, which mediates the uptake of iron. In T cells, the levels of CD71 have been directly associated with Myc expression implying, therefore, that the measurement of CD71 not only serves as an activation marker but also as a readout of the metabolic status of the cell (Preston et al., 2015). As measured by staining with conjugated antibodies and following FACS analysis, I observed that TGFβ did not affect the upregulation of CD25 and CD71 in T cells stimulated with OVA-N4. However, the expression of granzyme B was reduced by >80% in the same conditions (Fig. 4.1A). On the other hand, OT-I T cells stimulated with the lower affinity peptide OVA-T4 displayed an impaired upregulation of CD25, CD71

and granzyme B when treated with TGFβ indicating, therefore, that TGFβ is particularly suppressive against $CD8^+$ T cells primed with weak agonists (Fig. 4.1A). Interestingly, the expression of CD44, an adhesion receptor usually upregulated in activated T cells to promote migration, was not diminished but instead strongly induced in the presence of TGFβ (Fig. 4.1A), an observation that was also reported by Brownlie et al. (2017).

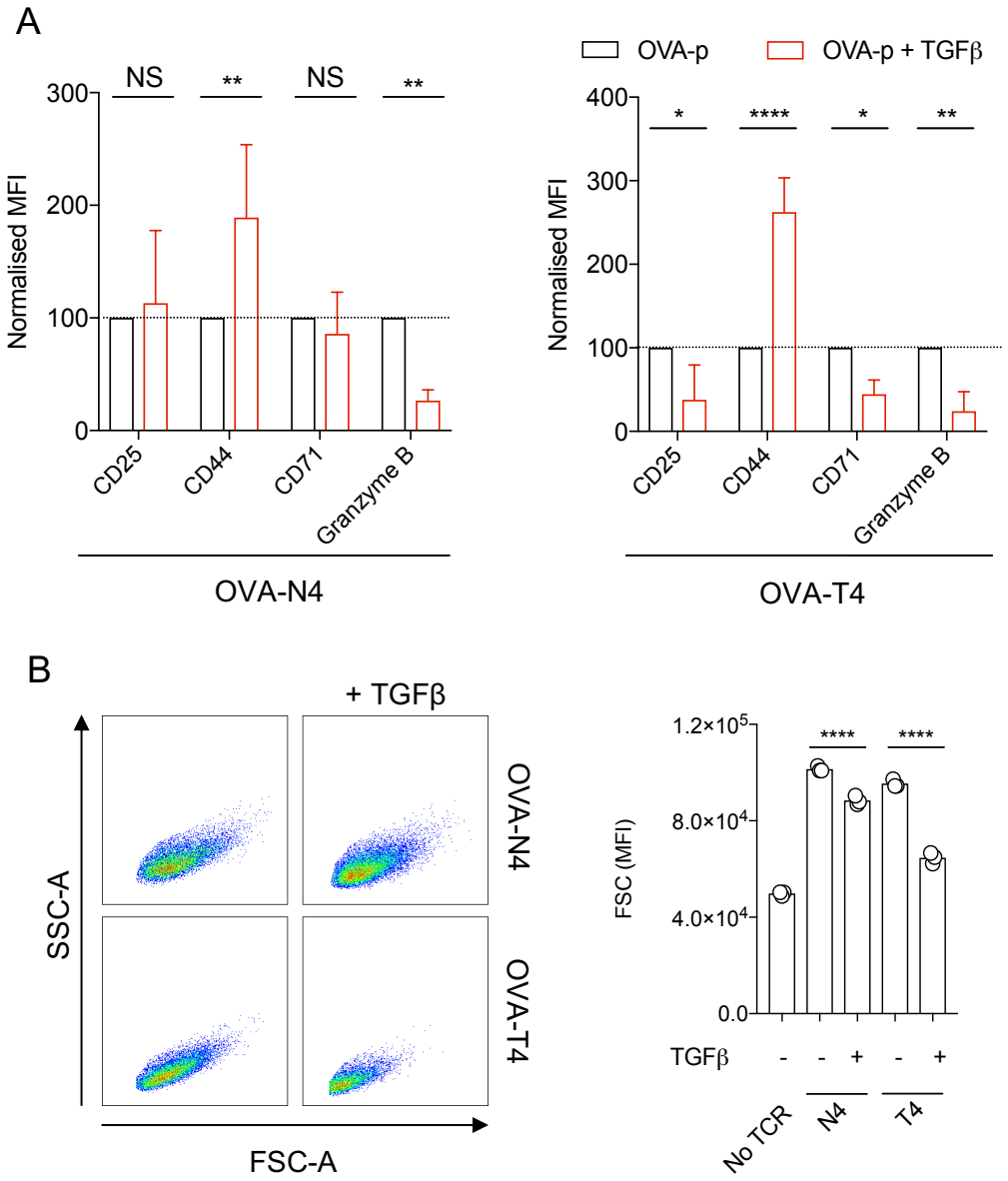


Figure 4.1: TGFβ suppresses CD8⁺ T cell activation and growth *in vitro.*

OT-I T lymphocytes were stimulated *in vitro* for 48h with OVA-p (10^{-6}M/10^{-8}M) ± TGFβ (5ng/ml). (A) After incubation, levels of the activation markers CD25, CD44, CD71 and granzyme B were assessed by FACS analysis. (B) Cell size was determined by FACS analysis. On the left, FSC-A/SSC-A dot plots of lymphocytes after exclusion of doublets and dead cells. On the right, FSC-A MFI values are shown. In (A), figures collect data from three independent experiments. In (B), data is from one of at least three experiments and dots in graph represent technical replicates. Data represents mean with SD. NS – Not significant; * - P<0.05; ** - P<0.005; **** - P<0.0001, as determined by one-way or two-way ANOVA with Tukey's or Sidak's multiple comparisons test, as appropriate.

Another key indication of T cell activation is an increase in cell size. As expected, I observed in FSC-A/SSC-A plots that T lymphocytes stimulated with only OVA-peptide, irrespective of the strength of the stimulation, at least doubled their size within the first 48h (Fig. 4.1B). The addition of TGFβ, consistently with the previous data, prevented the optimal growth of T cells upon TCR-stimulation, an effect that was found more severe in OVA-T4-stimulated cells (Fig. 4.1B).

To validate these results in the context of anti-tumour responses, I designed an *in vitro* approach that directly tested the effects of cancer cell-derived TGFβ on the activation of $CD8^+$ T cells responding to tumour associated antigens (TAAs). To do so, I utilised OVA-expressing ID8 cells, an ovarian carcinoma cell line that secretes TGFβ (Chen, S. et al., 2019). I co-cultured naïve OT-I T cells with OVA-N4, OVA-T4 or OVA-V4-expressing ID8 cells and determined T cell activation state. To determine the impact of tumour cell-derived TGFβ, cells were also treated with SB431542 (5μM), a selective TGFβRI kinase inhibitor. After 48h, approximately >95% of the OT-I T cells co-cultured with ID8-N4 cells expressed both GrB and IFNγ (Fig. 4.2A). In these co-cultures, the addition of SB431542 did not affect GrB and IFNγ levels indicating no effects of ID8-derived TGFβ against $CD8^+$ T cells primed with high affinity peptides (Fig. 4.2A). On the other hand, whilst ID8 cells expressing the very weak agonist OVA-V4 did not induce a T cell response, ID8 cells expressing the low affinity peptide OVA-T4 promoted T cell activation more efficiently in the presence of the TGFβRI inhibitor (Fig. 4.2A). In this condition, ~65% of the cells were $GrB^+IFN\gamma^+$ whereas only ~35% of the cells could upregulate these activation markers when the TGFβ signalling was not blocked (Fig. 4.2A). Similarly, the activation markers CD25 and CD71 were only differentially expressed in T lymphocytes cultured with ID8-T4 cells whilst no

differences were observed when cultured with high affinity ID8-N4 or very low affinity ID8-V4 cells (Fig. 4.2B). Altogether, this data suggests that tumour cell-derived TGFβ dampens initial CD8⁺ T cell activation only against low affinity antigens.

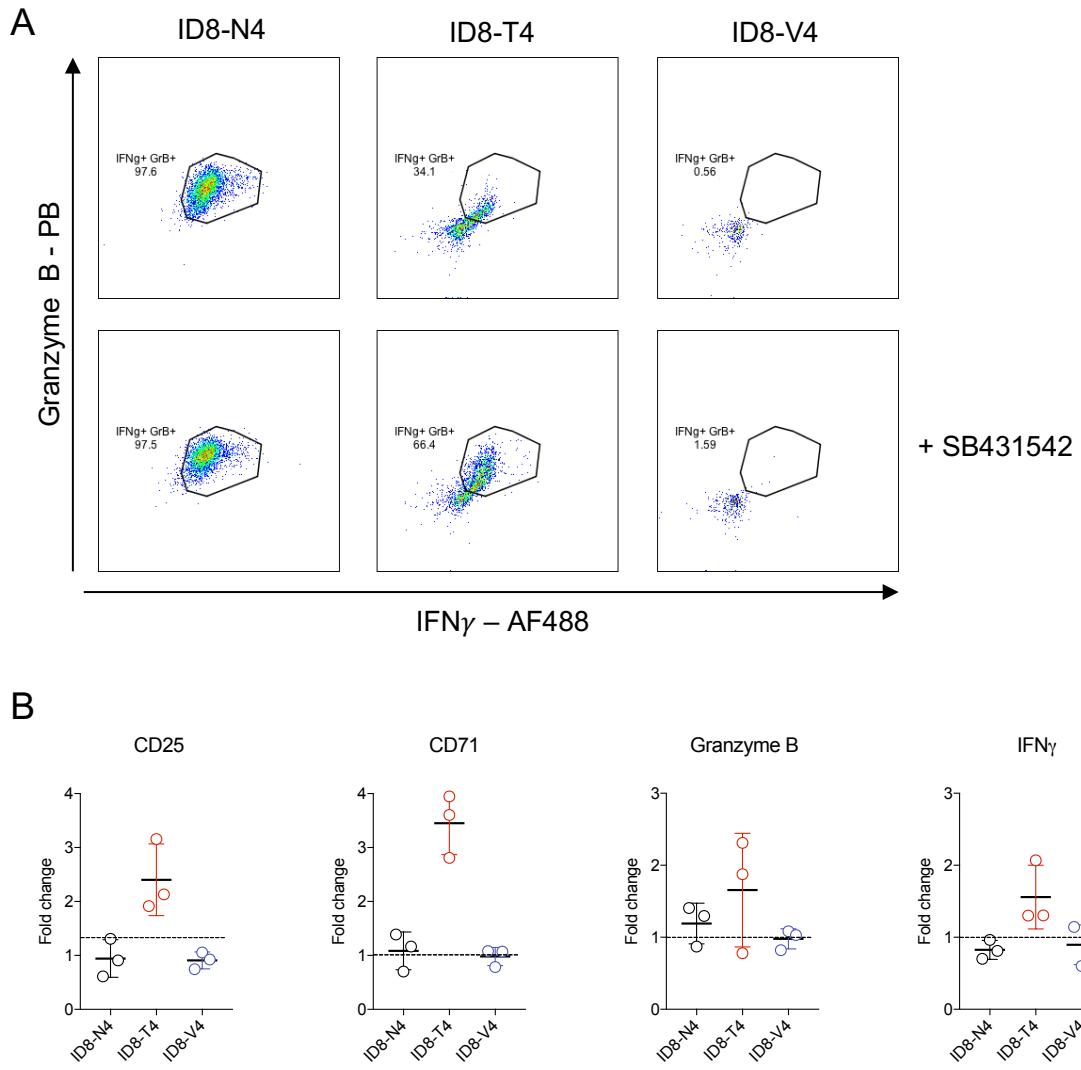


Figure 4.2: Tumour cell-derived TGFβ inhibits CD8⁺ T cell activation against weak agonists.

OT-I T lymphocytes were co-cultured with ID8-N4, ID8-T4 or ID8-V4 cells for 48h in the presence or absence of the TGFβRI blocker SB321542. Brefeldin A (2.5μg/ml) was added the last 4h of incubation and levels of GrB, IFNγ, CD25 and CD71 were assessed by FACS analysis. Data is representative from three independent experiments. In (B), summarised data from the three experiments are shown. Fold change ([ID8-OVA + OT-I + SB431542] / [ID8-OVA + OT-I]) is calculated based on MFI.

4.2.2 TGFβ inhibits growth and proliferation upon TCR-stimulation

Other investigations have described the restriction of IL-2 secretion as an important mechanisms by which TGFβ limits $CD8^+$ T cell responses (Thomas and Massagué, 2005; Brownlie et al., 2017). In order to validate these findings in the OT-I model, I stimulated OT-I cells with OVA-p ± TGFβ for 24h in the presence of anti-CD25 blocking antibody. Then, I evaluated IL-2 secretion as assessed by ELISA quantification of IL-2 levels in supernatants. Consistently, I observed that (1) OVA-N4 stimulation augments early IL-2 secretion when compared to OVA-T4 stimulated T cells and (2) that TGFβ hampers IL-2 secretion. Importantly, TGFβ-mediated inhibition of IL-2 secretion was performed irrespective of the strength of TCR-stimulation (Fig. 4.3A).

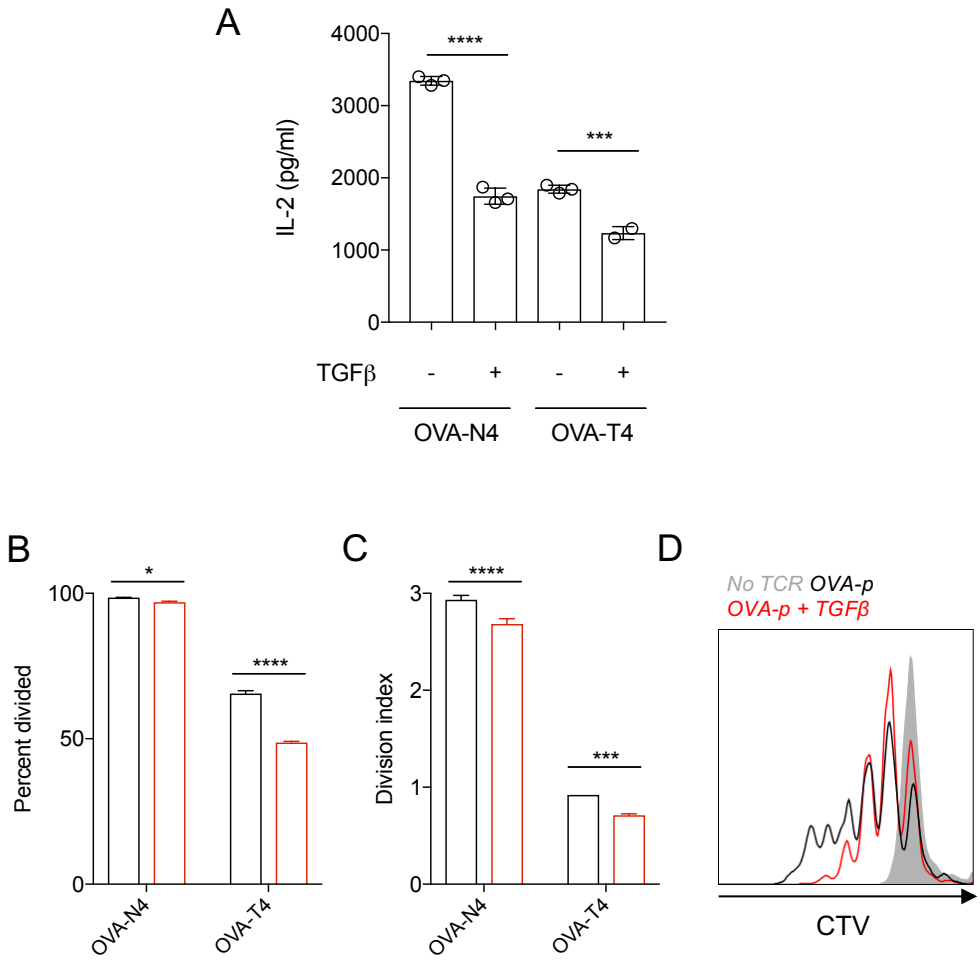


Figure 4.3: TGFβ restricts IL-2 production and proliferation upon TCR-priming.

OT-I T lymphocytes were stimulated *in vitro* for 24h (A) or 72h (B, C, D) with OVA-p (10^{-8}M) ± TGFβ (5ng/ml). In (A), secreted IL-2 levels in supernatants were assessed by ELISA. In (B, C, D), proliferative capacity was determined by dilution of CTV dye. The histogram shown in (D) represents CTV staining of OVA-T4 stimulated T cells ± TGFβ. Data is representative from one of three independent experiments. Data represents mean of technical triplicates and SD. * - P<0.05; *** - P<0.005; **** - P<0.0001, as determined by one-way or two-way ANOVA with Tukey's or Sidak's multiple comparisons test, as appropriate.

Previous studies have also well-stablished that TGFβ significantly dampens T cell proliferation, an effect that has been associated with inhibition of IL-2 secretion (Brownlie et al., 2017). To also validate these findings, I determined the proliferative capacity of T lymphocytes treated ± TGFβ using CTV dye. As expected, the strength of the TCR-stimulation determined the magnitude of the response. After 72h of stimulation, almost all T cells initiated their first division

when primed with OVA-N4 whilst only 65% did when primed with OVA-T4. Under TGFβ treatment, fewer T cells did undergo cell division, particularly in OVA-T4 stimulated lymphocytes (45% vs 65% in control conditions) (Fig. 4.3B and D). Moreover, as shown by a diminished division index (i.e. the average number of cell divisions that a cell undergoes), T cells stimulated in the presence of TGFβ were unable to go through as many divisions as compared to those stimulated with only OVA-p, indicating that TGFβ attenuated T cell expansion (Fig. 4.3C and D). This effect was observed in both OVA-N4 and OVA-T4 stimulated T cells (Fig. 4.3C and D).

Next, due to the essential role of IL-2 in promoting early T cell growth, activation and proliferation I questioned whether the inhibitory effects of TGFβ seen during early T cell activation (Fig. 4.1-3) were caused indirectly by the loss of IL-2. Thus, I stimulated OT-I T cells with OVA-T4 ± TGFβ and added hIL-2 (1ng/ml) from timepoint 0h to test if IL-2 replenishment would protect from TGFβ mediated suppression. After 48h, I measured the levels of CD25 and granzyme B and observed that whilst the presence of IL-2 alone seemed to improve the activation state of the cells (increased granzyme B expression), IL-2 replenishment did not prevent the downregulation of both markers when added together with TGFβ (Fig. 4.4A). Furthermore, to firmly demonstrate that the TGFβ-mediated inhibition of T cell activation was performed independently of IL-2 levels I treated the T cells with TGFβ only after 24h of TCR-stimulation. The rationale behind this experiment relies on the fact that the peak of IL-2 secretion takes place within the first 24h upon TCR-binding and, therefore, the cell culture media at this timepoint is enriched with plenty IL-2. My data indicates that, upon 48h of TCR-stimulation, both CD25 and granzyme B expression were still downregulated even when

addition of TGFβ is delayed for 24h (Fig. 4.4B). However, suppression was more pronounced when TGFβ is present from timepoint 0h (Fig. 4.4B). Altogether, these findings demonstrate that although the loss of IL-2 contributes to the suppressive effects, TGFβ also acts through IL-2 independent mechanisms to limit T cell activation.

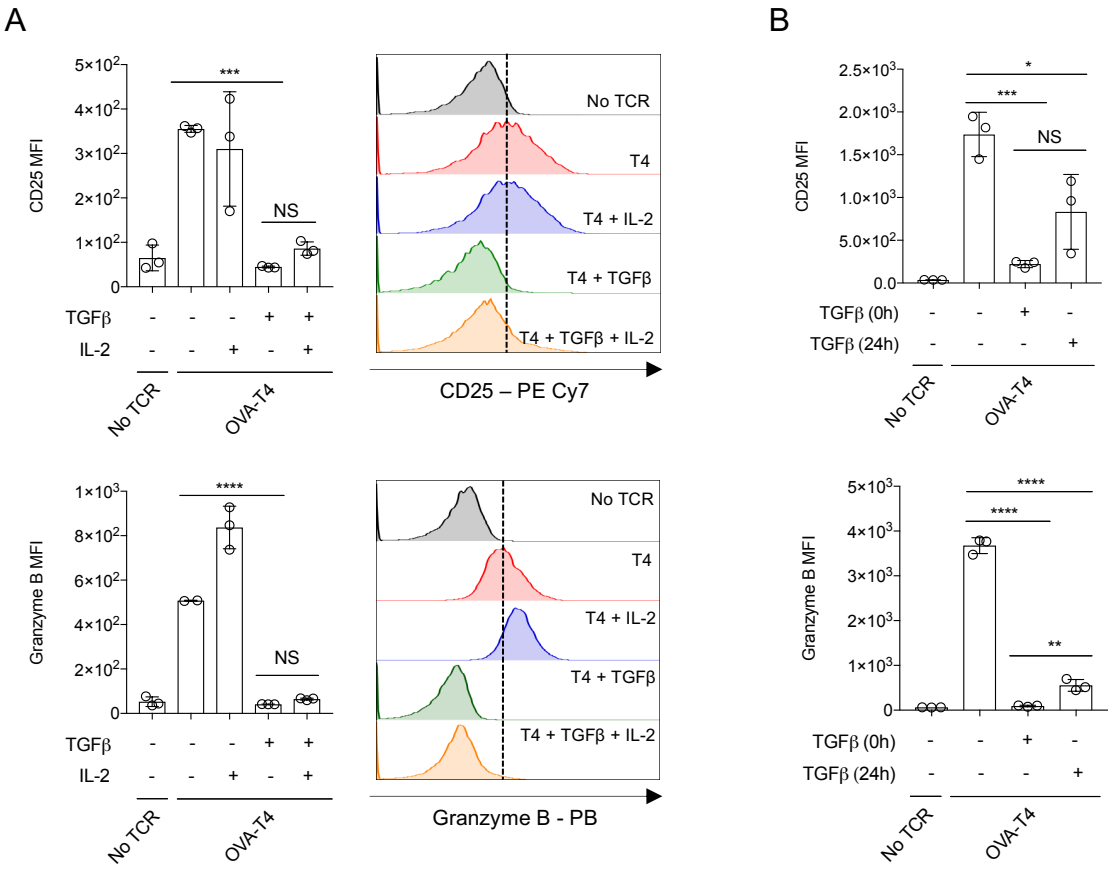


Figure 4.4: IL-2 replenishment does not prevent TGFβ-mediated suppression.

OT-I T lymphocytes were stimulated *in vitro* for 48h with OVA-p (10^{-8}M) ± TGFβ (5ng/ml). Where indicated, hIL-2 (1ng/ml) was added to the cells from timepoint 0h (A, B) or at 24h (B). After incubation, levels of CD25 and GrB were assessed by FACS analysis. Data is from one of three (A) or two (B) experiments with technical triplicates. Error bars represent SD. NS – Not significant; * - P<0.05; ** - P<0.005; *** - P<0.0005; **** - P<0.0001, as determined by one-way ANOVA with Tukey's multiple comparisons test.

4.2.3 TGFβ dampens cytokine secretion, but not immediate killing capacity, of CTLs

So far, I have described in detail the implications of TGFβ treatment during the priming of $CD8^+$ T cells, but how does the presence of TGFβ affect fully differentiated CTLs? Investigations by Tu et al. (2018) showed that $CD4^+$ T lymphocytes activated with strong agonists downregulate the expression of *Tgfbr1* and *Tgfbr2* upon TCR-binding to facilitate T_h1 lineage commitment. I hypothesised that a similar scenario could occur in $CD8^+$ T cells making effector CTLs less responsive to TGFβ, as compared to naïve cells. To test this, I compared levels of TGFβ-induced SMAD phosphorylation in CTLs, generated by initial priming with OVA-N4 for 2 days and expanded for 4 days further in the presence of hIL-2, and naïve OT-I cells. The TGFβRI inhibitor SB431542 was utilised as a negative control. I found that both naïve OT-I T cells and CTLs were able to trigger SMAD2/3 phosphorylation in response to TGFβ (Fig. 4.5). In fact, the abundance of pSMAD2/3 upon TGFβ stimulation was higher in CTLs than in naïve T cells (Fig. 4.5). Thus, my data indicates that, in contrast to results reported for effector $CD4^+$ T_h1 cells, CTLs are still responsive to TGFβ treatment.

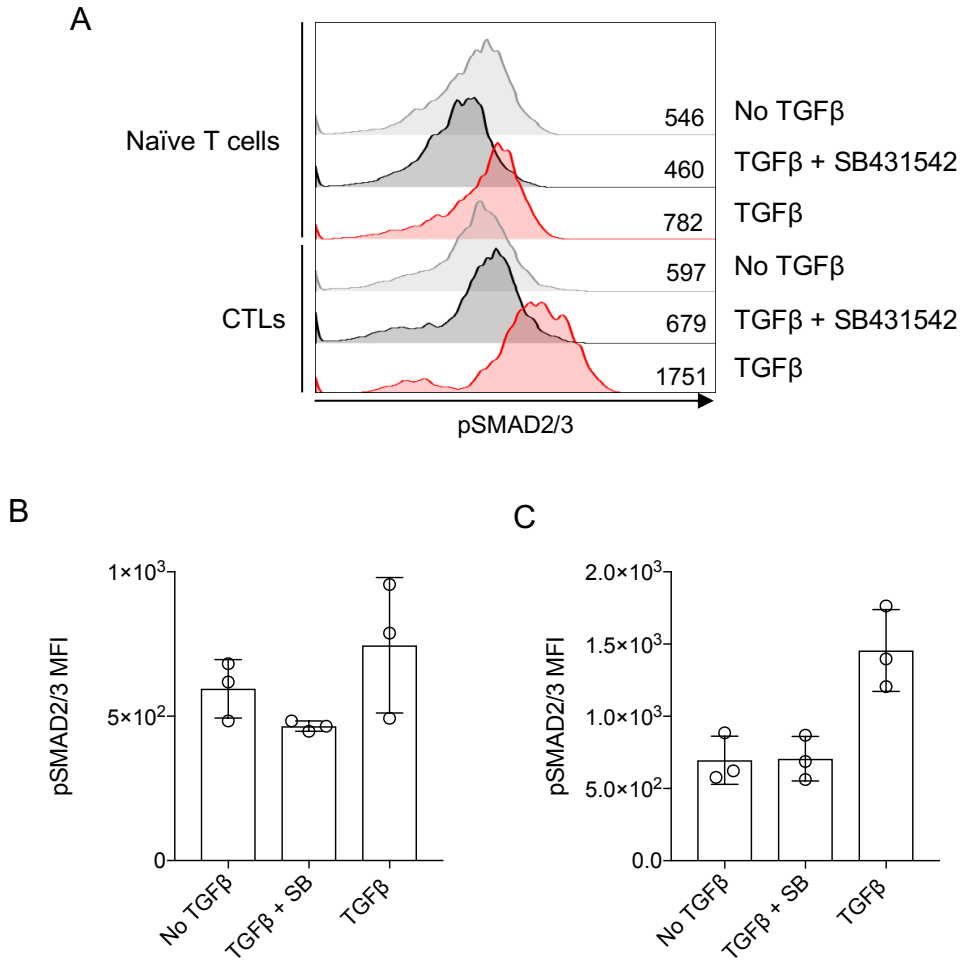


Figure 4.5: TGFβ triggers SMAD2/3 phosphorylation in both naïve and effector CD8⁺ T cells.

Naïve OT-I T lymphocytes or CTLs were stimulated *in vitro* for 1h with TGFβ (5ng/ml). The TGFβRI inhibitor SB431542 was utilised as a negative control. After incubation, levels of pSMAD2/3 (Ser465/467) were assessed by IC staining followed by FACS analysis. (A) Representative histogram of pSMAD2/3 levels in naïve T cells and CTLs. Values represent pSMAD2/3 MFI. Summarised data of pSMAD2/3 levels in naïve T cells (B) or CTLs (C) are indicated. Data is from one of three experiments with technical triplicates. Error bars represent SD.

Other groups (Thomas and Massagué, 2005), in accordance with the findings described previously, have clearly established that TGFβ is a strong inhibitor of granzyme B upregulation upon antigen detection and this has been linked to the increased tumour rejection observed in mouse models following TGFβ neutralisation (Gorelik and Flavell, 2001; Thomas and Massagué, 2005). However, there is little direct evidence indicating that TGFβ also impairs the

cytotoxicity of fully differentiated CTLs. Hence, I performed a killing assay by co-culturing luciferase-expressing ID8-T4 or ID8-V4 cells with OT-I CTLs overnight in the presence or absence of TGFβ. First, as expected, my data showed that the killing capacity of the CTLs correlated with the affinity of the OVA-peptide (Fig. 4.6). However, the proportion of targeted tumour cells (either ID8-T4 or ID8-V4) was equal irrespective of the presence of TGFβ (Fig. 4.6) suggesting, therefore, that TGFβ does not directly affect the cytotoxicity of CTLs.

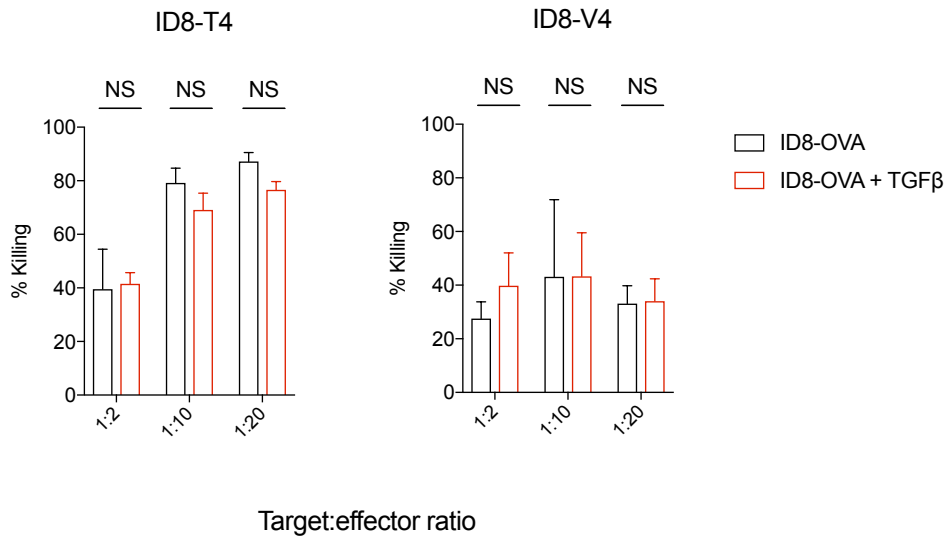


Figure 4.6: TGFβ does not impair CTL killing capacity.

