The Molecular Mechanisms of Cytokine-mediated Activation of Human CD8$^+$ T Cells

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Contents

Abstract

Background: $CD8^+$ T cells can be activated through both TCR-dependent and TCR-independent pathways, the former response was shown to be largely regulated by mTORC2 over mTORC1. This raises the question of whether TCR-independent $CD8^+$ T cell activation also involves the mTOR pathway and if so, which complex dominates. Additionally, because mTORC1 has been shown to regulate IRE1-α during ER stress, this study aims to explore whether the mTORC1-IRE1α pathway contributes to cytokine-induced $CD8^+$ T cell activation, especially in $NKG2A^+$ T cells and MAIT cells, known to be hyperresponsive to IL-12 and IL-18.

Methods: Human primary peripheral blood mononuclear cells (PBMCs), purified $CD8^+$ T cells and total T cells were used in this study. Small guide RNA and Cas9 protein or chemical inhibitors were used to study the function of proteins including mTORC1, mTORC2 and IRE1α. The knockout efficiency was assessed by western blotting. The activity of mTOR complexes was measured by phospho-flow cytometry (Phosflow). The expression of IFN-γ in $CD8^+$ T cells or total T cells were measured by enzyme-linked immunosorbent assay (ELISA) or flow cytometry. The phenotype and the activation of $CD8^+$ or $CD3^+$ subsets were assessed by flow cytometry.

Results: Chemical inhibition of mTORC1 had a more pronounced downregulation effect on cytokine-induced IFN-γ production than mTORC2 in total $CD8^+$ T cells. Both chemical inhibition and genetic knockout of IRE1α potently supressed XBP1s expression and reduced the production of cytokine-induced IFN-γ in total $CD8^+$ T cells. Regulation of cytokine-mediated activation by mTOR and IRE1α was further confirmed in CD8$^+$NKG2A$^+$CD161$^+$ T and MAIT cells. Despite the shared feature of hyperresponsiveness to IL-12 and IL-18 by CD8$^+$NKG2A$^+$CD161$^+$ T and MAIT cells, the former are less sensitive to glycolysis inhibition.

Conclusions: In contrast to TCR-dependent activation, cytokine-induced CD8$^+$ T cell activation is dependent more on mTORC1 than mTORC2 signalling. The regulation of cytokine-mediated activation by mTOR was partially dependent on IREα-XBP1s pathway. Additionally, NKG2A$^+$CD161$^+$ T and MAIT cells exhibit similar functional characteristics in response to cytokine stimulation but differed in energy utilisation.

List of Acronyms

Abbreviation	**Definition**
4E-BP1	4E-binding protein 1
AA-	Amino acids-free/low
AA+	Amino acids-supplemented/sufficient
ACACB	acetyl-CoA carboxylase beta
ACC1	Acetyl-coA-carboxylase 1
AKT	Protein kinase B
AMPK	AMP-activated protein kinase
AP-1	Activator protein 1
APC	Antigen-presenting cell
AR	Androgen receptor
ATAC-seq	Assay for Transposase-Accessible Chromatin using sequencing
ATF6	Activating transcription factor-6
Bcl-6	B-cell lymphoma 6
Bim	Bcl-2 interacting mediator of cell death
Blimp-1	B lymphocyte-induced maturation protein-1
BMDC	Bone marrow-derived dendritic cell
bZIP	Basic domain/leucine zipper
c-Jun	Jun proto-oncogene
CARMA1	Caspase recruitment domain-containing membrane-associated guanylate kinase protein 1
CRISPR	Clustered regularly interspaced short palindromic repeats
DAG	Diacylglycerol
DGAT2	diacylglycerol O-acyltransferase 2
DMSO	Dimethyl sulfoxide
EAA	Essential amino acid
ECAR	Elevated extracellular acidification rate
ECAR	Extracellular acidification rate
eIF2α	Eukaryotic translation initiation factor 2 alpha subunit
ELISA	Enzyme-linked immunosorbent assay
EOMES	Eomesodermin
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum-associated degradation
ERQC	endoplasmic reticulum quality control
ERSE	ER stress response elements
FAO	Fatty acid oxidation
FBS	Fetal bovine serum
FoxO1	Forkhead box protein O1
Foxp3	Forkhead box P3
GTP	Guanosine-5'-triphosphate
GTPase	Guanosine triphosphate
HLA-DR	Human leukocyte antigen-DR
Hrd1	Hydroxymethylglutaryl-CoA reductase degradation protein 1

IFN-γ	Interferon gamma
IGF-1	Insulin-like growth factor 1
IL-12	Interleukin-12
IL-12Rβ1/β2	IL-12 receptor beta1/beta2
IL-18Rα	IL-18 receptor alpha
IL-18Rβ	IL-18 receptor beta
iNKT	Invariant natural killer T
IRE1α	Inositol-requiring protein 1α
IRF4	Interferon regulatory factor 4
ITAMs	Immunoreceptor tyrosine-based activation motifs
JNK	c-Jun N-terminal kinases
KLRG1	Killer cell lectin-like receptor subfamily G member 1
LAR	Luminal androgen receptor
LAT	Linker for activation of T-cells
Lck	Lymphocyte-specific protein tyrosine kinase
LCMV	Lymphocytic choriomeningitis virus
LKB1	Liver kinase B1
MAIT	Mucosal-associated invariant T
MALT1	Mucosa-associated lymphoid tissue lymphoma translocation protein 1
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MKC	MKC8866
MPEC	Memory precursor effector cell
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
MYC	Myelocytomatosis oncogene
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NKG2A	Natural Killer cell Group 2 Member A
OCR	Oxygen consumption rate
OXPHOS	Oxygen phosphorylation
p-S6	Phosphorylated S6 ribosomal protein
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PD-L1	Programmed death-ligand 1
PERK	Protein kinase RNA-like ER kinase
PI3K	Phosphoinositide 3-kinases
PIKK	Phosphatidylinositol kinase-related kinase
PIP2	Phosphatidylinositol-4,5-bisphosphate
PIP$_3$	Phosphatidylinositol (3,4,5)-trisphosphate
PKC	Protein kinase C
PKCθ	Protein kinase C theta
PLCγ1	Phospholipase C gamma 1
PLZF	Promyelocytic leukemia zinc finger
qRT-PCR	Quantitative polymerase chain reaction
RA	Rheumatoid arthritis

Raptor	Regulatory-associated protein of mTOR
Rheb	Ras homolog enriched in brain
Rictor	Rapamycin-insensitive companion of mTOR
RIDD	Regulated IRE1-dependent mRNA decay
S6K1	S6 kinase 1
scRNA-seq	Single-cell transcriptomics
SGK1	Serum- and Glucocorticoid-regulated Kinase 1
SLC7A5	Solute carrier family 7 member 5
SLE	Systemic lupus erythematosus
SLEC	Short-lived effector cell
SLP76	SH2 domain-containing leukocyte phosphoprotein of 76 kD
SP	Sodium pyruvate
SREBP	Sterol regulatory element-binding protein
STAT4	Signal transducer and activator of transcription 4
T-bet	T-box expressed in T cells
TAB	T cell activation beads
TCR	T cell receptor
Tfh	Follicular helper T
TGF-β	Transforming growth factor beta
TNBC	Triple-negative breast cancer
Treg	Regulatory T cell
TRM	Tissue-resident memory T
TSC1/2	Tuberous sclerosis proteins 1/2
ULK1	Unc-51 like autophagy activating kinase 1
UPR	Unfolded protein response
Vam6	Vesicle associated membrane Protein 6
XBP1	X-box binding protein 1
XBP1s	Spliced XBP1
ZAP-70	Zeta-chain-associated protein kinase 70

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Chapter 1 Introduction

1.1 The diversity of T cell activation modes

T cells were first identified in the early 1960s through Miller's neonatal thymectomy experiments in mice, which demonstrated that the thymus is essential for immune competence (Miller 1962, Miller 1962). T cells are a type of lymphocytes that develop from bone-marrow precursors and then migrate to the thymus, where they mature (Kumar, Connors et al. 2018). They are a core component of the adaptive immune system, capable of specifically recognising and eliminating foreign antigens, while also playing a crucial role in inflammation regulation and tissue damage repair (Kumar, Connors et al. 2018). Traditionally, it was believed that T cell activation primarily depends on the recognition of antigenic peptides by the T cell receptor (TCR) in conjunction with the major histocompatibility complex (MHC), with co-stimulatory signals and cytokine signals synergistically inducing their proliferation, differentiation, and effector functions (Smith-Garvin, Koretzky et al. 2009). However, recent studies have revealed that specific cytokines such as Interleukin-12 (IL-12) and IL-18 can directly activate T cells, particularly memory CD8$^+$ T cells and innate-like T cells, to rapidly produce effector molecules such as interferon-gamma (IFN-γ) without antigen stimulation (Tominaga, Yoshimoto et al. 2000, Raué, Beadling et al. 2013, Ussher, Bilton et al. 2014, Schilbach, Welker et al. 2020, Garner, Amini et al. 2023).

This antigen-independent activation mechanism challenges the traditional understanding that T cells initiate immune responses only through TCR recognition of foreign antigens, suggesting that T cells can mediate rapid responses in innate immune contexts, particularly in scenarios such as early infection, tumour microenvironments, or barrier tissues (Whiteside, Snook et al. 2018). Additionally, this process typically does not depend on cell proliferation or transcriptional reprogramming but rather relies on rapidly available translational mechanisms and the activation of specific signalling pathways, such as mTOR signals, to achieve rapid synthesis of effector molecules (Munk, Sugiyama et al. 2011, Chi 2012).

Therefore, understanding the response mechanisms of T cells under different types of stimulation, especially the differences between classical antigen-dependent activation and cytokine-induced activation, can help reveal the diversity of T cell functions. This also helps us better understand the interactions between adaptive immunity and innate immunity and provides a theoretical basis for regulating T cell functions.

1.1.1 Adaptive immunity and TCR-dependent activation

Adaptive immunity is a crucial defence mechanism in vertebrates against specific pathogens, characterised by antigen specificity, clonal expansion, and the establishment of immune memory (Bonilla and Oettgen 2010). T cells, particularly CD8$^+$ T cells, play a central role in adaptive immunity by recognising and eliminating virus-infected cells or tumour cells, making them key effector cells in cytotoxic immunity (Sigal 2016, Raskov, Orhan et al. 2021, Meier, Satpathy et al. 2022).

The classical activation process of CD8$^+$ T cells begins with the TCR recognising peptides presented by antigen-presenting cells (APCs) on their surface in conjunction with MHC class I molecules. Effective activation typically also requires co-stimulatory signals (such as the binding of CD28 to B7-1/B7-2) and immune cytokines to jointly trigger a series of intracellular signal transduction pathways, thereby driving T cell clonal expansion, phenotypic changes, and functional differentiation (Smith-Garvin, Koretzky et al. 2009). Following activation, $CD8^+$ T cells begin to express and release key effector molecules, such as IFN-γ, granzyme B, and perforin, to exert direct cytotoxic effects (Zhang and Bevan 2011).

It has been shown that TCR signals not only determine whether T cells are activated but also regulate their subsequent fate decisions, including differentiation into short-lived effector cells (SLECs) or long-lived memory precursor effector cells (MPECs) based on differences in the expression of IL-7R and KLRG-1. High levels of the transcription factor T-bet promote SLEC differentiation, while low T-bet favours the MPEC fate, and this fate selection is regulated by the intensity of antigen stimulation and the concentration of inflammatory cytokines (Joshi, Cui et al. 2007).

This differentiation process is regulated by the integration of multiple signals, including the intensity and duration of antigen stimulation, co-stimulation intensity, cytokine environment, and precise regulation of intracellular transcription factors. Key transcription factors such as T-box expressed in T cells (T-bet), eomesodermin (EOMES), B lymphocyte-induced maturation protein-1 (Blimp-1), and B-cell lymphoma 6 (Bcl-6) determine the development of T cells toward effector or memory types at different stages (Hamilton and Jameson 2007, Kaech and Cui 2012, Chang, Wherry et al. 2014).

Additionally, TCR activation induces T cells to rapidly enter the cell cycle, enhances metabolic activity, and upregulates a series of functionally relevant gene expression programmes. Unlike the metabolic characteristics of resting cells, which primarily rely on oxidative phosphorylation, activated T cells exhibit a significant increase in glucose and amino acid uptake and initiate aerobic glycolysis and biosynthetic metabolic pathways, providing the necessary energy and raw materials for cell proliferation and effector functions (Pearce, Poffenberger et al. 2013). Although these metabolic changes are downstream effects of the activation process, increasing evidence in recent years suggests that they also reciprocally regulate T cell fate and function, making them an indispensable component of immune regulation.

In summary, TCR-dependent activation of CD8$^+$ T cells is not just an initiation mechanism for antigen responses but a complex process involving highly integrated signalling, metabolism, and gene expression programmes. Understanding the key characteristics of this classical activation pathway provides an important theoretical foundation for further exploring whether T cell activation under ‘non-classical’ stimuli (such as cytokine-mediated) follows the same principles and whether there are fundamental differences in their metabolic and signalling regulatory mechanisms.

1.1.2 Innate immunity and cytokine-induced T cell activation

Beyond classical TCR-dependent activation, cytokines such as IL-18 can trigger rapid effector responses in T cells independently of antigen recognition, a process often referred to as ‘innate-like’ activation. A well-studied mechanism is the synergistic stimulation of IL-12 and IL-18, which can rapidly induce CD8$^+$ T cells to secrete large amounts of IFN-γ even in the absence of TCR signals (Tominaga, Yoshimoto et al. 2000, Ussher, Bilton et al. 2014).

It has also been demonstrated that human mucosal-associated invariant T (MAIT) cells can be activated not only through TCR stimulation but also through inflammatory cytokines such as IL-12 and IL-18, enabling them to rapidly produce IFN-γ and other effector molecules even in the absence of antigen-specific recognition (Leng, Akther et al. 2019). This innate-like activation mechanism enables MAIT cells to rapidly respond to inflammatory signals and participate in tissue defence and repair before the adaptive immune response is fully activated.

Based on these findings and related studies, it is currently widely recognised that such TCR-independent effector responses typically occur during the early stages of infection or inflammation. At this point, antigen presentation has not yet been completed. These rapid responses help secure a critical time window for the initiation of the adaptive immune response.

Such responses are not uniformly present in all T cells but are enriched in specific subpopulations with innate characteristics, such as MAIT cells, γδ T cells, and CD8$^+$ T cells expressing CD161 or Natural Killer cell Group 2 Member A (NKG2A) (Ussher, Bilton et al. 2014, Kurioka, Jahun et al. 2017, Kurioka, Klenerman et al. 2018, Choi, Koh et al. 2023). CD8$^+$ NKG2A$^+$

T cells are distinctive innate cells that express high levels of IL-12 receptor beta1/beta2 (IL-12Rβ1/β2) and IL-18 receptor beta (IL-18Rβ), thus they are particularly sensitive to stimulation by IL-12 and IL-18. When stimulated by these cytokines, $CD8^+$ $NKG2A^+$ T cells rapidly produce IFN-γ in a TCR-independent manner. In addition, these cells express promyelocytic leukemia zinc finger (PLZF), which programs an innate-like effector state by upregulating IL-12R and IL-18R and tissue homing molecules, enabling rapid TCR independent responses(Choi, Koh et al. 2023).

T cells with predominate innate function are typically found in mucosal and barrier tissues, exhibit high sensitivity to cytokines, and have a low activation threshold, enabling them to rapidly secrete effector cytokines such as IFN-γ upon pathogen invasion or tissue damage (Kurioka, Jahun et al. 2017, Leng, Akther et al. 2019). For example, MAIT cells which highly express both IL-18 and IL-12 receptors can undergo bystander activation when pathogen-associated molecular patterns (PAMPs) activate monocytes to release IL-12 and IL-18. Through this mechanism, MAIT cells can rapidly produce effector cytokines and participate in early inflammatory responses and pathogen clearance (Ussher, Bilton et al. 2014).

Compared to traditional T cells, innate-like T cells such as MAIT cells and γδ T cells can rapidly initiate effector responses through cytokine signals in inflammatory environments without undergoing classic antigen-specific activation (Van Kaer, Postoak et al. 2022). It has been acknowledged that MAIT cells and invariant natural killer T (iNKT) cells acquire the ability to express key molecules such as PLZF, T-bet, and IL-18R during their development. These molecules maintain their expression after maturation, enabling them to be highly sensitive to cytokine signals such as IL-12 and IL-18, thereby allowing them to rapidly initiate effector functions in the absence of TCR signals (Hackstein and Klenerman 2023). Similar with MAIT cells, CD8$^+$NKG2A$^+$ T cells retain PLZF expression in adulthood, which enhances IL-12 and IL-18 receptor expression and enhances their ability to activate innate-type cells. Unlike CD8$^+$ KIR$^+$ T cells, which responded preferentially to IL-15 and exhibited NK-type cytotoxicity, CD8$^+$NKG2A$^+$ T cells specialised in IL-12+IL-18-induced cytokine production (Choi, Koh et al. 2023).

Although no experiments have yet demonstrated that innate-like T cells exhibit high basal metabolic activity in a resting state, human MAIT cells have been shown to rapidly upregulate glycolytic flux upon stimulation with IL-12+IL-18 or TCR, and promote the production of effector molecules such as IFN-γ through mammalian target of rapamycin complex 1 (mTORC1) signalling without requiring clonal proliferation (O'Brien, Loftus et al. 2019). Therefore, in terms of signal transduction, metabolic requirements to functional output, cytokine-induced innate-like T cell activation represents an activation pattern distinct from the classical adaptive response driven by the TCR.

In summary, cytokines such as IL-12 and IL-18 can rapidly induce innate-like CD8$^+$ T cells, particularly MAIT cells, to produce effector molecules such as IFN-γ in the absence of TCR signals. These innate-like T cells are enriched in mucosal and barrier tissues and are highly sensitive to cytokine signals. Compared to classical TCR-dependent activation, this cytokine-driven activation modality exhibits significant differences in signal pathways, metabolic requirements, and functional outputs. A deeper understanding of this non-classical activation mode could help elucidate the essence of T cell functional diversity and provide new strategies for immune intervention in diseases such as infection, inflammation, and tumours.

1.1.3 IL-12+IL-18-mediated rapid effector function in the absence of TCR

IL-12 and IL-18 are two important pro-inflammatory cytokines that can synergistically induce CD8$^+$ T cells to rapidly produce IFN-γ in the absence of TCR signals. Animal models and in vitro studies have confirmed that following Listeria infection in mice, CD8$^+$ T cells from mice that have not been exposed to the antigen can produce IFN-γ within 16 hours. This response is dependent on IL-12 and IL-18 rather than TCR activation (Berg, Cordes et al. 2002). Additionally, this phenomenon has been shown to be particularly pronounced in the human CD8$^+$ CD161^{++} T cell subpopulation, which can rapidly exert its effects even in the absence of antigen recognition (Tominaga, Yoshimoto et al. 2000, Ussher, Bilton et al. 2014). This type of response is fast and does not require antigen engagement, suggesting that it may be involved in early antiviral and antitumour immune responses.

One of the key mechanisms by which IL-12 and IL-18 synergizes to induce rapid IFN-γ production in CD8$^+$ T cells involves the upregulation of IL-18 receptor alpha (IL-18Rα) expression through STAT4 activation. It has been demonstrated that IL-12 stimulation enhances IL-18Rα expression on T cells, thereby increasing their sensitivity to IL-18 and enabling robust effector responses even in the absence of TCR engagement(Yoshimoto, Takeda et al. 1998). In this context, IL-12 not only primes T cells by regulating receptor expression but also activates downstream transcriptional programs required for effector function.

At the transcriptional level, IL-12 and IL-18 synergistically enhance IFN-γ gene expression through a cooperative mechanism involving signal transducer and activator of transcription 4 (STAT4) and the Jun proto-oncogene/activator protein 1 (c-Jun/AP-1) complex. IL-12 stimulation activates STAT4 through tyrosine phosphorylation, while IL-18 induces serine phosphorylation of c-Jun via the c-Jun N-terminal kinases (JNK) signalling pathway. Although STAT4 itself does not directly bind to the IFN-γ promoter, it's demonstrated that STAT4 physically associates with phosphorylated c-Jun to form a functional protein complex. This STAT4-c-Jun complex enhances the deoxyribonucleic acid binding ability of c-Jun to the AP-1 site within the IFN-γ promoter region, leading to a significant increase in promoter activity (Nakahira, Ahn et al. 2002). This process represents a key mechanism for cytokine signal integration, in which IL-12 amplifies the transcriptional effect of IL-18 on AP-1-dependent gene activation. Therefore, the synergistic effect of IL-12/IL-18 bypasses the requirement for TCR-mediated activation to drive the production of large amounts of IFN-γ in T cells, providing a molecular basis for antigen-independent effector cell responses.

In addition, IL-12 signalling activates STAT4, which rapidly induces the expression of T-bet in CD8$^+$ T cells. Early upregulation of T-bet is a key determinant for the initiation of the type 1 CD8$^+$ T cell differentiation program, which not only promotes lineage commitment but also stabilizes IFN-γ expression by promoting chromatin accessibility at effector gene loci such as IFNG. T-bet binds directly to distal enhancer elements within the IFNG locus, thereby facilitating histone modifications and recruitment of chromatin remodelling complexes that enable efficient transcriptional activation of IFN-γ and other effector genes. In addition, T-bet represses alternative lineage-associated transcriptional programs, ensuring that $CD8^+$ T cells acquire appropriate effector functions while preventing aberrant differentiation into Th2- or Th17-like profiles (Prier, Li et al. 2019).

In parallel, interferon regulatory factor 4 (IRF4) plays a key role in maintaining $CD8^+$ T cell expansion and functional stability. IRF4 supports the energy and synthetic material requirements of effector cells by upregulating the expression of metabolic genes such as glucose transporter proteins, while suppressing the expression of proapoptotic factor Bcl-2 interacting mediator of cell death (Bim) and cell cycle inhibitory factors to promote cell proliferation and survival (Yao, Buzo et al. 2013). These mechanisms have been well validated under TCR-dependent activation conditions. However, whether IRF4 is involved in regulation in the same way in cytokine-induced activation remains to be further investigated. Nevertheless, the transcriptional regulatory framework comprising STAT4, T-bet, and IRF4 are capable of synergistically promote effector function acquisition in $CD8^+$ T cells in different activation settings.

From a metabolic perspective, IL-12 and IL-18 stimulation can lead to rapid activation of the mTORC1 pathway and enhancement of glycolytic flux in innate-like $CD8^+$ T cells such as MAIT cells. This metabolic shift provides the necessary energetic and synthetic support for the immediate production of effector molecules, including IFN-γ, without the need for clonal proliferation. Notably, the current studies are mainly based on an in vitro model of TCR signalling and IL-12+IL-18 co-stimulation, showing that MAIT cells exhibit a significant elevation of S6 protein phosphorylation (a marker of mTORC1 activity) and glycolysis rate, accompanied by an efficient expression of IFN-γ under these conditions (Tao, Pan et al. 2021). Thus, although IL-12/IL-18 signalling plays a key role in the regulation of metabolism and effector functions, its ability to independently drive similar responses in the complete absence of TCR signalling remains to be further verified. Compared with the classical antigen-dependent activation model, this rapid activation response based on the synergy between cytokines and TCRs is more suitable for the immediate immune response of innate T-cell subsets in barrier tissues.

In summary, the combined stimulation of IL-12 and IL-18 constitutes a rapid activation pathway that is fundamentally different from the classical TCR-driven mechanism. This mechanism does not require antigen recognition, instead driving IFN-γ release via STAT4, accompanied by T-bet upregulation and metabolic activation, particularly prominent in innate-like CD8$^+$ T cell subsets such as MAIT and CD161$^+$ cells. This pathway provides the body with a first line of defence when antigen presentation has not yet been fully established, playing a crucial physiological role in early antiviral, antitumour, and mucosal tissue protection.

1.2 mTOR signalling as a central metabolic and functional regulator in T cells

The mammalian target of rapamycin (mTOR) is a conserved serine/threonine kinase that serves as a key integrator of extracellular environmental changes, playing a critical role in fundamental biological processes such as nutrient sensing, energy metabolism, protein synthesis, and growth regulation (Panwar, Singh et al. 2023). In the immune response, TCR and CD28 signalling are responsible for initiating T cell activation. The mTOR pathway is a central hub that integrates signals from TCR/CD28, cytokines, nutritional status and energy signals. These signals determine metabolic reprogramming of T cells, such as glucose uptake, protein synthesis and mitochondrial production. mTOR also determines the lineage decision between effector, regulatory, or memory T cells. At the same time, it controls the effector function output of T cells (Huang, Long et al. 2020).

When mTORC1 activity is inhibited, such as through rapamycin treatment, T cells are unable to efficiently expand or produce effector cytokines, although they are still able to receive activation signals from the TCR and CD28 and upregulate IL-2 expression (Powell and Delgoffe 2010). In this situation, T cells are more likely to enter a low response state or differentiate into regulatory T cells in response to environmental factors such as transforming growth factor beta (TGF-β) (Delgoffe, Kole et al. 2009, Zeng, Yang et al. 2013). Studies have also shown that high mTORC1 activity promotes CD8$^+$ T cell differentiation toward effector phenotypes by enhancing glycolysis and anabolism (Pollizzi and Powell 2015). In contrast, with low mTORC1 activity, T cells are more inclined to form long-lived memory or regulatory lineages, a process that is co-regulated by cytokines and the metabolic environment (Araki, Turner et al. 2009, Sukumar, Liu et al. 2013).

Previous studies have primarily focused on the mechanism of mTORC1 activation under synergistic stimulation by TCRs and cytokines, but it is unclear whether cytokines can independently drive the complete activation and metabolic reprogramming of T cell mTORC1 in the absence of significant TCR involvement. This question is particularly critical in innate-like T cells such as $CD161^{+}CD8^{+}$ T cells, which are highly sensitive to cytokine signalling. Therefore, clarifying how IL-12 and IL-18 regulate mTORC1 activity in the absence of TCR signalling context is an important part of understanding the nonclassical activation pathways and rapid effector functions of T cells.

In the following sections, I will review the functional characteristics of mTORC1 and mTORC2, summarise their established roles in TCR-mediated activation and highlight new insights into the regulation of mTOR signalling in the context of cytokine-induced activation.

1.2.1 Key roles of mTORC1 and mTORC2 in T cell biology

mTOR plays an important signalling role in the regulation of T cell metabolism and function by constituting two structurally and functionally distinct complexes, mTORC1 and mTORC2. mTORC1 has a core subunit, regulatory-associated protein of mTOR (Raptor), whose activity is easily inhibited by rapamycin, while mTORC2 contains a key subunit, rapamycin-insensitive companion of mTOR (Rictor), which is not sensitive to rapamycin for a short period of time. This structural difference determines the significant difference between the two in terms of signal reception and functional output (Saxton and Sabatini 2017, Panwar, Singh et al. 2023).

mTORC1 is a complex centred around Raptor and is sensitive to rapamycin. Its primary function is to promote protein synthesis, nucleotide synthesis, and lipid biogenesis by phosphorylating downstream effectors S6 kinase 1 (S6K1) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), while also enhancing key metabolic pathways such as glycolysis and pyruvate metabolism (Laplante and Sabatini 2012). In T cells, the activation of mTORC1 marks the critical switch from a resting state to an activated and proliferative state. It promotes cell cycle entry and enhances clonal expansion capacity while supporting the expression of effector factors such as IL-2 and IFN-γ. Even when TCR and co-stimulatory signals are present, inhibition of mTORC1 disrupts this process (Delgoffe, Kole et al. 2009). Hence mTORC1 signalling pathway plays a crucial role in the initial expansion and acquisition of effector functions in T cells.

In contrast, mTORC2, with Rictor as its key subunit, is insensitive to acute rapamycin treatment. In the regulation of T cell differentiation and function, mTORC2 plays a key role that distinguishes it from mTORC1. It was shown that mTORC2 mediates the phosphorylation modification of forkhead box protein O1 (FoxO1) through activation of protein kinase B (AKT) signalling, prompting the translocation of FoxO1 from the nucleus to the cytoplasm, thereby inhibiting the expression of genes associated with memory programs, such as IL-7R and CD62L (Zeng, Cohen et al. 2016). Although this study revealed the function of the mTORC2-AKT-FoxO1 axis in the regulation of migration and differentiation mainly in CD4$^+$ follicular helper T (Tfh) cells, considering that FoxO1 is a key transcription factor in CD8$^+$ T cell fate determination, this mechanism is expected to play a similar role in the regulation of effector-memory homeostasis in CD8$^+$ T cells, which needs to be validated by further studies.

Serum- and Glucocorticoid-regulated Kinase 1 (SGK1) is one of the key downstream effector molecules of mTORC2, which promotes effector differentiation in CD8$^+$ T cells by phosphorylating FoxO1, prompting its export from the nucleus, and repressing memory-associated genes, such as IL-7R and Eomes. SGK1 deficiency enhances nuclear FoxO1 activity, favouring the formation of memory precursors and improving long-term cell survival, emphasizing the mTORC2-SGK1-FoxO1 axis as a key regulator of effector-memory fate balance in CD8$^+$ T cells (Patel, Heikamp et al. 2022).

Furthermore, it has been shown that mTORC2 is involved in cytoskeletal remodelling and maintenance of mitochondrial function through the regulation of AKT, SGK1 and protein kinase C (PKC) signalling pathways (Oh and Jacinto 2011). Despite the lack of direct evidence, mTORC2 has been suggested to potentially influence T cell functional adaptation in microenvironments such as nutrient deprivation or high inflammatory load by regulating lipid metabolism and oxidative stress response (Fu and Hall 2020).

Overall, the roles of mTORC1 and mTORC2 in regulating T cell metabolism and function are both independent and highly synergistic. mTORC1 is mainly involved in the initial activation of T cells, sensing nutrient and signalling inputs to drive metabolic reprogramming and effector expression. While mTORC2 is involved in lineage fate determination and maintenance of functional homeostasis through the regulation of AKT, SGK1 and FoxO1 signalling axes and plays a key role in lipid metabolism, oxidative stress response and cytoskeleton remodelling. This coordination mechanism enables T cells to flexibly adjust their response strength and functional attributes according to microenvironmental changes, ensuring the effectiveness and adaptability of immune defence.

1.2.2 The links between TCR-dependent activation and mTOR

TCR-mediated antigen recognition is a key event in the initiation of an adaptive immune response. After synergistically interacting with CD28 co-stimulatory signalling, the TCR induces the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) on the CD3 complex through tyrosine kinases, such as lymphocyte-specific protein tyrosine kinase (Lck) and zeta-chain-associated protein kinase 70 (ZAP-70), which leads to the formation of the linker for activation of T-cells (LAT)-SH2 domain-containing leukocyte phosphoprotein of 76 kD (SLP76) signalling complex (Shah, Al-Haidari et al. 2021). This complex serves as a membrane proximal signalling integration platform that couples TCR/CD28 signalling to downstream metabolic pathways upon TCR activation (Myers et al., 2019). The complex not only recruits phospholipase C gamma 1 (PLCγ1) to amplify Ca^{2+} and diacylglycerol (DAG) signalling but also promotes phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) enrichment by recruiting phosphoinositide 3-kinases (PI3K) to membrane microregions, which in turn triggers AKT and mTOR signalling cascades (Shim, Jung et al. 2011, Shyer, Flavell et al. 2020).

In this signalling chain, activation of mTORC2 is dependent on the enrichment and spatial localization of PIP3 at the plasma membrane. PIP3 microregions provide binding sites for AKT's membrane anchoring, enabling mTORC2 to mediate phosphorylation of the AKT Ser473 site, completing the full activation of AKT (Myers, Wheeler et al. 2019). This membrane platform localization mechanism acts early in TCR activation, enabling mTORC2 to respond rapidly to external stimuli, mediate AKT Ser473 phosphorylation and trigger signal amplification and backbone reorganization, thereby providing T cells with migratory capacity and initial metabolic regulation. It has also been shown that active mTORC2 supports cell motility by regulating PKC, SGK1 and AKT, and by participating in the regulation of F-actin rearrangement and microtubule dynamics (Chantaravisoot, Wongkongkathep et al. 2023).

Compared with mTORC2, the activation of mTORC1 is not only dependent on TCR signalling intensity but also reflects the integration property for multiple signals. Upon TCR stimulation, AKT inhibits the tuberous sclerosis proteins 1/2 (TSC1/2) complex after phosphorylation of the Ser473 site mediated by mTORC2, deregulates its inhibition of the small guanosine triphosphate (GTPase) Ras homolog enriched in brain (Rheb), and promotes the enrichment of Rheb-guanosine-5'-triphosphate (GTP) on the surface of the lysosome and activation of mTORC1 (Shi and Sun 2015, Saxton and Sabatini 2017). In addition to the classical PI3K-AKT-TSC2-Rheb axis, TCR signalling also activates mTORC1 via the caspase recruitment domain-containing membrane-associated guanylate kinase protein 1 (CARMA1)-mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1) complex bypass (Hamilton et al., 2014). Upon synergistic stimulation by TCR and CD28, protein kinase C theta (PKCθ) phosphorylates CARMA1, prompting it to bind to MALT1 to form a signalling complex (Shi and

Sun 2015). In addition to mediating nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation, the CARMA1-MALT1 complex enhances the amplification effect of TCR signalling to mTORC1. Notably, although Bcl10 is essential for NF-κB, its deletion does not affect mTORC1 activation, suggesting that CARMA1-MALT1 has an independent regulatory role for mTORC1 (Hamilton, Phong et al. 2014).

However, mTORC1 activation requires not only continuous TCR signalling input, but also the integration of metabolic sensing signals such as intracellular amino acid concentration, energy supply and oxygen level. This dual regulation of TCR signalling intensity and cellular metabolic state enables mTORC1 to accurately coordinate the metabolic reprogramming and biosynthetic demands of T cells, while ensuring the precision of TCR-specific responses (Chapman and Chi 2014, Myers, Wheeler et al. 2019, Shi, Chapman et al. 2019).

In summary, TCR signalling tightly coordinates antigen recognition with cellular metabolic regulation through a spatial and intensity sensing mechanism, which ensures the specificity and precision of the mTOR pathway in T cell activation. As a central integration factor, mTORC1 senses both TCR signal intensity and combines with intracellular nutrient and energy status, realizing a dynamic match between metabolic programs and functional outputs. However, it was found that IL-12 and IL-18 induced IFN-γ production by resting CD4$^+$ T cells in the absence of antigenic stimulation could be blocked by rapamycin, suggesting the involvement of the mTOR pathway (Munk, Sugiyama et al. 2011). Although the exact molecular mechanism is not yet clear, these results suggest that cytokines are able to activate mTOR signalling in the absence of TCR activation. The next section will focus on the mechanism of cytokine-induced mTOR activation and its role in the nonclassical function of T cells.

1.2.3 mTOR activation under cytokine stimulation: emerging evidence

In addition to the classical TCR-mediated activation pathway, a growing number of studies have shown that inflammatory cytokines themselves can directly induce mTOR pathway activation in the absence of antigen recognition, conferring on T cells the ability to rapidly initiate immune functions.

For instance, it has been demonstrated that combined stimulation with IL-12 and IL-18 induced a rise in phosphorylation of the S6 protein in mouse CD8$^+$ T cells in the absence of TCR signalling activation, suggesting that the mTORC1 signalling pathway was effectively activated (Jergović, Thompson et al. 2019). On the other hand, in human NK cells, it’s also reported that combined stimulation of IL-12 and IL-18 was effective in inducing phosphorylated S6 ribosomal protein (p-S6) and promoting mTORC1 activation, thereby enhancing the metabolic activity and effector function of NK cells (Viel, Besson et al. 2017). This suggests that IL-12 and IL-18 can achieve rapid metabolic reprogramming and functional activation in different types of lymphocytes through the mTORC1 pathway.

Additionally, it was investigated that IL-33 induces activation of mTORC1 in mouse CD8$^+$ T cells in the absence of TCR stimulation by binding to the receptor ST2, as evidenced by enhanced phosphorylation of p-S6 (Liang, Wang et al. 2022). They also found that IL-33 stimulation significantly enhanced glycolytic activity which was indicated by elevated extracellular acidification rate (ECAR). And this metabolic effect was dependent on mTORC1 activation in CD8$^+$ T cells, which was significantly attenuated by inhibition with rapamycin. This result suggests that IL-33 can mediate glycolytic metabolic reprogramming of CD8$^+$ T cells through the mTORC1 signalling pathway independent of TCR signalling activation (Liang, Wang et al. 2022). This study suggests a non-traditional activation mechanism by which inflammatory cytokines directly regulate T cell function through the mTOR pathway.

In addition to IL-12, IL-18 and IL-33, the common gamma chain cytokine IL-15 has been shown to activate mTORC1 signalling in T cells under antigen-free stimulation conditions (Lee, Park et al. 2024). In a mouse model, it has been demonstrated that infection-induced IL-15 expression enables memory CD8$^+$ T cells to rapidly enter the cell cycle through the mTORC1 pathway without the need for re-exposure to antigen. This antigen-independent proliferation can be effectively blocked by mTORC1 inhibition with rapamycin (Richer, Pewe et al. 2015).

In summary, in addition to the classical TCR-mediated activation mechanism, inflammatory cytokines such as IL-12, IL-18, IL-33 and IL-15 can activate the mTOR signalling pathway through an antigen-independent pathway, driving metabolic reprogramming and effector function initiation. This non-classical mode of activation allows T cells to respond rapidly to infections or inflammatory environments before an adaptive immune response is established. Therefore, cytokine regulation of T cell function through the mTORC1 pathway reveals an important regulatory mechanism for the immune system to respond to early threats and provides a new target for immune intervention.

1.3 Heterogeneity in CD8⁺ T cell subsets and metabolic dependency

CD8⁺ T cells play a central role in adaptive and innate immunity, but they are not a homogeneous population. Under the combined influence of multiple factors such as antigenic stimulation, co-stimulatory signals, inflammatory environment and metabolic state, CD8⁺ T cells are able to differentiate into subpopulations with different phenotypic and functional properties, forming a complex and diverse immune cell lineage (Zhang and Bevan 2011, Kaech and Cui 2012). Recently, with the deepening of single-cell histology and immunometabolism studies, more and more studies have revealed the complex regulatory network of CD8⁺ T cell subpopulations in terms of functional specificity, metabolic dependence, and coupling mechanisms of signalling pathways (Hartmann, Mrdjen et al. 2021, Fernández-García, Franco et al. 2022, Choi, Koh et al. 2023).

Through single-cell transcriptome and metabolic flow analyses, innate-like CD8⁺ T cell subsets such as NKG2A⁺ and KIR⁺ subpopulations was found to rapidly initiate effector functions under inflammatory environments in the absence of antigenic stimuli through specific metabolic pathways (Choi, Koh et al. 2023). Whereas CD8⁺ T cell subpopulations with different differentiation states and functional orientations show high heterogeneity in metabolic requirements such as amino acid metabolism, glycolysis, and mitochondrial respiration (Hartmann, Mrdjen et al. 2021, Fernández-García, Franco et al. 2022).

These findings not only enrich our understanding of the functional heterogeneity of T cells but also provide a new theoretical basis and intervention strategies for targeting and regulating the immune metabolism of specific subpopulations. This section will provide a systematic discussion around the distribution patterns of $CD8^+$ T cell subsets, the innate-like characteristics in barrier tissues, and the differences in metabolic and signalling pathway dependencies of the different subpopulations.

1.3.1 $CD8^+$ T cell subsets and subset-specific features

$CD8^+$ T cells play a key role in viral infection, tumour immunity and inflammatory responses through direct killing of target cells and secretion of effector cytokines (Kaech and Cui 2012). With further research, $CD8^+$ T cells have been classified into functionally specific multiple subpopulations based on differences in developmental origin, surface markers, and activation modes, including effector T cells, memory T cells, tissue-resident memory T (TRM) cells, and nonclassical subpopulations with innate-like immune features (Koh, Lee et al. 2023).

The different subpopulations apply differentiated functional strategies in initiating immune responses: effector versus memory $CD8^+$ T cells rely on TCR-mediated specific antigen recognition and acquire effector function after clonal expansion, whereas TRM cells are characterised by residing in barrier tissues and achieving rapid local responses (Zhang and Bevan 2011, Szabo, Miron et al. 2019). In addition, recent studies have revealed that some $CD8^+$ T cell subpopulations expressing NKG2A and CD161 are able to directly initiate effector functions via inflammatory cytokine signals such as IL-12 and IL-18 in the absence of antigenic stimulation and exhibit innate-like immune response (Fergusson, Smith et al. 2014, Choi, Koh et al. 2023).

In recent years, the critical role of the mTOR signalling pathway and metabolic programs in $CD8^+$ T cell fate determination has been widely revealed. Classical effector and memory $CD8^+$ T cells are activated by TCR recognition of antigens, and their functional differentiation depends on differential regulation of mTOR signalling. In mice, enhanced mTORC1 activity drives the glycolytic program to support rapid proliferation and effector synthesis, contributing to cell differentiation in the short-lived effector direction, whereas restricted mTORC1 activity and preserved mTORC2 function facilitate metabolic adaptation and long-term survival of memory T cells (Pollizzi, Patel et al. 2015). In human $CD8^+$ T cells, mTOR activity supports sustained IFN-γ production and proliferation under TCR stimulation (Setoguchi, Matsui et al. 2015); however, direct causal separation of mTORC1 versus mTORC2 remains limited.

In contrast, $CD8^+$ TRM cells exhibit metabolic profiles that are highly adapted to their survival environment. TRM cells are permanently present in mucous membranes and tissue barriers and possess rapid response and local immune surveillance capacities. It has been shown that these cells tend to rely on fatty acid oxidation (FAO) and mitochondrial respiration for survival and function, adapting to the chronically nutrient-poor state of the tissue microenvironment (Pan and Kupper 2018). In a gastric adenocarcinoma model, it was further found that tumour-infiltrating $CD8^+$ TRM cells relied heavily on the FAO metabolic pathway to maintain their function, and that programmed death-ligand 1 (PD-L1) inhibitor treatment improved their lipid metabolism and stimulated antitumour activity (Lin, Zhang et al. 2020).

The above studies suggest that CD8$^+$ T cell subsets exhibit characteristic metabolic adaptations in different tissues and disease states. Differences in these metabolic pathways not only reflect the survival needs of different CD8$^+$ T cell subsets under physiological conditions, but also determine their response rate, effector capacity, and functional persistence in the immune response. mTORC1-glycolysis axis provides the basis for the rapid effects of acute antigenic responses, whereas the FAO-mitochondrial respiratory pathway supports the long-term residency of tissues and immune surveillance, forming an important metabolic basis for the functional heterogeneity of CD8$^+$ T cells.

1.3.2 Innate-like phenotypes in barrier immunity and infection

Barrier tissues such as the intestine and liver are enriched in populations of CD8$^+$ T cells with innate-like functions, such as MAIT cells and non-MAIT CD161^{hi} CD8$^+$ T cells. They broadly express IL-18R and IL-12R and can respond rapidly to inflammatory cytokines without the need for TCR activation, rapidly producing IFN-γ (Kurioka, Klenerman et al. 2018). These cells also express chemokine receptors such as CCR6 and CXCR6, which contribute to their enrichment in tissues such as liver, intestine and lung, and are highly activated in localized inflammation, as evidenced by the upregulation of phenotypic markers such as CD69 and human leukocyte antigen-DR (HLA-DR) (Kurioka, Klenerman et al. 2018). These features suggest that innate-like CD8$^+$ T cells may play a key role in barrier immunity and early infection response.