CTLs generated *in vitro* during 6d were co-cultured with luciferase-expressing ID8-T4 or ID8-V4 in the presence or absence of TGFβ. After an overnight incubation, cell cultures were washed in PBS and ID8 confluence was determined by bioluminescence imaging. % killing was determined based on negative control (no added CTLs). Data is from one of two repeated experiments with technical triplicates. Error bars represent SD. NS – Not significant, as determined by two-way ANOVA with Sidak's multiple comparisons test.

Next, I tested whether TGFβ affected cytokine production of CTLs. I re-stimulated CTLs with OVA-p ± TGFβ for 24h and quantified IFNγ levels in supernatants by ELISA. I observed that TCR-induced IFNγ levels were substantially diminished by TGFβ in both OVA-N4 (by ~60%) and OVA-T4 (by ~65%) re-stimulation

conditions (Fig. 4.7). These data indicate that TGFβ inhibits CTL IFNγ production irrespective of the strength of TCR stimulation.

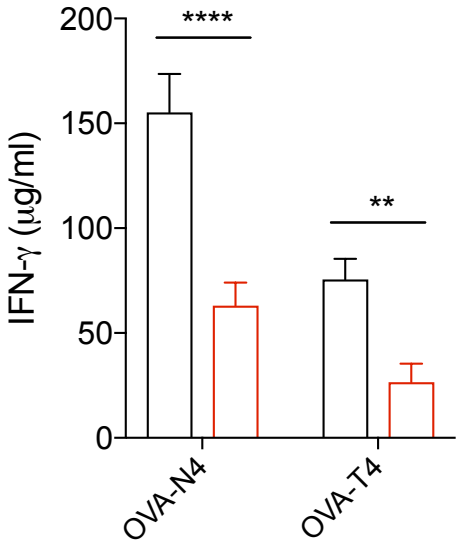


Figure 4.7: TGFβ limits IFNγ secretion of CTLs.

CTLs generated *in vitro* during 6d were re-challenged with OVA-p (10^{-8}M) ± TGFβ (5ng/ml). After 24h, Levels of IFNγ in SN were determined by ELISA. Data is from one of three experiments with technical replicates. Error bars represent SD. ** - P<0.005; **** - P<0.0001, as determined by two-way ANOVA with Sidak's multiple comparisons test.

4.2.4 TGFβ reshapes the transcriptome of activated CD8$^+$ T lymphocytes

In order to gain a broad understanding on the mechanisms that sustain the suppression of CD8$^+$ T cell priming by TGFβ, I decided to explore the transcriptomic profile of activated T cells ± TGFβ. Thus, I stimulated OT-I T cells from 4 biological replicates with OVA-T4 ± TGFβ for 24h. Then, cells were lysed, mRNA was isolated and mRNA-seq performed (all details are specified in Section 2.7). The number of differentially expressed transcripts (adjusted *p*-value < 0.05, determined as described in Section 2.8) between TGFβ treated and non-treated cells was 5800, with 3248 upregulated genes vs 2552 downregulated

genes in the presence of TGFβ (Fig. 4.8A). However, the number decreased from 3248 to 1224 and from 2552 to 333, respectively, when limiting the analysis to those transcripts differentially expressed with a fold-change (FC) > 2 (Fig. 4.8B). As indicated in the volcano plot (Fig. 4.8C), *Gzmb* and *Eomes*, which encode for granzyme B and the differentiation transcription factor Eomesodermin, were remarkably repressed by TGFβ . On the other hand, genes encoding for the chemokine receptor C-C Motif Chemokine Receptor 8 (*Ccr8*), the nucleotidase CD73 (*Nt5e*), the Acyl-CoA synthase ACBG1 (*Acsbg1*) or the TGFβ signalling associated *Ski* and *Tgbr1* are some of the highlighted transcripts when analysing the upregulated genes (Fig. 4.8B). In Table 4.1, the top 20 most upregulated and downregulated genes, based on FC, are shown.

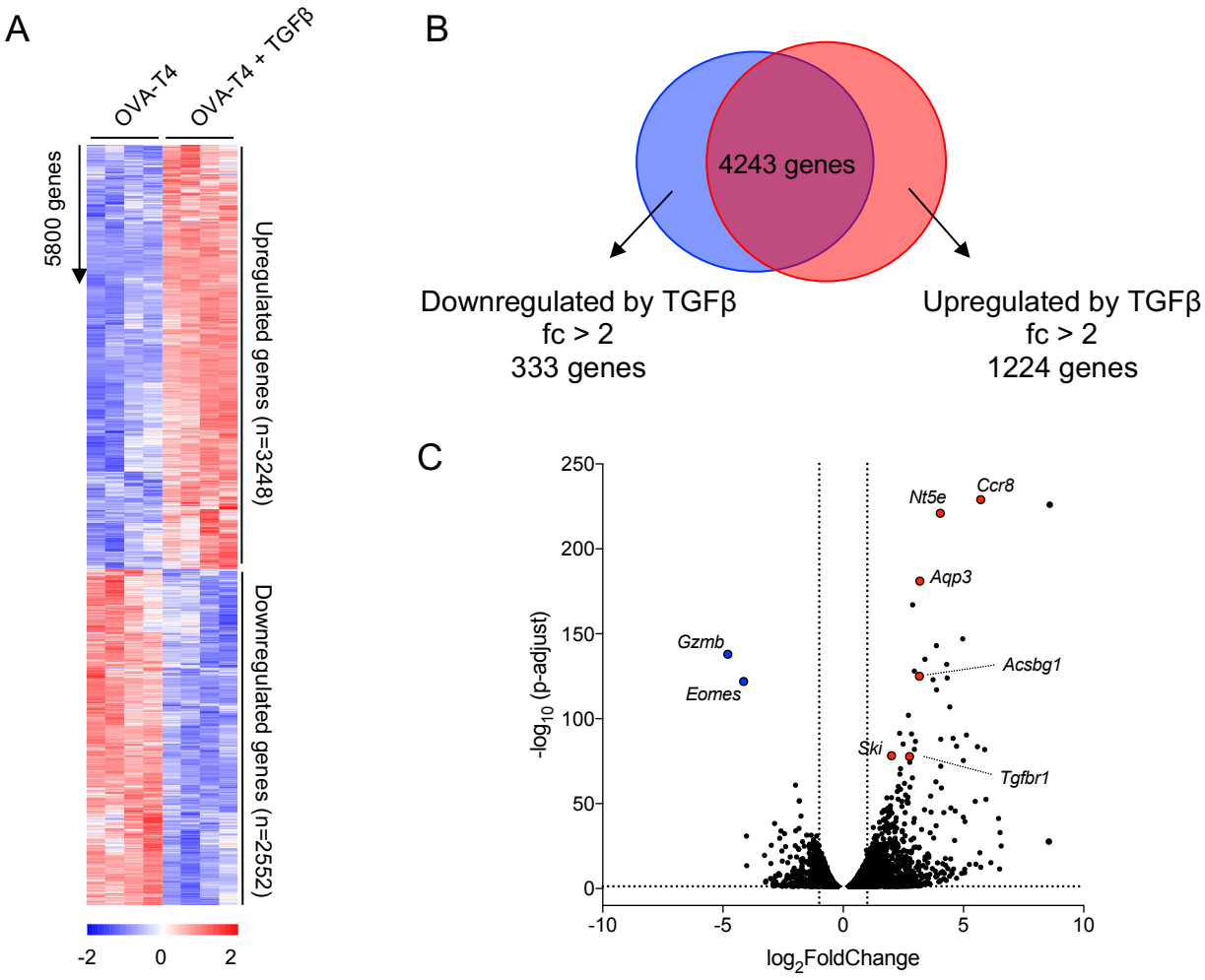


Figure 4.8: TGFβ modulates the transcriptome of CD8⁺ T lymphocytes upon TCR-priming.

OT-I T lymphocytes were stimulated *in vitro* for 24h with OVA-T4 (10^{-8}M) ± TGFβ (5ng/ml). After incubation, mRNA was isolated and mRNA-seq was performed. Only differentially expressed genes (adjusted *p*-value < 0.05) are displayed (n=5800). In (A), values represent $\log_2$(FC). In (C), dashed lines determine cut-offs – i.e. adjusted *p*-value < 0.05 (horizontal) and FC > 2 (vertical). Data from 4 biological replicates is included.

Gene	FC	p-value
Igha	385.76	1.76E-230
Apol9b	375.99	1.55E-30
Cx3cr1	94.80	7.12E-28
Lamc3	92.20	6.01E-36
Fn1	91.11	1.13E-13
Cdh1	88.00	2.45E-44
Gna14	70.39	1.03E-17
Olfm2	61.24	8.91E-56
Maf	59.07	1.90E-85
Bgn	52.80	1.03E-14
Ccr8	52.63	2.78E-234
Gja5	51.25	9.66E-24
Agap1	47.87	4.73E-87
Atp1a2	45.83	9.93E-17
Zfhx3	45.08	1.44E-54
5430421N21Rik	37.99	1.46E-16
Entpd1	34.81	4.74E-94
Osgin1	33.41	1.93E-42
Gm12503	33.19	1.89E-11

Gene	FC	p-value
Gzmb	27.71	2.24E-142
Eomes	17.59	3.81E-126
Ptpn5	16.18	8.57E-34
Wnt10a	16.17	9.24E-16
Zan	9.62	3.24E-22
Enam	9.37	2.24E-05
Bglap3	9.30	4.77E-06
Mapk12	8.14	9.21E-08
Ccr5	8.05	3.78E-17
Ak4	7.92	3.06E-28
Exph5	7.78	4.57E-07
Trpa1	7.57	2.46E-06
Spta1	7.50	2.22E-06
Ak3l2-ps	7.39	3.03E-07
Csf2	7.25	9.79E-06
Bspry	7.22	2.48E-41
Cnnm1	7.17	1.08E-05
Gstt1	7.04	2.22E-09
Gm26580	7.00	8.75E-06

Table 4.1: Most upregulated and downregulated transcripts by TGFβ.

Left: top 20 upregulated transcripts, FC = (OVA-T4 + TGFβ) / (OVA-T4). Right: top 20 downregulated transcripts, FC = (OVA-T4) / (OVA-T4 + TGFβ).

4.2.5 TGFβ profoundly modifies the metabolic transcriptome of activated CD8$^+$ T cells

To better understand how TGFβ inhibits CD8$^+$ T cell activation I performed Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis on the 5800 differentially expressed genes (adjusted $p < 0.05$, no FC cut-off). Genes associated with metabolic pathways were the most affected by TGFβ treatment, with a total of 464 genes (Fig. 4.9). Specifically, carbon metabolism (n=71), glycolysis (n=52) or the biosynthesis of amino acids (n=56) were the major metabolic pathways altered by TGFβ. Other pathways included genes involved in the regulation of RNA transport (n=99), the spliceosome (n=82) or ribosome biogenesis (n=62) (Fig. 4.9).

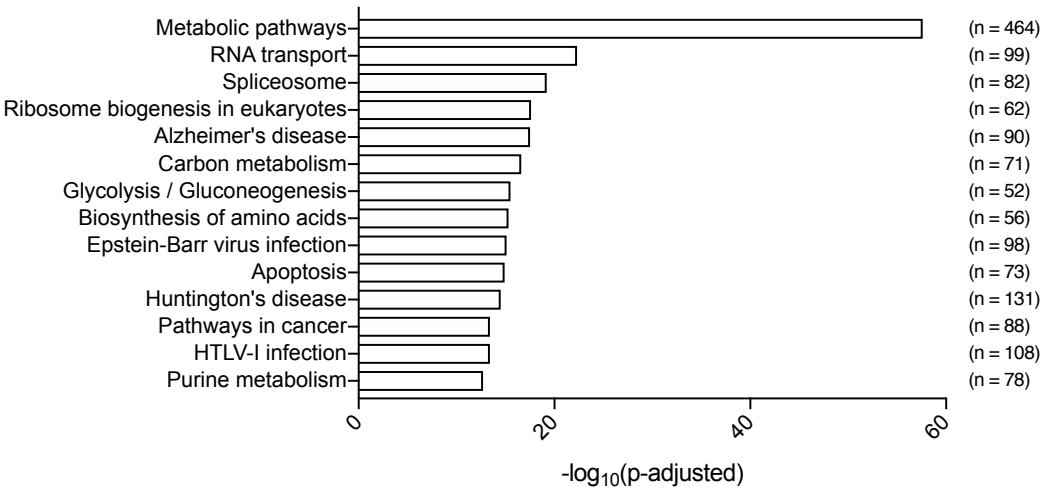


Figure 4.9: Metabolic pathways are the most affected by TGFβ.

List of the most influenced pathways by TGFβ, as assessed by KEGG analysis of the 5800 differentially expressed genes (adjusted *p*-value < 0.05), including upregulated and downregulated genes. On the right, *n* indicates number of genes identified in each pathway.

Then, I further explored which pathways were particularly repressed or induced by TGFβ performing KEGG analysis only of the differentially expressed genes with a FC > 2 (1224 upregulated and 333 downregulated genes, as specified in

Section 4.2.4). This analysis identified that genes involved in the regulation of inflammatory bowel disease, leishmaniasis, T_h17 cell differentiation, hematopoietic cell lineage and cytokine-cytokine receptor interaction were the most upregulated pathways by TGFβ (Fig. 4.10A). Within the 1224 upregulated transcripts, the most recurrent amongst the top 10 pathways included genes that encoded for MHC-II complex proteins (e.g. *H2-DMb2, H2-DMb1, H2-DMa),* cytokine receptors such as IFNGR2, TGFβRI, TGFβRII, IL7R and IL6RA or the integrins CD49d (*Itga4*) and CD11b (*Itgam*) (Fig. 4.10B). Considering that $CD8^+$ T lymphocytes do not express MHC-II, the upregulation of genes encoding for this molecule is surprising. However, it is important to note that, in spite of showing a high FC, these are expressed at very low levels (FPKM < 5). By contrast, genes associated with T cell cytotoxicity (*Fasl, Prf1, Gzmb*) or the metabolism of cysteine and methionine, as well as glycine, serine and threonine (*Cth, Cbs)*, were some of the most highlighted transcripts when analysing pathway enrichment of the 333 downregulated genes (Fig. 4.10A and B).

A

	Index	KEGG pathway identifier	p-value	# of genes
Upregulated by TGFβ (1224 genes)	1	Inflammatory bowel disease (IBD)	7.516×10^{-9}	18
	2	Leishmaniasis	6.809×10^{-8}	18
	3	Th17 cell differentiation	1.804×10^{-7}	22
	4	Hematopoietic cell lineage	8.137×10^{-7}	20
	5	Cytokine-cytokine receptor interaction	0.00004984	36
Downregulated by TGFβ (333 genes)	1	Cysteine and methionine metabolism	0.001424	5
	2	Autoimmune thyroid disease	0.001913	6
	3	TNF signalling pathway	0.002452	7
	4	Glycine, serine and threonine metabolism	0.004301	4
	5	Type I diabetes mellitus	0.005847	5

B

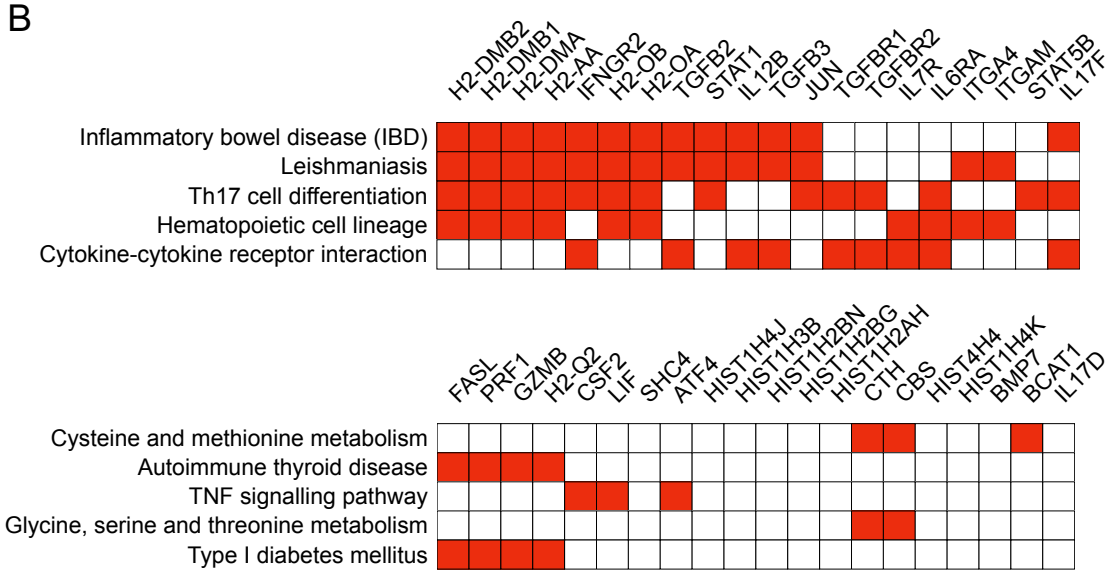


Figure 4.10: Pathway analysis of upregulated and downregulated genes by TGFβ.

(A) List of the most influenced pathways by TGFβ, as assessed by KEGG analysis (FC > 2, *p*-value < 0.05). (B) Clustergrams include most recurrent genes identified amongst the top 10 pathways. Genes associated with the specified pathway are indicated in red.

4.2.6 TGFβ transcriptionally regulates CD8$^+$ T cell activation, differentiation and acquisition of effector functions

As mentioned, the fate of T lymphocytes upon TCR-stimulation is dictated depending on the exposure to environmental signals. This fate is manifested through the expression of specific markers, such as surface proteins, TFs or cytokines. To better comprehend how TGFβ was defining the outcome of TCR-primed CD8$^+$ T cells I performed an in-depth analysis of the expression of the activation/differentiation markers.

First, I analysed expression levels of transcripts that encode for some of the most well established early activation markers including CD25, CD44, CD69 and CD71, usually induced upon TCR-ligation, or CD62L and the IL-7R, which are usually downregulated. Data indicated that TGFβ limited the upregulation of CD25, CD69 and CD71 transcripts whilst maintained the expression of CD62L and IL7R, indicating the suppression of T cell activation (Fig. 4.11). Consistent with my previous findings, CD44 expression was induced by TGFβ (Fig. 4.11). Regarding the expression of inhibitory receptors, the mRNA encoding for PD-1 (*Pdcd1*) and TIM3 (*Havcr2*) was induced in the presence of TGFβ. However, this was not observed for *Ctla4, Lag3* or *Tigit*, which were either repressed or unchanged. Furthermore, the effector molecules perforin, FasL, granzyme C and, particularly, granzyme B (with a striking 26-fold change), were strongly repressed upon TGFβ treatment. Other effector molecules such as granzyme A and M or the LT-α and β were little or not affected by TGFβ (Fig. 4.11).

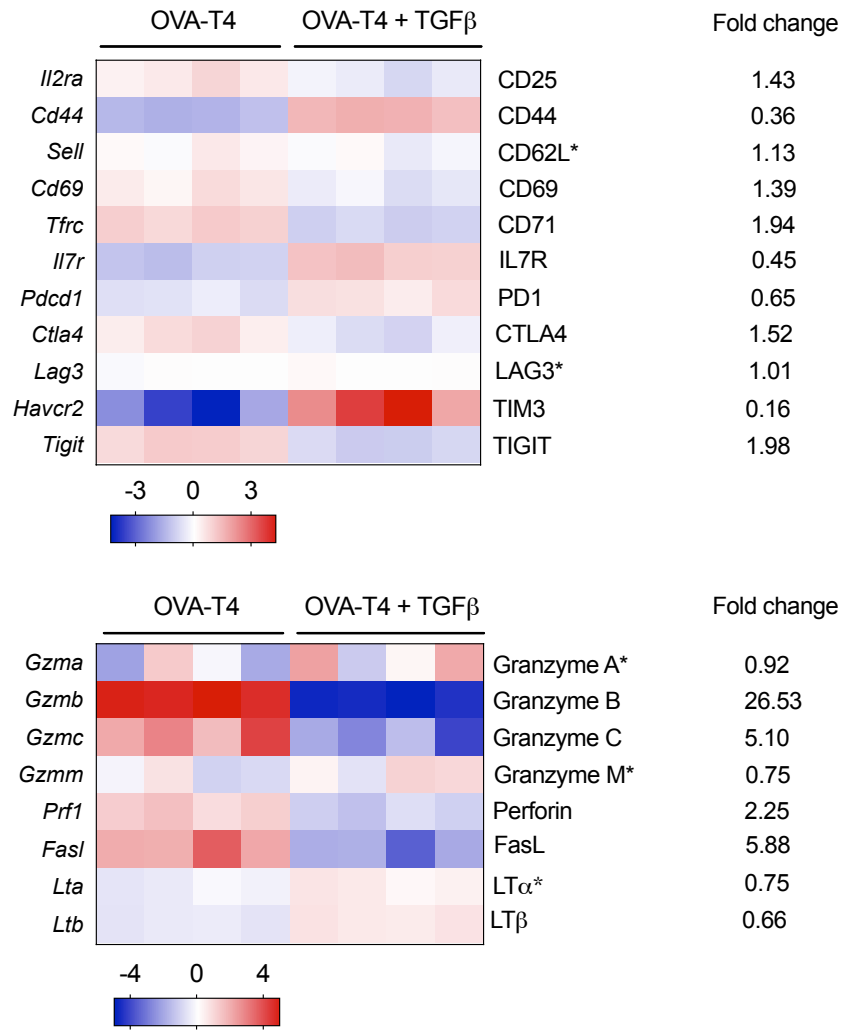
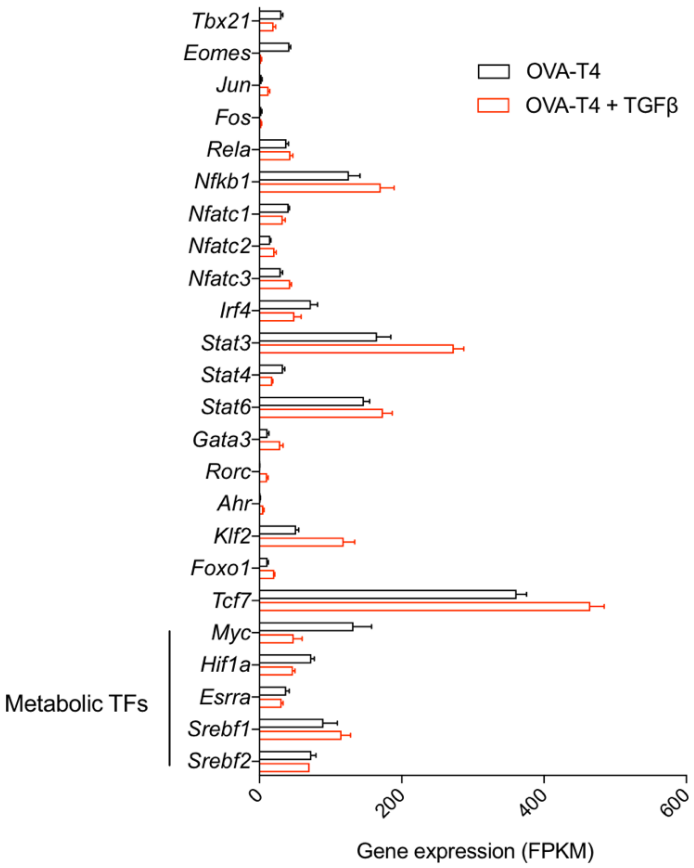


Figure 4.11: TGFβ alters the transcription of activation markers and effector molecules in CD8⁺ T cells upon TCR-priming.

mRNA expression of the indicated markers is displayed, as assessed by RNA-seq (n=4). Values in heatmaps represent $\log_2$(FC). Non-differentially expressed genes are indicated with (*). Indicated values for FC are calculated as FC = (OVA-T4) / (OVA-T4 + TGFβ).

Next, I focused on a myriad of TFs associated with T cell differentiation and metabolism. I observed that *Eomes* was the most downregulated TF-encoding gene by TGFβ, with a FC > 18 (Fig. 4.12). Moreover, genes encoding for other TFs involved in the acquisition of an effector phenotype, e.g. Tbet, IRF4 or STAT4, were also repressed in the presence of TGFβ. Nonetheless, this trend was not uniformly followed when analysing TFs associated with early TCR-signalling pathways, some of which were upregulated (e.g. *Jun, Nfkb1, Nfat2*), downregulated (*Nfatc1*) or unchanged (*Fos, Rela*) (Fig. 4.12). Interestingly, the expression of genes encoding for Forkhead box protein O1 (FOXO1) or transcription factor 1 (Tcf1; encoded by *Tcf7*), both downregulated during effector differentiation (Danilo et al., 2018), was higher in TGFβ-treated T lymphocytes (Fig. 4.12). *Rorc* expression, known to be induced by TGFβ in T_h17 cells (Zhu, J. et al., 2010), was also upregulated. As for the expression of metabolic TFs, *Myc* and *Hif1a,* associated with glycolysis, glutaminolysis and AA metabolism, were significantly reduced but no differences were observed in *Esrra, Srepf1* and *Srebp2* expression (Fig. 4.12), which are involved in mitochondrial and lipid metabolism, respectively.



Gene	FC	Significance
Tbx21	1.58	TRUE
Eomes	18.44	TRUE
Jun	0.24	TRUE
Fos	0.99	FALSE
Rela	0.91	FALSE
Nfkb1	0.77	TRUE
Nfatc1	1.25	TRUE
Nfatc2	0.71	TRUE
Nfatc3	0.69	TRUE
Irf4	1.5	TRUE
Stat3	0.60	TRUE
Stat4	1.83	TRUE
Stat6	0.84	FALSE
Gata3	0.39	TRUE
Rorc	0.03	TRUE
Ahr	0.22	TRUE
Klf2	0.45	TRUE
Foxo1	0.57	TRUE
Tcf7	0.80	TRUE
Myc	2.77	TRUE
Hif1a	1.54	TRUE
Esrra	1.2	FALSE
Srebf1	0.77	FALSE
Srebf2	1.07	FALSE

Figure 4.12: TGFβ treatment modifies the gene expression of key TFs.

Gene expression (FPKM) of the indicated TFs is displayed, as assessed by RNA-seq (n=4). Error bars indicate SD. On the table, blue indicates downregulated transcripts; red indicates upregulated transcripts; black indicates non-differentially expressed transcripts. Indicated values for FC are calculated as FC = (OVA-T4) / (OVA-T4 + TGFβ). Differences in gene expression are considered significant (i.e. “TRUE” in table) when adjusted *p*-value < 0.05.

Then, I looked into how TGFβ modulated cytokine transcription of activated T cells. I saw that, even in the presence of TGFβ, T cells induce the expression of *Ifng* and *Tnf*, typically represented in effector T cells. Expression of *Ifng* was slightly diminished but, surprisingly, did not reach statistical significance (Fig. 4.13A). Other cytokines were very low or not expressed and only a significant increase was seen in *Il17f* upon TGFβ-treatment (Fig. 4.13A). In regards to cytokine receptors, those involved with effector or memory differentiation, such as *Il2ra, Il12rb1*, *Il12rb2* and *Il15ra* were downregulated by TGFβ. On the other hand, *Il6ra, Ifngr2* and, particularly, *Tgfbr1*, were some of the receptor-encoding genes that were expressed at higher levels upon TGFβ-stimulation (Fig. 4.13B).

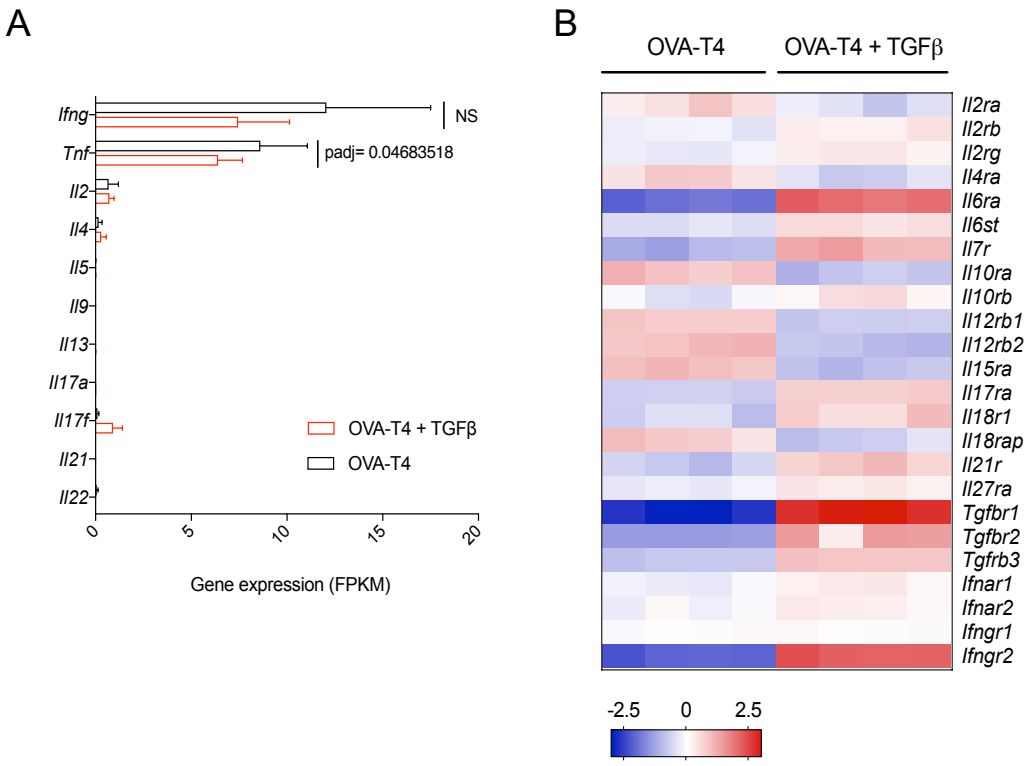


Figure 4.13: TGFβ remodels the mRNA expression of cytokines and cytokine receptors in CD8⁺ T cells.