NKG2A-expressing CD8$^+$ T cells are a subset of cells with a precursor-like phenotype that express NK-related receptors such as CD94, accompanied by tissue-resident markers for CD69$^+$ and CD103. These cells have been found to be innate-like T cells that are often found in barrier tissues such as the liver, lungs, and intestines, and are particularly enriched under conditions of chronic inflammation or infection (Chen, Xie et al. 2024). It's demonstrated that this subpopulation is highly expressive of IL-12 and IL-18 receptors and can rapidly produce IFN-γ in response to IL-12+IL-18 stimulation, whereas conventional memory T cells and CD8$^+$KIR$^+$ cells were less responsive. Conversely, CD8$^+$KIR$^+$ T cells exhibited NK-like cytotoxic activity when induced by IL-15 (Choi, Koh et al. 2023). In a variety of disease contexts, NKG2A$^+$ CD8$^+$ T cells are associated with an immunomodulatory or protective immune state, but their function is often limited by the NKG2A-HLA-E suppression axis (Wang, Xiong et al. 2022). NKG2A blockade has been shown to effectively deregulate its functional inhibition and enhance tumour vaccine-induced CD8$^+$ T-cell responses, suggesting that this subpopulation combines both innate defence and therapeutic intervention potential (André, Denis et al. 2018).

In addition, γδ T cells are scarce in secondary lymphoid organs but are significantly enriched in epithelial barriers such as skin, intestine and lungs, serving as the first line of immune defence and tissue repair functions (Nielsen, Witherden et al. 2017). They achieve immediate response to microbes and injury and maintain mucosal homeostasis through rapid production of cytokines (Ribot, Lopes et al. 2021). Represented by mouse Vγ4$^+$ and Vγ6$^+$ γδT17 cells and human CCR6$^+$Vγ9Vδ2$^+$ γδT17 cells, these γδT17 cells can be activated directly by IL-1β and IL-23 in the absence of TCR stimulation to rapidly secrete effectors such as IL-17 and IFN-γ, which play key roles in early immune response and maintenance of tissue homeostasis (Sutton, Lalor et al. 2009, Papotto, Reinhardt et al. 2018). IL-17 secreted by γδT17 cells promotes the secretion of antimicrobial peptides and chemokines by epithelial cells and enhances neutrophil recruitment, while IFN-γ activates macrophages and dendritic cells to enhance antigen presentation and bactericidal capacity (McKenzie, Kara et al. 2017). In Psoriasis, these cells are also highly expressive of CCR6 and CCR2, allowing them to be resident at the barrier site at homeostasis and to rapidly mobilize and migrate in a targeted manner to the focal area after the onset of inflammation (Qi, Wang et al. 2021). Their unique migratory and localization abilities, combined with purely innate cytokine induction, make γδT17 cells key effectors in early immune regulation and tissue repair.

Also, iNKT cells are CD1d-restricted, innate-like T cell subsets with invariant TCRα chains and a limited diversity of TCRβ chain combinations that develop with high expression of the transcription factor PLZF, directing innate-like phenotypic differentiation and enhancing effector response potential (Park, DiPalma et al. 2019). Upon maturation, these cells mainly reside in the liver, lung, intestine and adipose tissue and differentiate into functional subtypes such as NKT1, NKT2 and NKT17, which are known for their endogenous and rapid secretion of IFN-γ, IL-4 and IL-17, respectively (Crosby and Kronenberg 2018). They are not only activated by TCR recognition of CD1d-presented exogenous or endogenous lipid antigens, but also rapidly produce high levels of IFN-γ without TCR involvement in response to the combined action of pro-inflammatory cytokines such as IL-12 and IL-18 to accomplish a first effect response (Reilly, Wands et al. 2010, Holzapfel, Tyznik et al. 2014).

Barrier tissues are enriched with multiple innate-like CD8$^+$ T cell subpopulations, such as MAIT, CD161^{hi}, NKG2A$^+$, iNKT and γδT17 cells. These cells can rapidly respond to inflammatory cytokines under TCR-activation-free conditions, secreting IFN-γ, IL-17, IL-4, and other effector molecules to exert early immune defence and tissue homeostasis regulation. Existing studies focus on the activation characteristics and signalling pathways of a single subpopulation but lack the systematic analysis of cross-subpopulation functional comparisons and synergistic effects. Filling this gap will help to construct an integrative map of barrier immunity and provide a theoretical basis for multi-targeted immunomodulatory strategies.

1.3.3 Metabolic and signalling diversity across subsets of CD8$^+$ T cells

Different subpopulations of CD8$^+$ T cells exhibit highly differentiated metabolic programmes during their activation, differentiation, and functional maintenance (Bevilacqua, Li et al. 2022). For example, effector CD8$^+$ T cells rapidly enhance glycolysis upon activation to support their rapid proliferation and cytotoxicity, whereas tissue-resident memory CD8$^+$ T cells are more dependent on mitochondrial oxidative phosphorylation for long-term survival and local immune surveillance (Quinn, Hussain et al. 2020).

The rapid effector functions of MAIT cells are dependent on a unique glycogen-glycolysis metabolic pattern, and it has been investigated that MAIT cells mobilize endogenous glycogen upon activation to generate energy via the glycolytic pathway within hours, supporting IFN-γ and granzyme release, while inhibition of glycogen metabolism significantly diminishes their cytotoxicity (Cassidy, Kedia-Mehta et al. 2023, Kedia-Mehta, Pisarska et al. 2023). The proliferation of MAIT cells relies on the transcription factor myelocytomatosis oncogene (MYC) and the amino-acid transporter solute carrier family 7 member 5 (SLC7A5), which together drive amino acid uptake and glycolysis to support rapid proliferation (Kedia-Mehta, Pisarska et al. 2023). And in high-iron environments, MAIT cells regulate their metabolic activity and proliferative capacity through CD71-mediated iron uptake (Ryan, Clutter et al. 2024).

In terms of metabolic regulation, iNKT cell development and subtype differentiation are regulated by a balance of mTORC1 and mTORC2 signalling. Studies in mice show that Raptor deletion blocks early development and favors NKT2 differentiation, whereas Rictor deletion affects NKT17 differentiation (Yarosz, Chang et al. 2021). In the effector phase, iNKT cells are activated by TCR to simultaneously upregulate aerobic glycolysis and mitochondrial oxidative phosphorylation to support efficient secretion of cytokines such as IFN-γ and IL-4 (Fu, Zhu et al. 2019). Recent studies have shown that vesicle associated membrane Protein 6 (Vam6) recruits AMP-activated protein kinase (AMPK) to the lysosome by forming the Ras-related protein Rab7a-Vam6-AMPK complex in iNKT cells, thereby inhibiting the activation of mTORC1 and reducing the production of IFN-γ. This demonstrates the critical role of AMPK-mTOR in the regulation of iNKT function(Bai, Wu et al. 2022).

γδT1 and γδT17 cells exhibit significant metabolic differences, γδT1 (IFN-γ-producing γδT) cells rely primarily on glycolysis; γδT17 (IL-17-producing γδT) cells rely on mitochondrial oxidative phosphorylation and lipid oxidation to drive IL-17 secretion. γδT17 cells have higher mitochondrial mass and ROS production, a property critical for IL-17 gene transcription. This difference develops during the developmental stages of the thymus and persists in peripheral barrier tissues (Lopes, McIntyre et al. 2021). In the inflammatory environment of psoriasis, acetyl-CoA-carboxylase 1 (ACC1)-mediated de novo fatty acid synthesis enhances malonyl coenzyme A production and promotes cell membrane lipid remodelling, thereby further enhancing the effector function of γδT17 cells (Kao, Lauterbach et al. 2025).

In human solid tumours, NKG2A$^+$ CD8$^+$ T cells display a distinctive CD39$^+$CD103$^+$ TRM-like phenotype with high cytotoxic potential, and IL-12 plus TCR stimulation together upregulate NKG2A, but the energy metabolism profile has not yet been completely elucidated (Fesneau, Samson et al. 2024). Cytokines such as IL-12 and IL-15 have been shown to induce CD8$^+$ T cells and NK cells to acquire a memory-like functional state and influence their differentiation trajectory (Lugli, Hudspeth et al. 2016). It has also been shown that small molecules secreted by tumour cells induce activated CD8$^+$ T cells to upregulate NKG2A expression, while inhibiting mitochondrial respiration and impairing cytotoxicity, including production of cytokines, granzyme B, and perforin, and the ability to kill tumour cells, suggesting that this type of functionally limited phenotype is closely related to metabolic inhibition (Rodriguez-Garcia, Graves et al. 2024).

NKG2A$^+$ CD8$^+$ T cells exhibit distinct immune phenotypes and regulatory features in different chronic disease contexts. In chronic viral infections and tumour environments, CD8$^+$ T cells often show high expression of NKG2A, accompanied by decreased cytotoxicity and cytokine secretion, suggesting that they are in a suppressed functional status (van Montfoort, Borst et al. 2018). In contrast, in autoimmune diseases such as systemic lupus erythematosus, the proportion of peripheral blood CD8$^+$ T cells is elevated and NKG2A expression on the surface of $CD3^+$ T cells is down-regulated, whereas NKG2D expression is up-regulated, resulting in an increased NKG2D/NKG2A ratio (Li, Pan et al. 2010). Since NKG2A is an inhibitory receptor and NKG2D acts as a co-stimulatory molecule for $CD8^+$ T cells mainly in adaptive immunity, this imbalance in expression may enhance the effector function of $CD8^+$ T cells and promote immune activation. These differences suggest that NKG2A-related metabolic-signalling coupling mechanisms may play opposite regulatory roles in different pathological settings, suggesting the need to resolve the molecular basis and functional consequences for specific disease contexts.

Taken together, different innate-like T cell subpopulations have significant differences in metabolic programs and signalling regulation that shape their functional characteristics in barrier tissue defence, chronic infection control and immunopathology. Such tightly coupled metabolic-functional programmes also provide potential entry points for immune regulation in specific pathological settings. In-depth analysis of their subpopulation heterogeneity and metabolic-signalling regulatory mechanisms will provide a solid foundation for precise immune interventions in chronic inflammation, persistent infections, and tumours, as well as strategic guidance.

1.4 The unfolded protein response: IRE1α/XBP1s signalling in immune regulation

In immune cells, the metabolic state is not only regulated by energy and nutrient signals, but also closely related to endoplasmic reticulum (ER) stress. The endoplasmic reticulum plays a central role in protein folding, post-translational modification and secretion. And when protein load increases due to antigenic stimuli, cytokine signals, or tumour microenvironmental stress, the unfolded protein response (UPR) is initiated to coordinate the cell's folding capacity with its synthetic requirements (Grootjans, Kaser et al. 2016, Chen, Shi et al. 2023). The UPR uses three sensors: inositol-requiring protein 1α (IRE1α), protein kinase RNA-like ER kinase (PERK) and activating transcription factor-6 (ATF6).

In the subsequent part of this section, I will firstly review the three signalling branches of the UPR and their basic biological features, then summarise the key roles of IRE1α signalling pathway in the immune function of T cells and lastly explore the differential regulatory mechanisms of this pathway and its disease relevance in different T cell subpopulations. This will provide the theoretical basis for the study of the interaction between metabolism, UPR pathways and cell signalling in the context of specific pathologies in the subsequent chapters.

1.4.1 The unfolded protein response and its branches

The endoplasmic reticulum undertakes core functions such as protein folding, post-translational modification and secretion in immune cells. When stresses such as antigenic stimuli, cytokine signals or the tumour microenvironment result in protein loading that exceeds the folding capacity of the endoplasmic reticulum, the UPR is triggered to reconcile the folding capacity with the need for synthesis to maintain cellular homeostasis (Grootjans, Kaser et al. 2016, Hetz and Papa 2018). The UPR contains three classical signalling pathways:

IRE1α/XBP1s pathway

IRE1α undergoes dimerization and self-phosphorylation in response to endoplasmic reticulum stress, which activates its cytoplasmic-terminal RNase activity to catalyse the asymmetric splicing of X-box binding protein 1 (XBP1) mRNA, removing a 26-nucleotide intron and altering the reading frame to generate XBP1s, the transcriptionally active spliced isoform of XBP1. After entering the nucleus, XBP1s binds to cis-acting elements on specific promoters through its basic domain/leucine zipper (bZIP) structural domain and induces the expression of a variety of genes involved in the maintenance of endoplasmic reticulum homeostasis, including molecular chaperones, folding enzymes, and endoplasmic reticulum-associated degradation (ERAD) components, and regulates lipid synthesis pathways in order to support the expansion of endoplasmic reticulum membranes and to enhance the efficiency of protein folding, assembly, and secretion (Yoshida, Matsui et al. 2001, Calfon, Zeng et al. 2002).

This transcriptional program not only enhances the ability of cells to process and secrete proteins under high load but also regulates metabolic adaptations and cytokine production in immune cells, for example, affecting the expression of key immune molecules such as IL-6 and IFN-γ to maintain effector function and survival (Hetz and Papa 2018). In mammalian-derived cell models such as mouse embryonic fibroblasts, it’s demonstrated that XBP1 synergises with ATF6α to induce the transcription of a variety of endoplasmic reticulum quality control (ERQC) proteins, which significantly enhances the cellular ability to fold and secrete proteins under stress conditions (Yamamoto, Sato et al. 2007). Subsequent studies in immune cells further validated the importance of this pathway. For example, in ovarian cancer models, tumour microenvironment-induced activation of IRE1α-XBP1s pathway has been shown to inhibit glucose transport and glutamine metabolism in CD8$^+$ T cells, leading to decreased mitochondrial respiration and restricted IFN-γ production, while its antitumor effects are significantly enhanced under conditions of T cell-specific deletion of Xbp1 (Song, Sandoval et al. 2018). Furthermore, it’s demonstrated in a mouse model of asthma that XBP1 is required for Th2 polarisation and the establishment of airway inflammatory responses, suggesting that it has a regulatory role in different T cell subsets (Zeng, Xiao et al. 2022).

Overall, these findings highlight that the IRE1α-XBP1s axis links endoplasmic reticulum homeostasis to T cell metabolism and functional programming, thereby shaping the effector capacity of T cells in different pathological situations.

PERK pathway

Under endoplasmic reticulum stress conditions, PERK dimerises and self-phosphorylates, which activates the kinase activity of its cytoplasmic terminus and phosphorylates eukaryotic translation initiation factor 2 alpha subunit (eIF2α), leading to a temporary inhibition of overall protein translation and a reduction in the nascent polypeptide entry into endoplasmic reticulum load into the endoplasmic reticulum (Harding, Zhang et al. 1999, Harding, Novoa et al. 2000). At the same time, the pathway selectively promotes the translation of specific stress-adaptation-related transcription factors, such as ATF4, which induces genes for amino acid metabolism, antioxidant responses, and folding chaperone expression, thereby enhancing cell survival in stressful environments (Harding, Zhang et al. 2003).

Experimental evidence suggests that the PERK-eIF2α-ATF4 axis has an important role in the regulation of immune cell function. For example, in mouse regulatory T cells, endoplasmic reticulum stress has been reported to promote cell proliferation and survival in response to TCR signalling via PERK activation, while enhance inhibitory functions (Feng, Luo et al. 2021). This effect has been shown to be dependent on eIF2α phosphorylation and ATF4-mediated transcriptional responses, including upregulation of amino acid uptake, glutathione metabolism, and expression of genes related to antioxidant defences. Inhibition of PERK signalling has been found to markedly impair these processes, suggesting that this pathway is critical for the activation and maintenance of regulatory T cell (Treg) functions (Feng, Luo et al. 2021). In tumour cells, this pathway has also been reported to involve in the regulation of cellular functions. For example, in human luminal androgen receptor triple-negative breast cancer (LAR-TNBC) and prostate cancer cells, ER stress has been found to down-regulate androgen receptor (AR) expression through PERK-eIF2α-ATF4 signalling. ATF4 can directly bind to the AR promoter and repress its transcriptional activity, leading to impaired cell proliferation. This mechanism has also been demonstrated in a mouse transplantation tumour model (Li, Zhou et al. 2022).

Taken together, these findings suggest that the PERK pathway plays an important regulatory role in inflammatory, infectious and tumour microenvironments by decreasing endoplasmic reticulum loading and inducing ATF4-mediated stress adaptive gene expression, thereby linking endoplasmic reticulum homeostasis to metabolic adaptation and effector functions of immune cells.

ATF6 pathway

Under endoplasmic reticulum stress, activating transcription factor 6 (ATF6) transports from the endoplasmic reticulum membrane to the Golgi, where it is cleaved by site-1 and site-2 proteases, removing the membrane anchoring region and generating a transcriptionally active N-terminal fragment (ATF6-N) (Haze, Yoshida et al. 1999, Ye, Rawson et al. 2000). Activated ATF6-N enters the nucleus and binds to ER stress response elements (ERSE), to induce the expression of molecular chaperones, folding enzymes and components of the ERAD pathway, thereby enhancing protein folding and degradation of misfolded proteins (Yamamoto, Sato et al. 2007).

The function of the ATF6 pathway has been experimentally demonstrated in immune cells. Studies in mouse bone marrow-derived dendritic cells and B cells have shown that ATF6 activation synergistically with XBP1 induces endoplasmic reticulum quality control protein expression and significantly enhances cellular secretion under high load conditions (Yamamoto, Sato et al. 2007). In a model of chronic ileitis, ATF6 binds to the promoters of effector genes such as Granzyme B and Bcl-2 in CD8αβ$^+$ intraepithelial lymphocytes along with

ATF4 and XBP1s, linking ER stress to cytotoxic function and survival (Chang, Ocvirk et al. 2012). Furthermore, in an in vivo clustered regularly interspaced short palindromic repeats (CRISPR) screen, ATF6 was identified as a regulatory element that affects CD8$^+$ T cell differentiation (Chen, Arai et al. 2021). Taken together, these findings suggest that ATF6 combines ER homeostasis with functional programming of immune cells by increasing protein folding capacity and regulating expression of effector genes under stress.

Among these three branches, the IRE1α/XBP1s pathway is not only involved in the maintenance of endoplasmic reticulum homeostasis but is also thought to influence the differentiation trajectories and effector functions of a variety of immune cells such as T cells and dendritic cells by regulating gene transcription, lipid metabolism and cytokine production (Grootjans, Kaser et al. 2016, Chen, Shi et al. 2023). In the context of solid tumours, it has been reported that IRE1α activity in the tumour microenvironment can enhance CD8$^+$ T cell-mediated anti-tumour immune responses and boosts the efficacy of immunotherapies (Yang, Wang et al. 2024), suggesting that this pathway may be a potential target for intervention in immune modulation.

1.4.2 Immune functions of IRE1α/XBP1s in T cells

IRE1α is a coreceptor of the UPR, it plays an important regulatory role in multiple T cell subsets. Existing studies have shown that this pathway can support its function in CD4$^+$ T cell activation by relieving secretory stress and promoting proliferation (Pramanik, Chen et al. 2018).

In Th1 differentiation, current evidence suggests that the IRE1α/XBP1s pathway is not a major limiting factor for its effector function. This view stems mainly from the weak enrichment signals of Xbp1 under Th1 conditions in genome-wide CRISPR screens, but there’s still a lack of direct functional validation experiments (Henriksson, Chen et al. 2019), and therefore its Th1 cell effector formation still needs to be further investigated.

In contrast, Th2 cells differentiate in response to IL-4 stimulation and secrete IL-4, IL-5, IL-9, and IL-13 (Walker and McKenzie 2018). The IRE1α/XBP1s pathway is essential for maintaining optimal levels of cytokine secretion such as IL-5 and IL-13 in Th2 cells, and its inhibition or absence significantly reduces the expression of these factor expression (Zheng, Wu et al. 2018, Henriksson, Chen et al. 2019). During Th2 differentiation or activation, IRE1α RNase inhibitor 4μ8c inhibits the production of IL-5, IL-13 and leads to cellular arrest in the S or G2/M phase, but does not significantly affect IL-4 (Pramanik, Chen et al. 2018, Poe, Youngblood et al. 2019).

In Treg cells, deletion of the E3 ubiquitin ligase hydroxymethylglutaryl-CoA reductase degradation protein 1 (Hrd1) elevates IRE1α levels and activates p38 mitogen-activated protein kinase (MAPK), inhibiting forkhead box P3 (Foxp3) synthesis and impairing Treg stability (Xu, Melo-Cardenas et al. 2019). In autoimmune diseases, this pathway shows aberrant regulation. IRE1, PERK, and CHOP levels are reduced in peripheral blood mononuclear cells of systemic lupus erythematosus (SLE) patients, whereas XBP1 and its spliced form are elevated (Wang, Cheng et al. 2014). IRE1α/XBP1 activity is enhanced in synovial fibroblasts of patients with rheumatoid arthritis (RA), which can maintain the stability of inflammatory factor mRNAs by inflammatory factor mRNA stability to promote disease progression (Kabala, Angiolilli et al. 2017) and mediates autophagy via the IRE1α/JNK axis to enhance cell proliferation, migration and invasion (Wang, Fan et al. 2022).

In CD8⁺ T cells, IRE1α/XBP1s signalling is equally important in infection and tumour immunity. In acute viral infections such as lymphocytic choriomeningitis virus (LCMV) and bacterial infections such as Listeria monocytogenes infection, IRE1α-mediated XBP1 splicing occurs predominantly in the effector phase and is highly enriched in killer cell lectin-like receptor subfamily G member 1 (KLRG1) ^{hi} terminally differentiated effector cells. Genetic deletion of XBP1 or inhibition of IRE1α RNase activity reduces the proportion of this cell population (Kamimura & Bevan, 2008).

In addition, in the tumour microenvironment, recent studies have shown that tumour cells' own IRE1α-XBP1s signalling can drive the reprogramming of tumour-infiltrating myeloid cells to an immunosuppressive phenotype through the promotion of cholesterol synthesis, thereby inhibiting the infiltration and effector function of CD8⁺ T cells. Inhibition of XBP1 activity in tumour cells reduces the accumulation of immunosuppressive myeloid cells (Yang, Huo et al. 2022).