(A) Gene expression (FPKM) of the indicated cytokines is displayed, as assessed by RNA-seq (n=4). Error bars indicate SD. (B) Gene expression of the indicated cytokine receptors is displayed. Values represent $\log_2$(FC).

The effects of TGFβ on expression of chemokine receptor genes were variable. Canonically, naïve T lymphocytes express CCR7, which is downregulated upon TCR-stimulation while CCR5 and CXCR3, among others, are induced (Nolz et al., 2011). TGFβ impeded the downregulation of *Ccr7* upon TCR-stimulation while inhibiting the induction of *Ccr5.* However, *Cxcr3* expression was not repressed, but instead promoted, in the presence of TGFβ. Amongst other potentially interesting results, I observed a striking effect on *Ccr8* expression being, as mentioned in Section 1.2.5, one of the most upregulated genes by TGFβ (Fig. 4.14).

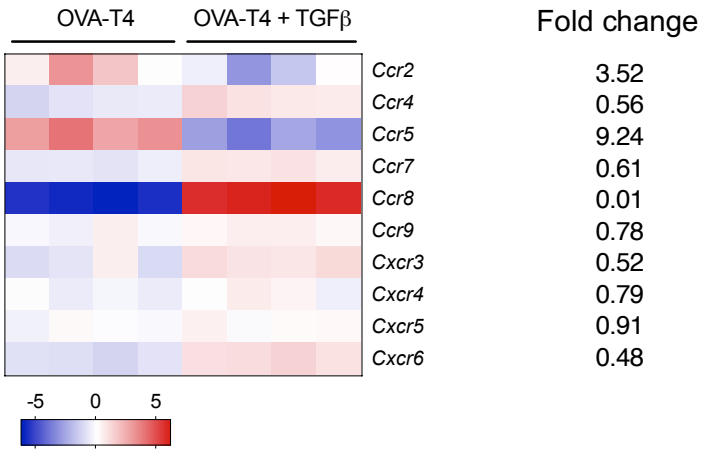


Figure 4.14: TGFβ changes the mRNA expression of chemokine receptors in CD8⁺ T cells.

mRNA expression of the indicated chemokine receptors is displayed, as assessed by RNA-seq (n=4). Values in heatmaps represent $\log_2$(FC). Indicated values for FC are calculated as FC = (OVA-T4) / (OVA-T4 + TGFβ).

4.3 Discussion

The mechanisms by which TGFβ dampens $CD8^+$ T cell responses remains to be fully understood. Specifically, as mentioned in Section 4.1, it still is uncertain whether TGFβ predominantly inhibits $CD8^+$ T cells *in vivo* through direct action or indirectly through effects on other cell types. In the present chapter, I have characterised in depth how TGFβ is able to directly shape the phenotype of $CD8^+$ T lymphocytes *in vitro*. Furthermore, I examined the transcriptomic changes induced by TGFβ, acquiring valuable new comprehension on its mechanisms of action.

First, the study of naïve $CD8^+$ T cells stimulated in the presence or absence of TGFβ confirmed that TGFβ negatively affects early events upon TCR-priming as shown by reduced growth, proliferation and expression of key activation markers and effector molecules (e.g. CD25, CD71, GrB), particularly in response to weak agonists (Fig. 4.1). This data supports previous studies that confirm that TGFβ strongly represses $CD8^+$ T cell expansion and cytotoxicity both *in vivo* and *in vitro* (Gorelik and Flavell, 2001; Thomas and Massagué, 2005; Arumugam et al., 2015; Brownlie et al., 2017). However, my findings differ from those observed by Thomas and Massagué (2005) who concluded that TGFβ provides a selective inhibition of the cytotoxic program rather than a general inhibition of T cell activation, as shown by comparable levels of CD44, CD69 and CD62L expression but increased GrB and IFNγ in tumour reactive $CD8^+$ T lymphocytes upon TGFβ neutralisation *in vivo*. However, it is important to note that they utilised tumour cell lines that expressed the high affinity peptide OVA-N4 to induce T cell anti-tumour responses. As shown in this chapter, and in accordance to other investigations (Arumugam et al., 2015; Brownlie et al., 2017), the strength of the

TCR-stimulation is key to determine the suppressive capacity of TGFβ. My experiments exploring the direct effects of tumour-derived TGFβ during $CD8^+$ T cell priming confirmed that TGFβ produced by tumour cells was sufficient to limit early GrB or IFNγ expression in T cells recognising OVA-T4 but not OVA-N4. Thus, my data supports a model where the TCR-signalling strength is crucial to determine the severity of TGFβ immunosuppression: whereas high levels of TGFβ selectively targets the cytotoxic and proliferative programs in those T cells stimulated by strong peptides *in vitro*, TGFβ induces a general inhibition in response to weak agonists that results in an unsuccessful exit of quiescence and an almost complete loss of T cell activity. Furthermore, these results suggest that not only the peptide affinity determines the response to TGFβ but that other variables, such as its concentration or its source, should be taken into consideration when investigating the role of TGFβ in the context of anti-tumour T cell responses.

As mentioned, I have shown that whilst mostly all the analysed markers, except CD44, are repressed by TGFβ upon OVA-T4-stimulation, only some specific markers (e.g. GrB, IL-2, IFNγ; Fig. 4.1, 4.3 and 4.7) are inhibited irrespective of the strength of the stimulation. It is possible that this selectivity relies on the direct role of SMAD proteins in regulating the transcription of these genes (McKarns et al., 2004; Thomas and Massagué, 2005) whereas other markers, such as CD25 and CD71 might be suppressed through indirect mechanisms. I tested whether the lack of IL-2 was one of those indirect mechanisms that was further depleting activation in OVA-T4-stimulated $CD8^+$ T cells. IL-2 replenishment has been shown to revert TGFβ-mediated suppression of T cell proliferation (Brownlie et al., 2017), whilst other studies confirm that the expression of GrB and IFNγ is not

recovered in IL-2 replete conditions (Thomas and Massagué, 2005), further reinforcing the hypothesis that only some effects are directly mediated by TGFβ signalling. My data corroborates that the addition of IL-2 does not prevent TGFβ-mediated repression of GrB or CD25 expression. This data indicates, therefore, that IL-2 depletion is not responsible for the TGFβ-mediated inhibition of CD25, but whether other mechanisms might be involved is still unknown.

So far, I have discussed and concluded that TGFβ particularly represses $CD8^+$ T cell responses against low affinity peptides *in vitro*. But, what are the likely consequences *in vivo*? Many reports that identified a TGFβ-mediated decrease in anti-tumour $CD8^+$ T cell responses utilised transplanted tumour models (Gorelik and Flavell, 2001; Thomas and Massagué, 2005) or models of spontaneous cancer (Donkor et al., 2011) that determine responses to well-characterised peptides with high T cell reactivity. Nonetheless, any study has directly compared responses to strong vs weak agonists in the context of TGFβ-mediated inhibition and, therefore, there is still a lack of evidence regarding to the existence of different magnitudes of T cell suppression by TGFβ *in vivo*. Future experiments could include, for example, the assessment of T cell responses against OVA-N4 vs OVA-T4-expressing tumours *in vivo* in the presence or absence of a TGFβ neutralising agent (e.g. a TGFβR blocker). Albeit not directly testing T cell responses to different affinity peptides, Brownlie et al. (2017) showed that *Ptpn22*$^{-/-}$ T cells, which exhibit an improved recognition of weak agonists and increased TCR-signalling strength, counteracted TGFβ inhibition during early stages of T cell activation *in vitro* resulting in enhanced tumour rejection upon ACT *in vivo*. This findings suggest that my *in vitro* observations are likely to translate into similar outcomes *in vivo*, where $CD8^+$ T cells would

respond even more poorly to low affinity peptides in the presence of TGFβ leading to very deficient, or even null, T cell responses.

Why is the previous statement particularly relevant in the field of cancer immunotherapy? If TGFβ represses T cell function more significantly against weak peptides, it is likely that TGFβ inhibits anti-tumour responses to low affinity TAAs to a greater extent than to high affinity neoantigens. Under this premise, it is reasonable to think that anti-TGFβ therapies would be particularly beneficial against aberrantly expressed self-TAAs. A current weakness of ICB therapies is that successful outcomes are low unless tumours are highly immunogenic (known as 'hot' tumours), a feature usually observed when tumour specific antigens (TSAs, neoantigens) are expressed (Lechner et al., 2013). Thus, it has been proposed that TGFβ neutralisation could potentially enhance anti-tumour responses against TAAs, transforming 'cold' tumours into 'hot' tumours and, ergo, promoting better anti-tumour responses when combined with ICB therapies. Accordingly, many investigations have demonstrated that the combination of ICB and anti-TGFβ therapies significantly reduces tumour burden when compared to ICB treatment alone (Terabe et al., 2017; Mariathasan et al., 2018; Tauriello et al., 2018; Lan et al., 2018; Ravi et al., 2018).

A key and novel contribution of this chapter is the characterisation of the transcriptomic profile of activated $CD8^+$ T cells in the presence or absence of TGFβ. Thomas and Massagué (2005) previously performed a gene micro array comparing the transcriptome of TGFβ treated and untreated $CD8^+$ T cells. However, the identified transcripts were restricted to a defined gene library and, therefore, the analysis was somewhat limited, with only 104 identified genes with a FC > 2. Conversely, my RNA-seq data has provided a much broader

understanding on how TGFβ reshapes the transcriptome of $CD8^+$ T cells (1553 genes FC > 2). This has allowed me to perform KEGG analysis to distinguish key pathways affected by TGFβ. In addition to the well-stablished repression of the cytotoxic program (*Gzmb, Prf1, Fasl*) (Thomas and Massagué, 2005), I have identified substantial changes in the translational machinery and, particularly, in metabolic pathways. Thus, I have found additional possible mechanisms by which TGFβ might be interfering with the activation of $CD8^+$ T lymphocytes, a key finding to better understand the intricacies of TGFβ activity. The impact of TGFβ on the metabolic reprogramming of $CD8^+$ T cells will be further explored in Chapter 5.

Defining the transcriptome of TGFβ-treated T cells is a powerful strategy to understand global changes in gene expression, but it is important to considerate that transcript levels not always correlate with protein levels (Plotkin, 2010; Vogel and Marcotte, 2012). Hence, the interpretation of the RNA-seq data should be taken with caution and, although is valuable to guide us and predict changes, those should always be confirmed at the protein level. Furthermore, the RNA-seq data has the limitation of not including an unstimulated negative control (T=0h). In this chapter and the following, I have focused on the validation genes of interest for this thesis (e.g., activation markers, effector molecules, cytokines or metabolic proteins), which were mostly downregulated, but the RNA-seq data revealed that a significant cluster of genes is also substantially upregulated. Nonetheless, due to the lack of T=0h negative control, whether TGFβ induces the expression of these genes or whether it prevents its downregulation upon TCR-stimulation is unclear. Further exploring these genes at a protein level in the presence of an

unstimulated negative control is required to better understand the mechanisms of action of TGFβ.

$CD8^+$ T cells can respond to TGFβ stimulation engaging an inhibitory (T_{reg}) or an IL-17 or IL-9-producing phenotype (known as T_c17 and T_c9 cells, respectively) (St. Paul and Ohashi, 2020). Thus, we could argue that the scarcity of a cytotoxic phenotype is due to an alternative T cell differentiation. However, there is no strong evidence indicating that this could be the case in my TGFβ-treated T cells. For example, in the context of T_{regs}, the RNA-seq data shows that there is no *Foxp3* or *Il10* expression. Similarly, there is no pattern suggesting a T_c9 phenotype. Although some T_c17 classic genes are moderately upregulated by TGFβ, such as *Il17f* or *Rorc,* these are expressed at very low levels, particularly when compared to classic T_c1 genes (e.g. *Ifng*, *Tnf* or *Tbx21*). Furthermore, it is important to note that these alternative phenotypes are acquired in combination of TGFβ with other cytokines (St. Paul and Ohashi, 2020). Considering that those were missing in the *in vitro* conditions utilised here, it is unlikely that a $T_{reg}/T_c9/T_c17$ phenotype would be optimally induced by TGFβ alone. Nonetheless, we cannot exclude that this could occur *in vivo* and, therefore, it should be taken into consideration in future studies.

An important discrepancy observed in the RNA-seq data when compared to previous investigations, is the lack of *Ifng* repression. It is well-stablished that *Ifng* is a direct target of SMADs (Thomas and Massagué, 2005) so it is surprising that I do not observe any significant changes upon TGFβ treatment. However, *Ifng* levels are very low within the first 24h upon TCR-stimulation, particularly with low affinity stimulations and, therefore, it is more likely for changes to be more apparent at later time-points, when substantial amounts of IFNγ are being

produced. Alternatively, it is important to note that SMADs often regulate transcription in association with another co-factors, a feature that allows TGFβ to respond differently depending on the cell type and context (Massagué, 2012; David and Massagué, 2018; Batlle and Massagué, 2019). For example, it has been described that *Ifng* is repressed by a complex formed by SMAD2/3/4 and AFT1, whilst *Gzmb* regulation requires an additional CREB binding (Thomas and Massagué, 2005). It is therefore possible that the *in vitro* approach utilised here is missing a signal, perhaps CD28, relevant for the specific inhibition of *Ifng*. Albeit the absence of transcriptional repression, I have shown that IFNγ levels are diminished after 48h of T cell activation suggesting that (1) transcriptional changes appear at later-timepoints or (2) TGFβ also regulates IFNγ expression post-transcriptionally.

Finally, the study of the effect of TGFβ on fully differentiated CTLs has shed light into some surprising findings. Although TGFβ repressed IFNγ production by CTLs, I have found that the presence of TGFβ did not dampen their killing capacity. However, we have to be cautious with the interpretation of these results. CTLs generated *in vitro* contain pre-formed cytotoxic granules that could be secreted at the moment of encountering the target cells, regardless of the presence of TGFβ. Thus, although I can confirm that TGFβ does not prevent the immediate killing of target cells, it is likely that a delayed inhibitory effect, when effector molecules require to be re-synthesised, could occur. An alternative explanation to my results is that the deficient cytotoxic capacity observed in *in vivo* studies (Gorelik and Flavell, 2001; Thomas and Massagué, 2005; Donkor et al., 2011) is (1) only present when experiencing TGFβ signalling during early stages of activation or (2) not caused by a direct effect of TGFβ on CTL

cytotoxicity but rather by a loss of cytokine secretion or through its influence in other immune cells (Fridlender et al., 2009; Flavell et al., 2010; Tu et al., 2014). My results confirming SMAD2 phosphorylation and IFNγ repression in CTLs exclude the loss of responsiveness to TGFβ as a possible explanation.

In summary, I have clearly established that TGFβ directly interferes with the activation of $CD8^+$ T lymphocytes, particularly against weak agonists. Furthermore, the characterisation of the transcriptome upon TGFβ-treatment has revealed some possible underlying mechanisms. In the next chapter, I investigate in detail the major event altered by TGFβ: the engagement of the metabolic reprogramming.

Chapter 5 The influence of TGFβ on CD8$^+$ T cell metabolic reprogramming

5.1 Introduction

In the previous chapter, I described the suppressive consequences of TGFβ on the functionality of naïve and effector CD8$^+$ T cells after TCR stimulation. Furthermore, the RNA-seq analysis provided new insight into the mechanisms of action of TGFβ in CD8$^+$ T lymphocytes. Amongst the major hallmarks of those CD8$^+$ T cells primed in the presence of TGFβ I found: (1) the repression of translation, (2) the inhibition of the cytotoxic program and, particularly, (3) the alteration of metabolism.

Our current knowledge on TGFβ and its modulation of immune cell metabolism is limited. Studies in NK cells have reported that TGFβ inhibits their response to IL-15 through repression of mTOR activity and bioenergetic metabolism (Viel et al., 2016). Moreover, Zaiatz-Bittencourt et al. (2018) later described also in NK cells that TGFβ prevents IL-2-induced metabolic reprogramming by suppressing OXPHOS, glycolytic capacity and upregulation of the transferrin receptor CD71. Dimeloe et al. (2019) defined that, in CD4$^+$ T cells, Smad proteins are able to directly interact with the mitochondria leading to a reduced respiratory capacity and IFNγ secretion. However, how TGFβ shapes CD8$^+$ T cell metabolism is less well described. In this chapter, in order to further understand how TGFβ induces CD8$^+$ T cell dysfunction, I aimed to investigate the influence of TGFβ on the metabolic reprogramming of activated CD8$^+$ T cells.

5.2 Results

5.2.1 TGFβ inhibits T cell activation by repressing the Myc-induced transcriptional program

Chromatin immunoprecipitation (ChIP) Enrichment Analysis (ChEA) is a gene-set database that compiles the targets of 199 transcription factors based on ChIP-X data (Lachmann et al., 2010). I utilised this tool to identify key transcriptional programs that could be modulated by TGFβ. ChEA analysis of the genes downregulated by TGFβ (FC > 1.5, adjusted *p*-value < 0.05) identified a significant overlap with the Myc-induced transcriptional program (Table 5.1). This finding suggested, therefore, that much of the TGFβ-mediated T cell dysfunction might be caused by the repression of Myc expression.

Index	ChEA identifier	*p*-value
1	MYC 18555785 ChIP-Seq MESCs Mouse	7.262×10^{-51}
2	MYC 19030024 ChIP-ChIP MESCs Mouse	8.264×10^{-49}
3	MYC 18358816 ChIP-ChIP MESCs Mouse	1.937×10^{-46}
4	EKLF 21900194 ChIP-Seq ERYTHROCYTE Mouse	1.863×10^{-39}
5	MYC 19079543 ChIP-ChIP MESCs Mouse	4.787×10^{-17}

Table 5.1: ChEA analysis of downregulated genes by TGFβ.

In fact, as mentioned in the previous chapter, TGFβ strongly inhibited *Myc* expression based on my RNA-seq data (FC = 2.77; Fig. 5.1A). This was also observed at the protein level. I stimulated OT-I T cells with OVA-p for 48h and observed that, as assessed by flow cytometry, Myc expression was significantly reduced in the presence of TGFβ, irrespective of the strength of the TCR-stimulation (Fig. 5.1B).

As Myc stabilisation is highly dependent on IL-2 levels (Preston et al., 2015) I questioned whether TGFβ-induced suppression of IL-2 (see Fig. 4.3A) had an

important role in Myc decline. Thus, OT-I cells were stimulated with OVA-p ± TGFβ in the presence of exogenous IL-2 (1ng/ml). Data indicated that IL-2 replenishment had no effect on the levels of Myc expression in T cells stimulated with OVA-N4 and TGFβ, whereas it was slightly but not significantly increased in OVA-T4 stimulated T cells (Fig. 5.1C). To further reinforce these results, I delayed treating OT-I cells with TGFβ until after 24h from TCR-priming, i.e., after the IL-2 secretion peak. Again, I saw that, even when added at 24h, TGFβ still suppressed Myc expression although in a lesser extent as compared to TGFβ-treated T cells from timepoint 0h (Fig. 5.1D). Altogether, this data implied that TGFβ dampened Myc expression and its subsequent transcriptional program through IL-2 independent mechanisms.

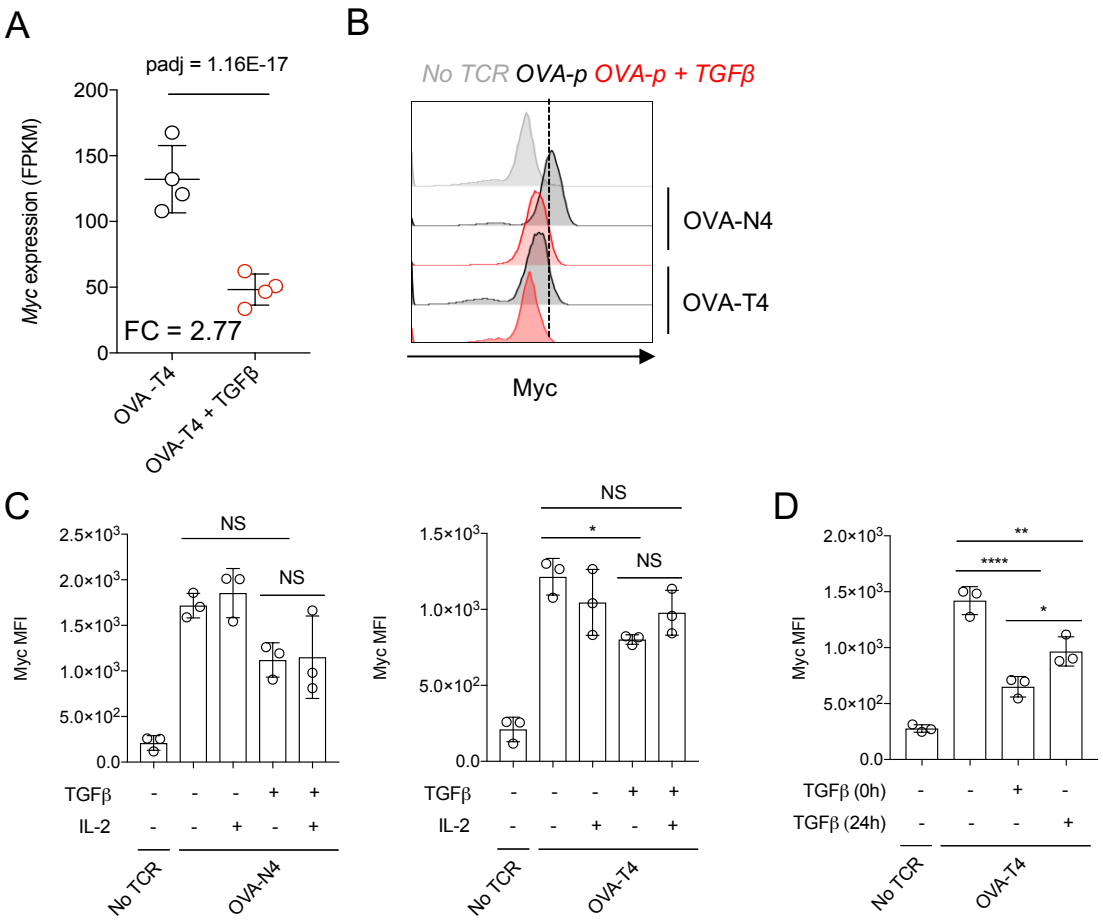


Figure 5.1: TGFβ represses Myc expression in activated CD8⁺ T cells.

OT-I T lymphocytes were stimulated *in vitro* for 24h (A) or 48h (B, C, D) with OVA-p (10^{-8}M) ± TGFβ. Unstimulated T cells (No TCR) were cultured in the presence of IL-7 and used as negative controls. Where indicated, hIL-2 (1ng/ml) was added. Myc mRNA (A) or protein (B, C, D) levels were acquired by RNA-seq or FACS analysis, respectively. In (A), dots represent biological replicates. In (B, C, D), dots represent technical triplicates. Data is from one of at least two repeated experiments. NS – Not significant; * - P<0.05; ** - P<0.005; **** - P<0.0001, as determined by one-way ANOVA with Tukey's multiple comparisons test.

5.2.2 TGFβ suppresses the glycolytic metabolism of activated CD8⁺ T cells

The engagement of aerobic glycolysis is one of the key events of the TCR-induced metabolic reprogramming. Glycolysis is mainly regulated in early activated T cells by upregulation of Myc and HIF-1α, both of which are suppressed by TGFβ (Fig. 5.1 and 4.12). Thus, I hypothesised that T cells would inefficiently promote glycolysis after TCR-stimulation in the presence of TGFβ.

First, I used the RNA-seq data to look into the mRNA levels of the glycolytic enzymes, as well as glucose and lactate transporters. There was a modest decrease in mRNA expression of almost all glycolytic enzymes, except *Hk1* and *Aldoc,* which in fact displayed a mild but significant upregulation, and *Ldhb* (Fig. 5.2). *Gapdh* was strongly repressed in 3 out of 4 biological replicates but the lack of consistency concluded in non-significant changes (Fig. 5.2).

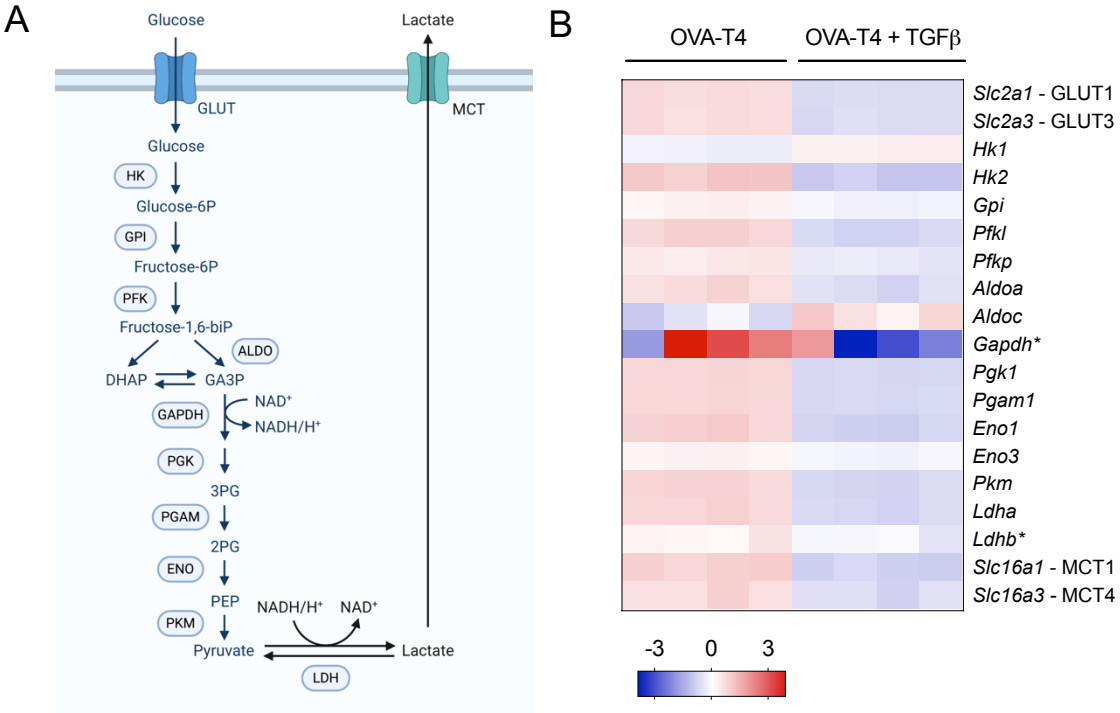


Figure 5.2: TGFβ downregulates the expression of glycolysis associated genes.

(A) Schematic representation of aerobic glycolysis. (B) mRNA expression of glycolytic enzymes upon 24h of TCR-stimulation with OVA-T4 (10^{-8}M) ± TGFβ, as assessed by RNA-seq (n=4). Values represent $\log_2$(FC). Non-differentially expressed genes are indicated with (*).

The glucose transporters GLUT1 and GLUT3, encoded by *Slc2a1* and *Slc2a3* respectively, were significantly downregulated (Fig. 5.2B and 5.3A). I confirmed that GLUT1 was also diminished by TGFβ at the protein level after 48h of TCR-

stimulation (Fig. 5.3B). These phenotypes were associated with decreased uptake of the glucose analogue 2-NBDG (Fig. 5.3C).

TGFβ also prevented the upregulation of *Ldha*, the enzyme that ultimately converts pyruvate into lactate (Fig. 5.2 and 5.3D). To determine the magnitude of the consequences of *Ldha* downregulation, together with the decreased glucose uptake and expression of other glycolytic enzymes, I tested the glycolytic capacity of T cells based on their ability to modify the extracellular pH. Using the Seahorse XFe96 Analyser I saw that T lymphocytes stimulated in the presence of TGFβ had an attenuated ECAR increase in response to glucose administration (Fig. 5.3E). This phenomenon was observed in both OVA-N4 and OVA-T4 stimulated T cells, indicating that TGFβ intervenes with glycolysis regardless of the strength of the initial TCR-stimulation (Fig. 5.3E). Importantly, a decrease was also observed in the expression of the lactate transporter *Slc16a1* (Fig. 5.2B) which functions as a rate limiting step for the glycolytic flux due to the negative feedback provoked by lactate accumulation (Tanner et al., 2018).

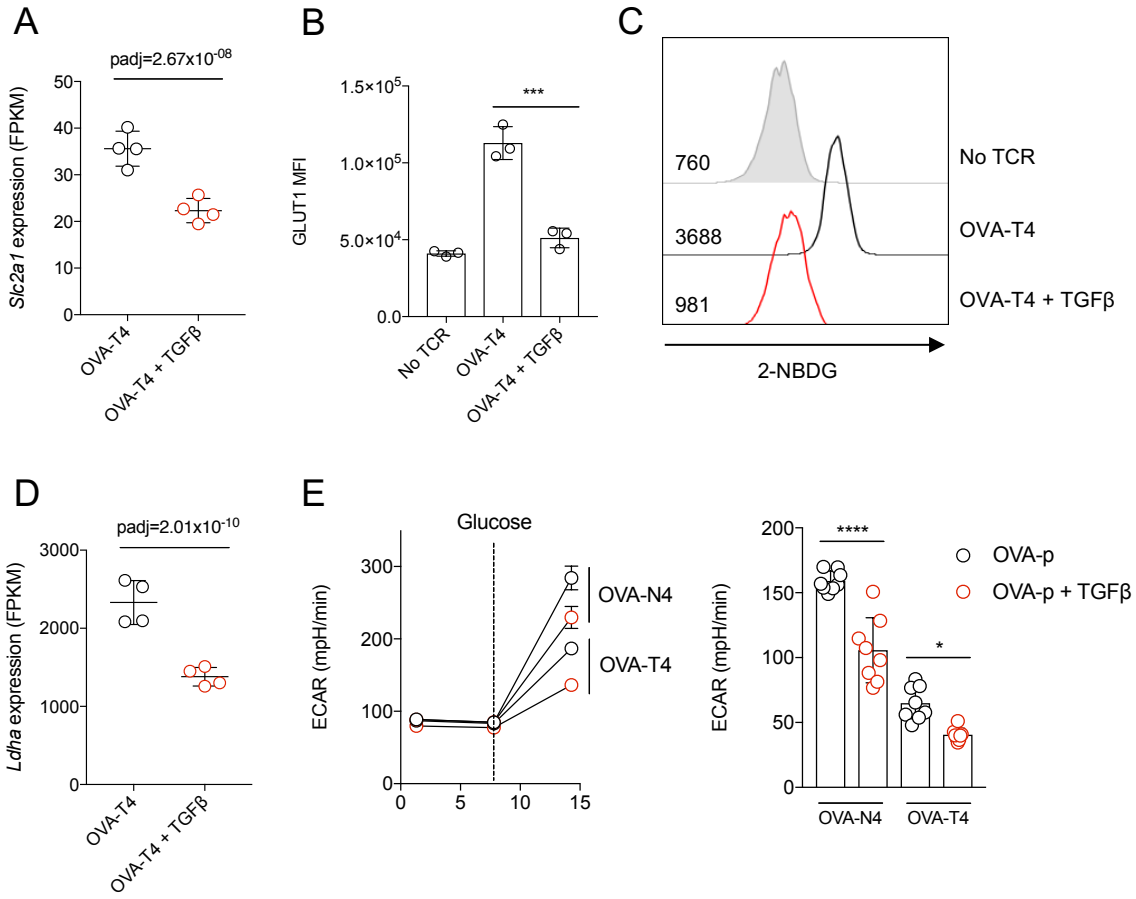


Figure 5.3: TGFβ treated T lymphocytes display an impaired glycolytic capacity.

OT-I T lymphocytes were stimulated *in vitro* for 24h (A, D) or 48h (B, C, E) with OVA-p (10^{-8}M) ± TGFβ. Unstimulated T cells (No TCR) were cultured in the presence of IL-7 and used as negative controls. (A, D) *Slc2a1* and *Ldha* expression was assessed by RNA-seq. (B) GLUT1 protein levels were assessed by intracellular staining followed by FACS analysis. (C) 2-NBDG uptake was assessed by FACS analysis upon incubation. (D) ECAR values were acquired using the Seahorse XFe96 analyser. Bar graph (right), represents ECAR values after glucose injection. In (A, D), dots represent biological replicates. In (B, E), dots represent technical triplicates. Data is from one of at least three repeated experiments. * - P<0.05; *** - P<0.0005; **** - P<0.0001, as determined by one-way ANOVA with Tukey's multiple comparisons test.

5.2.3 TGFβ alters amino acid metabolism of activated CD8$^+$ T lymphocytes

Myc-induced metabolic reprogramming has been strongly associated with its ability to induce AA uptake, particularly via upregulation of LAT1 expression (i.e. Slc7a5) (Wang, R. et al., 2011; Marchingo et al., 2020). Thus, my next step consisted of testing the effects of TGFβ on AA transport. Using my RNA-seq data, I looked into the expression of genes encoding for some of the main AA transporters in T cells including ASCT2 (*Slc1a5*), the sodium-independent cationic AA transporters (CAT)1 and 3 (*Slc7a1* and *Slc7a3*), CD98 (*Slc3a2*), LAT1 (*Slc7a5*), y$^+$LAT2 (*Slc7a6*) and the sodium-coupled AA transporters (SNAT)1 and 2 (*Slc38a1* and *Slc38a2*). All of them, except *Slc38a2*, were substantially downregulated (Fig. 5.4B). In Fig. 5.4A, a schematic representation of some key T cell AA transporters and their substrates, as reviewed by Wang, W. and Zou (2020), is shown.

In order to link the transcriptional changes to functional outcomes, I focussed on the activity of Slc7a5, of which expression was reduced almost by half (Fig. 5.4C). First, I measured the protein levels of CD98, which binds to Slc7a5 to form LAT1 as well as to Slc7a6 to form y$^+$LAT2 (Fig. 5.4A). Consistent with mRNA levels, CD98 was diminished by TGFβ (Fig. 5.4D). Furthermore, I directly assessed the functionality of the Slc7a5/CD98 heterodimer based on the uptake of kynurenine, a tryptophan metabolite with self-fluorescent properties that enters the cell using this specific transporter (Sinclair et al., 2018) (Fig. 5.4A). As expected, kynurenine uptake and, therefore, Slc7a5-mediated AA uptake, was significantly suppressed in the presence of TGFβ after 48h of TCR-stimulation (Fig. 5.4E).

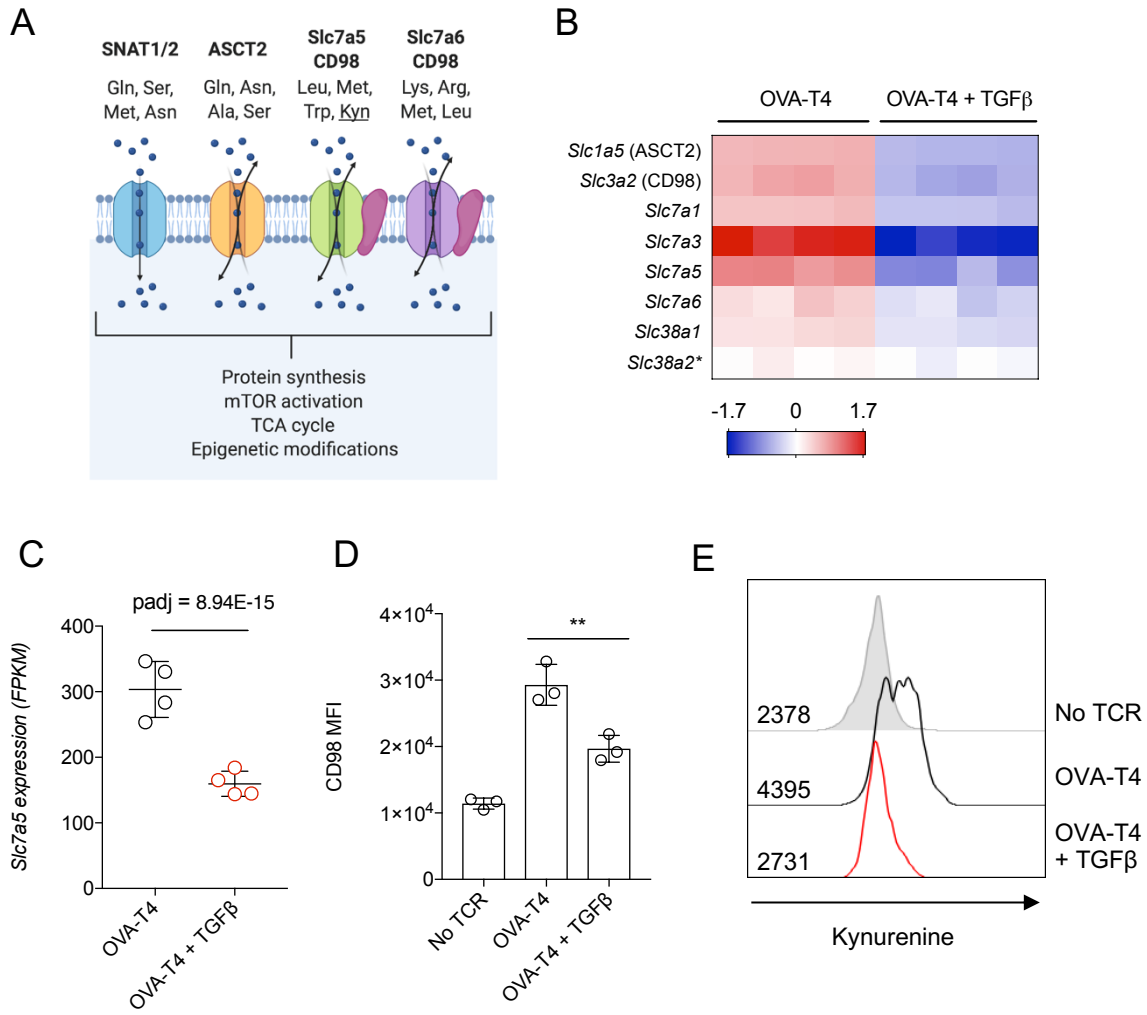


Figure 5.4: TGFβ disrupts upregulation of amino acid transporters and uptake of AAs into activated CD8⁺ T cells.

(A) Schematic representation of main amino acid transporters and their functions in activated T lymphocytes. (B, C, D, E) OT-I T lymphocytes were stimulated *in vitro* for 24h (B, C) or 48h (D, E) with OVA-p (10^{-8}M) ± TGFβ. Unstimulated T cells (No TCR) were cultured in the presence of IL-7 and used as negative controls. mRNA levels (B, C) are from RNA-seq data (n=4). In (B), values represent log_2(FC) and non-differentially expressed genes are indicated with (*). In (D), CD98 expression was assessed by FACS analysis. In (E), kynurenine uptake was assessed by FACS analysis upon incubation. Values in histogram represent geometric MFI. Dots represent biological (C) or technical (D) replicates. In (D, E), data is from one of at least three repeated experiments. ** - P<0.005, as determined by one-way ANOVA with Tukey's multiple comparisons test.

I next focused on the study of glutamine (Gln), the most consumed AA in activated T cells. In T cells, Gln uptake is mostly mediated by ASCT2, whilst TCR-induced ASCT2 expression is repressed both at a transcriptional (Fig. 5.4B) and

protein level by TGFβ, although only significantly when primed with OVA-T4 (Fig. 5.5B). Then, I further explored how TGFβ affected the expression of enzymes involved in the multiple pathways by which Gln is utilised upon TCR-stimulation. RNA-seq data showed that enzymes involved in the conversion of Gln into intermediaries of the hexosamine pathway (*Gfpt1*) were not affected, whilst those associated with nucleotide synthesis (*Pfas, Ppat, Cad*) were significantly downregulated by TGFβ (Fig. 5.5C). Furthermore, the synthesis of Asn mediated by ASNS, key to act as an exchange factor for the uptake of essential AAs (EAAs) in transformed cells (Krall et al., 2016) and potentially in T lymphocytes (Hope et al., 2020), was severely impaired (Fig. 5.5C). These EAAs exchanged for Asn support mTOR activation and prevent the expression of activation transcription factor 4 (ATF4), an stress-induced transcription factor regulated by nutrient deprivation (Yang et al., 2018). However, *Atf4* was not induced, but instead repressed, in the presence of TGFβ. Importantly, the expression of glutaminolysis-associated genes (*Gls1, Gls2, Got1, Got2*), the pathway that fuels the TCA cycle in the absence of glycolysis-derived pyruvate, was also diminished in TGFβ-treated T cells (Fig. 5.5C). Glutamine-derived glutamate also acts as a substrate for glutathione (GSH) synthesis, but no alterations were shown in the enzymes that mediate this process (Fig. 5.5C). Altogether, this findings suggest that TGFβ limits glutamine uptake and its utilisation to a great extent upon TCR-stimulation.

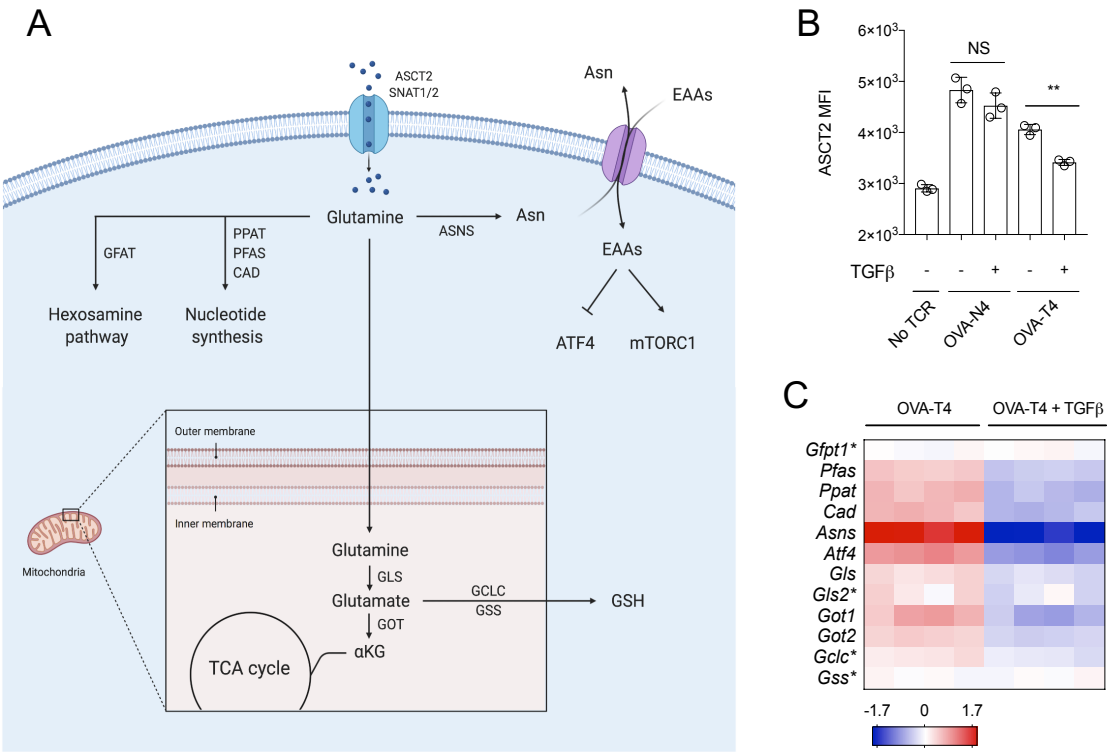


Figure 5.5: TGFβ impairs glutamine metabolism upon TCR-stimulation.

(A) Schematic representation of glutamine fates in activated T lymphocytes. (B, C). OT-I T lymphocytes were stimulated *in vitro* for 24h (B, C) or 48h (D, E) with OVA-p (10^{-8}M) ± TGFβ. Unstimulated T cells (No TCR) were cultured in the presence of IL-7 and used as negative controls. In (B), ASCT2 expression was determined by FACS analysis. In (C), mRNA levels are from RNA-seq data (n=4). Values represent log_2(FC) and non-differentially expressed genes are indicated with (*). Dots represent technical replicates. Data is from one of three repeated experiments. NS – Not significant; ** - P<0.005, as determined by one-way ANOVA with Tukey's multiple comparisons test.

5.2.4 TGFβ restricts protein synthesis during CD8⁺ T cell activation

Amino acid uptake is crucial to permit the high rates of protein synthesis required during T cell activation. The previous findings (Fig. 5.4 and 5.5), added to the fact that some of the most affected pathways by TGFβ were associated with ribosome biogenesis and RNA transport (see Section 4.2.5), led to the hypothesis that TGFβ could interfere with T cell activation through translational repression. Thus, I tested the ability of T cells to synthesise new proteins in the presence or absence of TGFβ. As assessed by OPP incorporation, I observed that, after 48h of TCR-

stimulation with OVA-N4, TGFβ did not limit protein synthesis (Fig. 5.6). Nonetheless, OPP-incorporation in T cells stimulated with OVA-T4 was suppressed, suggesting that TGFβ impedes T cell activation against weak agonists, at least in part, by limiting the translational program (Fig. 5.6).

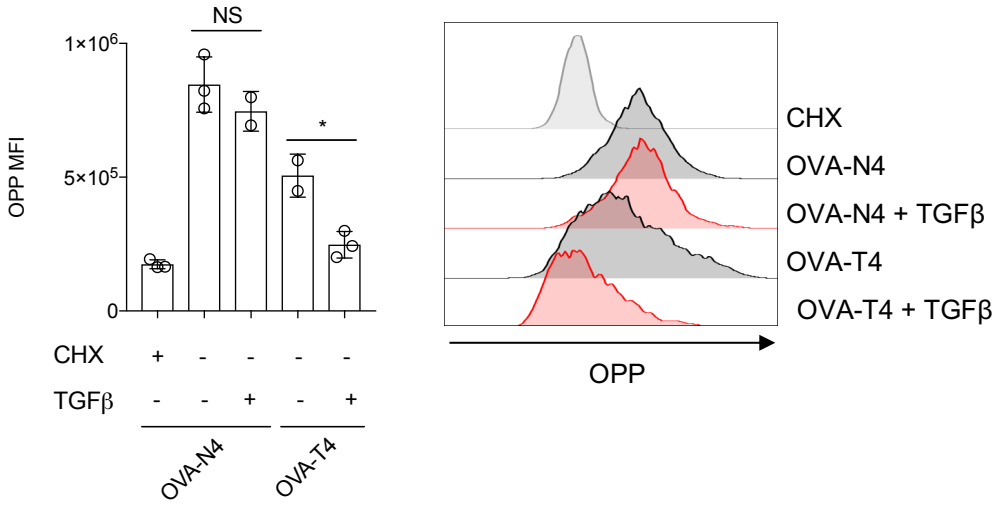


Figure 5.6: TGFβ inhibits protein synthesis in CD8⁺ T cells.

OT-I T lymphocytes were stimulated *in vitro* with OVA-p (10^{-8}M) ± TGFβ. Cycloheximide (CHX) was used as negative control. After 48h of TCR-stimulation, protein synthesis was assessed by OPP incorporation, determined by FACS analysis. Dots represent technical replicates. Data is from one of three repeated experiments. NS – Not significant; * - P<0.05, as determined by one-way ANOVA with Tukey's multiple comparisons test.

5.2.5 TGFβ does not affect mTORC1 activity in CD8⁺ T lymphocytes

Another key function of AAs is to maintain mTORC1 activity (Kelly and Pearce, 2020; Wang, W. and Zou, 2020). Furthermore, recent investigations have reported that mTORC1 also senses the glycolytic metabolite DHAP to be active (Orozco et al., 2020). Also, previous research has stablished that TGFβ suppresses mTOR activation in NK cells (Viel et al., 2016). Hence, I wondered whether defective AA and glucose uptake caused upon TGFβ exposure (Fig. 5.3C and 5.4E) would lead to a diminished mTORC1 activity. To address this question I stimulated OT-I T cells with OVA-p ± TGFβ and measured mTORC1

activity based on the phosphorylation of one of its substrates, the ribosomal protein S6. After 24h, I unexpectedly observed that in either OVA-N4 or OVA-T4-stimulated T cells TGFβ did not restrict S6 phosphorylation (Fig. 5.7A) indicating, therefore, that TGFβ does not affect mTORC1 activity, at least, within the first 24h of T cell activation. Consistently, the expression of *Mtor, Rptor, Mlst8, Akt1s1* and *Deptor*, components of the mTORC1 complex, was not altered by TGFβ (Fig. 5.7B).

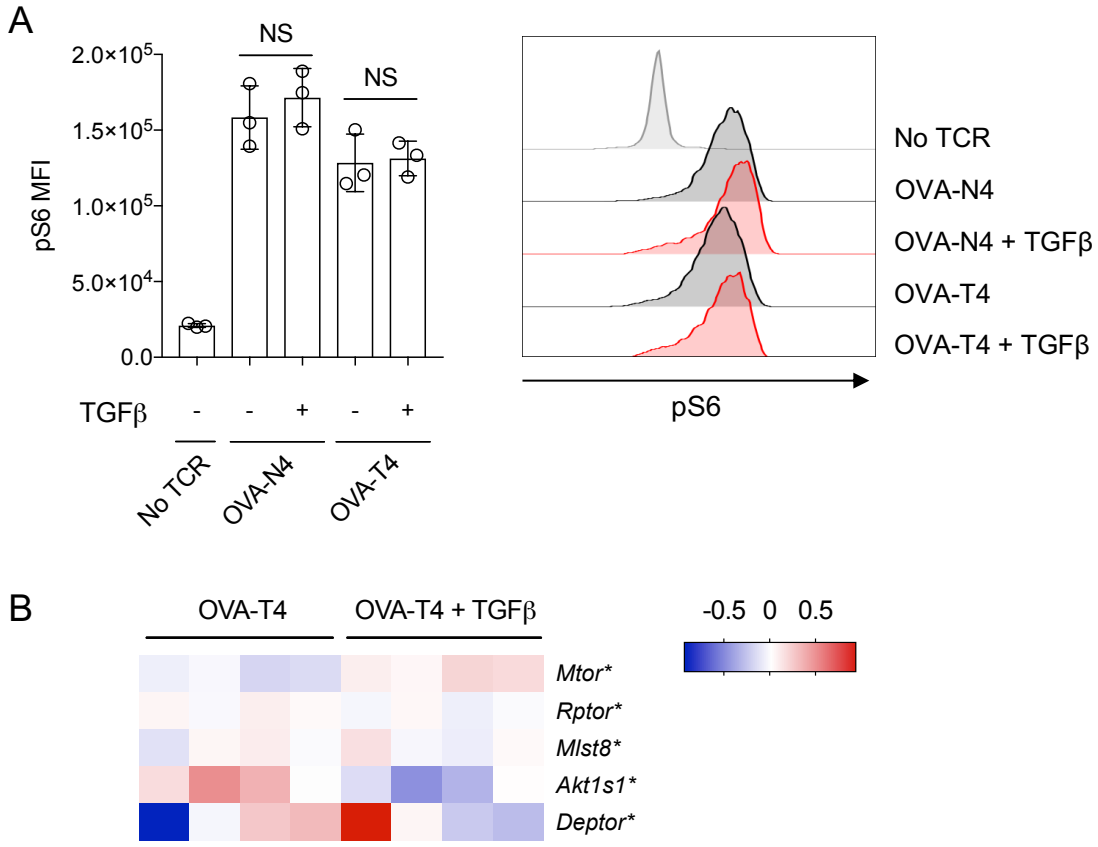


Figure 5.7: mTORC1 activity is not altered by TGFβ in CD8⁺ T cells.

OT-I T lymphocytes were stimulated *in vitro* with OVA-p (10^{-8}M) ± TGFβ for 24h. Unstimulated T cells (No TCR) were cultured in the presence of IL-7 and used as negative controls. After 24h of TCR-stimulation, phosphorylation of ribosomal protein S6 at sites Ser240/244 was quantified by intracellular staining and FACS analysis. In (B), mRNA expression was assessed by RNA-seq (n=4). Values represent $\log_2$(FC) and non-differentially expressed genes are indicated with (*). Dots represent technical replicates. Data is from one of three repeated experiments. NS – Not significant, as determined by one-way ANOVA with Tukey's multiple comparisons test.

5.2.6 TGFβ-treated CD8$^+$ T cells remodel mitochondrial metabolism

So far, I investigated how TGFβ affects the Myc/HIF1α-driven metabolism of glucose and AAs in activated CD8$^+$ T cells but, how was TGFβ modulating mitochondrial metabolism?

T lymphocytes promote mitochondrial biogenesis within the first 24h of TCR-stimulation in order to support the increased mitochondrial activity (Ron-Harel et al., 2016). Thus, I first analysed the expression of a panel of TFs that have been associated with the regulation of mitochondrial biogenesis including *Tfam, Ppargc1, Pprc1, Nrip1, Gabpb1, Nrf1* and *Esrra* (ERRα). I saw that two of them, *Tfam* and *Pprc1*, which encode for MTFA and the PGC-1-related co-activator respectively, were significantly downregulated by TGFβ. This data suggested, as previously reported in other immune cells (Zaiatz-Bittencourt et al., 2018; Dimeloe et al., 2019), that TGFβ could suppress CD8$^+$ T cell function through modulation of mitochondrial activity (Fig. 5.8).

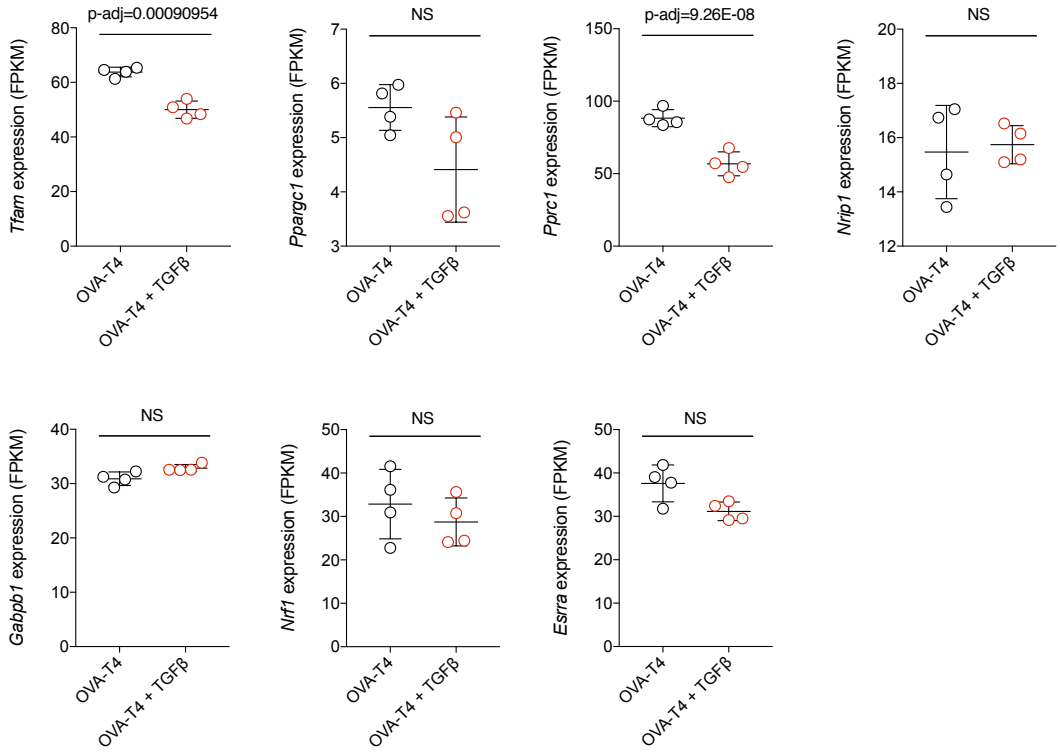


Figure 5.8: The expression of mitochondrial biogenesis related genes varies upon TGFβ treatment.

OT-I T lymphocytes were stimulated *in vitro* for 24h with OVA-p (10^{-8}M) ± TGFβ. mRNA expression was assessed by RNA-seq (n=4).

The effect of TGFβ on the mitochondria has been generally associated with an OXPHOS deficiency (Zaiatz-Bittencourt et al., 2018; Dimeloe et al., 2019). Taking this into account, I focused on the study of this particular mitochondrial function. To study the consequences of TGFβ treatment on OXPHOS activity I first looked at the expression of genes encoding for TCA cycle related enzymes, key to provide a high amount of the NADH and FADH2 utilised by the ETC to synthesise ATP (Fig. 5.9). As shown in Fig. 5.10, TGFβ generally reduces their expression. However, the inhibition was rather mild with only *Pck2*, an enzyme that converts oxaloacetate into PEP and, therefore, not directly a TCA cycle enzyme, showing a FC > 2. Similarly, the expression of the ETC complex subunits were slightly decreased by TGFβ (Fig. 5.10). However, this was not observed consistently. For

example, *mt-Co1*, *mt-Co2* and *mt-Co3*, which encode for the subunits of the cytochrome C oxidase (complex IV) were not repressed but rather increased in the presence of TGFβ (Fig. 5.10).

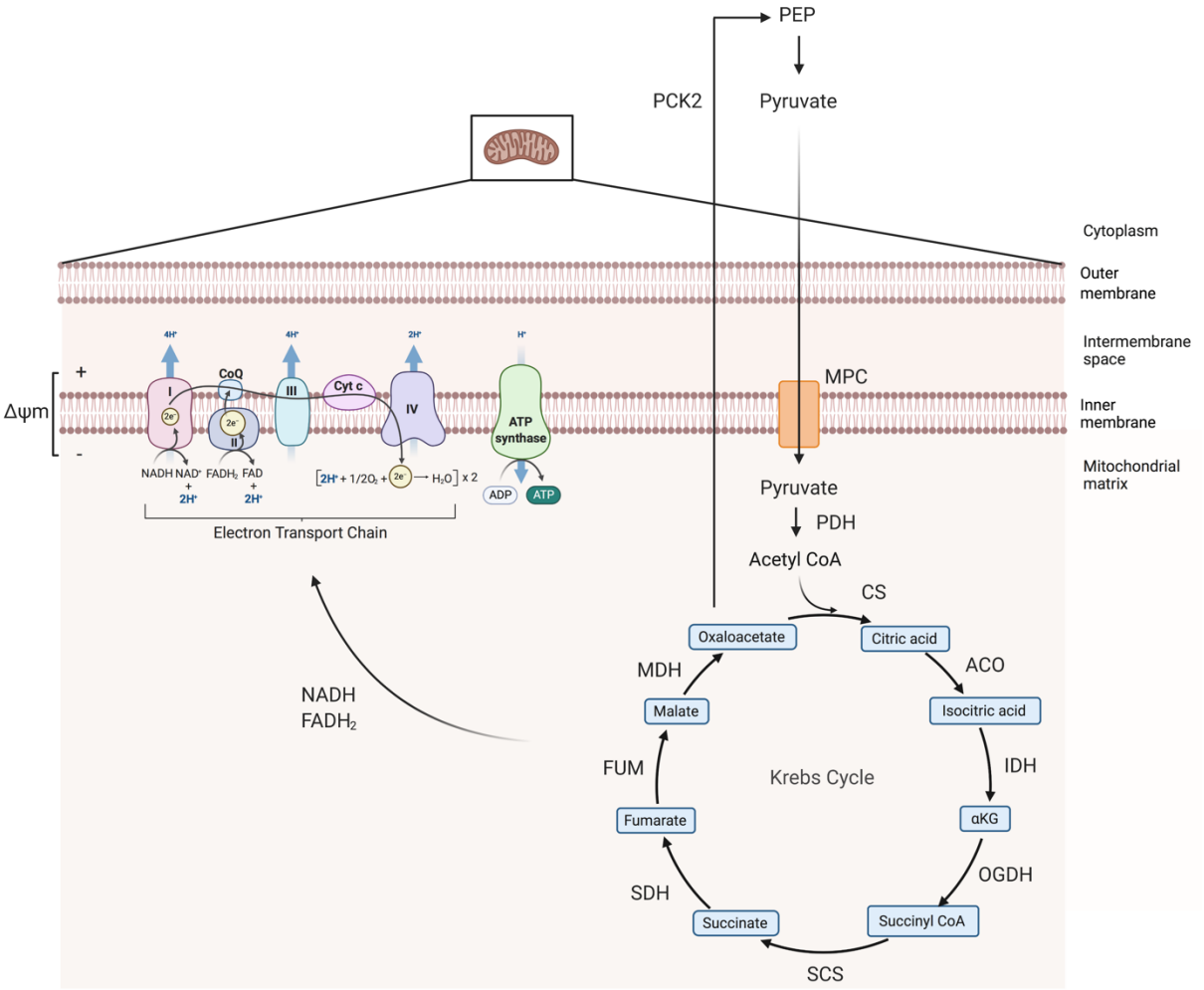


Figure 5.9: Schematic representation of the ATP-producing mitochondrial activity.