Overall, these results suggest that the IRE1α/XBP1s pathway plays different roles in different T cell subpopulations, supporting CD4⁺ T cell activation, promoting cytokine production and proliferation in Th2 cells, plays a dispensable role in Th1 effector function and maintaining Treg stability. In CD8⁺ T cells, it can directly promote terminal effector cell differentiation during infection or indirectly regulate their effector formation and protective activity in tumours by reshaping the immune microenvironment.

1.4.3 Unknowns in the context of TCR-independent activation of CD8⁺ T cells

While the IRE1α/XBP1s pathway is known to regulate stress responses and support immune cell function, its specific roles in CD8⁺ T cells are not yet fully understood. Most of the current studies on the IRE1α/XBP1s pathway in T cells are based on TCR-dependent classical activation conditions. For instance, it’s investigated that sustained antigenic stimulation remodels the transcriptional and metabolic programs of CD8⁺ T cells, thereby limiting their effector functions (Unal, Kuzu et al. 2024). It's also reported that the IRE1α/ XBP1s axis maintains T cell function in the ovarian cancer setting by regulating mitochondrial activity, reflecting its important role in sustained TCR signalling (Song, Sandoval et al. 2018). In contrast, TCR-independent innate-like activation patterns such as IL-12+IL-18-mediated activation are still very limitedly studied, and it is unclear whether these cytokines can trigger endoplasmic reticulum stress responses, particularly activation of the IRE1α/XBP1s pathway.

The IRE1α-XBP1 pathway has been shown to involved in the regulation of protein folding, lipid metabolism, and immune function. It’s reported that the IRE1α/XBP1s pathway regulates mitochondrial activity and metabolic adaptations in T cells and can influence reactive oxygen species (ROS) production under stress conditions (Song, Sandoval et al. 2018). In acute infection models such as LCMV, XBP1s supports the differentiation of CD8⁺ T cells into terminal effector cells by increasing KLRG1 expression, although it is not required for the overall differentiation state (Kamimura and Bevan 2008). However, these mechanistic insights are primarily derived from contexts such as nutrient deprivation, chronic antigenic stimulation or tumour-associated stress. There is still a lack of investigation as to whether the pathway maintains short-term function through mitochondrial metabolism or fatty acid oxidation in the context of innate-like activation. Additionally, whether this pathway affects T cell memory formation, immune synapse stability, and long-term survival potential has not been elucidated.

In terms of the indirect roles, it has been shown that activation of IRE1α in bone marrow-derived dendritic cells (BMDCs) enhances CD8$^+$ T-cell initiation (Medel, Costoya et al. 2018), but whether it also functions as an antigen-independent intracellular regulator in T cells themselves remains to be experimentally confirmed. In particular, whether IRE1α is involved in initial cytokine perception and signalling integration in the absence of co-stimulatory signals has not been systematically resolved.

In addition, it has been revealed that the IRE1α/XBP1s pathway plays an important role in immune cell differentiation by alleviating secretory stress, accelerating proliferation and orchestrating metabolic and transcriptional networks (Acosta-Alvear, Karagöz et al. 2018). In a model of acute viral infection, high expression of the IL-7 receptor CD127 was shown to mark effector CD8$^+$ T cells that will be transformed into long-lived memory cells (Kaech, Tan et al. 2003). This observation provides important background for the identification of memory precursors, but it’s not yet clear whether the IRE1α/XBP1s pathway affects IL-7 receptor expression or the formation of memory programmes. These questions also remain unanswered under cytokine-driven activation conditions.

1.5 Potential crosstalk between mTOR and IRE1α/XBP1s pathways

In the previous sections, I discussed the functional roles and dynamics of mTOR and the IRE1α/XBP1s pathway in the activation of innate-like CD8$^+$ T cells. In this section, I will explore how mTOR signalling aggravates endoplasmic reticulum loading and activates the IRE1α/XBP1s pathway by enhancing protein and lipid synthesis from the perspective of endoplasmic reticulum stress. This section will introduce the potential interaction between the two pathways in cellular stress adaptation and signalling integration, offering mechanistic insight into innate-like CD8$^+$ T cell regulation.

1.5.1 mTOR-induced protein synthesis and ER stress

mTORC1 is a key regulator of cellular protein and lipid synthesis. It activates S6K1 and phosphorylates eukaryotic translation initiation factor 4E-BP1 to relieve eIF4E inhibition. It promotes cap-dependent translation and helps eIF3 and eIF4F assemble into the translation pre-initiation complex (Holz, Ballif et al. 2005). In parallel, mTORC1 enhances lipogenesis by controlling lipin-1-dependent sterol regulatory element-binding protein (SREBP) activity (Peterson, Sengupta et al. 2011). It also increases lipid synthesis gene expression at the post-transcriptional level via the S6K1-SRPK2 pathway, thus providing lipids for membrane production (Lee, Zheng et al. 2017).

UPR is activated when the folding demand exceeds the processing capacity of the endoplasmic reticulum. The three main branches are IRE1α/XBP1s, PERK, and ATF6, which are used to enhance folding and clearance and restore homeostasis (Hetz and Papa 2018). In mouse B cells, an early mTORC1-dependent increase in UPR-related transcripts probably occurs before high secretory load and without XBP1s activity. At this stage, PERK, ATF6, and IRE1α have not been activated (Gaudette, Jones et al. 2020). Other studies report differences between early stress and recovery stages in how mTOR relates to the UPR. A rise in IRE1α -JNK signalling is seen early in stress, and inhibition of mTORC1 reduces this response (Kato et al., 2012). During recovery, AKT-mTOR promotes endoplasmic reticulum-mitochondria contacts and attenuates IRE1α RNase activity, both mTORC1 inhibition and dual mTORC1/2 blockade delay contact reformation. The relative roles of mTORC1 and mTORC2 in this process remain to be determined (Sanchez-Alvarez, del Pozo et al. 2017). However, these results are mostly from non-T cell models, and their applicability in T cells requires further validation.

Membrane lipid changes provide another route of control. mTORC1-SREBP axis enhances phospholipid and neutral lipid synthesis and can change endoplasmic reticulum membrane composition and tension (Peterson, Sengupta et al. 2011). Lipid bilayer stress can be recognized by transmembrane receptor domains that activate IRE1 and PERK (Volmer, van der Ploeg et al. 2013, Halbleib, Pesek et al. 2017). Thus, mTORC1 may affect UPR initiation and strength through translational load and membrane load.

1.5.2 UPR-mediated regulation of mTOR through lipid and metabolic control

UPR can affect mTORC1 principally in two ways, through membrane lipid remodelling and through metabolic control and autophagy. When protein synthesis rises, UPR increases ER chaperones such as BiP and upregulates ER-associated degradation genes mainly via ATF6 and XBP1s. It can also promote autophagy through PERK–eIF2α–ATF4 branch, which helps to clear misfolded proteins (Ogata, Hino et al. 2006, Hetz, Zhang et al. 2020). The UPR reduces translation via the PERK-eIF2α pathway and can inhibit mTORC1 signalling via AMPK and ATF4 targets such as REDD1 and Sestrin2 (Harding, Zhang et al. 2000, Gwinn, Shackelford et al. 2008, Whitney, Jefferson et al. 2009, Kim, Kundu et al. 2011).

At the membrane lipid level, XBP1s induce lipid synthesis-associated genes in the hepatic system, and reported direct targets include diacylglycerol O-acyltransferase 2 (DGAT2) and acetyl-CoA carboxylase beta (ACACB) (Lee, Scapa et al. 2008). ER membranes could thus amplify, and their composition may change accordingly. These changes may affect the environment for recruitment and activation of mTORC1 at the lysosomal membrane (Lee and Glimcher 2009, Peterson, Sengupta et al. 2011).

At the level of metabolism, ER stress often involves energy stress, which can activate AMPK. AMPK then inhibits mTORC1 via phosphorylating TSC2 and Raptor, which lowers translation and biosynthesis (Inoki, Zhu et al. 2003, Gwinn, Shackelford et al. 2008). UPR can act directly on autophagy-associated kinases via PERK. In hepatic lipotoxicity context, PERK phosphorylates p62 and unc-51 like autophagy activating kinase 1 (ULK1), driving the p62-ULK1 axis and promoting KEAP1-NRF2 activation (Lee, Park et al. 2022). ER stress also induces autophagy and contributes to homeostatic recovery (Ogata, Hino et al. 2006). When energy stress inhibits mTORC1, phosphorylation of ULK1 at Ser757 is reduced, AMPK then activates ULK1 at Ser317 and Ser777, initiating and maintaining autophagy (Kim, Kundu et al. 2011). In addition, regulated IRE1-dependent mRNA decay (RIDD) can degrade metabolism-related mRNAs, for example DGAT2, which may indirectly shift lipid and nutrient signals and thereby influence mTORC1. RIDD targets differ by cell type and need to be validated in the models used (So, Hur et al. 2012, Almanza, Mnich et al. 2022).

Current evidence favours indirect regulation of mTORC1 by the UPR through energy sensing, autophagy, and mRNA degradation. Consistent primary evidence for a direct action of UPR sensors on the mTORC1 complex is still lacking. Most of these studies have been performed on non-T cells. In cytokine-driven human CD8$^+$ T cells, especially in innate-like subpopulations such as NKG2A$^+$CD161$^+$ and MAIT cells, it is not clear whether feedback from the UPR to mTORC1 exists, how strong, or when it occurs during activation and recovery. Clarifying these relationships will help understand how cytokine stimulation maintains IFN-γ production while maintaining protein folding and quality control.

1.6 Hypothesis and objectives

1.6.1 Central hypothesis

The central hypothesis of this thesis is that in the absence of antigen recognition, inflammatory cytokines IL-12 and IL-18 can induce rapid effector function in all $CD8^+$ T cells by activating the mTOR signalling pathway, and that the innate-like $CD8^+$ T cell subsets are more metabolically responsive to this process. Furthermore, the synergistic activation of the endoplasmic reticulum stress pathway IRE1α/XBP1s may further regulate mTOR-dependent metabolic reprogramming and functional output.

This hypothesis is based on three key observations presented in this thesis. First and foremost, combined IL-12 and IL-18 stimulation rapidly activates the mTORC1 pathway in $CD8^+$ T cells in the absence of TCR signalling and induces the production of effectors such as IFN-γ. Additionally, $NKG2A^+CD161^+$ and other innate-like $CD8^+$ T cell subpopulations showed stronger mTOR activity and metabolic response capacity in this process, suggesting that they possess higher cytokine sensitivity. Finally, IRE1α/XBP1s stress signalling co-activates with the mTOR pathway upon cytokine stimulation, which may play a bridging role between rapid activation of cellular functions and homeostatic regulation.

1.6.2 Project aims

To test the above hypotheses, the following three specific objectives were set in this study:

Objective 1: To compare the differences in functional and metabolic characteristics of CD8$^+$ T cells in response to IL-12+IL-18 versus TCR-dependent stimuli.

Objective 2: To analyse differences in the nutrient dependence of mTOR signalling across CD8$^+$ T cell subpopulations, with particular focus on the metabolic response characteristics of innate-like subpopulations.

Objective 3: To explore the role of the IRE1α/XBP1s pathway in cytokine-induced activation of CD8$^+$ T cells, and its synergistic regulatory mechanism with mTOR signalling.

Elucidating how cytokines integrate mTORC1 with IRE1α/XBP1s in innate-like CD8$^+$ T cells could help to explain the organisation of the rapid IFN-γ response when antigen presentation is limited and suggest potential targets for intervention that are finely regulated by subpopulation.

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Chapter 2 Methodology

2.1 Mononuclear cell separation from human peripheral blood

Human peripheral blood mononuclear cells (PBMCs) were isolated from fresh human blood cones. Firstly, the fresh blood cone was collected in 50μL tube and diluted 1:10 with phosphate-buffered saline (PBS) to reduce viscosity. Then 30mL diluted blood was carefully layered over 15mL Ficoll-Paque, ensuring minimal mixing of the layers. The sample was then centrifuged at 800 × g for 20 minutes at room temperature without braking, allowing for the formation of distinct layers. The PBMCs were the white, buffy coat layer just above the Ficoll layer and were carefully aspirated using a sterile pipette. These cells are transferred to a new tube and washed with RPMI-1640 medium, followed by centrifugation at 400 × g for 10 minutes to remove residual Ficoll and plasma contaminants. After two or three wash steps, the cell pellet was resuspended in R10 medium (RPMI 1640 medium (Sigma) supplemented with 10% Fetal Bovine Serum (Sigma) and 1% GlutaMAX (Gibco)), and cell counting was performed using a hemocytometer. The isolated PBMCs were then used immediately or suspended in freezing medium containing 90% fetal bovine serum (FBS) and 10% dimethyl sulfoxide (DMSO), followed by controlled rate freezing before transfer to liquid nitrogen for long-term storage.

2.2 T cell isolation

Human primary CD8$^+$ T or CD3$^+$ T cells were isolated from whole PBMCs by negative selection using CD8$^+$ T cell isolation kit (Miltinyi Biotec) or T cell isolation kit (Miltinyi Biotec) based on magnetic separation. Human PBMCs were washed twice with MACS buffer, and the total cell number was determined using a hemacytometer. Cells was then centrifuged at 500×g for 4 minutes at 4°C and the supernatant was carefully discarded. The cell pellet was resuspended in 40 µL of MACS buffer per 10^7 total cells, followed by incubation with 10 µL per 10^7 total cells of Biotin-Antibody Cocktail that selectively binds non-CD8$^+$ T cells. After incubation at 2–8°C for 5 minutes, the CD8$^+$ or CD3$^+$ T Cell MicroBead Cocktail was added in a 20 µL per 10^7 total cells ratio, allowing magnetic labelling of non-CD8$^+$ or non-CD3$^+$ T cells. After incubation at 2–8°C for 10 minutes, the labelled cell suspension was then applied to an LS Column placed in a MACS Separator, where magnetically labelled non-CD8$^+$ or non-CD3$^+$ T were attached, while unlabelled CD8$^+$ or CD3$^+$ T cells passed through and were collected. To ensure high purity, the column was washed with additional 3mL MACS buffer twice, and the combined flow-through fractions were collected as the enriched CD8$^+$ or CD3$^+$ T cells, which were used immediately for downstream experiments.

2.3 T cell culture

Human primary CD8$^+$ or CD3$^+$ T cells were cultured in R10 medium, consisting of RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum (FBS, Sigma) and 1% GlutaMAX (Gibco). Cells were maintained at 37°C in a humidified incubator with 5% CO_2 and were subsequently counted using a hemacytometer under a light microscope following Trypan blue (Sigma) staining.

2.4 In vitro cell treatment

Human primary CD8$^+$ T cells were cultured in 96-well round-bottom plates (Corning) and subjected to chemical inhibition prior to cytokine stimulation. The cells were pretreated with the following compounds according to different investigating aims.

For chapter 3 and 4, different mTOR inhibitors were used: Rapamycin (0, 10, 50, or 100 nM), OSI-027 (0, 5, 10, or 20 µM), or a combination of Rapamycin (10 nM) and OSI-027 (10 µM). For Chapter 3, I also used glycolytic inhibitor 2-Deoxy-D-Glucose (2-DG) (1, 2.5, 5, or 10 µM) and mitochondrial oxygen phosphorylation (OXPHOS) inhibitor Oligomycin (0.001, 0.01, 0.1, or 1 µM). For chapter 5, I further applied IRE1α RNase inhibitors: MKC8866 (1, 2, 4 or 8 µM), 4µ8C (5, 10 or 20 µM), and IRE1α kinase inhibitor: KIRA6 (1, 2, 4 or 8 µM). All mTOR inhibitors, glycolysis inhibitor and OXPHOS inhibitor were added 1 hour prior to stimulation. All IRE1α inhibitors were added 15 hours prior to stimulation. The cells were then stimulated for 7 hours with IL-12 and IL-18 (each at 50 ng/mL). Unstimulated cells with or without inhibitor treatment served as negative controls.

After stimulation, both cells and culture supernatants were collected for further analyses, including flow cytometry for intracellular IFN-γ staining and XBP1s detection, as well as quantification of cytokines in the supernatant, where applicable.

2.5 Inhibitor vehicle controls and control groups

All chemical inhibitors, including Rapamycin, OSI-027, 2-DG, Oligomycin, MKC8866, 4μ8C, and KIRA6, were dissolved in DMSO and diluted in complete medium to the indicated concentrations. To control for solvent effects, all treatment groups contained an equal final concentration of DMSO (≤0.1%). Control conditions included unstimulated untreated cells (negative control), and cytokine-stimulated cells treated with DMSO alone (vehicle control). These controls allowed for the assessment of both baseline activation and the specific effects of inhibitors. Inhibitor-treated groups were stimulated with IL-12 (50 ng/mL) and IL-18 (50 ng/mL) following a pre-treatment for 1 hour with the indicated compounds. Cells and supernatants were collected after 7 hours for subsequent analysis.

2.6 Enzyme-linked immunosorbent assay (ELISA)

The protein concentration of pro-inflammatory cytokine IFN-γ in cell culture supernatants was assayed using standard ELISA kits (Invitrogen) in accordance with the protocol set out by the manufacturer. The Multiskan Ascent ELISA reader was employed to read the ELISA plates at a test wavelength of 450 nM and a background wavelength of 620 nM. Then, the standard curve of ELISA was plotted using Microsoft Excel software, while the corresponding concentrations of samples were calculated using the absorbance value and standard curve.

2.7 Preparation of amino acid media

To directly examine the effect of amino acid starvation and refeed on human CD8$^+$ T cells, amino acid-deficient (AA-) and -sufficient (AA+) medium were made. AA- medium was generated from RPMI 1640 powder (US Biological Life Science) and sodium phosphate dibasic (5.6 mM, matching the concentration in commercially available RPMI 1640 medium, GIBCO) and supplemented with 10% (v/v) dialyzed FBS (Thermo Fisher Scientific). AA+ medium was prepared by supplementing AA- medium with MEM amino acids solution (essential amino acids, 50x), MEM non-essential amino acids solution (NEAA, 100x) and 200 mM L-Gln (all from GIBCO) to achieve a final concentration of 1xEAA, 1xNEAA and 2 mM L-glutamine. AA+ medium was also supplemented with 10% (v/v) dialysed FBS. The optimised R0 AA+ medium was generated by supplementing R0 with MEM essential amino acids and MEM non-essential amino acids, together with L-glutamine. Relative to R0, AA+ therefore contains the following supplemented amino acids.

2.8 T cell intracellular staining

After incubation, cells were centrifuged for 5 minutes at 500x g, washed then stained with CD3-BV786, CD8-BV711, CD161-FITC, TCRVa7.2-PE Dazzel (or PE Cy5.5), NKG2A-PE Cy7 (all from Biolegend) at 4°C for 30 minutes to determine different cell types. At the meantime, cells were stained with LIVE/DEAD® Fixable Violet Dead Cell Stain Kit (Life Technologies) to identify living cells. Cells were then washed twice, permeabilized (BD Biosciences) at RT for 20 minutes, washed again and stained for 30 minutes at room temperature (RT) with: IFNγ-

BV650 (Biolegend). Afterwards, Cells were washed twice more and stored in FACS buffer prior to analysis on a flow cytometer (BD LSRFortessa).

2.9 Phos-Flow cytometry

Cells were centrifuged at 500g for 5 mins and resuspended in 50 µL of a master mix of cell-surface-staining antibodies diluted in FACS buffer (0.2% BSA, 2 mM EDTA in PBS with incubation for 30 minutes at 4°C in dark, LIVE/DEAD® Fixable Violet Dead Cell Stain Kit (Life Technologies) was used at the meantime to identify living cells. Cells were washed in FACS buffer and fixed using 100 µL BD Cyto-fix Fixation Buffer (BD Biosciences) for 20 minutes at 4°C in dark. Cell permeabilization was performed by washing cells in 100 µL 1X BD Phos-flow Perm/Wash Buffer I (BD Biosciences), followed by incubation in 100 µL permeabilization buffer for 10 minutes at room temperature in the dark. Intracellular antibody staining was performed overnight at 4°C in the dark in 50 µL BD Phos-flow Perm/Wash Buffer I. Cells were washed twice more and stored in FACS buffer prior to analysis on a flow cytometer (BD LSRFortessa).

2.10 Flow cytometry and statistics

The geometrical mean fluorescence intensities of cells were measured and analysed by were analysed using a BD LSR Fortessa flow cytometer calibrated with BD FACSDiva CS&T Research Beads (BD Biosciences). Results were analysed using FlowJo 10.8. All summarised graphs were performed using GraphPad Prism 10, and statistical significance between two groups was evaluated using a two-tailed Student's t-test, while comparisons among multiple groups were conducted using one-way ANOVA. Where ANOVA was used, Tukey's multiple comparisons test was performed as the post hoc test to compare all conditions; the post hoc test is stated in the relevant figure legends. Significance levels were defined as follows: no significance (ns)=p>0.05; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

2.11 Quantitative polymerase chain reaction (qRT-PCR)

Human primary CD8^{+} T cells were collected by centrifugation and resuspended in TRIzol reagent (Ambion, 15596026). Total RNA was isolated using the Direct-zol RNA MiniPrep Kit (Zymo Research, R2052) following the manufacturer's protocol. Subsequently, RNA was reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4368813). TaqMan Gene Expression Assay probe Hs1060665_g1 for ACTB were used as control, and probe Hs00989291_m1 for IFNG were used as the experimental readout (Applied Biosystems). Quantitative real-time PCR was performed using the Applied Biosystems 7500 Fast system. Reactions were set up with TaqMan Fast Universal Master Mix (Applied Biosystems, 4352042). The mRNA expression was quantified using the 2–ΔΔCt method.

2.12 CRISPR/Cas9-mediated ERN1 knockout

To precomplex Cas9/RNP, 6 uL 30 µM guide RNA (AAVS1 and ERN1 from IDT) and 2 µl 30 µM TrueCut Cas9 Protein v2 (Thermo Fisher Scientific) were gently mixed by pipetting up and down in a PCR strip and incubated at room temperature for 20 min. For nucleofection, 200 µl complete R10 media per well of a 96-well plate was prewarmed. 1 million CD8^{+} T cells were re-suspended in 20 µL primary cell nucleofection solution (P2 Primary Cell 96-well Nucleofector kit, Lonza) and mixed with 8 µL precomplexed Cas9/RNP. The cell/RNP mix was transferred to Nucleofection cuvette strips. Cells were electroporated using a 4D nucleofector (4D-Nucleofector Core Unit: Lonza, AAF-1002B; 4D-Nucleofector X Unit: AAF-1002X; Lonza) with the EH100 pulse condition. After nucleofection, cells were moved to prewarmed R10 media and cultured for 3 days before the cytokine stimulation assay.

2.13 Western blotting

Cells were lysed with RIPA buffer containing protease inhibitors (Sigma). Protein concentrations were measured using the BCA assay. Afterward, proteins were separated by electrophoresis on NuPAGE 4-12% Bis-Tris Protein Gels (Life Technologies) and transferred onto nitrocellulose membranes (Thermo Fisher Scientific) for immunoblot analysis. Primary antibodies used included rabbit anti-IRE1α (1:1000, Cell Signaling, 14C10) and mouse anti-β-Actin (1:1000, Sigma-Aldrich, AC-15). Secondary antibodies were IRDye 800CW goat anti-mouse IgG and IRDye 680RD goat anti-rabbit IgG (1:10000, LI-COR Biosciences). Membrane bands were scanned with the LI-COR Odyssey Imager (LI-COR, USA) and analyzed using Image Studio and ImageJ software (Wayne Rasband, NIH, USA).

2.14 Study approval

Venous blood was obtained under protocols approved by the Oxford Research Ethics Committee (ethics reference number 06/Q1606/139).

Chapter 3 The mTOR Pathway in Cytokine-Driven Activation of Human CD8$^+$ T Cells

3.1 Introduction

The mammalian target of Rapamycin (mTOR) is a highly conserved serine/threonine protein kinase and a key member of the phosphatidylinositol kinase-related kinase (PIKK) protein family. mTOR is so named because it is inhibited by Rapamycin, which was first isolated from a soil bacterium on Rapa Nui (the Easter Island) (Saxton and Sabatini 2017, Hobby, Clark et al. 2022). mTOR acts as a central coordinator, processing signals from growth factors, nutrients, and energy levels to regulate cell metabolism, migration and proliferation. By balancing anabolic and catabolic processes, it plays a vital role in maintaining metabolic stability across various cell types, including immune cells like T lymphocytes (Delgoffe, Kole et al. 2009, Powell and Delgoffe 2010, Chi 2012, Panwar, Singh et al. 2023).

There are two distinct catalytic subunit complexes of mTOR, mTORC1 and mTORC2, which play different roles in regulating metabolism and cell proliferation(Liu and Sabatini 2020, Szwed, Kim et al. 2021). mTORC1 is a key regulator of environmental and hormonal signals, activating anabolic processes and inhibiting catabolic processes when nutrients such as amino acids, glucose, cholesterol and nucleotides are abundant. It has been reported that mTORC1 regulates cell growth through mRNA translation, ribosome biogenesis and autophagy(Panwar, Singh et al. 2023). mTORC2 acts primarily as an effector of PI3K signalling, regulating cell proliferation, survival and cytoskeletal organisation, and activates PI3K in response to insulin, insulin-like growth factor 1 (IGF-1), and leptin, by enhancing the activation of other downstream effectors of the AKT and insulin signalling pathways (Laplante and Sabatini 2012, Szwed, Kim et al. 2021, Simcox and Lamming 2022, Panwar, Singh et al. 2023).

In T cells, mTOR signalling plays a crucial role in directing differentiation into effector or memory subpopulations, regulating gene expression, metabolic programs and cytokine production. Our current understanding mainly comes from studies of TCR-dependent activation. For example, Powell and Pollizzi systematically described the regulatory mechanism of TCR and IL-2/IL-7/IL-12 signals via the PI3K-Akt-mTOR pathway, which regulates the effector/memory differentiation of CD4$^+$ or CD8$^+$ T cells (Pollizzi and Powell 2014). However, cytokines can also activate mTOR in T cells. For instance, in Th2 and Th17 cells, IL-4 and IL-1 activate mTOR respectively, promoting cell cycle progression (Stephenson, Park et al. 2005, Gulen, Kang et al. 2010).

Once activated, mTOR regulates important functional and metabolic programmes in CD8$^+$ T cells, independently of TCR signalling (Chi 2012). When these cells are stimulated by cytokines such as IL-12 and IL-18, they upregulate effector molecules such as IFN-γ to enable cytotoxic activity (Berg, Cordes et al. 2002, Barbarin, Cayssials et al. 2017). Previous studies have shown that IL-12 alone can activate mTORC1 and promote the phosphorylation of S6 and 4E-BP1(Rao, Li et al. 2010, Delgoffe, Pollizzi et al. 2011). Additionally, IL-18 can also activate mTORC1, and mechanistic studies have indicated that it indirectly enhances mTORC1 signalling by promoting amino acid transport (Almutairi, Ali et al. 2019). Therefore, the combination of IL-12 and IL-18 is likely to further enhance mTORC1 activation, evidenced by increased phosphorylation levels of S6 and 4E-BP1.

Activated mTOR increases cellular energy metabolism and growth rate by promoting glucose degradation, which is also known as aerobic glycolysis, mitochondrial activity and amino acid metabolism. Cytokine signalling also upregulates transcription factors such as T-bet, IRF4 and XBP1s and directs the cells towards effector identity. For example, IL-12 and IL-18 are known to increase T-bet expression. In addition, cytokines such as IL-15 and IL-2 have been shown to promote CD8$^+$ T cell survival and differentiation in a mTOR-dependent manner. Overall, these studies suggest that cytokines can activate mTOR to trigger similar responses as antigen stimulation does, although with different signals and timing. This supports the idea that mTOR plays a central role in helping CD8$^+$ T cells respond to antigens and cytokines.

mTOR plays a crucial role in CD8$^+$ T cell function, both in response to TCR activation and cytokines (Rao, Li et al. 2010, Huang, Long et al. 2020). These cytokines, especially IL-12 and IL-18, act synergistically to enhance CD8$^+$ T cell activation, proliferation and IFN-γ production, with IL-12 leading to a stronger cellular response to IL-18 (Yoshimoto, Takeda et al. 1998, Sareneva, Julkunen et al. 2000, Raué, Brien et al. 2004). In this context, activation of mTOR drives effector T cell differentiation, but its regulation is critical for balancing effector and memory cell development. In cancer, excessive IL-18-driven mTOR signalling leads to CD8$^+$ T cell exhaustion, reduces cytotoxicity and impairs the immune response to tumours (Lutz, Hellmund et al. 2023).

A recent study has shown that amino acids, functioning via the small G proteins Rag and Rheb, are essential for licensing mTORC1 activity in T cells (Shi, Chapman et al. 2019). In regulatory T cells, amino acids initiate and support TCR-induced mTORC1 signalling, especially arginine and leucine, which are necessary for their proliferative and inhibitory functions. Importantly, deletion of RagA/B or Rheb1/2 in Treg cells results in impaired mTORC1 activation and autoimmune disease in mice. These results suggest that mTORC1 can respond not only to cytokine and receptor signals but also to signals from intracellular nutrients, suggesting that amino acids may also influence the mTOR-dependent response in cytokine-activated CD8$^+$ T cells.

Previous studies have shown that mTOR signalling is often dysregulated in human diseases because of mutations, abnormal upstream signals or loss of negative regulation. In cancer, for example, hyperactivation of the PI3K-AKT-mTOR axis is often observed, contributing to uncontrolled cell growth, metabolic reprogramming, and resistance to apoptosis (Zou, Tao et al. 2020). Similarly, abnormal mTOR activity has been linked to metabolic disorders such as type 2 diabetes, as well as to immune-related disorders, where it influences lymphocyte development and inflammatory responses (Zoncu, Efeyan et al. 2011). These findings highlight mTOR as a central role in disease pathogenesis and a promising therapeutic target across multiple pathological setting.

Understanding cytokine-induced mTOR-dependent activation is critical in many real-world situations, such as in viral infections or tumours, CD8$^+$ T cells may be activated by cytokines before encountering antigens, and in autoimmune diseases or cytokine storms, this pathway may lead to harmful T cell activation. Drugs that block mTOR, such as Rapamycin, are already used in medicine (Ballou and Lin 2008) and could be improved to target the pathway more precisely. This type of research could help develop better immunotherapies or reduce the side effects of an overactive immune response.

3.2 Aims

To achieve my main goal of exploring whether and how the mTOR pathway contributes to cytokine activation of $CD8^+$ T cells, I propose the following specific aims:

Aim 1: Compare the in vitro responses of $CD8^+$ T cells to TCR-dependent and -independent stimulation.

Aim 2: Determine whether the mTOR pathway is involved in cytokine-induced $CD8^+$ T cell activation.

Aim 3: Investigate whether the presence of amino acids affects mTOR activation and effector function in cytokine-stimulated $CD8^+$ T cells.

Aim 4: Assess the role of glycolysis in mTOR-regulated cytokine-induced $CD8^+$ T cell activation.

3.3 Results

This study focused on human CD8$^+$ T cells obtained from the peripheral blood of healthy donors. Many studies have shown that CD8$^+$ T cells, including certain subpopulations such as MAIT cells, can be activated either by their TCR or by cytokines alone, particularly IL-12+IL-18. This means that these cells do not always need to recognise a specific antigen to be activated. Before investigating the signalling pathways involved, it was first examined whether cytokine-only activation could be reproduced in the experimental system used in this study.

3.3.1 CD8$^+$ T cells were responsive to both IL-12+IL-18 and T cell activation beads

To test the response of CD8$^+$ T cells to different types of stimuli, CD8$^+$ T cells were isolated from fresh or frozen PBMCs from healthy donors and stimulated for 15 hours with IL-12+IL-18 or T cell activation beads (TAB). IFN-γ production under these conditions was measured using ELISA and relative live cell counts were detected by flow cytometry (Figure 3.1A).

CD8$^+$ T cells exposed to IL-12+IL-18 or TAB produced significantly more IFN-γ compared to unstimulated controls (Figure 3.1B). This showed that CD8$^+$ T cells could be activated and functioning in both stimulation settings. These results support previous studies, confirming that CD8$^+$ T cells can be activated in vitro via cytokine signalling or TCR-dependent pathways. With this confirmation, the next stage of the study focused on defining how these activation pathways depend on specific signalling mechanisms, especially the mTOR pathway.

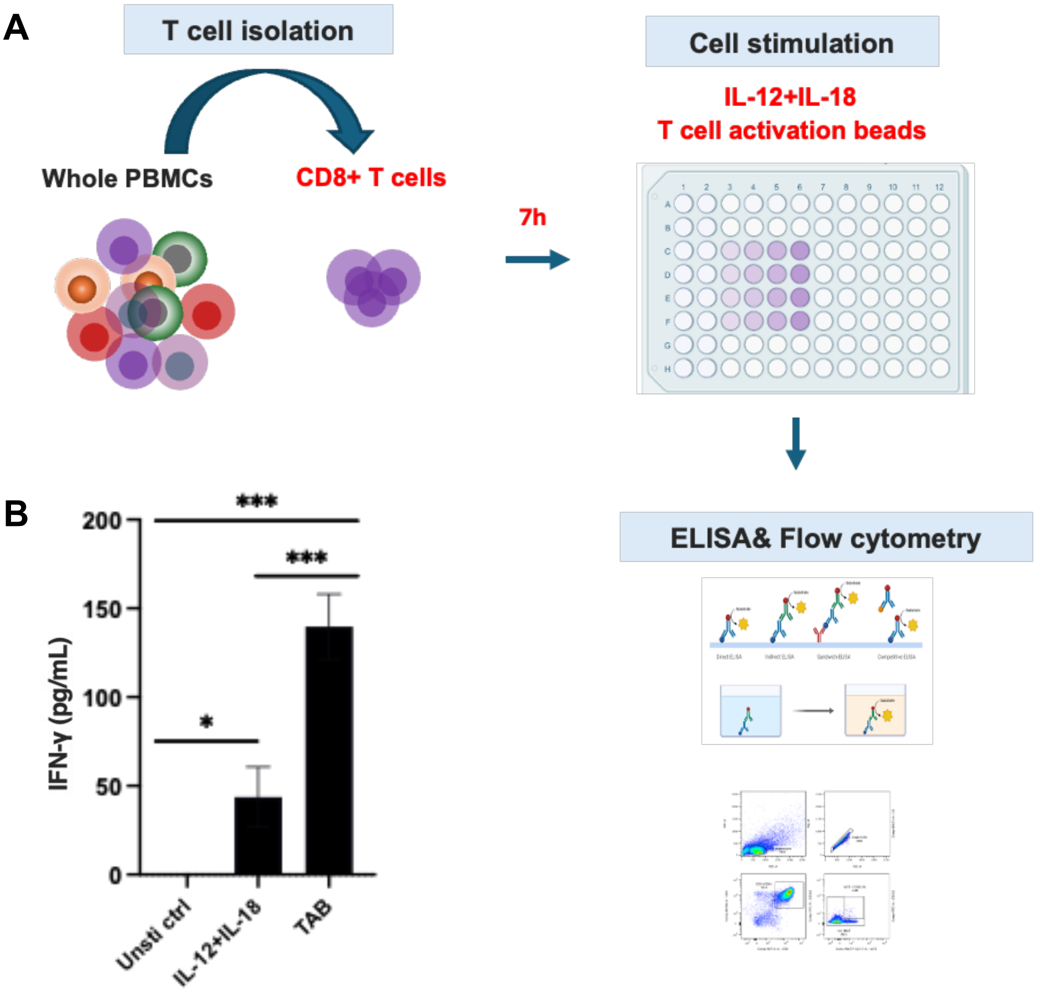


Figure 3.1. CD8^{+} T cells respond to both IL-12+IL-18 and T cell activation beads.

(A) The experimental workflow. Human CD8^{+} T cells were isolated from fresh or frozen whole PBMCs, followed by IL-12+IL-18 or T cell activation beads (TAB) stimulation for 15 hours. And the supernatant or cells were then collected for ELISA or FACS test. (B) The IFN-γ production in cytokine or TAB activated CD8^{+} T cells. n=4 biological replicates. Data were analysed using one-way ANOVA followed by Tukey's multiple comparisons test. $^{*}p < 0.05$, $^{***}p < 0.001$.

3.2.2 mTOR chemical inhibitors downregulate IFN-γ production by CD8$^+$ T cells more efficiently than AKT inhibitors

mTOR is the primary regulatory factor of T cell metabolism, growth, and function. It controls how T cells utilise energy and synthesise proteins during immune responses. mTOR consists of two complexes: mTORC1 and mTORC2. Figure 3.2 shows an overview of mTOR signalling pathways and the targets of chemical inhibitors used in this study. mTORC1 interacts with nutrients, growth factors, and cytokines, promoting protein synthesis and glycogen synthesis through S6 kinase and 4E-BP1 (Ma and Blenis 2009). mTORC2 supports long-term cell survival and regulates cell structure by activating the Ser473 site of AKT (Fu and Hall 2020). AKT is a key molecule that activates mTOR, especially under the influence of TCR and co-stimulatory signals(Fu and Hall 2020). The PI3K-AKT-mTOR axis plays a key role in T cell activation and fate determination, particularly in cytokine production regulation and metabolic reprogramming (Chi 2012, Glaviano, Foo et al. 2023).

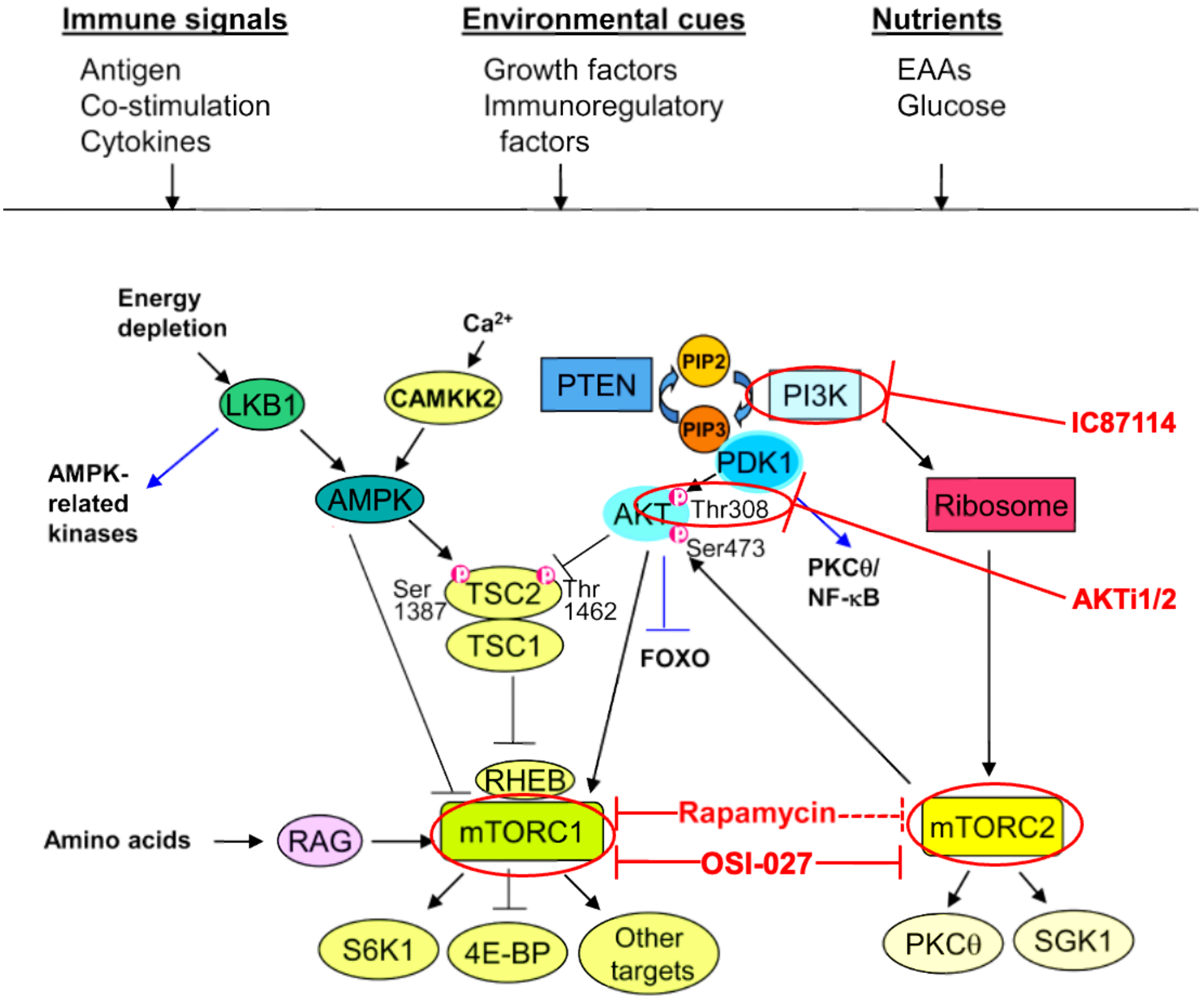


Figure 3.2 Overview of mTOR signalling pathways and the targets of chemical inhibitors used in this study.

This diagram was adapted from (Chi 2012), it shows how immune signals, environmental factors, and nutrients activate the mTOR signalling pathway in T cells. The phosphoinositide 3-kinase (PI3K) pathway leads to the activation of AKT, which is phosphorylated at two key sites: Thr308 (phosphorylated by PDK1) and Ser473 (phosphorylated by mTORC2). Activated AKT inhibits the tuberous sclerosis 1 (TSC1)-TSC2 complex, allowing Ras homologue enriched in brain (RHEB) to activate mTORC1. Subsequently, mTORC1 promotes protein synthesis through downstream targets such as S6 kinases (S6Ks) and eIF4E-binding proteins (4E-BPs). mTORC2 also promotes the activation of AKT and controls other proteins such as serum- and glucocorticoid-inducible kinase 1 (SGK1) and protein kinase C θ (PKCθ). The chemical inhibitors used in this study are marked in red. IC87114 targets PI3K, blocking upstream signalling transduction; AKTi1/2 directly inhibits AKT activity, affecting both mTORC1 and mTORC2 downstream signalling; Rapamycin specifically inhibits mTORC1, disrupting S6K1/4E-BP-mediated translational pathways; OSI-027 is a well-established dual inhibitor of mTORC1 and mTORC2, resulting in broader suppression of mTOR signalling. *LKB1, liver kinase B1; AMPK, AMP-activated protein kinase; EAA, essential amino acid; PIP2, phosphatidylinositol-4,5-bisphosphate; PIP3, phosphatidylinositol-3,4,5-triphosphate.*

To test the functional role of these pathways in cytokine-induced activation of human CD8$^+$ T cells, cells were treated with chemical inhibitors targeting mTOR or AKT for 15 hours, followed by IL-12+IL-18 stimulation in the presence of inhibitors overnight. The intracellular IFN-γ expression response was measured by flow cytometry.

It was observed that treatment of CD8$^+$ T cells with mTOR inhibitors (Rapamycin, OSI-027) resulted in a significant reduction in IFN-γ production (Figure 3.3 A-B), which was detected via flow cytometry. This further confirms that mTOR activity is critical for cytokine-induced IFN-γ production. This result is consistent with previous studies, indicating that mTOR is involved in the expression of proinflammatory cytokine in both TCR-dependent and TCR-independent contexts (Kaech and Cui 2012, Gubser, Bantug et al. 2013, Liang, Wang et al. 2022). Subsequently, it was investigated that AKT inhibition resulted in a mild decrease in IFN-γ levels, but less potent than mTOR inhibition (Figure 3.3 C-D). The inhibitory effect of AKTi1/2 was not strong enough initially, possibly due to low concentration.