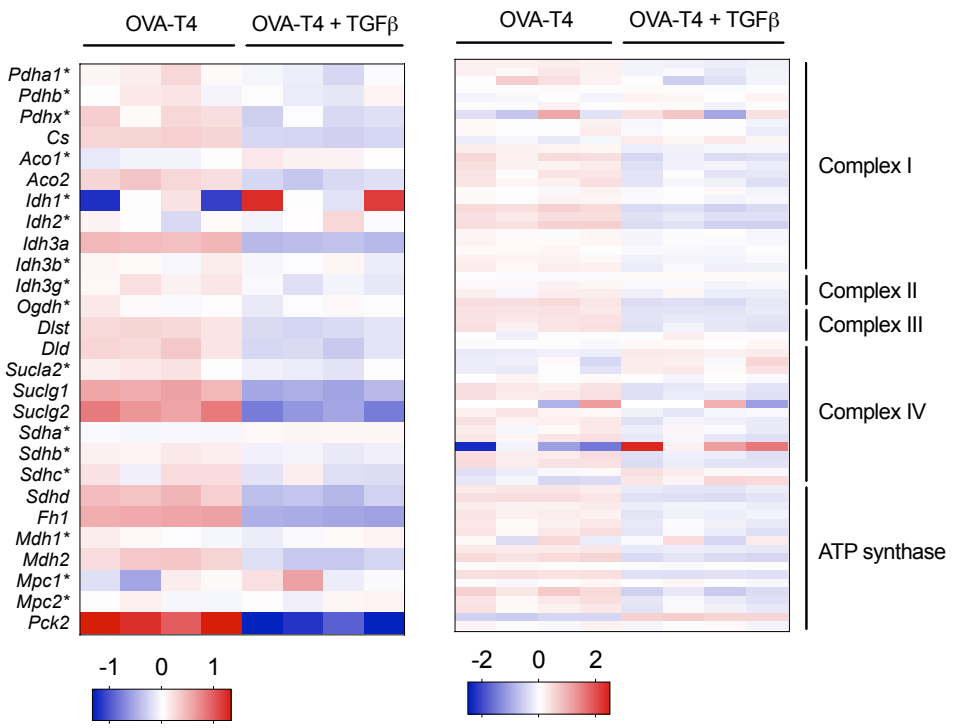


Figure 5.10: TGFβ mildly affects TCA cycle and ETC associated gene expression.

OT-I T lymphocytes were stimulated *in vitro* for 24h with OVA-p (10^{-8}M) ± TGFβ. mRNA expression was assessed by RNA-seq (n=4). Values represent $\log_2$(FC) and non-differentially expressed genes are indicated with (*).

Next, I investigated how the transcriptional repression of the TCA cycle and ETC, added to *Tfam* and *Pprc1* inhibition, translated to mitochondrial performance. Some key readouts of active ETC/OXPHOS include (1) ATP production mediated by the ATP synthase and (2) oxygen consumption mediated by the complex IV (Fig. 5.9). I hypothesised that, considering the transcriptomic data and the repression of mitochondrial metabolism in previous reports (Zaiatz-Bittencourt et al., 2018; Dimeloe et al., 2019), the oxygen consumption rate (OCR) and ATP synthesis would decline upon TGFβ treatment. However, I unexpectedly observed that TGFβ did not impair either ATP synthesis nor basal oxygen

consumption after 48h of TCR-stimulation, as assessed by a luminescent-based assay or using the Seahorse XFe96 analyser, respectively (Fig. 5.11A and B).

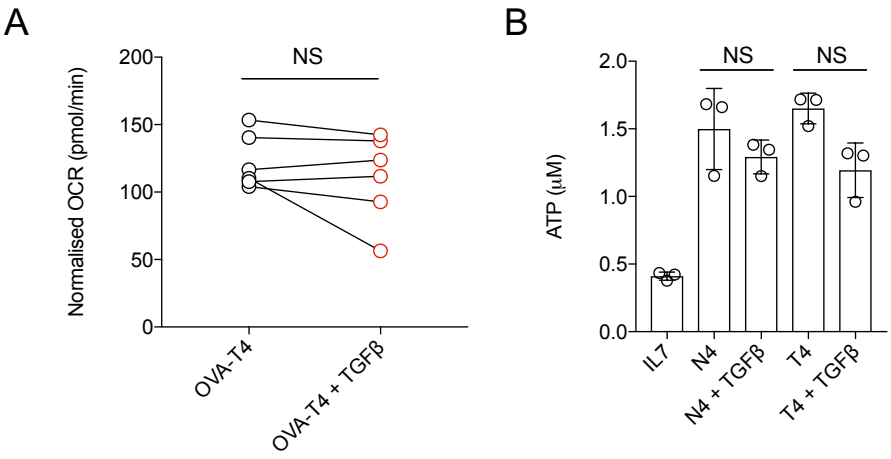


Figure 5.11: TGFβ does not alter ETC and OXPHOS activity of CD8⁺ T cells.

OT-I T lymphocytes were stimulated *in vitro* for 48h with OVA-p (10^{-8}M) ± TGFβ. Unstimulated T cells (No TCR) were cultured in the presence of IL-7 and used as negative controls. (A) Basal oxygen consumption rates (OCR) were determined using the Seahorse XFe96 analyser. (B) ATP levels were quantified using a luminescence-based assay. Dots represent biological (A) or technical (B) replicates. In (A), data is pooled from two independent experiments and in (B) data is from one of four repeated experiments. NS – Not significant, as determined by one-way ANOVA with Tukey's multiple comparisons test.

5.2.7 TGFβ alters lipid metabolism of activated CD8⁺ T lymphocytes

I have previously shown that TGFβ inhibited glucose and AA uptake (Fig. 5.3C and 5.4E). However, ATP production was not significantly affected by TGFβ and I wondered whether TGFβ-treated cells could use fatty acids as an alternative source to fuel energy production. Using the dye Bodipy-C16, I determined that fatty acid uptake was also diminished in TGFβ-treated T lymphocytes when stimulated with OVA-T4 during 48h, whilst no differences were observed in OVA-N4-stimulated T cells (Fig. 5.12A). To further study how fatty acids were utilised, I looked into the RNA-seq data to analyse the expression of enzymes that mediate β-oxidation (*Acadm, Echs1, Hadh, Acaa2*) but no significant changes were seen upon TGFβ-treatment (Fig. 5.12B). However, *Cpt1* and, particularly,

Acsbg1 were significantly upregulated by TGFβ (Fig. 5.12B). Cpt1 mediates fatty acid translocation into the mitochondria, whereas the LCFA-CoA ligase ACBG1 activates LCFAs by binding them to Acyl-CoA which leads to the initiation of β-oxidation or the synthesis of more complex lipids. Whether *Cpt1* and *Acsbg1* upregulation results in a more efficient utilisation of LCFAs in T cells needs to be further explored.

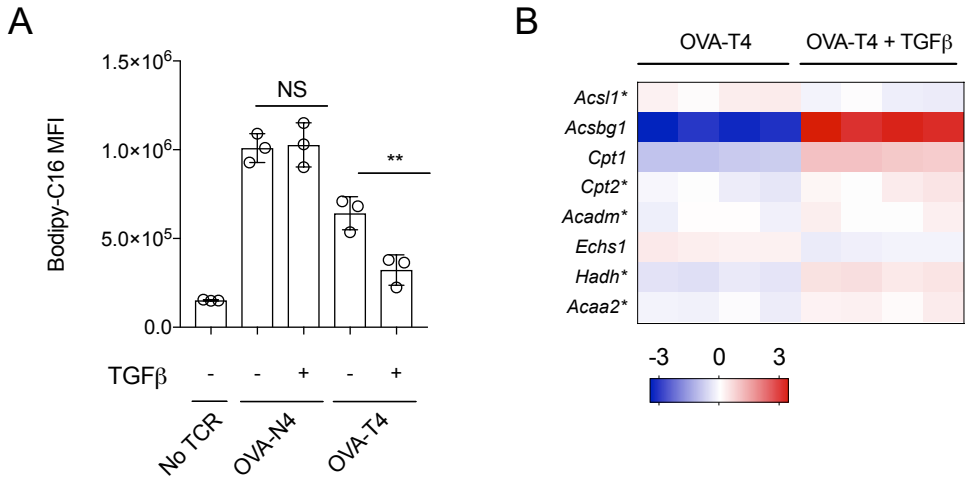


Figure 5.12: TGFβ modifies lipid metabolism in CD8⁺ T lymphocytes.

OT-I T lymphocytes were stimulated *in vitro* for 24h (B) or 48h (A) with OVA-p (10^{-8}M) ± TGFβ. Unstimulated T cells (No TCR) were cultured in the presence of IL-7 and used as negative controls. (A) Fatty acid uptake was assessed using Bodipy-C16 followed by FACS staining. (B) mRNA expression was assessed by RNA-seq (n=4). Values represent $\log_2$(FC) and non-differentially expressed genes are indicated with (*). In (A), dots represent technical replicates. Data is from one of three repeated experiments. NS – Not significant; ** - P<0.005, as determined by one-way ANOVA with Tukey's multiple comparisons test.

5.3 Discussion

In Chapter 4, I described that the metabolic transcriptome was severely affected by TGFβ during early stages of T cell activation. Here, I have shown that those transcriptomic modifications ultimately translate into a deficient TCR-induced metabolic reprogramming that leads to poor T cell activation.

First, the performed ChEA analysis unravelled that the Myc-driven transcriptional program, essential to initiate the metabolic reprogramming of T lymphocytes (Wang, R. et al., 2011; Marchingo et al., 2020), was one of the most altered by TGFβ. These findings suggested, therefore, that Myc could be a central target of TGFβ signalling to lead the suppression of T cell responses. In transformed cells, Myc repression has actually been described as a key event during the TGFβ cytostatic response (Zhang, Y. et al., 2017). In fact, it is well established that Smad3 works in conjunction with E2F4/5 and p107 to directly bind the *Myc* promoter (Chen, C.R. et al., 2002). In regards to T lymphocytes, Thomas and Massagué (2005) have previously shown that, consistent with my results, Myc expression is downregulated by TGFβ. However, the exact mechanisms and whether or not SMADs directly target *Myc* also in T lymphocytes remains to be elucidated.

As discussed in the previous chapter, I believe that those markers repressed by TGFβ irrespective of the strength of the TCR-stimulation are more likely to be direct SMAD targets, whilst those suppressed only against weak agonists are probably mediated by indirect mechanisms. Here, I have shown that Myc is inhibited in both OVA-N4 and OVA-T4 stimulated T cells suggesting therefore that Myc might be a SMAD target. However, it is important to consider that Myc expression is highly dependent on IL-2 levels (Preston et al., 2015) and, as shown

earlier (Fig. 4.3), TGFβ strongly inhibits IL-2 production also in both OVA-N4 and OVA-T4 stimulated T cells. Although I have shown that Myc repression also occurs regardless of IL-2 abundance, IL-2 replenishment had a modest effect on TGFβ-mediated inhibition of Myc (Fig. 5.1D). These findings suggest that, despite not being the unique mechanism by which TGFβ inhibits Myc, the lack of IL-2 supposes an additional obstacle to its upregulation and, therefore, whether the effects observed in Myc expression in both OVA-N4 and OVA-T4 stimulations are due to SMAD-direct or other indirect mechanisms is still uncertain.

The relatively modest inhibition of Myc expression as compared to granzyme B, a well-defined SMAD target gene, and the implications of other signals (e.g. IL-2) in Myc expression suggest that other mechanisms besides the canonical TGFβR-SMAD pathway might be involved in its regulation. For example, investigations by Gu et al. (2015) have reported that Smad4 plays an essential TGFβR-independent role in the induction of T cell proliferation, a phenomenon that, interestingly, is mediated by Myc. In this study, they show that Smad4-deficient T cells fail to optimally express Myc after activation, ultimately leading to defective proliferation. Thus, it is reasonable to speculate that the recruitment of Smad4 upon triggering of TGFβ receptor signalling prevents Smad4-mediated regulation of Myc expression. Altogether, these findings indicate that much is to be discovered about the specific mechanisms by which TGFβ controls Myc expression. Better understanding of these mechanisms may help to identify potential targets to revert the TGFβ-mediated T cell suppression during anti-tumour responses.

Consistent with reduced Myc expression, Myc-driven T cell metabolic reprogramming was also impaired by TGFβ, both at a transcriptional and

functional level, as shown by altered glucose and AA metabolism (Fig. 5.2-5). In the context of glucose metabolism, TGFβ-treated T cells had reduced 2-NBDG uptake and glycolysis, an effect that was observed in both OVA-N4 and OVA-T4 stimulated T cells (Fig. 5.3). This data coincides with the effects of TGFβ on NK cells and $CD4^+$ T cells (Viel et al., 2016; Zaiatz-Bittencourt et al., 2018; Dimeloe et al., 2019), although in these studies the glycolytic deficiency has not directly been associated with Myc repression.

With regards to AA metabolism, a relevant finding in this chapter is the severe diminishment in the Slc7a5-mediated AA uptake, as assessed utilising the tryptophan metabolite kynurenine (Fig. 5.4). In T lymphocytes, Slc7a5 deficiency results in a metabolic catastrophe that leads to a complete impairment of activation (Sinclair et al., 2013). Furthermore, the effects observed in Slc7a5-null and Myc-null T cells upon TCR-stimulation highly overlap suggesting, therefore, that a great extent of the Myc-induced metabolic reprogramming is caused by the lack of Slc7a5 expression (Marchingo et al., 2020). My results indicate that *Slc7a5* expression and subsequent AA uptake is highly compromised by TGFβ, further reinforcing that the loss of Myc expression is a central event during the TGFβ-mediated suppression of T cell responses.

The engagement of glycolysis and the uptake of AAs during T cell activation is essential to sustain the elevated rates of protein synthesis required for growth and proliferation (MacIver et al., 2013; Buck et al., 2015; Buck et al., 2017; Geltink et al., 2018). Consistently with my previous results, I have further shown that protein synthesis is dampened by TGFβ. Moreover, in Chapter 4 I also identified that ribosome biogenesis, based on the RNA-seq data, was strongly affected by

TGFβ, implying that protein synthesis might not only be affected by the lack of substrates but also by the lack of translational machinery.

An unexpected result observed in this chapter is the absence of altered mTOR activity. Considering that mTOR activation can be regulated by IL-2 and the abundance of AAs and glycolytic intermediates (Chapman and Chi, 2015; Orozco et al., 2020; Kelly and Pearce, 2020), seems surprising that the levels of pS6 are comparable in TGFβ treated and untreated T cells. Furthermore, mTOR has been recognised as a key target of TGFβ signalling in NK cells, where TGFβ and rapamycin treatment share a very similar effect in NK function and metabolism (Viel et al., 2016). mTOR inactivation has also been shown in human $CD4^+$ effector memory T cells (Dimeloe et al., 2019). On the contrary, Zaiatz-Bittencourt et al. (2018) demonstrated in human NK cells that mTOR inhibition is not the main mechanisms by which TGFβ suppressed NK metabolism. In fact, they also showed that mTOR activity was not affected within the first 18h upon cytokine-stimulation, but rather only during extended time periods (i.e. 5 days). Thus, I speculate that a similar scenario could occur in $CD8^+$ T cells: whilst mTOR activity is unaltered within the first 24h, the lack of IL-2, glucose and AAs might provide a delayed mTOR inactivation that I have not observed in my assay. These results suggest that the metabolic deficiencies in T cells treated with TGFβ are likely to be independent of mTOR activity, further validating the hypothesis of Myc repression being the major cause of the T cell metabolic paralysis.

My findings have shown that $CD8^+$ T cell mitochondrial metabolism was not impaired by TGFβ. This data contradicts the consensus in previous studies that corroborates a TGFβ-mediated inhibition of OXPHOS in other immune cells (Zaiatz-Bittencourt et al., 2018; Viel et al., 2016; Dimeloe et al., 2019). Particularly

in $CD4^+$ T cells, the loss of OXPHOS activity is directly mediated by the translocation of SMADs to the mitochondria (Dimeloe et al., 2019). However, we have to take into account the complexities of TGFβ activity and compare my data with other studies with caution. Specifically, the reported findings by Dimeloe et al. (2019) do not directly study how TGFβ impairs the metabolism of T cells during priming, but rather, they investigate the metabolism of activated effector memory $CD4^+$ T cells. Considering that, in $CD8^+$ T cells, Myc expression is transient and it is down-regulated after differentiation (Chou et al., 2014), it is possible that the effects of TGFβ on metabolism will differ depending on the stage of the T cell response. In terms of the Myc-induced metabolic reprogramming during T cell priming, Myc upregulates aerobic glycolysis and glutaminolyisis whilst downregulating β-oxidation (Wang, R. et al., 2011). Thus, it is reasonable to think that, in my assays, TGFβ-treated T cells may display higher rates of lipid oxidation as compared to controls, compensating for the lack of glucose and AAs and being sufficient to fuel mitochondrial metabolism. To test this hypothesis it will be of interest to perform a Seahorse Mito Fuel Flex Test, a metabolic assay that measures the capacity of cells to oxidise the three main mitochondrial fuels, i.e., glucose, glutamine and LCFAs. If confirmed, albeit being explanatory of my data, whether this mechanism will be sufficient to temporarily support T lymphocytes and overcome the TGFβ-induced Myc repression is still unknown.

An important aspect that I have not explored here is how TGFβ-treatment affected other metabolic pathways beyond glycolysis and OXPHOS. In the previous chapter, KEGG pathway analysis of all DEGs showed that one-carbon metabolism is one of the most affected pathways by TGFβ. Furthermore, when specifically performing KEGG pathway analysis of genes differentially expressed

with a FC > 2, those involved in the metabolism of serine, glycine or methionine were the most downregulated. These genes include *Cbs, Cth, Shmt1, Psat1, Mthfd2* or *Phgdh* and belong to the so-called SGOC metabolism, which has been proven as one of the key fates of glucose *in vivo* during the TCR-induced metabolic reprogramming and its engagement its essential to promote T cell differentiation and proliferation (Ma et al., 2017a; Ma et al., 2019). The upregulation of the proteins encoded by these genes upon TCR-priming is completely dampened in Myc KO T cells (http://www.immpres.co.uk), suggesting that Myc is an important regulator of SGOC metabolism. Further exploring the impact of TGFβ on SGOC metabolism will be of high interest to better comprehend the mechanisms by which the loss of Myc expression inhibits $CD8^+$ T cell activation.

Altogether, the findings in this chapter robustly confirm a TGFβ-mediated suppression of T cell metabolism. Mechanistically, TGFβ represses the expression of the key metabolic transcription factor Myc, resulting in an inadequate metabolic reprogramming during TCR-priming characterised by a poor engagement of aerobic glycolysis, Slc7a5-mediated AA transport and protein synthesis that ultimately leads to a deficient activation of $CD8^+$ T cells. These results provide valuable new understanding on the mechanisms that regulate TGFβ-mediated suppression and shed light into the attenuation of the T cell metabolic loss of motion as a potential target for TGFβ resistance and improvement of anti-tumour responses.

Chapter 6 The role of asparagine in T cell function

6.1 Introduction

Another key environmental determinant of T cell activation, in addition to signals 1, 2 and 3 and the cytokine milieu, is the availability of nutrients. Particularly, amino acids are highly demanded by activated T lymphocytes to sustain growth, proliferation and differentiation (Sinclair et al., 2013; Kelly and Pearce, 2020). The non-essential AA asparagine (Asn) can be intracellularly synthesised via asparagine synthetase (ASNS), an enzyme that catalyses the conversion of glutamine (Gln) and aspartate (Asp) into Asn and glutamate (Glu) (Lomelino et al., 2017). The role of Asn metabolism in cancer has been extensively studied, where it has been defined as a potential target due to its role in tumour growth and metastasis (Zhang, Ji et al., 2014; Krall et al., 2016; Knott et al., 2018). In fact, high ASNS expression is associated with poor prognosis in several cancer types (Lomelino et al., 2017; Chiu et al., 2020). Furthermore, asparaginases (ASNases), which break down Asn into Asp, have been widely used in the clinic as a chemotherapeutic adjuvant for the treatment of acute lymphoblastic leukaemia (ALL) (Lomelino et al., 2017; Chiu et al., 2020). A hallmark of ALL blasts is that they rely on an extracellular source of Asn due to the lack of ASNS expression. The action of ASNases deprives the cells of an extracellular source of Asn leading to a severe lack of Asn that results in cell death (Chiu et al., 2020).

The role of Asn and ASNS in immune responses remains largely unknown. Torres et al. (2016) described that treating naïve T cells with Salmonella-derived ASNases suppresses exit from quiescence upon TCR-priming implying that T lymphocytes are Asn auxotrophs. Nevertheless, ASNases also contain

glutaminase activity (Lomelino et al., 2017; Zhu, W. et al., 2019) and, therefore, whether the effects are caused by the extracellular deprivation of Asn or Gln is uncertain. Moreover, it is not well described whether ASNS is expressed in T lymphocytes and, if so, at which stage of activation this occurs. Thus, the implications of Asn-deprivation in the development of T cell responses is still unclear. In this chapter, I aim to:

1. Define the levels of ASNS expression in $CD8^+$ T lymphocytes.
2. Assess the requirements for extracellular Asn in $CD8^+$ T cell survival and growth.
3. Assess the requirements for extracellular Asn in $CD8^+$ T cell activation, proliferation and acquisition of effector functions.
4. Determine the impact of Asn-deprivation on the TCR-induced metabolic reprogramming of $CD8^+$ T cells.
5. Investigate the demands for extracellular Asn in CTL cytokine secretion.
6. Explore the role of Asn in other immune cells, such as B lymphocytes.

6.2 Results

6.2.1 ASNS expression is upregulated after TCR-stimulation

First of all, our group (Robert J. Salmond and Lynette P. Steele, University of Leeds) investigated ASNS expression in T cell subsets and showed that ASNS was not present either in thymocytes nor naïve T cells. However, ASNS expression was highly increased after 24h of TCR-stimulation in an mTOR-dependent manner (Fig. 6.1) (Hope et al., 2020).

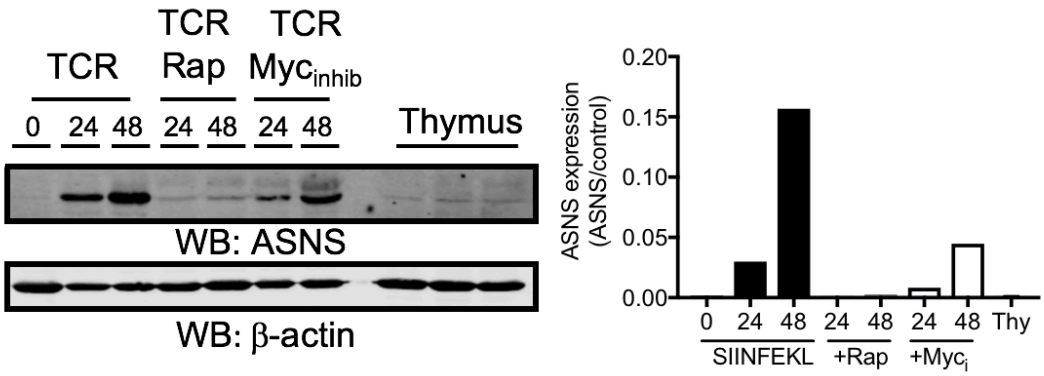


Figure 6.1: Naïve CD8$^+$ T lymphocytes do not express ASNS but it is highly upregulated upon TCR-stimulation.

On the left, western blots show ASNS protein levels in lysates from OT-I thymocytes, naïve T cells or T cells activated in nutrient-replete conditions with OVA-peptide ± rapamycin (Rap) ± Myc inhibitor (Myc$_i$) for the indicated time-points. β-actin serves as a control for protein loading. On the right, bar graph representation of ASNS expression (ASNS/β-actin). Data is representative of two independent experiments.

These findings led to the hypothesis that, whereas Asn availability would significantly limit early T cell activation, the Asn requirements would diminish as long as ASNS was upregulated. Hence, I designed a time-course experiment to explore the effects of Asn-deprivation during both ASNS-low (24h) and ASNS-high (48h, 72h) stages. To do so, naïve OT-I T cells were stimulated *in vitro* with OVA-N4 for 24, 48 and 72 hours in DMEM supplemented ± Asn ± Gln. This

approach allowed us to (1) comprehend the importance of ASNS contribution to the Asn pool, (2) differentiate the effects of the deprivation of both AAs (as assessed in previous publications that used ASNases) vs Asn alone and (3) test the interplay between Gln and Asn to promote survival, a phenomenon already observed in transformed cells (Zhang, Ji et al., 2014). After appropriate stimulation, the repercussion of Asn-depletion on T cell survival, growth, activation, proliferation and metabolism was assessed.

6.2.2 Asn availability determines survival and growth of $CD8^+$ T lymphocytes upon TCR-stimulation.

Amino acid deprivation induces a stress response mediated by GCN2 and ATF4 that leads to apoptosis when AA availability is not restored (Balasubramanian et al., 2013; Yang et al., 2018). Thus, I first tested how Asn deprivation affected the survival of $CD8^+$ T lymphocytes.

After 24h of culture, as assessed using the viability dye LD Aqua, only 50% of the T cells stimulated in the absence of Asn were alive (Fig. 6.2). Similarly, TCR stimulation in the absence of Gln highly induced T cell death, whilst the combined absence of both AAs had detrimental effects and T cell viability was reduced by 90% (Fig. 6.2). After 72h, >80% of the T cells stimulated without Gln were dead. However, no significant differences were observed between those T cells cultured in replete media conditions and those lacking Asn, suggesting that the latter were able to overcome the initial stress response seen at earlier timepoints (Fig. 6.2).

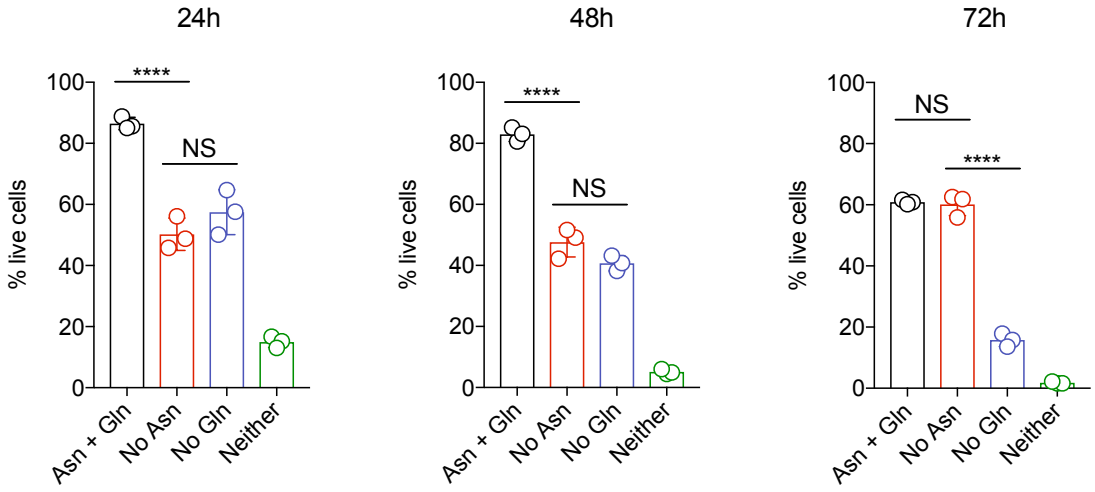


Figure 6.2: Asn-deprivation induces cell death of CD8⁺ T lymphocytes during early stages of activation.

OT-I T cells were stimulated in DMEM ± Asn ± Gln with OVA-peptide for 24 (left), 48 (middle) or 72 (right) hours. Cell viability is represented as assessed by FACS and LD Aqua staining. Data is from one of three repeated experiments. Dots represent technical triplicates. NS – Not significant; **** - P<0.0001, as determined by one-way ANOVA with Tukey's multiple comparisons test.

Next, I examined the blasting capacity of T cells. As determined by flow cytometry and analysis of FSC-A/SSC-A plots, the majority of viable T cells (70-80%) had blasted after 24h of TCR-stimulation when both AAs were present whereas only a small proportion (20-30%) grew in Asn-deprived conditions (Fig. 6.3A and B). At 48h, around 70-80% of the live cells in the 'No Asn' conditions displayed a blast phenotype which was observed in all T cells after 72h (Fig. 6.3A and B). Without Gln, despite a proportion of the cells (40%) being able to survive within the first 48h, T cell growth was totally impaired at all timepoints (Fig. 6.3B). As AAs are building blocks for protein synthesis (Kelly and Pearce, 2020), I questioned how the lack of extracellular Asn affected this process. As measured by OPP incorporation, protein synthesis was severely disrupted after 24h of stimulation when depriving T cells of Asn and/or Gln (Fig. 6.4). No significant

differences were seen when comparing 'No Asn' and 'No Gln' conditions. However, a small proportion of T cells under Asn-depleted conditions showed higher rates (similar to control cells) of newly synthesised proteins, a characteristic that was not observed without Gln (Fig. 6.4). This intermediate phenotype is comparable to previous results indicating that T cells stimulated in an Asn-deprived environment are unable to optimally initiate blastogenesis due to an incapacity to engage protein synthesis.