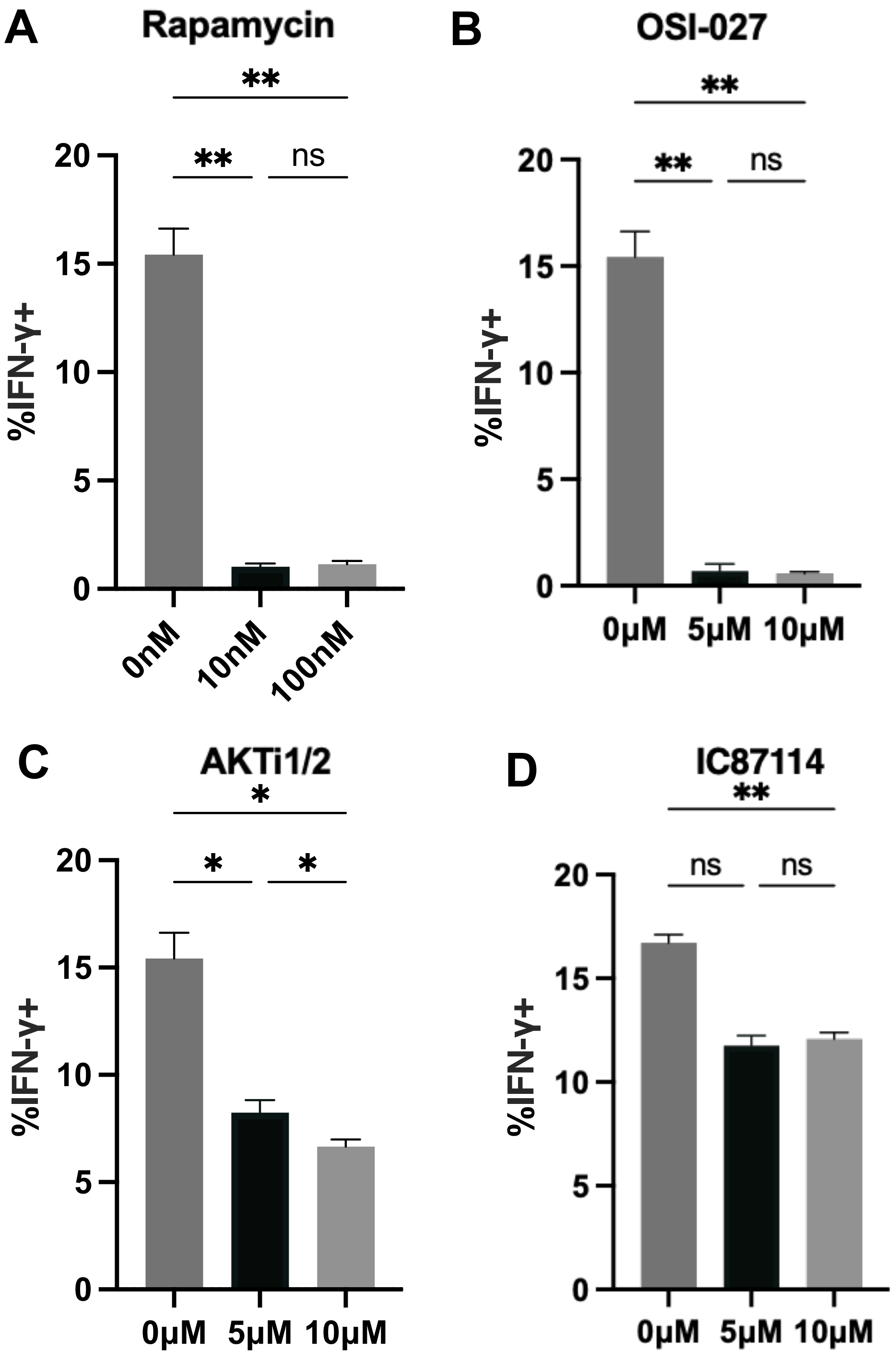


Figure 3.3 Effect of the mTOR and AKT chemical inhibitors on IFN-γ production by $CD8^+$ T cells.

Lymphocytes were first gated on $CD3^+CD8^+$ T cells. (A) to (D) Under different chemical inhibition of mTOR, the expression level of IFN-γ in cytokine stimulated $CD8^+$ T cells was detected by ICS flow cytometry. Data are represented as mean ± SEM. The p values were calculated by One-way ANOVA analysis followed by Tukey's multiple comparisons test. n=3 biological replicates. ns, no significance ($p > 0.05$), $^*p < 0.05$, $^{**}p < 0.01$.

The dose was then increased to examine its effect on IFN-γ expression (Figure 3.4A). When 10 µM and 20 µM AKTi1/2 were compared, IFN-γ expression decreased further at 20 µM, suggesting that maximal inhibition had not yet been reached. The dose was therefore increased to 40 µM. At 40 µM, IFN-γ expression was reduced more strongly. However, the relative number of live cells decreased sharply as well. This suggests that while stronger AKT inhibition can better suppress cytokine production, high doses may also impair cell viability. This indicates that AKT is critical for the survival of $CD8^+$ T cells, consistent with previous studies on the role of AKT in maintaining T cell survival (Kelly, Won et al. 2002, Su, Lin et al. 2007, Hähnel, Thaler et al. 2008).

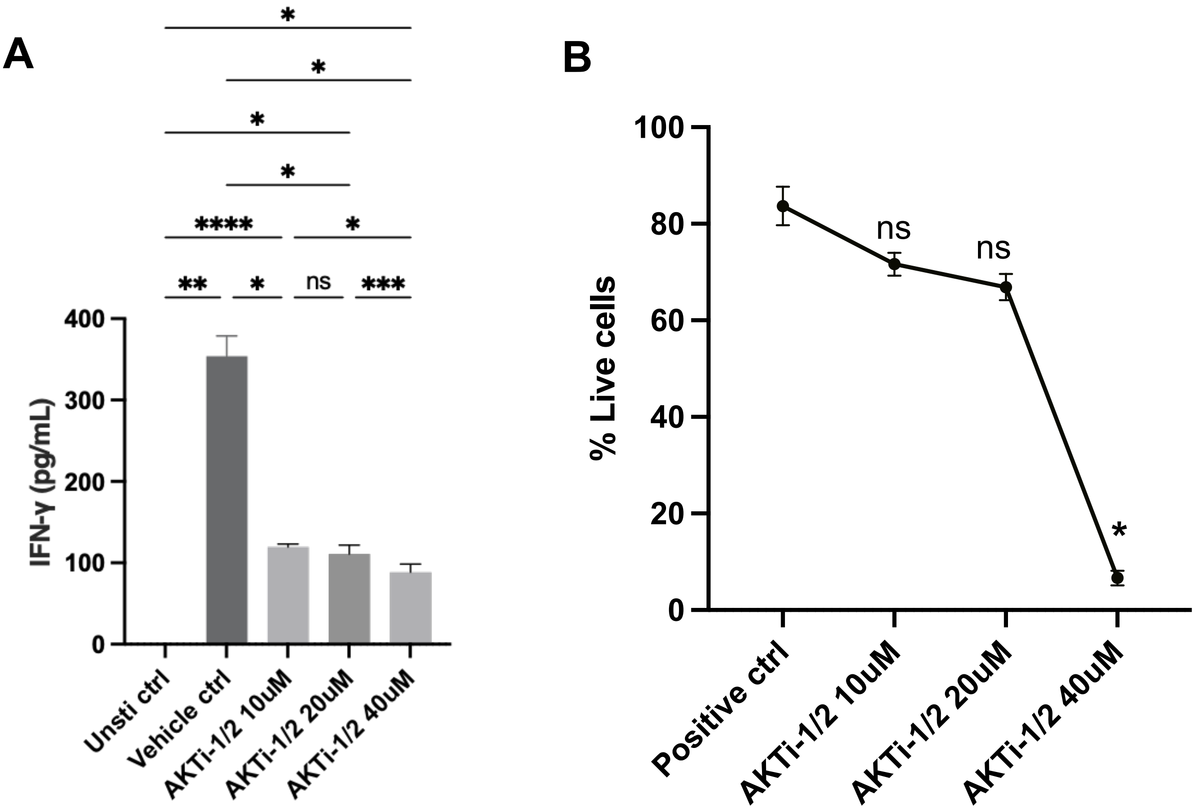


Figure 3.4 AKT inhibition reduces IFN-γ production in cytokine-stimulated CD8⁺ T cells but at higher concentrations it also affects the viability of the cells.

(A) Under different AKT inhibition condition, the expression level of IFN-γ by IL-12+IL-18-stimulated CD8⁺ T cells was detected by ELISA. n=3 biological replicates. (B) Percentage of live cells under the different experimental conditions shown in (A). Data are represented as mean ± SEM. The p values were calculated by One-way ANOVA analysis followed by Tukey's multiple comparisons test in A and by Dunnett's multiple comparisons test in B. ns, no significance ($p > 0.05$), *$p < 0.05$.

Taken together, these results suggest that the mTOR pathway plays a more direct role in regulating cytokine-induced effector functions in CD8⁺ T cells, while the AKT pathway may be more involved in supporting cell viability. Treatment with AKTi-1/2 partially reduced IFN-γ production, but at higher concentrations also led to a significant decrease in cell viability (Figure 3.4B). This raises the possibility that the observed effects are not solely due to pathway-specific inhibition, but may also reflect cytotoxicity, cell cycle disruption, or off-target effects. Further analysis would be needed to unravel these possibilities. In contrast, mTOR inhibition suppressed IFN-γ production more effectively, while cell viability remained at a higher level, supporting its role as a more proximal regulator of cytokine-driven activation. Based on these observations, subsequent experiments focused on optimising mTOR blockade.

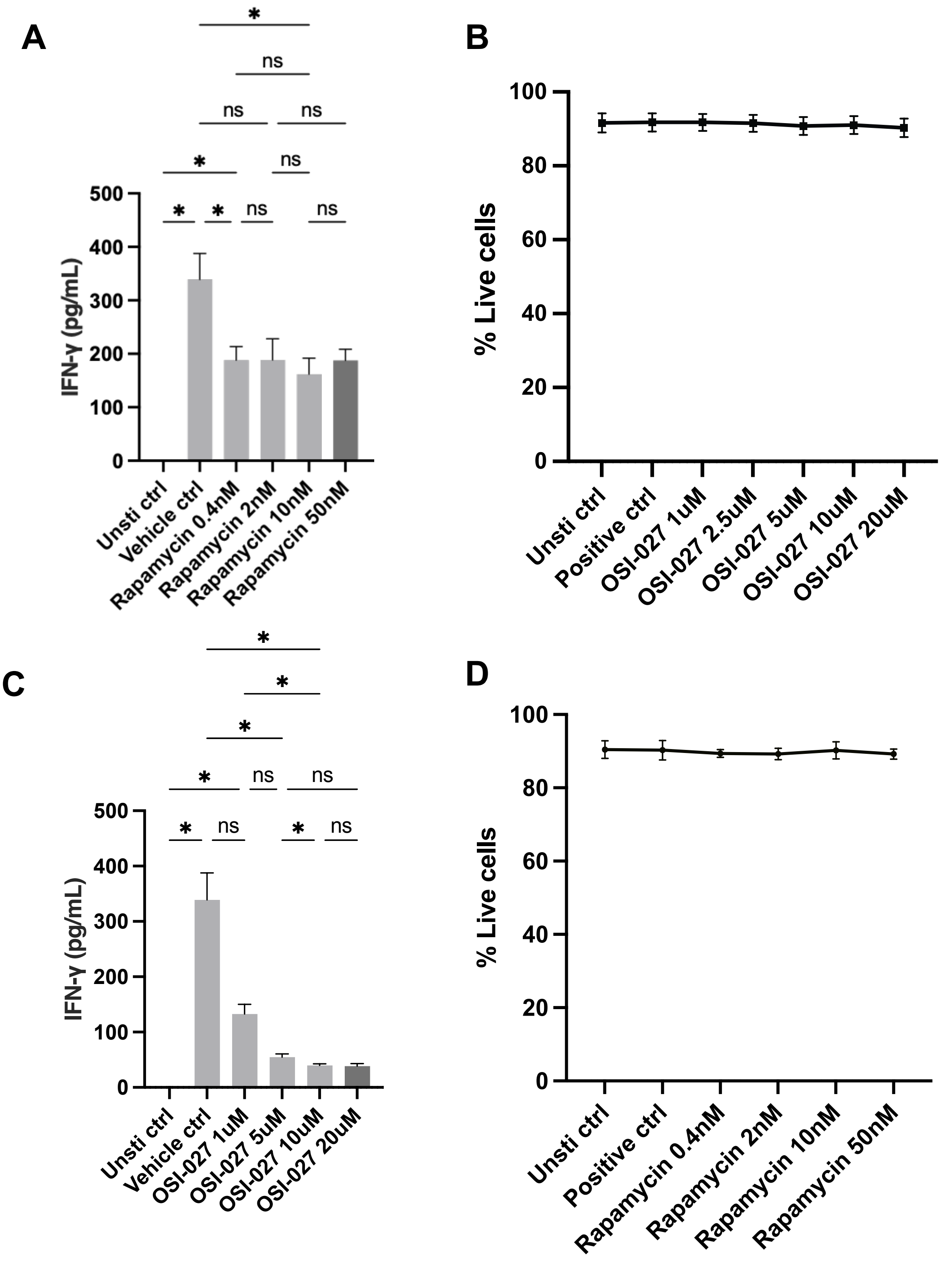


Figure 3.5 Dose-dependent inhibition of IFN-γ production in $CD8^+$ T cells by Rapamycin and OSI-027.

(A) Under different Rapamycin inhibition concentrations, the expression level of IFN- γ in IL-12+ IL-18 stimulated $CD8^+$ T cells was detected by ELISA. n=3 biological replicates. (B) Percentage of live cells under the different experimental conditions shown in (A). (C) Under different OSI-027 inhibition concentrations, the expression level of IFN-γ in cytokine stimulated $CD8^+$ T cells was detected by ELISA. n=3 biological replicates. (D Percentage of live cells under the different experimental conditions shown in (C). Data are represented as mean ± SEM. The p values were calculated by One-way ANOVA analysis followed by Tukey's multiple comparisons test in A &C. ns, no significance ($p > 0.05$), $^*p < 0.05$.

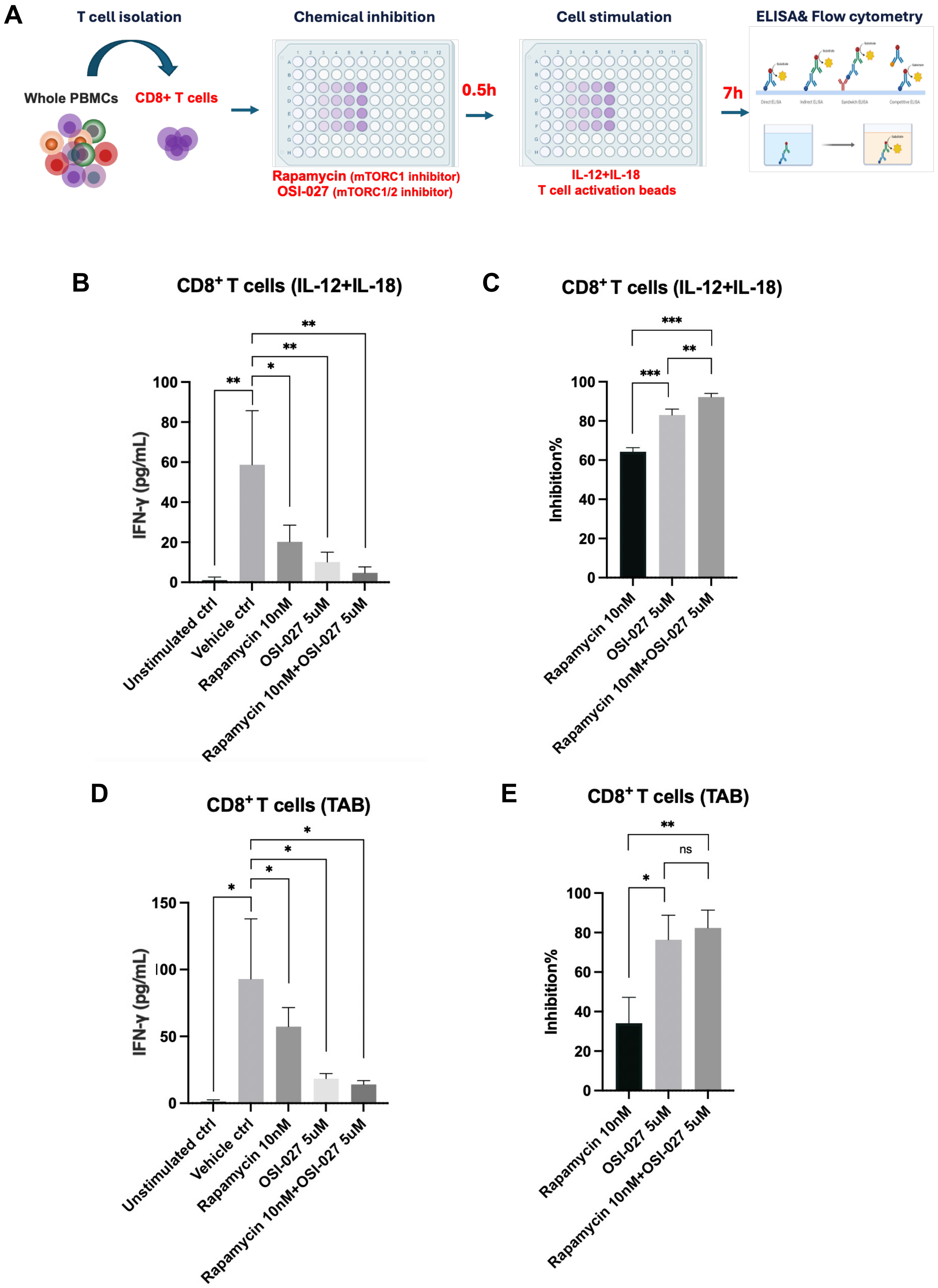
After observing that the mTOR inhibitors have a more pronounced regulatory effect on IFN-γ expression than the AKT inhibitors, the conditions for mTOR inhibitor use were further optimised. Since subsequent experiments required separate analysis of the functions of mTORC1 and mTORC2, a working concentration was selected that robustly inhibited cytokine-induced IFN-γ expression in $CD8^+$ T cells without materially affecting viability. To this end, dose-response experiments were conducted with the mTORC1 inhibitor Rapamycin and the mTORC1/2 dual inhibitor OSI-027.

Previous studies have shown that Rapamycin was effective to inhibit mTORC1 at low nanomolar concentration, such as 10 nM (Foster and Toschi 2009, Batista, Barata et al. 2011, Yin, Mitson-Salazar et al. 2017). To confirm this in the experimental system used here, rapamycin was tested across a range spanning below to above 10 nM (0.4-50 nM), and its effects on IFN-γ expression in IL-12+IL-18-stimulated $CD8^+$ T cells were assessed. The results showed that as the concentration of Rapamycin increased, the level of IFN-γ gradually decreased (Figure 3.5A). However, there was no significant difference between 10 nM and 50 nM conditions, suggesting that 10 nM was sufficient for maximal mTORC1 inhibition under these conditions. Additionally, the number of live cells remained almost the same at all concentrations (Figure 3.5B), indicating that Rapamycin did not measurably impair $CD8^+$ T cell survival within this range. Accordingly, 10 nM Rapamycin was selected for subsequent mTORC1 inhibition experiments.

OSI-027 was then evaluated across a range of concentrations. Unlike Rapamycin, OSI-027 appears to inhibit both mTORC1 and mTORC2 at concentrations of approximately 1-10 µM (Bhagwat, Gokhale et al. 2011, Gupta, Hendrickson et al. 2012). In my system, OSI-027 reduced IFN-γ expression at 1-5 µM compared to the vehicle control (Figure 3.5C). Increasing the concentration to 10 µM did not result in a further decrease in IFN-γ levels, suggesting that maximum inhibition was achieved within this range. Cell viability remained stable throughout the process (Figure 3.5D), indicating that OSI-027 did not compromise the survival of $CD8^+$ T cells. Based on these data, 10 µM OSI-027 was selected as the working concentration for dual mTORC1/2 inhibition in subsequent experiments.

3.2.3 Different from TCR-mediated $CD8^+$ T cell activation, mTORC1 pathway contributed more to cytokine-mediated $CD8^+$ T cell activation compared with mTORC2 pathway

Previous studies have shown that mTORC1 and mTORC2 play distinct roles under different immune stimuli. For example, mTORC1 is crucial for the differentiation of Th1 and Th17 cells, while mTORC2 primarily participates in Th2 cell differentiation (Delgoffe, Pollizzi et al. 2011). This suggests that mTOR complexes have functional specialisation in immune activation.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T cell isolation
Whole PBMCs
CD8+ T cells
Chemical inhibition
Rapamycin (mTORC1 inhibitor)
OSI-027 (mTORC1/2 inhibitor)
0.5h
Cell stimulation
IL-12+IL-18
T cell activation beads
7h
ELISA& Flow cytometry
Direct ELISA
Indirect ELISA
Sandwich ELISA
Competitive ELISA
B
CD8⁺ T cells (IL-12+IL-18)
IFN-γ (pg/mL)
0
20
40
60
80
100
**
*
**
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Unstimulated ctrl
Vehicle ctrl
Rapamycin 10nM
OSI-027 5uM
Rapamycin 10nM+OSI-027 5uM
C
CD8⁺ T cells (IL-12+IL-18)
Inhibition%
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Rapamycin 10nM
OSI-027 5uM
Rapamycin 10nM+OSI-027 5uM
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OSI-027 5uM
Rapamycin 10nM+OSI-027 5uM

Figure 3.6 Both mTORC1 and mTORC2 regulate cytokine-induced and TCR-mediated activation of CD8$^+$ T cells.