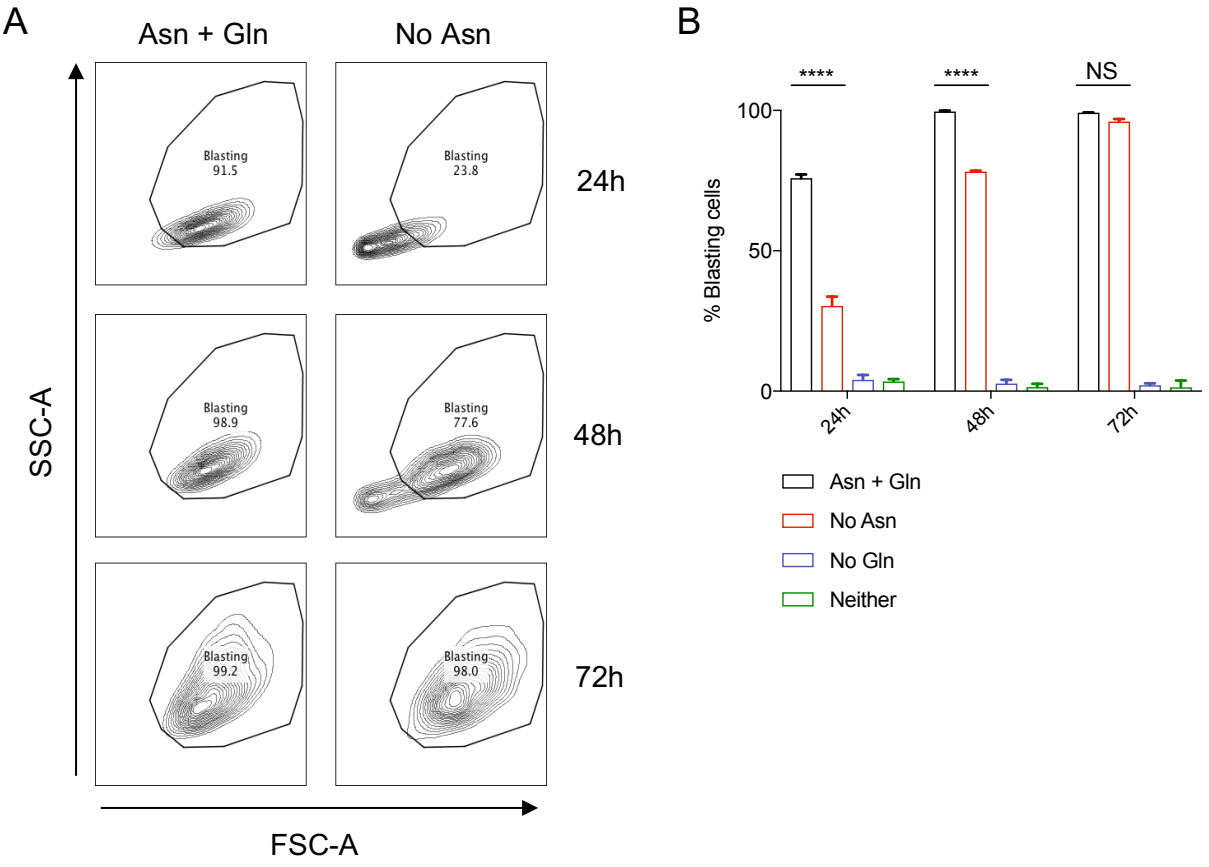


Figure 6.3: Asn-deprivation disrupts blastogenesis within the first stages of CD8⁺ T cell activation.

OT-I T cells were stimulated in DMEM ± Asn ± Gln with OVA-peptide for the indicated time-points. (A) Blasting population is gated as determined by cell size. (B) Bar chart represents summarised data of the proportion of T cells that have undergone blastogenesis at the indicated conditions and time-points. Data is from one of three repeated experiments. Error bars represent SD (N=3). NS – Not significant; **** - P<0.0001, as determined by two-way ANOVA with multiple comparisons.

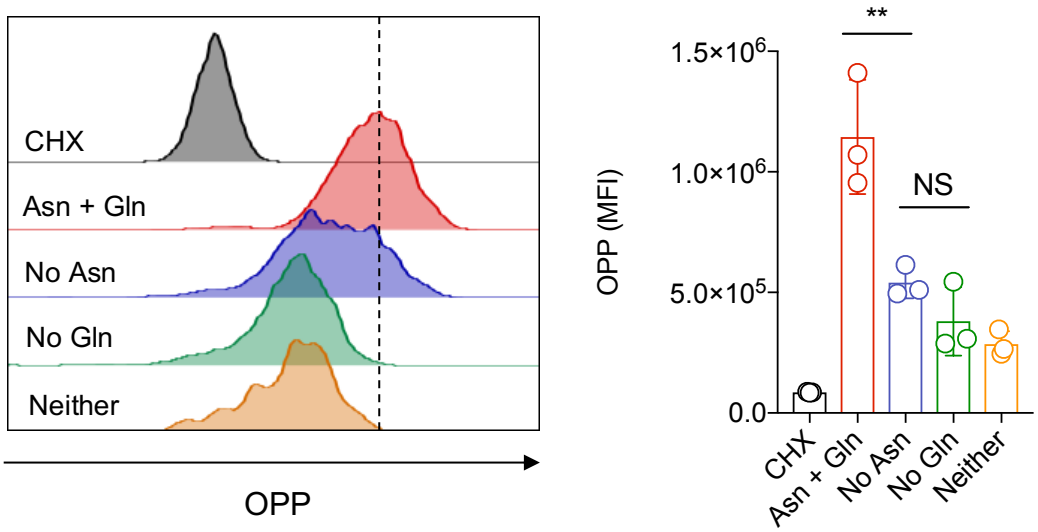


Figure 6.4: The lack of extracellular Asn limits *de novo* protein synthesis in early activated CD8⁺ T lymphocytes.

OT-I T cells were stimulated in DMEM ± Asn ± Gln with OVA-peptide for 24 hours. Protein synthesis capacity is represented as assessed by OPP incorporation and subsequent detection using Click-iT™ reagents. Cycloheximide (CHX), a protein synthesis inhibitor, was utilised as negative control. Data is from one of three repeated experiments. Dots represent technical triplicates. NS – Not significant; ** - P<0.005, as determined by one-way ANOVA with Tukey's multiple comparisons test.

6.2.3 CD8⁺ T cells are poorly activated in the absence of Asn

To better understand how the lack of extracellular Asn affected antigen-induced responses I investigated the activation state of T lymphocytes. After 24-72h of TCR-stimulation, the expression of the activation markers CD25, CD69 and CD71 was assessed. Upon 24h of antigen encounter, CD25 and CD71 upregulation was severely impaired in Asn-free conditions when compared to control but, interestingly, no effect was observed in CD69 expression despite the lack of Asn and/or Gln (Fig. 6.5A). Whereas most of the cells were $CD25^{+}CD71^{+}$ in AA replete media conditions at this time-point, only ~30% expressed both markers when Asn was absent (Fig. 6.5B). Nonetheless, at 72h, T cells lacking extracellular Asn were able to acquire a fully activated state comparable to those T cells grown

with both Gln and Asn (Fig. 6.5A and B). Without Gln, no upregulation of CD25 was seen at any stage (Fig. 6.5A and B). However, a reduced but consistent level of CD71 expression was observed at 24h in all T cells stimulated in the absence of Gln (Fig. 6.5C).

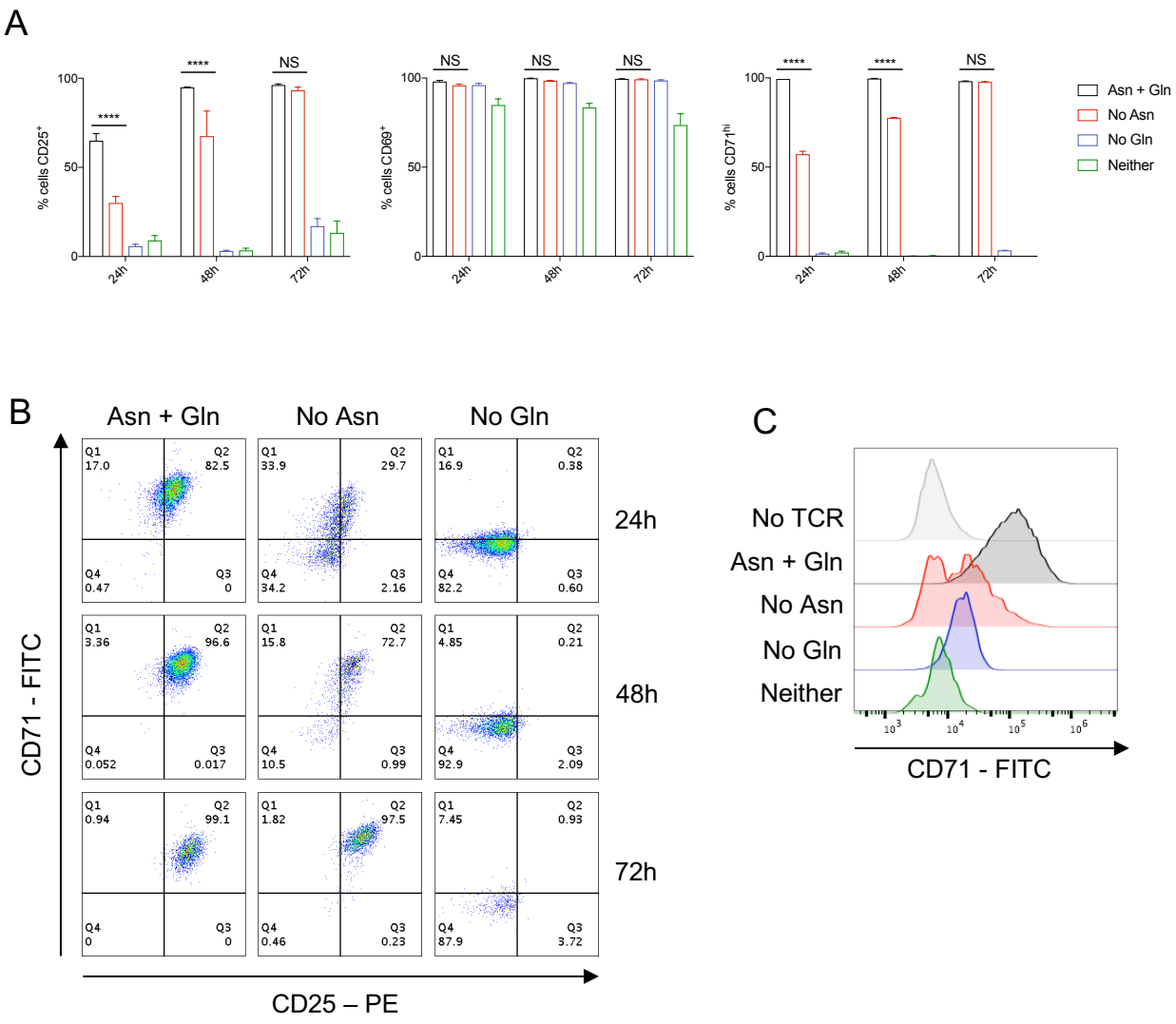


Figure 6.5: The lack of Asn compromises early T cell activation.

OT-I T cells were stimulated in DMEM ± Asn ± Gln with OVA-peptide for the indicated time-points. (A) Summarised data representing $CD25^{+}$, $CD69^{+}$ and $CD71^{hi}$ T lymphocytes at the indicated time-points and condition, as assessed by FACS analysis in live gated cells. (B) Proportion of T cells expressing the activation markers CD25 and CD71. (C) Histogram shows cell surface expression of CD71 after 24h of TCR-stimulation. Data is from one of three repeated experiments. Error bars represent SD. NS – Not significant; **** - $P<0.0001$, as determined by two-way ANOVA with multiple comparisons.

Similar phenotypes were observed when I assessed the levels of the transcription factors Eomes and Tbet (Fig. 6.6): whereas the absence of Gln completely suppressed their upregulation at all timepoints, Asn-depletion was suppressive only at early stages but T cells recovered upon longer antigen-stimulation. Consistently, the lack of Asn significantly reduced the proportion of GrB^+ T cells within the first 48h, an effect that withdrew at 72h (Fig. 6.7). GrB levels were not tested at 24h due to its low or null expression at early time-points. It is important to highlight that, at 24h (or 48h for GrB), although a proportion of T cells upregulated the different markers in the absence of Asn, their expression levels tend to be lower than those seen in complete conditions when analysed at a single-cell level (Fig. 6.5B and C, 6.6A, 6.7).

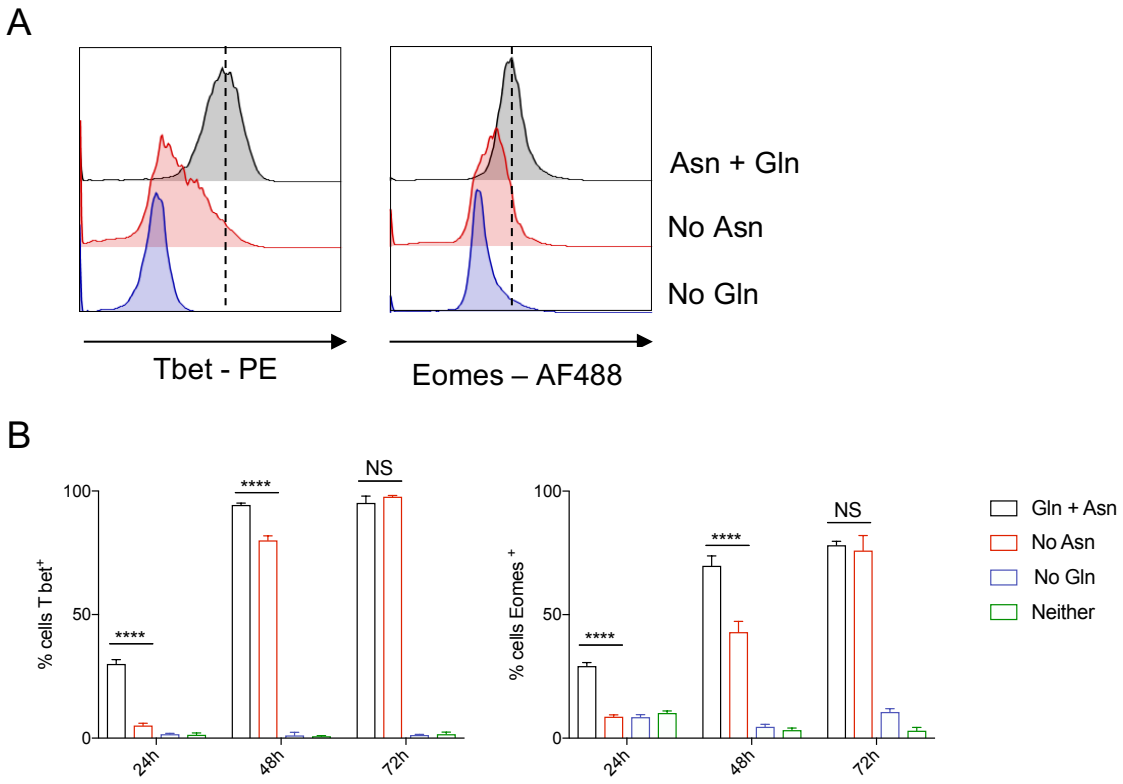


Figure 6.6: Asn-depletion delays the upregulation of Tbet and Eomes.

OT-I T cells were stimulated in DMEM ± Asn ± Gln with OVA-peptide for the indicated time-points. (A) Histogram shows intracellular expression of Tbet and Eomes after 24h of TCR-stimulation. (B) Summarised data representing Tbet$^+$ and Eomes$^+$ T lymphocytes at the indicated time-points and condition, as assessed by FACS analysis in live gated cells. Data is from one of three repeated experiments. Error bars represent SD. NS – Not significant; **** - P<0.0001, as determined by two-way ANOVA with multiple comparisons.

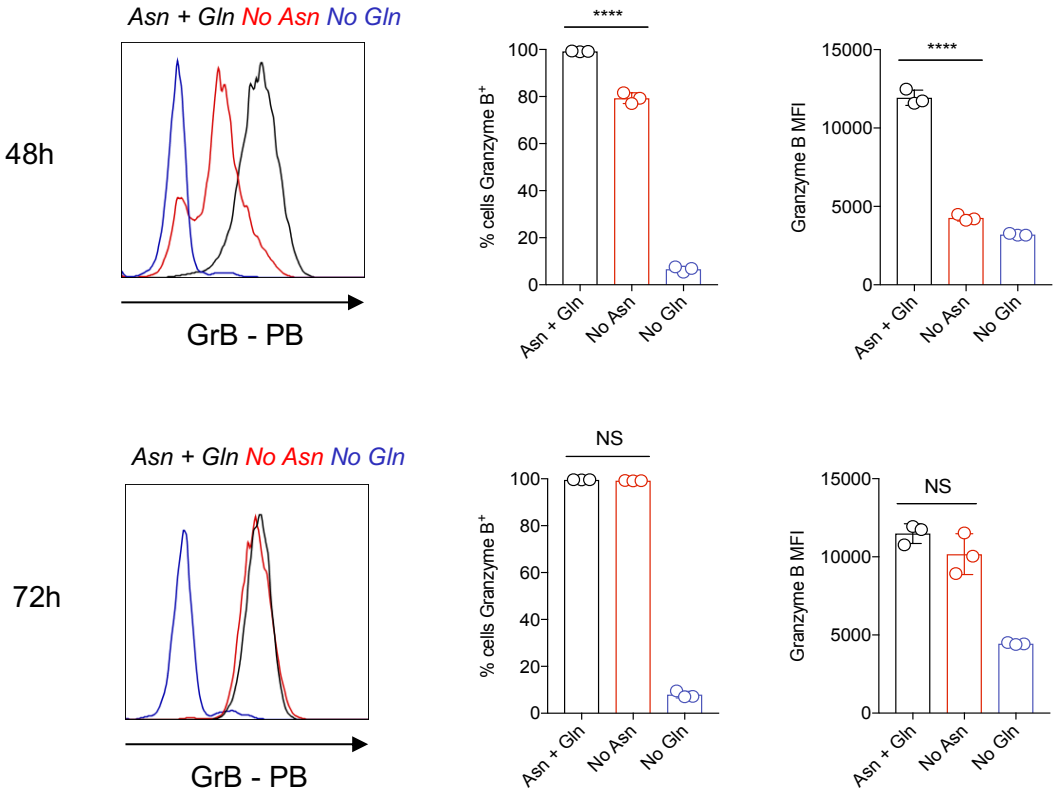


Figure 6.7: The absence of extracellular Asn restricts granzyme B expression.

OT-I T cells were stimulated in DMEM ± Asn ± Gln with OVA-peptide for 48 or 72 hours, as indicated. Representative histograms of GrB expression (left) and the proportion of GrB$^+$ T cells (middle) were determined by FACS analysis. Intracellular levels of GrB were quantified as assessed by mean fluorescence intensity (MFI). Data is from one of three repeated experiments. Dots represent technical triplicates. NS – Not significant; ** - P<0.005, as determined by one-way ANOVA with Tukey's multiple comparisons test.

Additionally, I showed, as measured by dilution of the CTV dye, that TCR-induced T cell proliferation was completely blocked in the absence of extracellular Gln but not without Asn, where >70% of T lymphocytes were able to initiate cell division after 72h of stimulation (Fig. 6.8A and B). Nonetheless, T lymphocytes cultured in Asn-deprived conditions displayed a reduced division index (~1 vs ~1.5 in control T cells) (Fig. 6.8C). Whereas the majority of the cells had undergone 2 or 3 divisions in control conditions only 30-40% of T cells were at this stage in the

absence of Asn (Fig. 6.8D) indicating a slower rate or delayed initiation of proliferation.

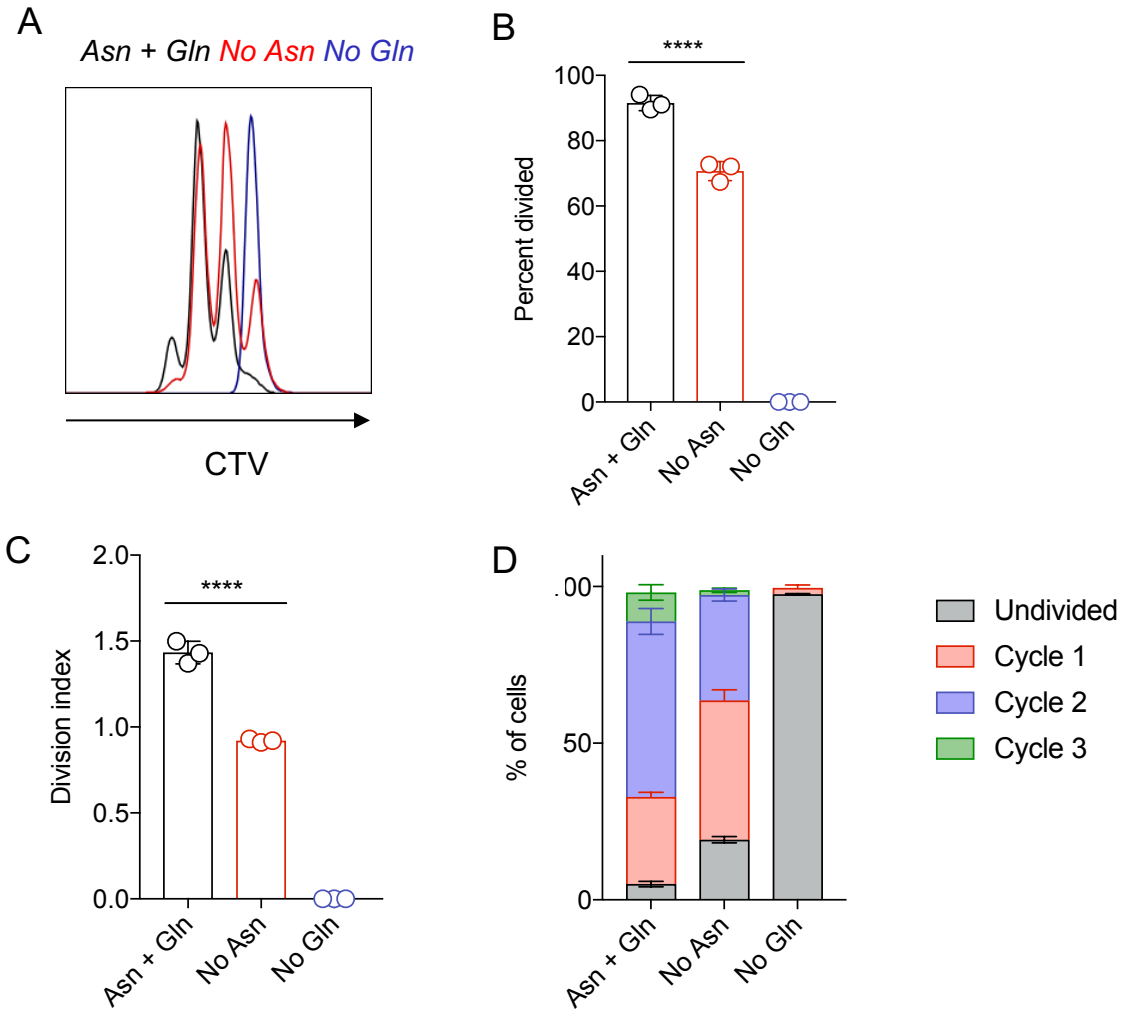


Figure 6.8: Antigen-induced CD8⁺ T cell proliferation is impaired by the absence of extracellular Asn.

OT-I T cells were stimulated in DMEM ± Asn ± Gln with OVA-peptide for 72 hours. Proliferative capacity was assessed by Cell Trace Violet (CTV) labelling of naïve T cells. Data is from one of three repeated experiments. Dots represent technical triplicates. NS – Not significant; **** - P<0.0001, as determined by one-way ANOVA with Tukey's multiple comparisons test.

Altogether, my findings indicate that Asn-deprivation compromises proliferation as well as the upregulation of activation markers, transcription factors and effector proteins during early stages upon TCR-stimulation. Nonetheless, and consistently with previous results (see Section 6.2.2), lymphocytes are able to

overcome these effects and ultimately acquire a fully activated state comparable to control cells.

6.2.4 $CD8^+$ T lymphocytes are unable to optimally engage antigen-induced metabolic reprogramming in the absence of extracellular asparagine

To further comprehend the mechanisms underlying defective early activation in the absence of extracellular Asn, I investigated the implications of Asn-deprivation on TCR-induced metabolic reprogramming. When measured at 24h of activation, TCR-induced expression of nutrient transporters was decreased in the absence of Asn and, consequently, nutrient uptake was substantially reduced. Specifically, only 50% of T cells upregulated GLUT1 whilst almost 80% expressed the glucose transporter in control conditions (Fig. 6.9A). These results were mirrored in the reduced ability of T cells to take up the fluorescent glucose analogue 2-NBDG in conditions lacking Asn (Fig. 6.10A). Similarly, the upregulation of the scavenger receptor CD36 and amino acid transporter CD98 was inhibited (Fig. 6.9B and C). Consequently, the uptake of AAs mediated by Slc7a5 and free fatty acids was severely impaired, as determined by the import of kynurenine and Bodipy-C16, respectively (Fig. 6.10B and C). The lack of extracellular Gln completely prevented GLUT1, CD98 and CD36 upregulation, leading to a failure to uptake nutrients (Fig. 6.9 and 6.10). To be noted, Gln-depletion suppressed CD98 expression more profoundly than Asn-deprivation did, an effect that was not reflected in the kynurenine assay, which revealed no differences when comparing ‘No Asn’ and ‘No Gln’ conditions (Fig. 6.9 and 6.10).

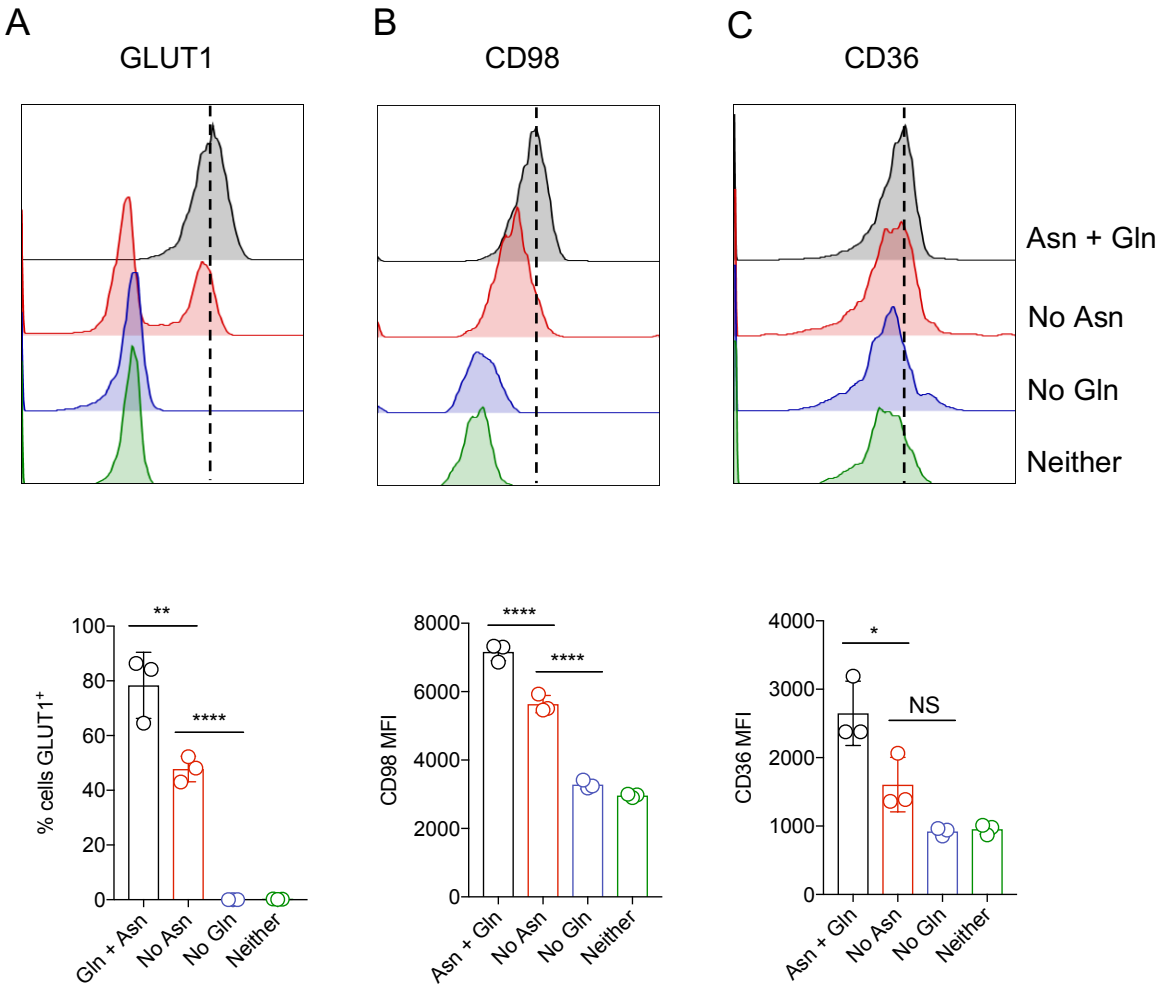


Figure 6.9: The lack of extracellular Asn limits the upregulation of the nutrient transporters GLUT1, CD98 and CD36 after TCR-stimulation.

OT-I T cells were stimulated in DMEM ± Asn ± Gln with OVA-peptide. After 24h, GLUT1, CD98 and CD36 was determined by FACS analysis using conjugated antibodies. Data is from one of three repeated experiments. Dots represent technical triplicates. NS – Not significant; **** - P<0.0001; ** - P<0.005; * - P<0.05, as determined by one-way ANOVA with Tukey's multiple comparisons test.

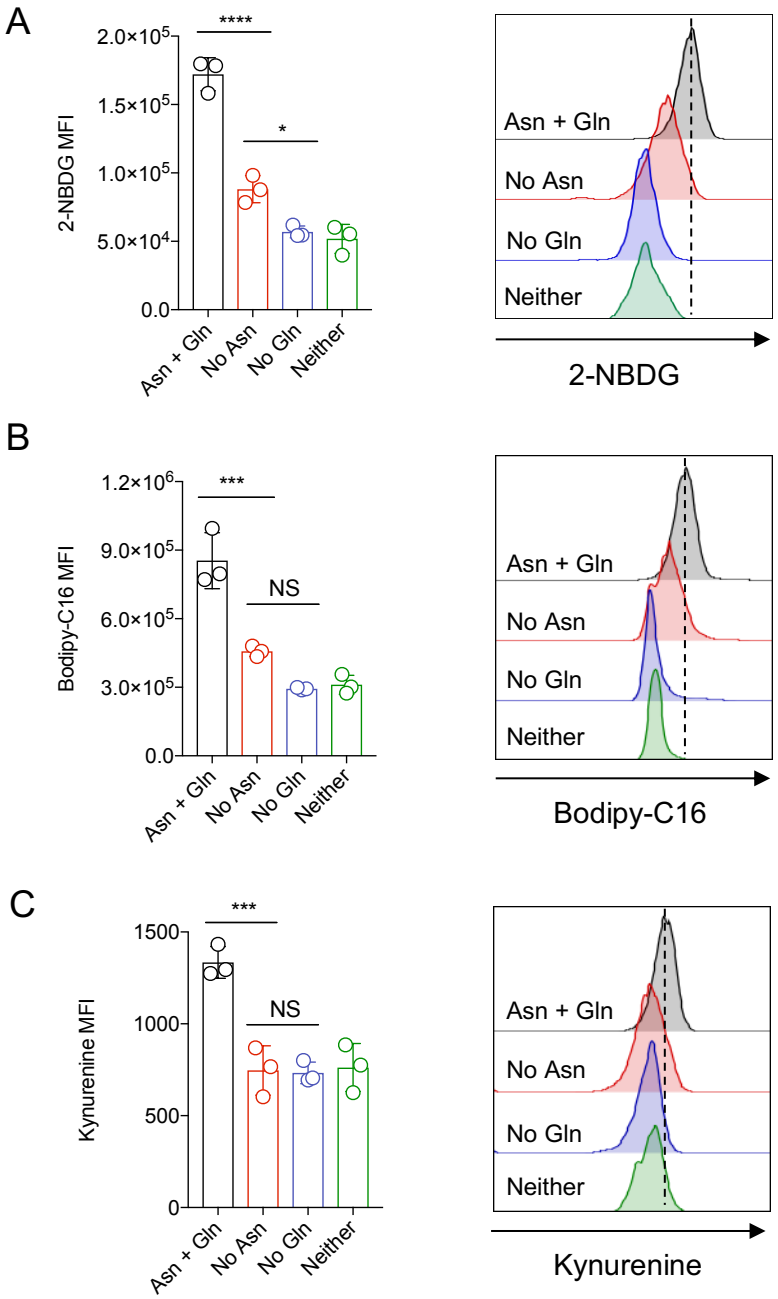


Figure 6.10: CD8⁺ T lymphocytes display reduced nutrient uptake when stimulated in the absence of extracellular Asn.

OT-I T cells were stimulated in DMEM ± Asn ± Gln with OVA-peptide. After 24h, T cells were cultured with 2-NBDG, Bodipy-C16 or kynurenine to evaluate glucose (A), free FA (B) and Slc7a5-mediated AA uptake (C), respectively, by FACS analysis. Data is from one of three repeated experiments. Dots represent technical triplicates. NS – Not significant; **** - P<0.0001; *** - P<0.0005; * - P<0.05, as determined by one-way ANOVA with Tukey's multiple comparisons test.

Next, I analysed OCR and ECAR values in T cells stimulated ± Asn to define their metabolic activity. Results indicated that T cells maintained in the absence of Asn were inefficient in engaging glycolysis and OXPHOS as indicated by reduced acidification and oxygen consumption, respectively (Fig. 6.11A). Lymphocytes stimulated without Asn displayed a quiescent phenotype when compared to control cells which exhibited an energetic phenotype characterised by elevated ECAR and OCR values (Fig. 6.11B). Consistent with these data, their ability to synthesise ATP was severely compromised, to the same extent as in Gln-deprived conditions (Fig. 6.11C).

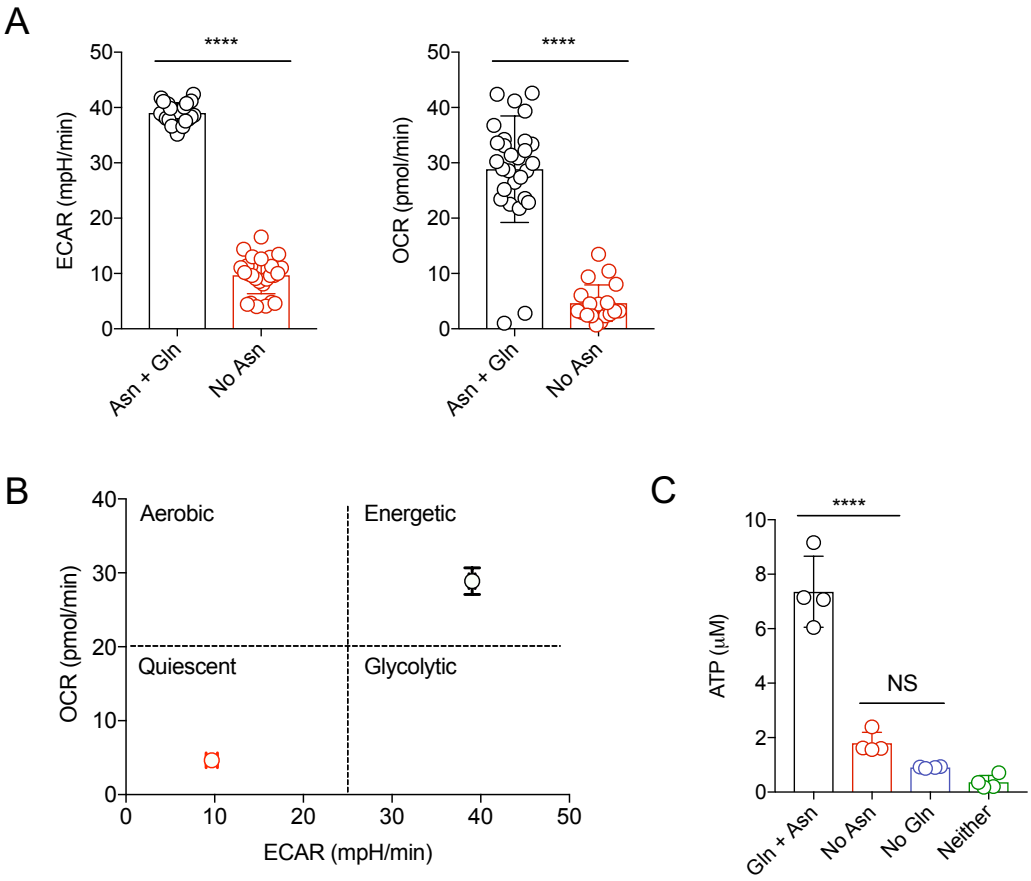


Figure 6.11: CD8⁺ T lymphocytes are unable to increase their metabolic activity after TCR-stimulation in Asn-deprived conditions.

OT-I T cells were stimulated in DMEM ± Asn ± Gln with OVA-peptide for 24h. (A, B) ECAR and OCR were determined utilising Seahorse XFe96 metabolic analyser. (C) ATP synthesis capacity was tested using a luminescence-based assay. Data is from one of two (A, B) or three (C) repeated experiments. In (A, C), dots represent technical replicates. In (B), dots represent mean values. Error bars indicate SD. NS – Not significant; **** - P<0.0001, as determined by two-tailed Student's *t*-test or one-way ANOVA with Tukey's multiple comparisons test, as appropriate.

I wondered whether upon prolonged responses, consistent with previous results (see Section 6.2.2 and 6.2.3), T cells were ultimately capable of undergoing efficient metabolic reprogramming. As expected, T lymphocytes stimulated with OVA-peptide for 72h in the absence of Asn displayed a metabolic profile comparable to control cells. No differences were observed with respect to 2NBDG and Bodipy-C16 uptake or ATP production between those T cells activated in nutrient-replete and Asn-deprived conditions (Fig. 6.12) confirming that, upon

prolonged stimulation, lymphocytes are able to complete the metabolic reprogramming regardless of extracellular Asn levels.

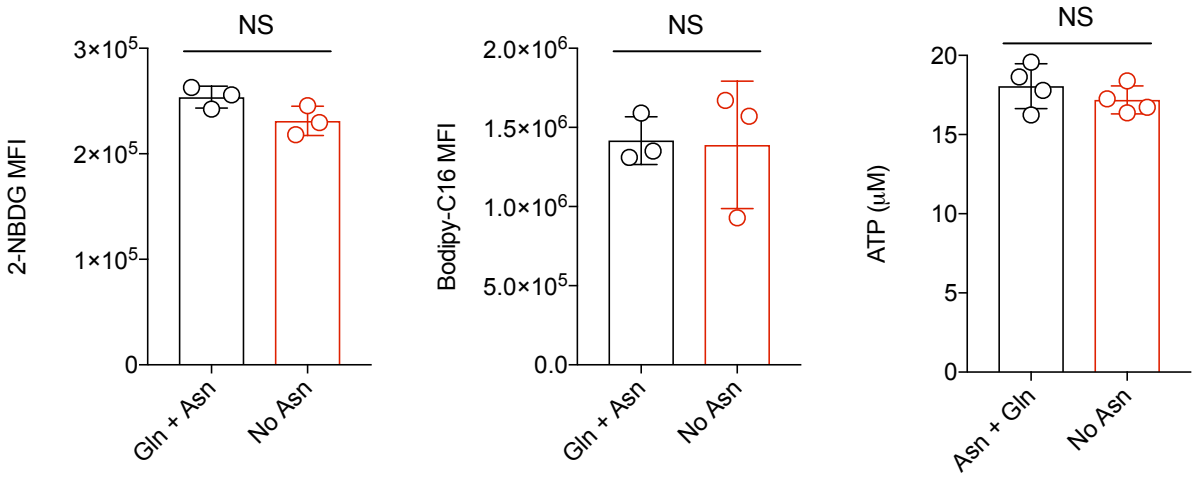


Figure 6.12: CD8⁺ T lymphocytes are able to undergo the TCR-induced metabolic reprogramming at later stages regardless of extracellular Asn levels.

OT-I T cells were stimulated in DMEM ± Asn with OVA-peptide for 72h. Then, 2-NBDG uptake (left), Bodipy-C16 uptake (middle) and ATP production (right) was assessed. Data is from one of at least two repeated experiments. Dots represent technical replicates. Error bars indicate SD. NS – Not significant, as determined using Mann-Whitney's test.

6.2.5 ASNS upregulation is required, and sufficient, to sustain CD8⁺ T cell responses in the absence of extracellular Asn

Altogether, these findings indicated that extracellular Asn was required by naïve CD8⁺ T lymphocytes within the first stages of activation. The lack of extracellular Asn restricted *de novo* protein synthesis resulting in high rates of cell death added to an incapacity to successfully engage the metabolic reprogramming that sustains blastogenesis, activation and proliferation. However, no phenotypic differences were observed at later time-points when comparing Asn-deprived and control conditions suggesting that CD8⁺ T cells lose its requirement for Asn upon prolonged antigen-induced responses.

$CD8^+$ T cells sharply increase ASNS expression after 24h of TCR-stimulation (Fig. 6.1) (Hope et al., 2020). Thus, our group reasoned that the mechanism by which T cells were able to overcome the inefficient initial response was due to an emergent ASNS-derived source of Asn. To verify this interpretation, we used a genetically-modified *Asns*^{Tm1a} mouse model. This strain contains an hypomorphic *Asns* allele that reduces >90% or ~60% of ASNS protein levels in homozygous and heterozygous mice, respectively (Fig. 6.13A). As expected, *Asns*$^{Tm1a/Tm1a}$ $CD8^+$ T lymphocytes stimulated in Asn-free media were incapable to induce blastogenesis after 24h of stimulation (Fig. 6.13B). On the other hand, and consistent with previous results (Fig. 6.3), a proportion of *Asns*$^{WT/WT}$ T cells was able to initiate blastogenesis in spite of Asn-deprivation (Fig. 6.13B). These results point out at ASNS expression as the fundamental event that allows T cell activation in the absence of Asn indicating that ASNS upregulation is sufficient to sustain $CD8^+$ T cell responses (Hope et al., 2020). All the data included in Fig. 6.14 was generated by other members in our laboratory (Robert J. Salmond and Lynette P. Steele, University of Leeds).

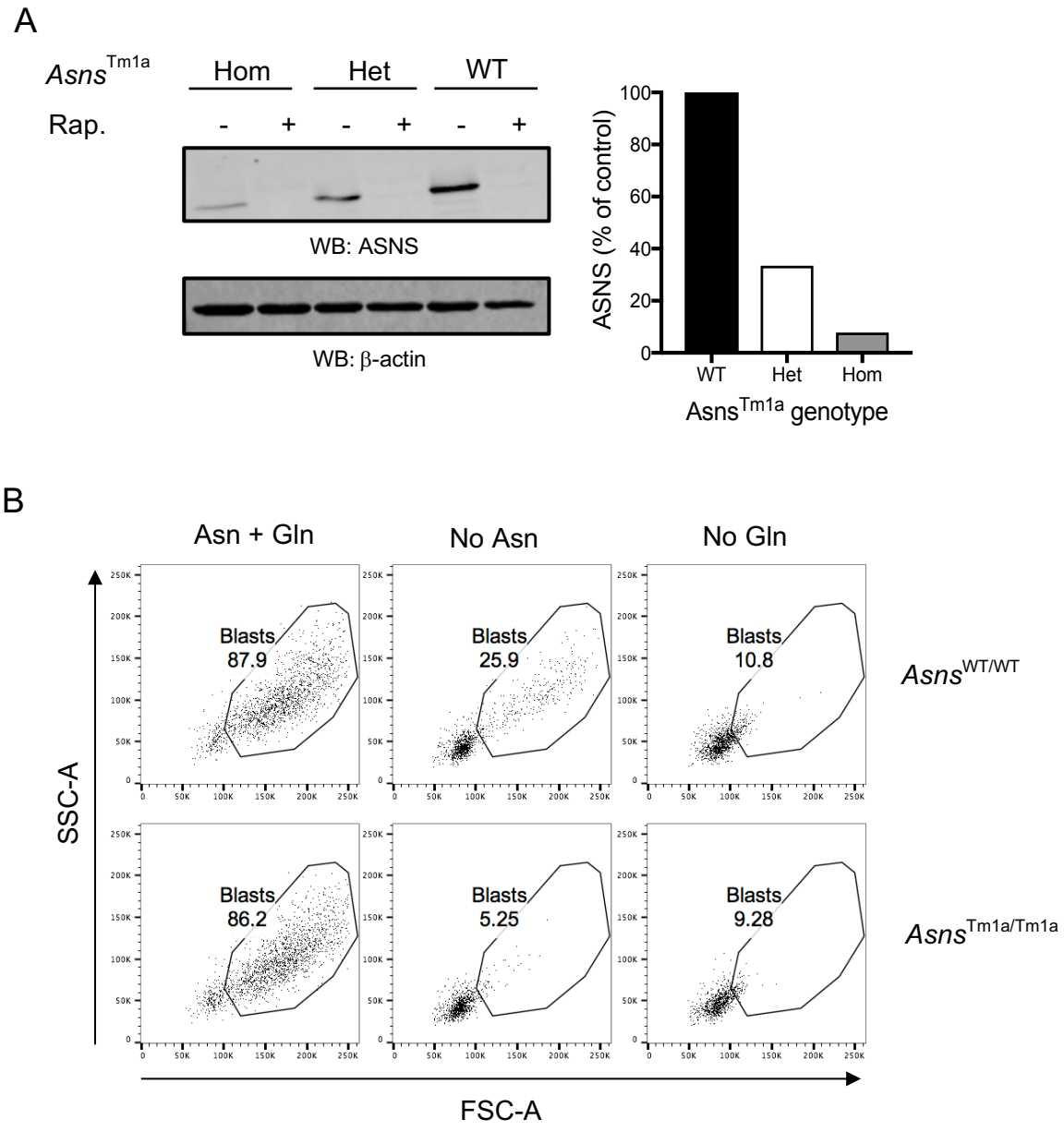


Figure 6.13: Asns$^{Tm1a/Tm1a}$ CD8^{+} T lymphocytes are unable to undergo blastogenesis in the absence of extracellular Asn.

(A) Immunoblot analysis of ASNS levels in *Asns*$^{WT/WT}$, *Asns*$^{WT/Tm1a}$ and *Asns*$^{Tm1a/Tm1a}$ activated T cells ± rapamycin (Rap). (B) *Asns*$^{WT/WT}$ and *Asns*$^{Tm1a/Tm1a}$ CD8^{+} T cells were MACS-sorted and stimulated *in vitro* with αCD3 and αCD28. After 24h of stimulation in DMEM ± Asn ± Gln, blasts were detected by FACS analysis with FSC/SSC plots.

6.2.6 The potency of TCR ligands determines survival and T cell activation in Asn-depleted environments

As mentioned above, the progression of antigen-induced responses in the absence of extracellular Asn is dependent on ASNS upregulation (Hope et al., 2020). So far, all experiments were performed using the high affinity OVA-peptide N4 for TCR-stimulation. However, the response rate of $CD8^+$ T cells depends on ligand potency (Richard et al., 2018). Hence, I hypothesised that under suboptimal TCR-stimulation scenarios fewer T cells would be able to trigger ASNS upregulation and, therefore, to survive and differentiate in Asn-depleted conditions. To address this question I stimulated OT-I T cells with the lower affinity OVA-peptide T4 and saw that, when Asn was absent, <20% of the cells survived vs 40% in OVA-N4-stimulated T lymphocytes (Fig. 6.14A). Moreover, their activation state was clearly compromised as the lack of Asn reduced the proportion of $CD25^+CD71^+$ T cells from a 45% to a 15% in OVA-N4 vs OVA-T4 stimulated cells (Fig. 6.14B). Although cell survival was not affected, CD25 and CD71 expression was proportionally impaired in nutrient-replete conditions when decreasing ligand potency (Fig. 6.14B). Thus, my data suggest that antigen affinity entails an added obstacle to successful T cell responses in Asn-deprived conditions.

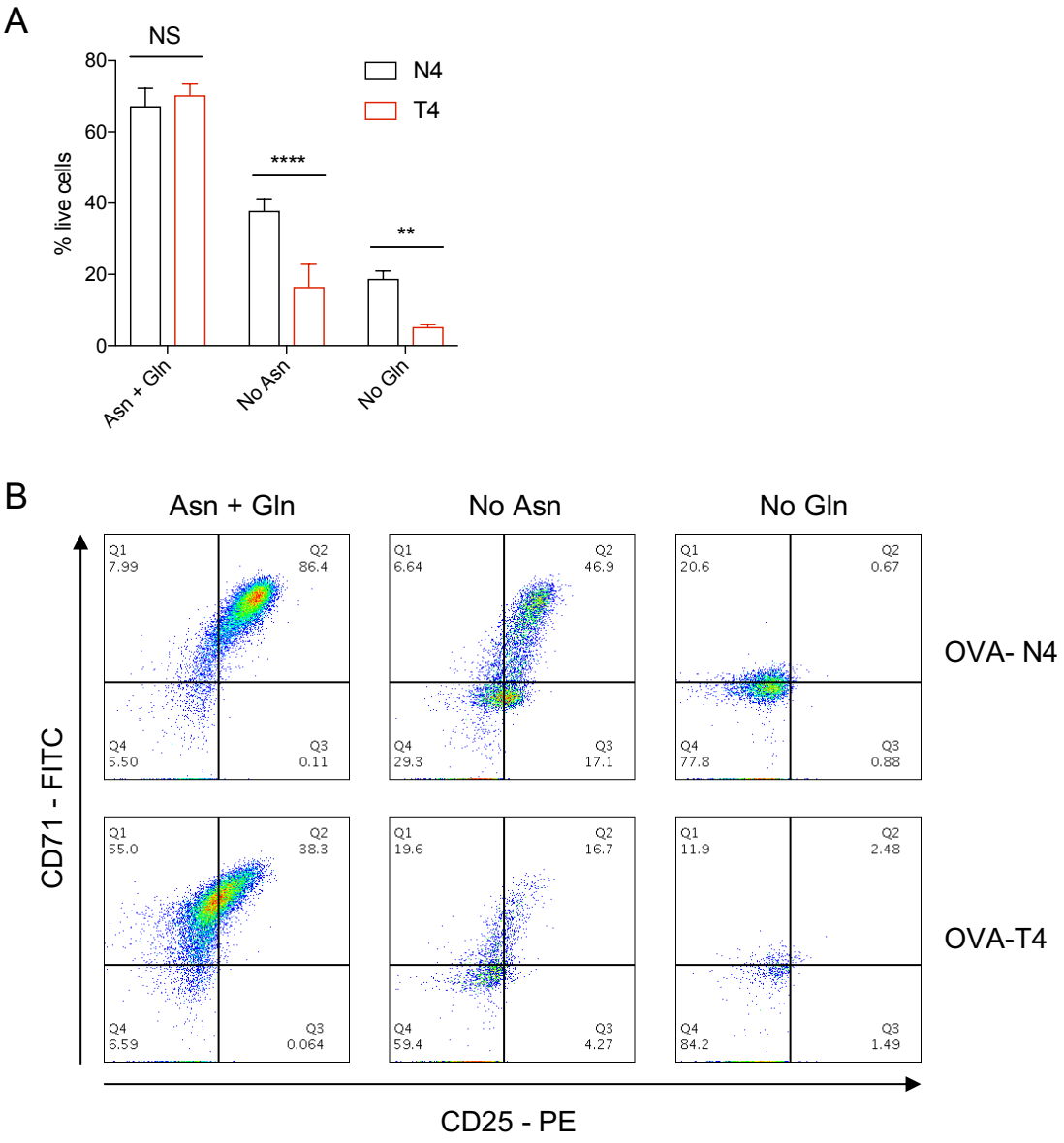


Figure 6.14: CD8⁺ T cell responses in Asn-deprived environments are limited by peptide affinity.

OT-I T cells were stimulated in DMEM ± Asn ± Gln with OVA-N4 or the lower affinity peptide OVA-T4. After 24h, cell viability (A) and cell surface expression of the activation markers CD25 and CD71 (B) were assessed by FACS analysis. Error bars represent SD. NS – Not significant; **** - P<0.0001; ** - P<0.005, as determined by two-way ANOVA with multiple comparisons.

6.2.7 Lack of extracellular asparagine suppresses early T cell activation independently of the loss of IL-2 secretion

Previous results in our group revealed that the lack of extracellular Asn severely suppresses IL-2 production within the first 24h (Hope et al., 2020). IL-2 signalling is associated to a complex transduction network that induces protein synthesis and an effector-like transcriptional program (Ross and Cantrell, 2018; Rollings et al., 2018). Hence, I questioned if the effects seen under Asn-deprivation circumstances could be partially caused by the loss of autocrine IL-2 signalling. OT-I T lymphocytes were stimulated as described above + IL-2 supplementation from timepoint 0h. My data established that IL-2 replenishment did not sustain survival when Asn and/or Gln were absent (Fig. 6.15A). Equally, the levels of GrB or the activation markers CD25 and CD71 were not rescued by IL-2 (Fig. 6.15, B and C) indicating that the impact of Asn-depletion on these parameters is independent of reduced IL-2 production.

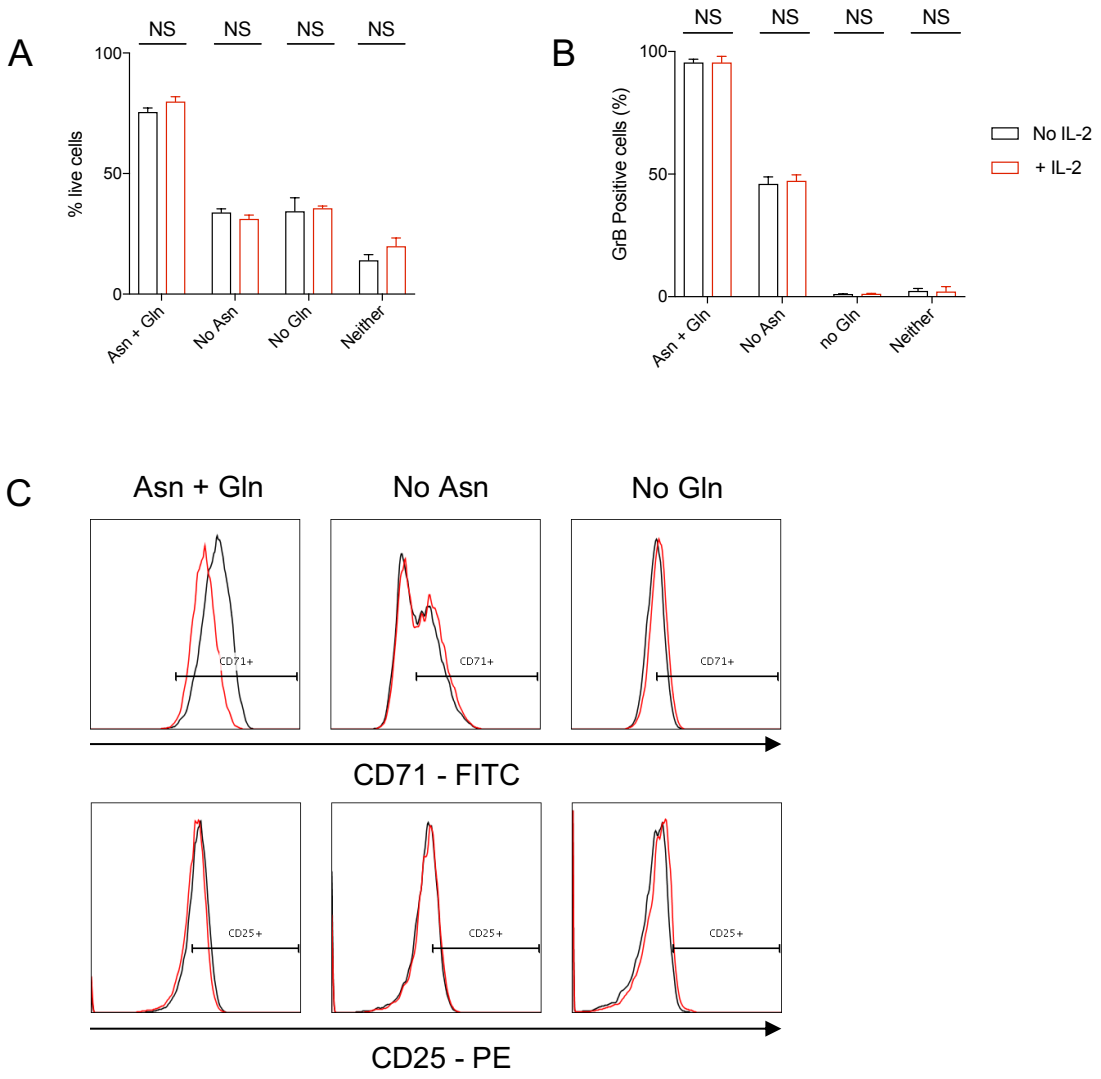


Figure 6.15: IL-2 replenishment does not rescue CD8⁺ T cell function in the absence of extracellular Asn.

OT-I T cells were stimulated in DMEM ± Asn ± Gln with OVA-N4 ± hIL-2 (1ng/ml). After 24h, cell viability (A) and expression of GrB (B) and the activation markers CD25 and CD71 (C) were assessed by FACS analysis. Data is from one of three repeated experiments. Error bars represent SD (N=3). NS – Not significant, as determined by two-way ANOVA with multiple comparisons.

6.2.8 CTLs are not asparagine auxotrophs

ASNS upregulation was key to promote CD8$^+$ T cell differentiation in the absence of extracellular Asn (Hope et al., 2020) but whether or not its expression was enough to sustain the function of fully differentiated CTLs was unclear. To address this question I generated OT-I CTLs *in vitro* in IMDM (nutrient-rich conditions) and re-stimulated them after 6 days in Asn-free media. To be noted, CTLs were transferred on day 5 to media lacking Asn to ensure depletion of intracellular Asn levels at the moment of re-stimulation. After 24h, CTLs maintained in replete and Asn-deprived conditions displayed the same levels of IFNγ and IL-2 secretion (Fig. 6.16) suggesting that ASNS upregulation is sufficient to sustain CTL function regardless of extracellular Asn availability. Thus, CD8$^+$ T lymphocytes were not Asn auxotrophs.

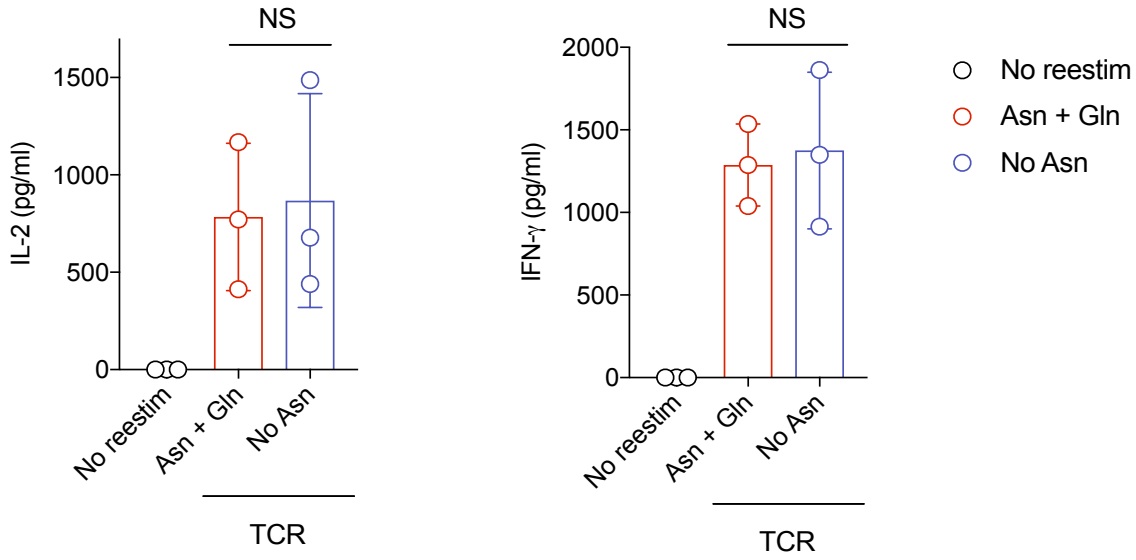


Figure 6.16: CTLs do not require extracellular Asn to sustain IFNγ and IL-2 secretion.