(A) The experimental workflow. Human CD8$^+$ T cells were isolated from fresh or frozen PBMCs and cultured at 0.5 million cells per well in 96-well round bottom plates. The cells were pretreated with chemical inhibitors and then stimulated with IL-12+IL-18 or TAB. (B) IFN-γ expression in CD8$^+$ T cells was measured after 7 hours of stimulation with IL-12+IL-18, with pretreatment with mTOR pathway inhibitors for 1 hour. Rapamycin, a selective mTORC1 inhibitor, and OSI-027, a dual mTORC1/mTORC2 inhibitor, were used to dissect the respective contributions of each mTOR complex to cytokine-induced effector function. (C) Percentage of IFN-γ reduction in CD8$^+$ T cells following cytokine stimulation and chemical inhibition of mTOR complexes. n=4 biological replicates. (D) IFN-γ expression in CD8$^+$ T cells following T cell activation beads (TAB) stimulation and chemical inhibition of mTOR complexes. (E) Percentage of IFN-γ reduction in CD8$^+$ T cells following TAB stimulation and chemical inhibition mTOR complexes. n=4 biological replicates. Data are represented as mean ± SEM. The p values were calculated by One-way ANOVA analysis followed by Tukey's multiple comparisons test. ns=no significance ($p > 0.05$), $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

To further compare the functional differences between mTORC1 and mTORC2 under different activation conditions, CD8⁺ T cells were pretreated with either Rapamycin to inhibit mTORC1 or and OSI-027 to simultaneously inhibit mTORC1 and mTORC2 for 1 hour, followed by stimulation with cytokines (IL-12+IL-18) or T cell activation beads (Figure 3.6A).

Under IL-12+IL-18 stimulation conditions, Rapamycin (10 nM) treatment resulted in around 65% inhibition of IFN-γ expression, while OSI-027 (5 μM) increased the inhibition rate to approximately 80%. The combination of Rapamycin and OSI-027 further increased the inhibition rate to nearly 90% (Figure 3.6B-C). Statistical analysis revealed a significant difference between Rapamycin and OSI-027, suggesting that while mTORC1 is the primary regulatory pathway, mTORC2 also contributed to this process.

Differently, under TAB stimulation conditions, Rapamycin inhibited approximately 35% of IFN-γ expression, while OSI-027 and the combination treatment both achieved approximately 80% inhibition (Figure 3.6D-E). Notably, there was no significant difference between the OSI-027 and combined treatment groups, but both were significantly higher than the Rapamycin monotherapy group, indicating that mTORC2 contributes more to CD8⁺ T cell activation under TAB stimulation.

In summary, mTORC1 plays a dominant role in cytokine-induced CD8⁺ T cell activation, while mTORC2 exerts stronger regulation in TCR-mediated activation. This difference suggests that

T cells activate different signalling and metabolic programmes in response to different types of immune signals such as cytokine-mediated stimulation and TCR-engaged activation.

3.2.4 CD8$^+$ T cells display distinct amino acid, glycolytic, and OXPHOS profiles under cytokine versus TCR activation

Recently, the mutual regulation between metabolism and immunity has gradually been focused more on. After activation, T cells need to rapidly initiate anabolic processes to support proliferation and effector differentiation, and amino acids, as key nutritional factors in cellular metabolism, play an important role in this process.

Previous studies have shown that specific amino acids not only provide energy and synthetic substrates for T cells but also participate in the regulation of T cell effector functions through sensing mechanisms, such as regulating mTOR activity. For example, arginine and leucine were found to enhance mTORC1 activation, while glutamine influences oxidative metabolism and the cell cycle (Shi, Chapman et al. 2019). Studies also showed that CD8$^+$ T cells are highly sensitive to amino acid environments, and amino acid deficiency significantly reduces IFN-γ expression and cytotoxic activity, thereby limiting their anti-infective and anti-tumour capabilities (Wang and Zou 2020, Bopp, Martinez et al. 2024). Building on previous findings, this study investigated the role of amino acids and glucose metabolism in the activation of CD8$^+$ T cells and IFN-γ expression in humans, with a particular focus on the interaction between these metabolic inputs and mTORC1 and mTORC2 signalling.

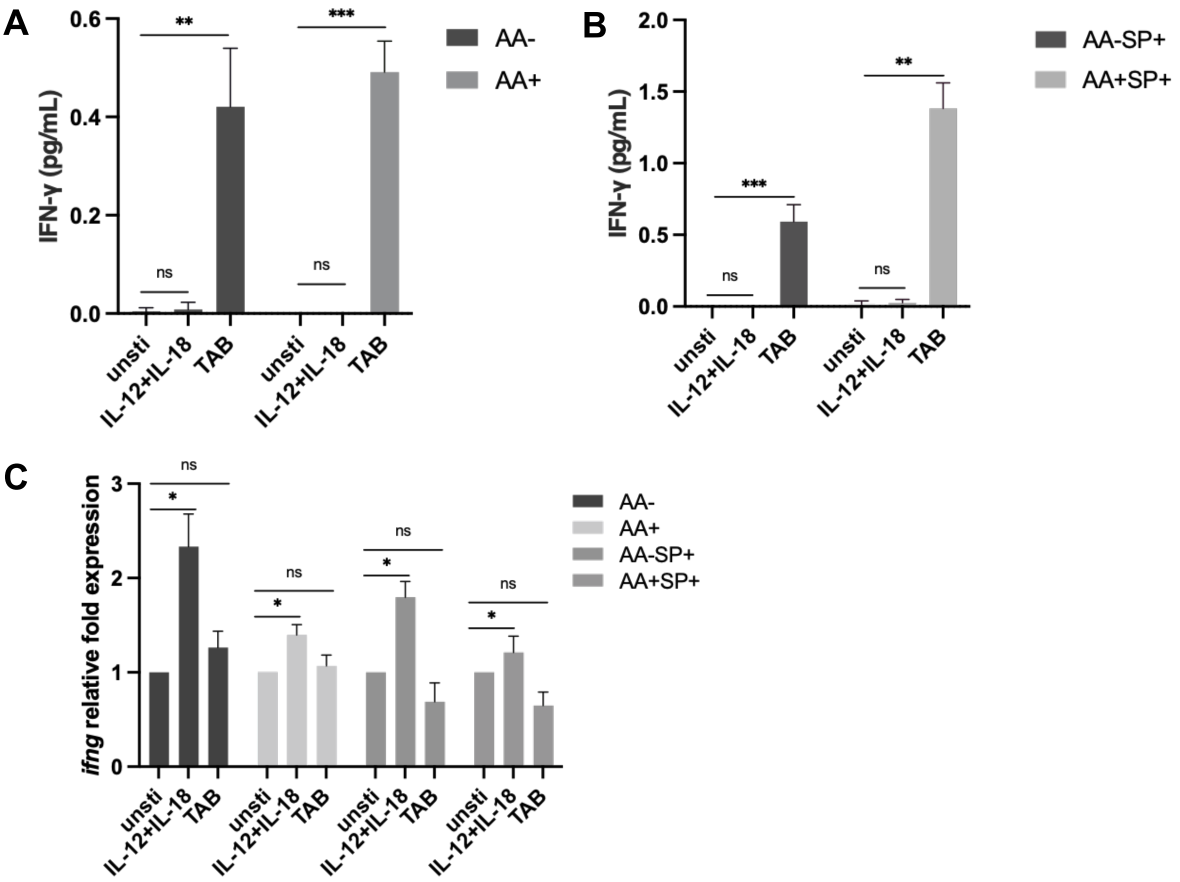


Figure 3.7 IFN-γ expression in activated CD8$^+$ T cells across amino-acid availability under IL-12+IL-18 or TAB stimulation.

(A) IFN-γ expression was measured by ELISA in CD8$^+$ T cells cultured in amino acid-free (AA-) or amino acid-sufficient (AA+) media and stimulated with cytokines or T cell activation beads, using 0.5 million cells per well. n=3 biological replicates. (B) In AA-SP+ and AA+SP+ cell culture media, the expression level of IFN-γ in cytokine or T cell activation beads stimulated CD8$^+$ T cells (0.5 million cells per well) was detected by ELISA. n=4 biological replicates. SP: Sodium pyruvate (C) The mRNA level of IFN-γ in CD8$^+$ T cells was measured by qPCR after 7 hours of stimulation with IL-12+IL-18 or TAB. IFN-γ transcript levels were normalised to GAPDH. Both stimuli induced IFN-γ expression compared to unstimulated controls, with TAB-stimulated cells generally showing higher transcript levels. Unstimulated cells were included as negative controls. n=3 biological replicates. Data are represented as mean ± SEM. The *p* values were calculated by two tailed t-test. ns=no significance ($p > 0.05$), $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.001$.

To clarify the effect of amino acids on IFN-γ expression in activated human CD8$^+$ T cells CD8$^+$ T cells were cultured in amino acids-free (AA-) and amino acids-supplemented (AA+) culture medium and treated cells with either IL-12+IL-18 or TAB (Figure 3.8A). However, the IFN-γ levels detected by ELISA were uniformly low under all conditions, including the IL-12+IL-18 positive control. This indicates that the system did not support robust activation and that no conclusions can be drawn from this experiment about the role of amino acids. To improve the system, sodium pyruvate (SP) was added to both the AA− and AA+ media (Figure 3.8B). Although this improved IFN-γ levels slightly, the overall response remained weak and unreliable. At the same time, qPCR analysis (Figure 3.8C) showed variable IFN-γ transcript levels, including a clear increase in AA+ conditions when cells were stimulated with IL-12 and IL-18, although the baseline was too low and comparisons were not clearly interpretable. Together, these results suggest that the current culture conditions were suboptimal for assessing the impact of amino acid deficiency and that further optimisation is needed.

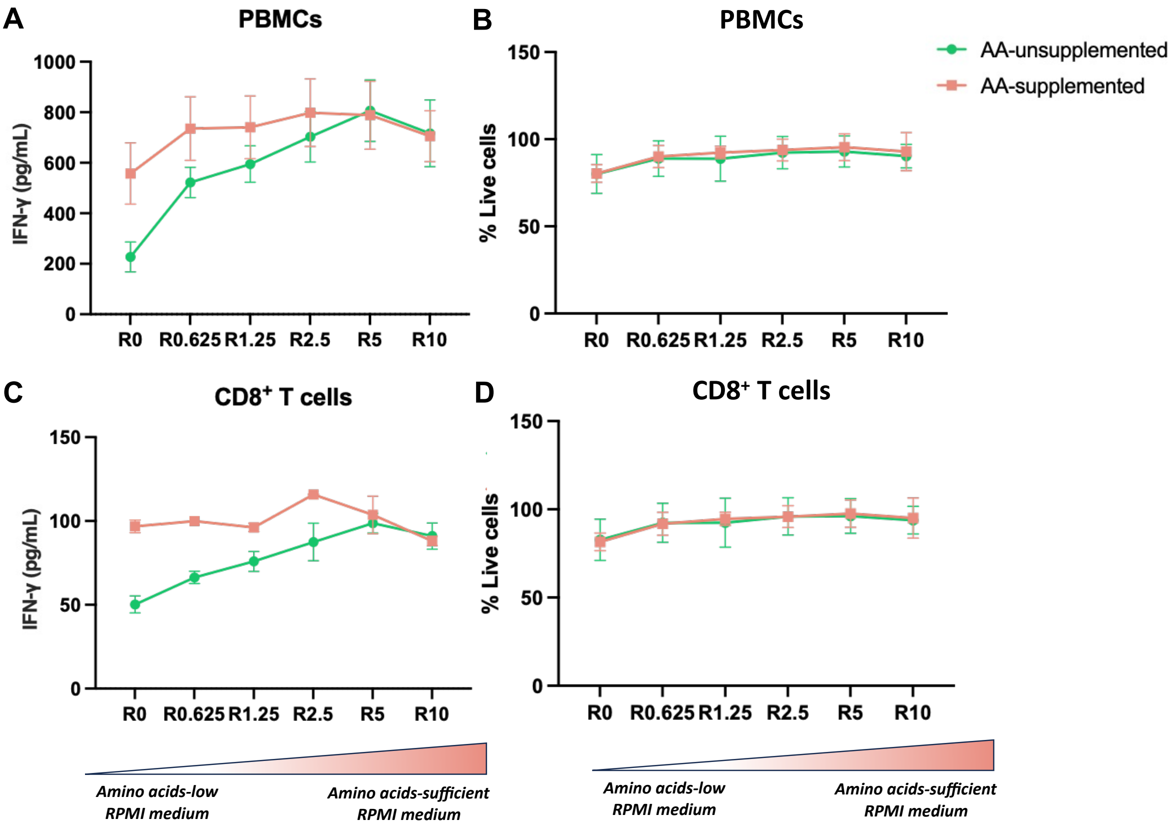


Figure 3.8 FBS titration to model amino acid-limited conditions for T cell functional assays.

(A) IFN-γ levels in IL-12+IL-18-stimulated PBMCs (0.5 × 10^6 cells per well) were quantified by ELISA in RPMI media containing different percentages of FBS, with or without additional amino acid supplementation. n=3 biological replicates. (B) Percentage of live cells under the different experimental conditions shown in (A). (C) In different FBS% RPMI culture media, the expression level of IFN-γ in cytokine stimulated CD8$^+$ T cells (0.5 million cells per well) was detected by ELISA. n=3 biological replicates. (D) Percentage of live cells under the different experimental conditions shown in (C). Data are represented as mean ± SEM.

Given the technical limitations of the previous experiment, particularly the failure of the IL-12+IL-18 stimulation condition to induce IFN-γ expression, a more suitable amino acid-restricted culture system was established. The aim was to maintain the basic reactivity of T cells while obtaining a clearer assessment of the influence of amino acid availability on activation. Therefore, the original AA- medium was discontinued and an alternative culture condition was developed to model a low-amino acid state without excessively restricting nutrients.

To this end, conventional RPMI 1640 medium without FBS was defined as the ‘R0’ baseline condition. This system contains low level of amino acids and glucose but lacks serum-derived nutritional supplementation. FBS contains a certain number of amino acids and growth factors, reduced FBS concentrations were used as a strategy to approximate nutrient-limited conditions. Considering that completely remove FBS might also impair cell activity, a gradient of FBS concentrations was established, and candidate conditions were screened by measuring IFN-γ expression levels and cell viability under cytokine stimulation.

CD8$^+$ T cells are typically cultured in RPMI 1640 medium supplemented with FBS (R10), which provides abundant amino acids and nutrients, maintaining normal cellular activity. However, the higher concentrations of serum-derived amino acids and their metabolic products in R10 will mislead the true regulatory effects of exogenous amino acid changes on T cell function. Therefore, this condition is not suitable for studying the immune response mechanisms under amino acid-deficient conditions. A gradient concentration of FBS supplementation (0%, 0.625%, 1.25%, 2.5%, 5%, 10%) was established and IFN-γ expression levels and changes in cell viability induced by 7-hour cytokine stimulation were compared under amino acid-supplemented (AA-supplemented) and amino acid-unsupplemented (AA-unsupplemented) conditions (Figure 3.8).

It was shown that in human PBMCs, IFN-γ expression gradually increased with increasing FBS concentration. In the meanwhile, IFN-γ production levels in the AA-supplemented condition consistently higher than those in the AA-unsupplemented condition, especially in R0 condition. The difference between the two conditions tended to decrease when FBS concentration reached 2.5% or higher (Figure 3.8A). There were no significant changes in total live cell numbers under different conditions (Figure 3.8B), indicating that the culture conditions had no impact on cell survival.

A similar trend was also observed in purified CD8$^+$ T cells. IFN-γ secretion was consistently higher under AA-supplemented conditions than under AA-unsupplemented conditions and increased with rising FBS concentrations, especially in R0 condition (Figure 3.8C). This suggests that R0 condition was the most suitable condition to study the role of amino acids in cytokine-induced T cell activation. The total live cell count remained stable across all groups (Figure 3.8D), further confirming that these conditions did not impair cell viability in my experimental setting.

Based on these optimisation results, R0 medium (0% FBS) was selected as the standard low-amino acid condition. The R0 culture system does not contain exogenous amino acids and only provides the minimum support necessary for cell survival. This enables a more accurate assessment of how amino acid availability and related signalling pathways regulate CD8$^+$ T-cell activation and function under controlled metabolic conditions.

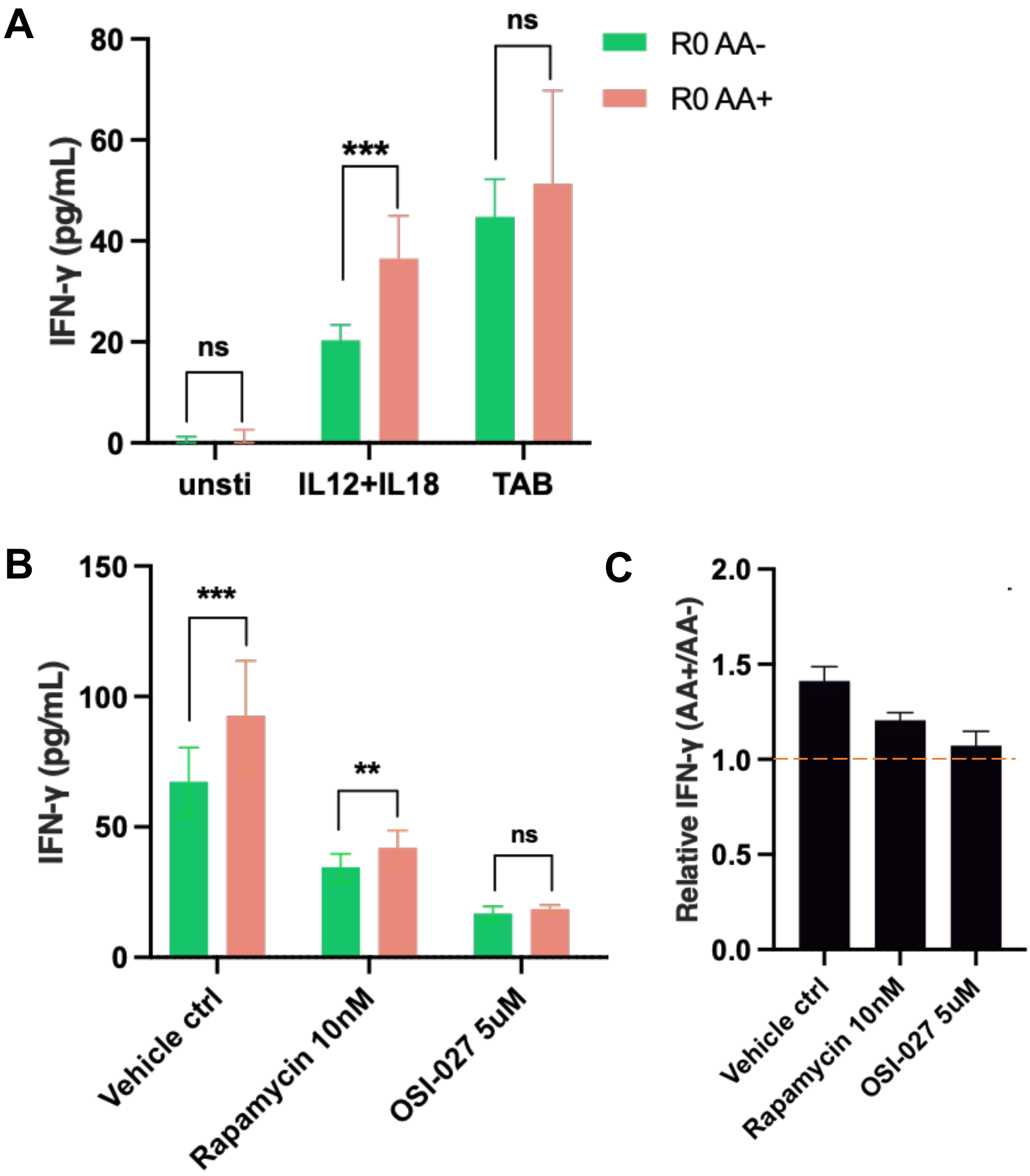


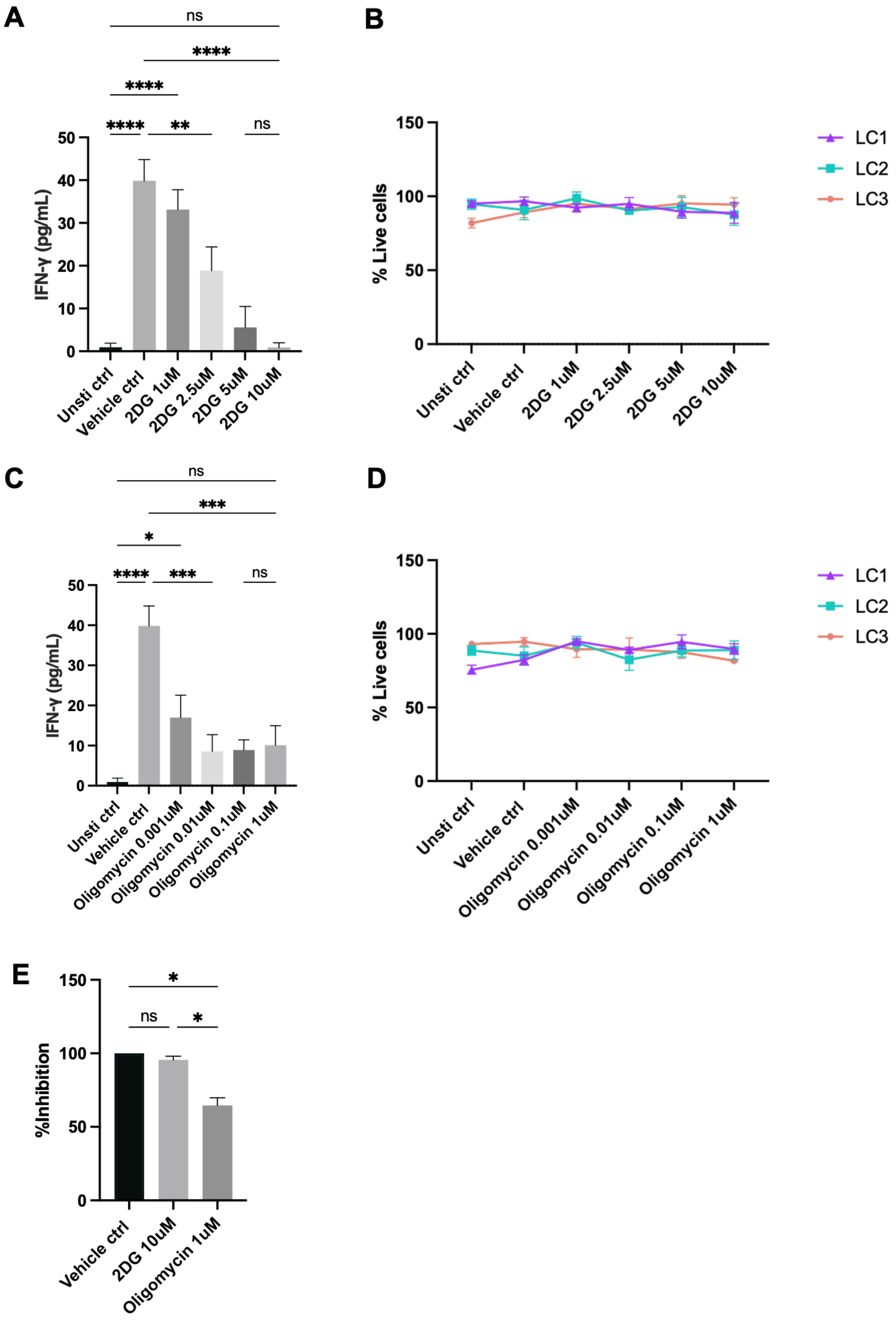
Figure 3.9 Amino acids increase cytokine-induced IFN-γ in CD8$^+$ T cells, with sensitivity to mTORC1 and mTORC1/2 inhibition.