CTLs were generated *in vitro* during 5d in IMDM, then transferred DMEM ± Asn for 24h. On day 6, CTLs were re-stimulated with OVA-peptide for 24h and levels of IFNγ and IL-2 in supernatants were determined by ELISA. Data is from one of at least two repeated experiments. Dots represent technical replicates. NS – Not significant, as determined by one-way ANOVA with Tukey's multiple comparisons test.

6.2.9 ASNS-deficiency does not affect IgM and IgG secretion by plasma cells after *in vivo* immunisation

To further understand the role of Asn synthesis during immune responses I explored the phenotype of *Asns*^{Tm1a} mice beyond the development of effector T lymphocytes. In order to do it, and *in vivo* immunisation with the hapten 4-Hydroxy-3-nitrophenyilacetyl (NP) conjugated to OVA, which acted as a carrier protein, was performed. NP-OVA immunisation provides T-cell dependent formation of germinal centres and class-switched, affinity matured NP-specific antibodies. *Asns*$^{WT/WT}$ *Asns*$^{WT/Tm1a}$ and *Asns*$^{Tm1a/Tm1a}$ were injected with NP-OVA emulsified in alum, a common vaccine adjuvant, on day 0 and boosted on day 35 to induce primary and memory responses, respectively. After 13 and 43 days, blood samples were collected to quantify hapten-specific IgM and IgG levels in serum. IgM and IgG antibodies were detected on day 13 but, surprisingly, all mice displayed comparable levels regardless of their genotype (Fig. 6.17). Consistently, IgM and IgG secretion did not seem to be altered by the lack of ASNS when analysed on day 43 (Fig. 6.17). Thus, these results suggest that the amounts of Asn in SLOs and the BM are sufficient to sustain antibody secretion in spite of ASNS-deficiency.

To be noted, all *in vivo* work (i.e. NP-OVA injections and collection of blood samples) was performed by Rebecca J. Brownlie (University of Leeds).

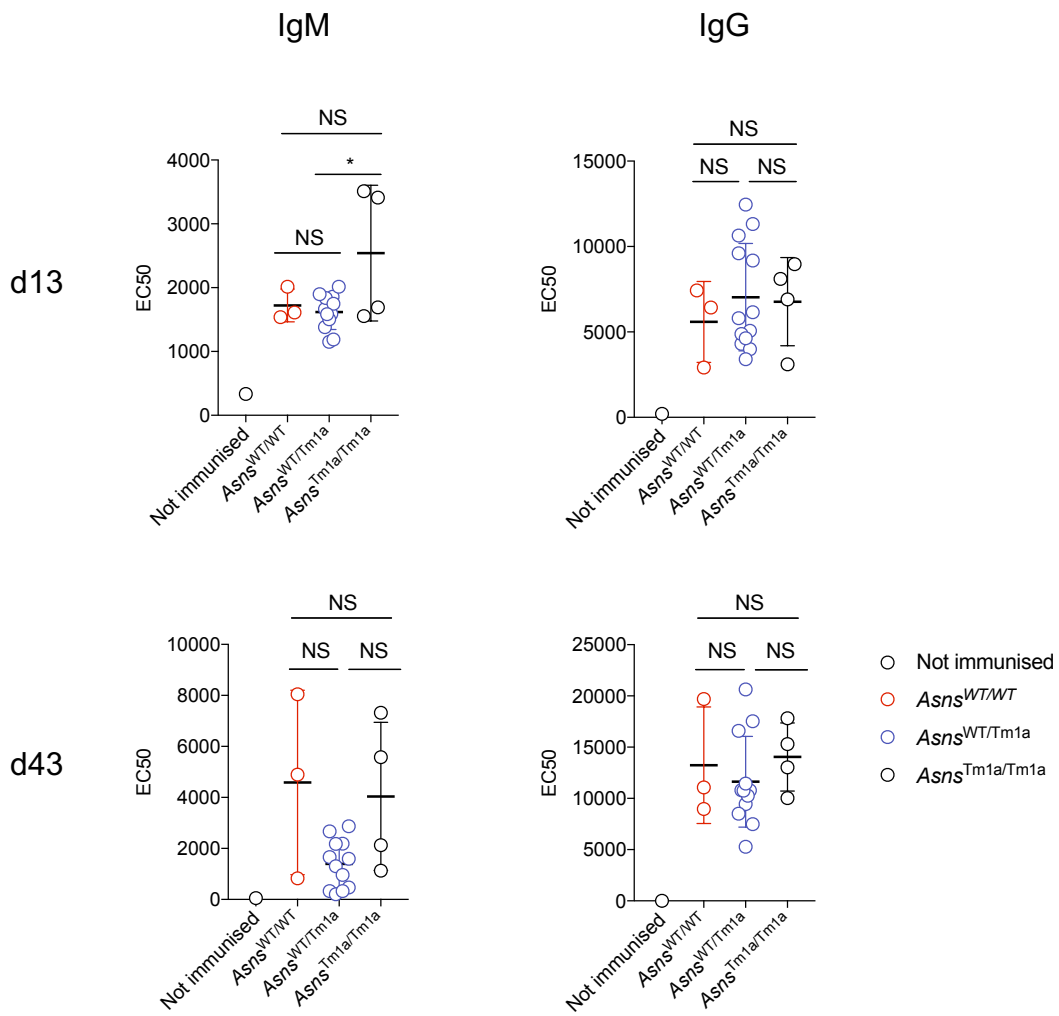


Figure 6.17: Secretion of IgM and IgG upon NP-OVA immunisation is not affected in ASNS-deficient mice.

Asns$^{WT/WT}$ (n=3), *Asns*$^{WT/Tm1a}$ (n=12) and *Asns*$^{Tm1a/Tm1a}$ (n=4) littermate mice were injected i.p. with NP-OVA in alum on day 0 and 35. Mice were bled on day 13 and 43. Serum IgM (left) and IgG (right) levels on day 13 (top) or 43 (bottom) were quantified using NP-specific ELISAs and EC50 values calculated by non-linear regression analysis. Dots represent biological replicates. NS – Not significant; * - P<0.05, as determined by one-way ANOVA with Tukey's multiple comparisons test.

6.3 Discussion

Antigen-induced T cell responses are driven by a metabolic reprogramming that sustains growth, clonal expansion and differentiation. One of the major events that occurs upon TCR-stimulation is an increase in nutrient uptake, including AAs. In this chapter, I have unravelled the importance of Asn uptake in the acquisition of a fully differentiated T cell effector phenotype. I have shown that Asn availability is required within the first stages of activation but, upon ASNS upregulation, $CD8^+$ T lymphocytes are able to respond independently of extracellular Asn levels.

Previous investigations reported that Asn deprivation generated by bacterial ASNases, which also have glutaminase activity, inhibits T cell activation (Kullas et al., 2012; Torres et al., 2016). My results endorse these studies and utilises a methodology that excludes ASNase-induced Gln-depletion as a possible suppressive mechanism in this scenario. Furthermore, my approach provides insight on the interplay between Gln and Asn to foster $CD8^+$ T cell responses. Studies performed in transformed cells have stablished that Asn plays an important role promoting survival when Gln is absent (Zhang, Ji et al., 2014). My data shows that, within the first 24h upon TCR-stimulation, the lack of both AAs is detrimental whilst Asn supplementation partially protects from the Gln-depletion induced cell death and vice versa, supporting this hypothesis also in T lymphocytes. Further, Gln enrichment is indispensable to initiate T cell activation in the absence of Asn indicating that Asn production via ASNS might be an important Gln contribution during T cell responses in addition to protein synthesis, anaplerosis or maintenance of redox balance (Kelly and Pearce, 2020).

Unlike previous reports, my data elucidates the importance of Asn availability beyond early T cell activation. I have shown that, as the antigen-induced T cell response progresses, $CD8^+$ T lymphocytes lose their requirement for

extracellular Asn. Indeed, T cells successfully differentiate into CTLs even when Asn is absent (Hope et al., 2020). In this context, I have provided evidence to confirm that ASNS upregulation is sufficient to maintain T cell activation, differentiation and effector functions. Although this suggests that extracellular Asn is not essential in $CD8^+$ T cell responses, it is important to consider that the lack of Asn might limit the magnitude of the response. The majority of viable T cells upon 24h of TCR-stimulation without Asn represent a non-blasting population that disappears at later timepoints. The lack of phenotypic differences observed after 72h between T cells cultured in nutrient-complete and Asn-deprived conditions could be interpreted in two ways: (1) the non-blasting population is initially negative for ASNS, which is subsequently upregulated leading to a delayed blastogenesis, or (2) the non-blasting population is incapable to promote ASNS expression and eventually prompts cell death. The latter would imply an early all-or-none response where only the small blasting population observed at 24h in Asn-deprived conditions (~30%) would ultimately become CTLs. Considering that 50% of the cells have undergone apoptosis at this timepoint, just ~15% of the whole initial population would be able to actually respond, indicating that the lack of extracellular Asn diminishes the probability of a T cell to get activated. To elucidate the fate of the non-blasting population it will be of interest to sort these cells and further study their potential phenotype, i.e., whether they undergo differentiation, cell death or both. It is important to highlight that these hypotheses are under the assumption that non-blasting cells do not express ASNS. I believe that the lack of blastogenesis is associated to a protein synthesis deficit that can only be rescued when ASNS is upregulated. The rationale behind this interpretation relies on our experiments performed with *Asns*^{Tm1a} mice which have demonstrated that the lack of ASNS expression

prevents the formation of the blasting population in Asn-free media. However, investigating ASNS levels in non-blasting vs blasting cells upon sorting is still required for confirmation.

The impairment of T cell activation by Asn-depletion and its implications *in vivo* are unclear. It is well stablished that the magnitude of primary T cell responses is dictated by the amount and duration of antigen presentation by APCs within secondary lymphoid organs. Moreover, it has been suggested that the size of the naïve T cell population that responds to antigen is also an important factor (Jenkins and Moon, 2012). Hence, as fewer T cells respond under Asn-deprived conditions, it is possible that the absence of Asn represents a further limiting factor of T cell responses. Also, we have to contemplate that *in vivo* environments are less favourable than my reductionist *in vitro* conditions, which can entail an additional obstacle for successful T cell responses when Asn is absent. In fact, my results showed that T cell activation is even more suppressed in Asn-free media when stimulated with the lower-affinity peptide OVA-T4. Thus, Asn availability might represent an extra piece into the complex inner workings that determines the extent of $CD8^+$ T cell responses.

Nonetheless, it is undescribed at the moment physiological situations in which the Asn levels drop within secondary lymphoid organs. Only the action of ASNases, produced either by bacterial pathogens or following parenteral administration, reduce the concentration of Asn in blood (Bunpo et al., 2008; Kullas et al., 2012; Knott et al., 2018). Both scenarios have been associated with dysfunctional immune responses (Kafkewitz and Bendich, 1983; Kullas et al., 2012; Hijiya and van der Sluis, 2016; Song et al., 2017). However, no evidence directly links immunosuppression with the inhibition of T cell activation within lymph nodes or spleens. Instead, other stages within the immune response, such

as the development of precursors in the bone marrow, could be altered by ASNases (Friedman, 1971; Bunpo et al., 2008; Hijiya and van der Sluis, 2016). Furthermore, it is likely that the intrinsic glutaminase activity of ASNases significantly contributes to its deleterious effects (Durden and Distasio, 1981; Kafkewitz and Bendich, 1983; Bunpo et al., 2008; Nguyen et al., 2018) and, therefore, whether Asn-depletion induces immunosuppression is still under debate. The development of glutaminase-free ASNases will elucidate the impact of Asn restriction in immune responses.

Cancer cells reprogram their metabolism to maintain aberrant proliferation (Pavlova and Thompson, 2016). Due to elevated metabolic activity, many cancer cells are auxotrophs for nutrients that are often otherwise non-essential and, therefore, the modification of nutrient availability has been proposed as a promising target for cancer therapy (Lukey et al., 2017; Kanarek et al., 2020). A key inconvenience of this approach is the side effects that nutrient deprivation might cause in healthy cells and, particularly, to the anti-tumour immune response (Chang et al., 2015; Kedia-Mehta and Finlay, 2019). Targeting Asn levels has not only been of interest in ALL or other ASNS-low tumours (Chiu et al., 2020). Several studies strongly sustain that asparagine synthesis is a key feature of aggressive tumours. ASNS expression is regulated by two of the most common mutated genes in cancer: p53 and KRAS (Gwinn et al., 2018; Deng et al., 2020). The former directly suppresses ASNS expression entering a reciprocal regulatory loop where p53 activation is sustained by a mechanism involving LKB1/AMPK. p53-null cells display increased ASNS expression and Asn synthesis which importantly contributes to proliferation while protecting from senescence (Deng et al., 2020). The latter induces ATF4 expression via Akt which directly upregulates ASNS driving to Asn accumulation, mTORC1 activation and

proliferation (Gwinn et al., 2018). Furthermore, Asn availability has been described as a crucial driver of EMT transition in breast cancer models (Knott et al., 2018). Altogether, these findings indicate that ASNS expression and subsequent Asn synthesis importantly contribute to tumour growth and metastasis. ASNS upregulation provides to tumour cells a key mechanism of metabolic adaptation to a nutrient-depleted environments derived by the lack of vascularisation (DeBerardinis et al., 2008; Zhang, Ji et al., 2014; Nakamura et al., 2018; Pathria et al., 2019). Thus, interfering with Asn synthesis, either by the utilisation of ASNases or seeking for ASNS inhibitors (Zhu, W. et al., 2019), might yield to a promising therapeutic strategy in cancer. Importantly, host cells for some viruses or bacteria rely on Asn metabolism and, therefore, the application of ASNases has also been proposed in these contexts (Ren et al., 2018; Lee et al., 2019). My results indicate that neither approaches are likely to disrupt the function of CTLs within the TME/site of infection, thereby combating tumour cells/pathogens without negatively affecting $CD8^+$ T cell responses. Furthermore, as a result of the interplay between Asn and Gln in tumour progression (Zhang, Ji et al., 2014), recent reports have pointed out their dual depletion as one of the strategies with better prospects (Kanarek et al., 2020). Nonetheless, my findings indicate that restricting both AAs might be catastrophic for T cell responses as the lack of Asn and Gln totally dampens T cell activation and differentiation. Thus, further investigations are required in order to determine whether depletion of Asn and Gln or Asn alone is more beneficial when taking into account the implications for both tumour growth and anti-tumour immune responses.

Understanding the mechanisms by which ASNS is regulated in T lymphocytes will provide insight into novel ways of manipulating T cell responses. Our findings indicate that ASNS is highly upregulated after 24h upon TCR-stimulation and,

consistently with previous reports (Howden et al., 2019), that its expression is highly sensitive to rapamycin. ASNS regulation has been mainly attributed to ATF4 but this has been mostly studied in the context of stress responses. In $CD4^+$ T lymphocytes, the increase in *Asns* mRNA levels upon 24h of TCR-binding disappears when knocking down *Atf4* even in non-stress conditions suggesting that ASNS regulation, at least during early stages of T cell activation, is exclusively under the control of ATF4 (Yang et al., 2018). In transformed cells, ATF4 regulation has been associated to Akt (Gwinn et al., 2018) and the mTORC1 substrate 4E-BP (Park et al., 2017). I speculate that, in $CD8^+$ T cells, a similar mechanism involving PI3K/Akt/mTOR activation might be sufficient to upregulate ASNS in an ATF4-dependent fashion upon TCR-stimulation. On the other hand, ATF4 is scarcely expressed in activated $CD8^+$ T cells suggesting that other mechanisms might be implicated. ASNS upregulation is altered in activated T cells lacking Myc (Marchingo et al., 2020) or upon treatment with Myc inhibitors (see Fig. 6.1). These findings point out at Myc as a possible transcription factor associated to ASNS expression after TCR-stimulation. However, Myc-deficiency also dampens the upregulation of AA transporters (Marchingo et al., 2020), a phenomenon that could severely impact mTORC1 activity and, therefore, whether Myc regulates ASNS directly or indirectly is still unclear.

I have demonstrated that Asn availability is crucial to initiate protein synthesis but whether it has ancillary functions is unknown. Other investigations performed in transformed cells have identified a role for Asn as signalling molecule where directly binds LKB1 to modulate AMPK activity (Deng et al., 2020). Furthermore, a report by Krall et al. (2016) revealed that Asn controls mTOR activation acting as an exchange factor for other AAs such as leucine or Gln. Our data shows that the absence of extracellular Asn limits mTOR activation (Hope et al., 2020)

suggesting that Asn might have a comparable function also in T lymphocytes. However, T cells stimulated in the absence of Asn display a decreased expression of nutrient transporters and, therefore, whether the loss of mTOR activation is associated with a deficient AA-exchange or to a general disruption of AA and glucose uptake is inconclusive. Under the assumption that Asn fine-tunes mTOR activity and considering that memory T cell differentiation is favoured when mTOR is attenuated (Nabe et al., 2018; Hope and Salmond, 2019), it will be of interest to study how the restriction of extracellular Asn or the limitation of Asn biosynthesis (e.g. *Asns*$^{Tm1a/WT}$ T cells) influences memory formation and the acquisition of long-term responses. In fact, CTLs grown in the presence of IL-15, key driver of memory phenotypes, display reduced ASNS expression (http://www.immpres.co.uk) further reinforcing a link between ASNS, mTOR and memory.

Another important question is whether ASNS upregulation supports T cell function exclusively through Asn synthesis. A number of metabolic enzymes have been associated with signalling roles above and beyond their metabolic functions, that are key to control T cell activation and effector function (Shyer et al., 2020). No reports have related ASNS with extra purposes other than Asn synthesis. However, it is important to consider that the levels of other AAs, such as Glu and Asp, are affected by the ASNS-mediated reaction. Nonetheless, our results clearly sustain that Asn replenishment is sufficient to recover $CD8^+$ T cell function in the *Asns*$^{Tm1a/Tm1a}$ model denoting that ASNS is not the only and major source of those AAs and that its contributions to T cell function are mainly a side effect to the ASNS-reaction.

Lastly, I have also explored how the lack of ASNS and/or Asn availability influences the function of other immune cells. In B lymphocytes, the loss of ASNS

does not affect antibody secretion upon *in vivo* immunisation. Plasma cells strongly upregulate ASNS expression in normal conditions suggesting that Asn requirements for protein synthesis are elevated (http://www.immgen.org). However, the findings described here indicate that exogenous Asn levels are plenty to sustain B cell function *in vivo*. An alternative hypothesis is that even <10% of ASNS expression in $Asns^{Tm1a/Tm1a}$ mice is able to synthesise enough Asn to maintain B cell function. The lack of a suitable ASNS inhibitor or a genetic model that completely abrogates *Asns* expression limits our further study and understanding regarding the significance of ASNS contribution to the total Asn pool. Furthermore, some preliminary analysis (data not shown) suggest that T_h1 cells, which do not upregulate as high amounts of ASNS when compared to CTLs (http://www.immpres.co.uk), are more sensitive to extracellular depletion of Asn. Nevertheless, at the moment, only $Asns^{Tm1a/WT}$ or $Asns^{Tm1a/Tm1a}$ T cells have been used to assess the impact of Asn-deprivation in T_h1 lymphocytes. Whether complete ASNS upregulation in $Asns^{WT/WT}$ T_h1s is sufficient to counterbalance the lack of an extracellular Asn source is unknown.

Altogether, these results indicate that the relevance of Asn supplementation is cell specific, seemingly to be dependent on the capacity of the cell to upregulate ASNS expression. The development of cell-type-specific ASNS KOs will allow the further study of the role of Asn and ASNS in T lymphocytes and other immune cell subsets *in vivo* and, ultimately, will provide valuable insight on our current understanding of the implications of nutrient availability in the regulation of immune responses.

Chapter 7 Discussion

7.1 Concluding remarks

The aim of this PhD project was to better understand how environmental cues shape $CD8^+$ T cell function and metabolism. So, what can we conclude from this research?

First, I have shown that signals 1, 2 and 3 contribute to the full activation of T lymphocytes through the regulation of distinct functions and metabolic pathways. Thus, I have reinforced the idea that, whilst signal 1 is required to trigger an antigen specific response, signals 2 and 3 also cooperate to promote T cell expansion and differentiation. Metabolically, this seems to be reflected in the magnitude or the type of metabolism that is engaged (e.g. glycolytic vs mitochondrial in IL-12- or IFNα-treated T cells, respectively), further emphasising that T cell activity and metabolism are tightly linked (Fig. 7.1A). Second, I have demonstrated that the action of the anti-inflammatory cytokine TGFβ represses $CD8^+$ T cell activation through transcriptional regulation of metabolic pathways. Specifically, I have shown that TGFβ significantly inhibits Myc expression and its induced metabolic reprogramming. Consequently, TGFβ-treated T cells present an unsuccessful engagement of glycolysis added to a deficient Slc7a5-mediated amino acid uptake that results in a poor translational activity that ultimately leads to the inhibition of T cell activation (Fig. 7.1B). Lastly, I have proven that $CD8^+$ T lymphocytes require extracellular Asn within the first stages upon TCR-stimulation, but that it becomes dispensable when ASNS is upregulated (Fig. 7.1C).

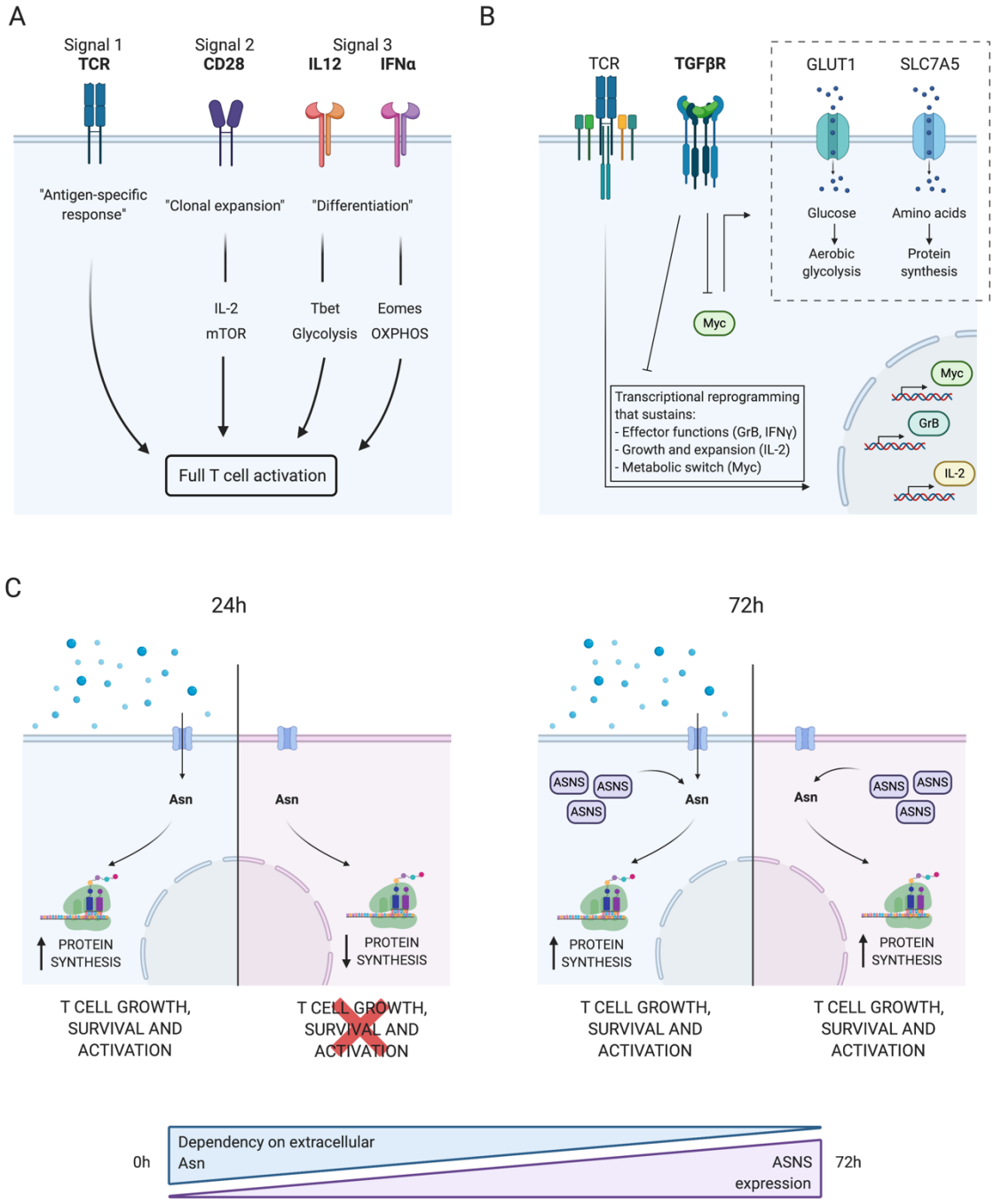


Figure 7.1: Graphical summary of the findings of this thesis.

Here, some of the major findings described in this thesis are shown. In (A), the influence of signals 1, 2 and 3 (Chapter 3). In (B), the impact of the anti-inflammatory cytokine TGFβ (Chapters 4 and 5). In (C), the effect of Asn-deprivation (Chapter 6).

7.2 'Alright, so what is next?'

While doing my PhD, I used to frequently meet with my main supervisor, Dr. Salmond, to show him my latest results. After discussing my data, he would always conclude the meeting with a brief silence followed by an 'alright, so what is next?', and I cannot finish this thesis without asking myself again this same question.

The findings exposed here have shed light into novel mechanisms that improve our understanding on the regulation of $CD8^+$ T cell function, which is key to comprehend immune associated diseases and find strategies to combat them. Only focusing on the elements investigated within this thesis, we can find several prospective questions that serve as an example of the value of this research and how could be exploited. For example: as T cell adaptation to Asn-deprivation is reduced against weak peptides, would T cells with an enhanced TCR signalling strength (e.g. *Ptpn22*$^{-/-}$ T cells) provide better responses in this type of environment? As TGFβ represses *Asns* expression, would CTLs be sensitive to the lack of extracellular Asn in the presence of TGFβ (e.g. in the TME)? As TGFβ is more suppressive against low affinity peptides, would the manipulation of the TCR signalling offer a suitable approach to counteract the TGFβ-mediated T cell suppression?

In spite of the contributions of this research, it is important to take into account that I have primarily focused on the study of $CD8^+$ T cells in the context of T cell priming. However, $CD8^+$ T cells navigate through multiple stages (e.g. naïve vs effector vs memory) and locations (e.g. SLOs vs sites of infection or TME) that might change the responsiveness to the certain signals. Moreover, we should also think outside the box (in this case, our model or approach) and acknowledge

that the studied signals could interfere with other cells *in vivo*, ultimately affecting to the final response of $CD8^+$ T cells. So, how would these signals affect other T cell subsets or immune cells? Are these findings comparable in other cell types? Would these effects on other cell types consequently modify $CD8^+$ T cell responses? Investigating how these environmental cues act on other immune cells and/or contexts comprise some of the loose ends that need to be tied in the near future in order gain a systemic perspective of their impact on the regulation of immune responses.

Continuing with the previous point, I should highlight that the findings described here are almost exclusively based on reductionist *in vitro* strategies. Following the rationale of this thesis, T cell responses are determined by the sum of integrated inputs, suggesting, therefore, that the influence of the studied signals might vary to some extent in an *in vivo* scenario. Furthermore, a recent report by Ma et al. (2019) found that the metabolic profile of $CD8^+$ T cells activated *in vivo* is slightly different to what has been previously established *in vitro*: whereas T cells activated *in vitro* shift from OXPHOS towards glycolysis, *in vivo* activated T cells are highly dependent on oxidative metabolism and SGOC metabolism. These statements highlight the importance of validating our findings *in vivo* in future research. The utilisation of recently developed technologies oriented to the study of the metabolic state of immune cells *in vivo* will provide further, more accurate and more detailed comprehension on the metabolic behaviour of T cells under the influence of the studied signals (Artyomov and Van den Bossche, 2020). These include, among others, the use of stable isotopes to track nutrient consumption and the engagement of metabolic pathways (Ma et al., 2019) or the performance of 'omic analysis of immune cells sorted *ex vivo* including metabolomics, transcriptomics and proteomics (e.g. mass spectrometry or RNA-

seq), both in a population and single-cell level (Artyomov and Van den Bossche, 2020; Hartmann et al., 2020; Argüello et al., 2020). Furthermore, we should also contemplate that our *in vitro* approaches and metabolic assays have been somewhat limited and some might present some weak spots. For example, Sinclair et al. (2020) recently reported that the utilisation of 2NBDG as a readout of glucose uptake is not a reliable tool in murine T cells indicating that the experiments carried out here using this fluorescent glucose analogue should be interpreted with caution.

In conclusion, the field of immunometabolism has evolved tremendously during the last decade. The realisation that immune regulation and metabolism are inextricably tied has given rise to a wave of studies exploring the metabolism of immune cells in health and disease which has led to the identification of new regulatory mechanisms and therapeutic targets. In fact, some metabolic-targeted drugs (e.g. dimethyl fumarate or methotrexate) are already in the market for the treatment of some autoimmune diseases, indicating the bench-to-bedside potential of this area of study. Moreover, although in this thesis I have focused on the research of metabolism in the context of immunity, emerging investigations situate metabolism at the spotlight as a hub connecting multiple disciplines including cancer, cellular development or aging. All seems to point at metabolism as the path to follow to build a solid strategy to modulate cell function to our liking. Further research on both cell-intrinsic and systemic metabolism and its connection to cell function (or dysfunction) will provide invaluable new knowledge on our understanding of the regulation of cellular processes in health and disease. I believe that the findings presented here will contribute and become the basis of future research focused on the translation of this knowledge to the development of new therapeutic strategies.

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