(A) IFN-γ expression in CD8$^+$ T cells following cytokine or TAB stimulation with or without amino acids in R0 media. n=4 biological replicates. (B) IFN-γ expression in CD8$^+$ T cells following cytokine stimulation and mTOR chemical inhibition with or without amino acids in R0 media. (C) Percentage of IFN-γ reduction in CD8$^+$ T cells following cytokine stimulation and chemical inhibition of mTOR complexes with or without amino acids. Data are derived from the absolute IFN-γ values shown in (B). n=4 biological replicates. Data are represented as mean ± SEM. Statistical analysis was performed using paired t-test in A-B. ns=no significance, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

In the optimised R0 culture environment, it was investigated whether amino acids regulate the response of $CD8^+$ T cells to cytokine stimulation, and whether this regulation was through the mTOR signalling pathway. To address this, human primary $CD8^+$ T cells were cultured in either R0 AA− or R0 AA+ media in the presence of IL-12+IL-18 or TAB for 7 hours, and IFN-γ levels were measured by ELISA. The results showed that under amino acids-low condition, IL-12+IL-18-induced IFN-γ expression was significantly reduced, amino acid supplementation restored IFN-γ expression levels (Figure 3.9A). This change was not seen under TAB stimulation, further supporting the focus of this study on cytokine-mediated activation responses.

To determine the role of amino acids in mTOR signalling in this context, cells were pretreated with Rapamycin (selectively inhibiting mTORC1) and OSI-027 (jointly inhibiting mTORC1 and mTORC2) and then stimulated with IL-12 and IL-18 for 7 hours. Under amino acid-sufficient conditions, IFN-γ expression was significantly reduced by rapamycin, whereas a stronger inhibition was observed with OSI-027 (Figure 3.9B). Further analysis of inhibitory efficiency indicated that, under amino acid-supplemented conditions, both mTORC1 and mTORC2 are involved in regulating IFN-γ expression (Figure 3.9C), suggesting that the amino acid-enhanced activation effect depends on the synergistic action of the mTORC1 and C2 pathways.

After determining that amino acid availability enhances IFN-γ expression in $CD8^+$ T cells via the mTOR signalling and given that mTOR is closely associated with metabolic status, the potential energy sources required for this response was investigated. Metabolic reprogramming is a key event of T cell activation, and effector function is closely associated with changes in glycolysis and mitochondrial respiration (Shi, Zhang et al. 2025). However, it remains unclear whether cytokine-induced activation shares the same metabolic dependence as classical TCR-engaged activation. To further understand the metabolic dependence of T cells under different activation modes, the relative contribution of major energy-producing pathways was assessed, with focus on whether there are differences between cytokine stimulation and TAB stimulation.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2DG 10uM
Oligomycin 1uM

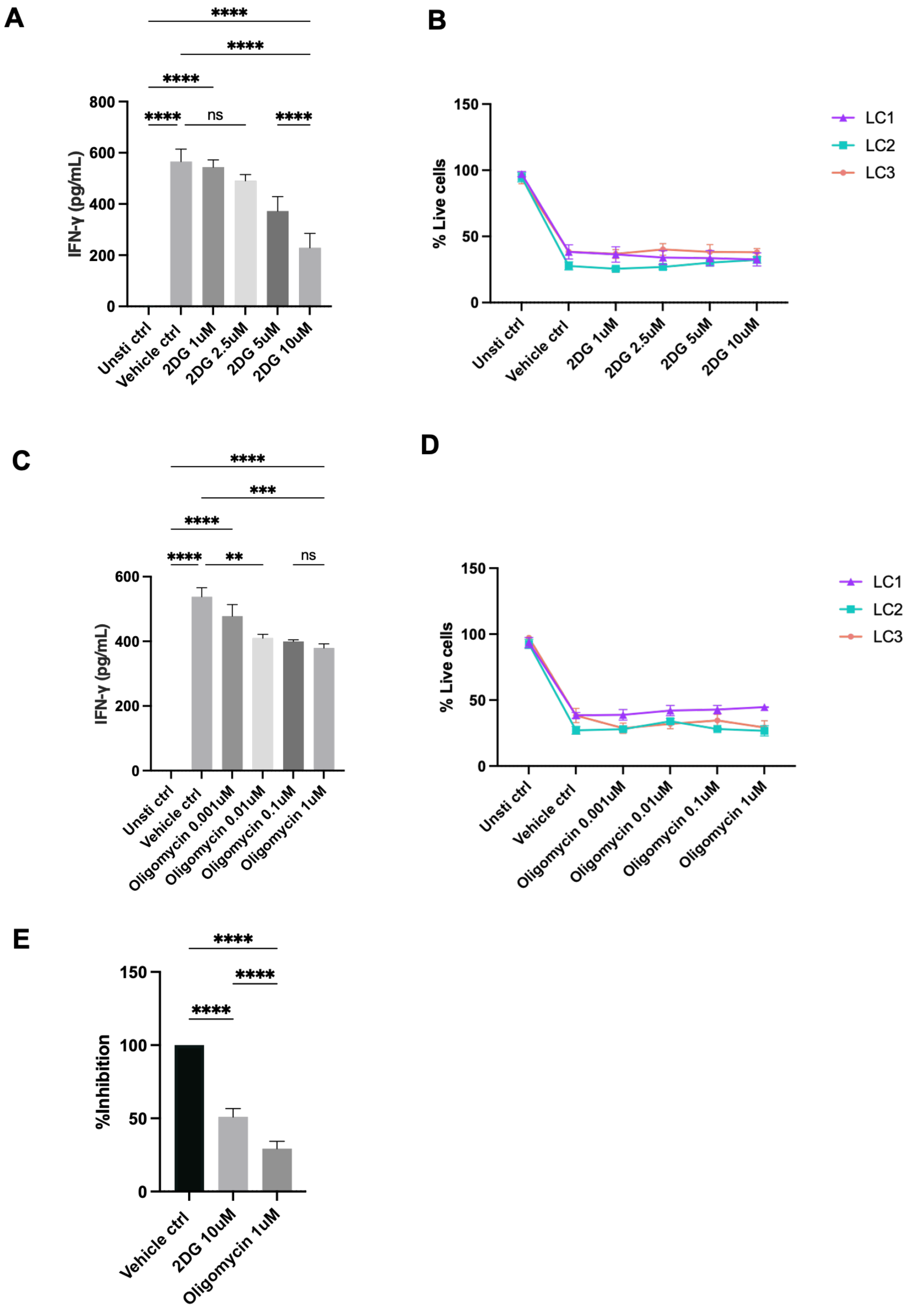
Figure 3.10 Effects of glycolysis and OXPHOS inhibition on cytokine-activated CD8$^+$ T cells.

(A) IFN-γ expression in CD8$^+$ T cells following cytokine stimulation with 2-DG. n=3 biological replicates. (B) Percentage of live cells under the different experimental conditions shown in (A), LC: leucocyte cone. (C) IFN-γ expression in CD8$^+$ T cells following cytokine stimulation with Oligomycin. n=3 biological replicates. (D) Percentage of live cells under the different experimental conditions shown in (C). (E) Percentage of IFN-γ reduction in CD8$^+$ T cells following cytokine stimulation and chemical inhibition. Data are represented as mean ± SEM. The p values were calculated by one-way ANOVA analysis followed by Tukey's multiple comparisons test. ns=no significance, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

To determine the primary energy source relied on by $CD8^+$ T cells during activation, cells were pretreated with the glycolytic inhibitor 2-DG or the mitochondrial oxygen phosphorylation inhibitor Oligomycin under IL-12+IL-18 and TAB stimulation conditions, respectively.

In cytokine-stimulated $CD8^+$ T cells, IFN-γ production was gradually decreased with increasing 2-DG doses. At a 2-DG concentration of 10 μM, IFN-γ levels were nearly completely inhibited (Figure 3.10A). Cell viability remained stable across all treatment groups (Figure 3.10B), indicating that reduced IFN-γ was unlikely to be driven by cytotoxicity.

Similarly, Oligomycin treatment (Figure 3.10C) also reduced IFN-γ expression, though to a lesser extent. Quantitative analysis of the inhibitory effects (Figure 3.10E) showed that glycolysis inhibition resulted in over 90% inhibition, while OXPHOS inhibition only caused around 50% inhibition, indicating that glycolysis is the primary metabolic pathway supporting cytokine-induced IFN-γ expression.


A
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IFN-γ (pg/mL)
800
600
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0
Unsti ctrl
Vehicle ctrl
2DG 1uM
2DG 2.5uM
2DG 5uM
2DG 10uM
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% Live cells
150
100
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LC3
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Vehicle ctrl
2DG 1uM
2DG 2.5uM
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2DG 10uM
Oligomycin 1uM

Figure 3.11 Sensitivity of TAB-stimulated $CD8^+$ T cells to glycolytic and mitochondrial inhibition.

(A) IFN-γ expression in $CD8^+$ T cells following TAB stimulation with 2-DG. n=3 biological replicates. (B) Percentage of live cells under the different experimental conditions shown in (A), LC: leucocyte cone. (C) IFN-γ expression in $CD8^+$ T cells following TAB stimulation with Oligomycin. n=3 biological replicates. (D) Percentage of live cells under the different experimental conditions shown in (C). (E) Percentage of IFN-γ reduction in $CD8^+$ T cells following TAB stimulation and chemical inhibition. Data are represented as mean ± SEM. The p values were calculated by one-way ANOVA analysis followed by Tukey's multiple comparisons test. ns=no significance, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

Different from cytokine-induced activation, in TAB-stimulated CD8$^+$ T cells, although 2-DG still reduced IFN-γ levels in a dose-dependent manner (Figure 3.11A), the inhibitory effect was less pronounced, with an inhibition rate of approximately 55% at 10 μM (Figure 3.11E). Oligomycin also reduced cytokine expression (Figure 3.11C), showing a similar inhibition of approximately 35%. In addition, live cell counts were unaffected by any of the inhibitors (Figure 3.11B, D), confirming that the observed effects reflect functional impairment rather than cell death.

Taken together, these results indicate that TAB-activated CD8$^+$ T cells rely on glycolytic and mitochondrial pathways, consistent with broader metabolic involvement in TCR-induced activation processes. This aligns with the previously mentioned more important role of mTORC2 in TAB activation, further indicating that T cells use different metabolic pathways to support their functions under distinct activation modes.

In summary, these data reveal fundamental differences in metabolic dependency among CD8$^+$ T cells under different activation signals. IL-12+IL-18 stimulation drives a highly glycolysis-dependent metabolic programme with less reliance on mitochondrial OXPHOS. In contrast, TAB stimulation involves both glycolysis and OXPHOS to maintain effector function.

Given this, it was examined whether these metabolic dependencies are consistent across all CD8$^+$ T cells or differ between functionally distinct subpopulations. The analysis was focused on two phenotypically and functionally distinct populations, CD161^{hi} and NKG2A$^+$ T cells.

CD161^{hi} cells are known for their innate nature and sensitivity to cytokines, while NKG2A^{+} cells, as demonstrated in recent studies, exhibit a strong IFN-γ response to IL-12+IL-18, potentially representing a balanced effector cell population.

The next chapter will utilise the experimental system established in this chapter to explore the differences between these subpopulations in terms of cytokine-induced activation, metabolic dependency, and signal transduction features.

3.4 Discussion

In this chapter the regulation of human CD8⁺ T cell effector function by mTOR signalling and the metabolic environment was examined under different stimuli, including cytokines such as IL-12+IL-18 and TAB which mimicked TCR-engaged activation. Past studies have focused on antigen-specific TCR pathways, but recent studies have shown that cytokines such as IL-12 and IL-18 can activate T cells in the absence of antigens, especially inducing them to produce IFN-γ (Tominaga, Yoshimoto et al. 2000, Barbarin, Cayssials et al. 2017, Leng, Akther et al. 2019). This antigen-free rapid response was quite important during viral infection, tumour inflammation, or rapid immune responses in barrier tissues (Raeber, Zurbuchen et al. 2018, Sun, Su et al. 2023). On this basis, this study deeply explores the differences in metabolic requirement and immune signal dependence between the two activation pathways.

To elucidate the metabolic requirements of this activation condition, it was systematically compared cytokine-induced responses with TCR-induced responses using chemical inhibitors, combined with time gradient and nutritional condition regulation, and metabolic blockade. IL-12+IL-18 stimulation was found to primarily activated mTORC1 rather than mTORC2, with high sensitivity to glycolytic inhibition and to amino-acid availability. Both IL-12+IL-18 and TAB stimulate IFN-γ production in CD8⁺ T cells but have different regulatory properties. Also, TAB stimulation activates mTORC1 and mTORC2, while the cytokine-induced response depends mainly on mTORC1. This difference may reflect the nature of upstream signal transduction. Cytokine-induced pathways primarily involve STAT-mediated transcriptional responses (O'Shea and Murray 2008, Hu, li et al. 2021), while TCR signalling activates broader pathways, including PI3K/AKT, mTOR, and calcium signalling, all of which influence cellular metabolism(Chi 2012, Wang, Tao et al. 2020).

Additionally, rapamycin markedly inhibited cytokine-induced IFN-γ, whereas dual mTORC1/2 inhibition was more effective under TCR-engaged activation, reflecting different signalling dependencies. Notably, 5 µM OSI-027 is already a high concentration, it was therefore used as the upper dose limit for cross-comparisons. At this dose, IL-12+IL-18 stimulation with showed almost complete suppression of IFN-γ, while stimulation with TAB did not reach an inhibitory plateau. This indicates differential sensitivities to mTOR inhibition between the two activation pathways and supports their distinct upstream signalling dependencies, highlighting the dominant role of mTORC1 in cytokine-induced responses.

AKT inhibition disrupted effector function while impairing cell survival, suggesting its dual role in metabolism and survival. Since AKT is upstream of mTOR and also regulates survival and glucose metabolism, these results indicated the importance of careful control of experimental conditions to distinguish functional from cytotoxic effects. An optimised strategy for pretreatment with inhibitors (Figure 3.5) allows analysis of signal transduction while minimising off-target effects.

In addition, under AA-low conditions, IFN-γ expression was severely reduced, but supplementing amino acids under low-nutrient conditions partially restored the cellular function of CD8$^+$ T cells, and amino acid availability also regulated mTOR-dependent response.

In this study, amino acid deprivation in the original AA- medium was shown to substantially impair cytokine-induced IFN-γ production, but these stringent conditions also reduced cell viability. To solve this problem, RPMI without FBS (R0) was implemented to maintain a minimum level of basic supply. In this system, amino acid supplementation restores IFN-γ production, confirming that amino acids are important regulators of cytokine-induced function, likely through Rag-dependent activation of mTORC1 (Shi, Chapman et al. 2019).

Importantly, both mTORC1 and mTORC2 were found to be sensitive to amino acid supplementation, indicating a broader metabolic impact. These results show that limited access to nutrients can alter the fate and function of T cells and suggests that cytokine-stimulated T cells are metabolically flexible but sensitive to environmental signals. This difference is also evident in energy consumption. As shown in Figures 3.11 and 3.12, cytokine-stimulated IFN-γ expression is highly sensitive to glycolytic blockade, while TAB-induced activation shows sensitivity to both glycolytic and mitochondrial OXPHOS inhibition.

In summary, these results indicate that cytokine-induced effector responses proceed via rapid and targeted metabolic programmes, whereas TCR-driven responses involve cell proliferation and broad transcriptional changes. This suggests that different immune signals not only shape the features of T cell responses but also set different metabolic demands. Understanding these differences is key for designing therapeutic strategies targeting diseases dominated by cytokine-driven or antigen-specific T cell responses.

3.5 Limitations and Future Directions

Although the findings of this chapter are compelling, there are still some limitations that cannot be ignored and need to be improved and explored further.

Firstly, while the use of chemical inhibitors to study signalling pathways is a convenient method, their lack of specificity remains a significant limiting factor. Most inhibitors may act on multiple targets simultaneously, introducing non-specific effects that are difficult to distinguish. For example, we observed that AKT inhibitors, while reducing IFN-γ expression, also significantly affected the survival of $CD8^+$ T cells. This makes it challenging to determine whether the functional inhibition stems directly from metabolic regulation or is an indirect result of cytotoxic effects. Since there is no well-established specific inhibitor for mTORC2, a dual inhibitor that targets both mTORC1 and mTORC2 was used to indirectly assess the contribution of mTORC2. Therefore, future studies should consider incorporating more targeted genetic intervention methods, such as CRISPR or siRNA, to specifically knockout signalling components and further clarify the relationship between metabolic pathways and functional effects.

Secondly, the current in vitro culture systems used still exhibit significant differences from real tissue environments in terms of nutrient composition and microenvironmental conditions. While the nutrient-restricted model applied helps investigate the importance of amino acids and metabolic pathways, its extreme nutrient-deprived condition may not accurately reflect the actual conditions in pathological tissues. For example, in microenvironments such as tumours, infections, or chronic inflammation, T cells often exist under hypoxic, hypoglycaemic, or lactic acid-accumulating conditions, which may shift their sensitivity to metabolic inhibition and nutrient availability (Maimela, Liu et al. 2019, Gong, Yang et al. 2024). Therefore, future studies should consider constructing in vitro models that more closely mimic physiological conditions or utilise mouse models for in vivo validation to enhance the clinical relevance of research findings.

Additionally, $CD8^+$ T cells are not a homogeneous population but consist of multiple subpopulations with distinct phenotypic and functional characteristics, such as MAIT and MAIT-like cells and cells expressing the inhibitory receptor NKG2A (Godfrey, Koay et al. 2019, Choi, Koh et al. 2023). However, the samples used in this study represent the overall $CD8^+$ T cell population and do not further elucidate the subset-specific metabolic sensitivities and signalling responses. Therefore, future studies should perform subpopulation strategies to systematically compare the metabolic behaviours and functional consequences of different subpopulations under similar experimental settings in this chapter.

In summary, this chapter systematically analyses the signalling contributions and metabolic sensitivities of human $CD8^+$ T cells under IL-12+IL-18 or TAB stimulation conditions, clarifying the key role of the mTORC1 pathway and amino acid sensing in cytokine-induced activation processes. While this study provides new insights into the metabolic and signalling aspects of cytokine-induced $CD8^+$ T cell activation, further exploration and adjustment are needed in terms of target specificity, the physiological relevance of experimental models, and differences at the subpopulation level.

3.6 Conclusion

This study shows that human CD8$^+$ T cells employ different metabolic strategies under different activation conditions. Cytokine stimulation by IL-12 and IL-18 is characterised by strong mTORC1 involvement and high sensitivity to glycolytic inhibition, while TCR-mediated activation engages both mTORC1 and mTORC2 and is sensitive to inhibition of both glycolysis and mitochondrial metabolism. The availability of amino acids plays a crucial role in the production of IFN-γ, especially under cytokine stimulation, highlighting the sensitivity of these responses to nutrient supply. These findings demonstrate that cytokine-driven and TCR-dependent responses are regulated by distinct metabolic and signal transduction requirements. Understanding these differences helps explain how T cells adapt to different immune environments and provides insights for the development of therapeutic approaches for diseases like infections, cancer, and inflammatory diseases.

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Chapter 4 Functional Characterization of Cytokine-responsive CD8$^+$ T Cell Subsets: Interrogating the Roles of mTOR and Energy Metabolism

4.1 Introduction

In the previous chapter, it was shown that combined stimulation with the proinflammatory cytokines IL-12 and IL-18 rapidly induces IFN-γ production in human CD8$^+$ T cells, independent of TCR engagement. This finding aligns with previous reports showing that IL-12 and IL-18 can bypass conventional antigen-specific activation pathways to elicit effector responses, particularly in memory or innate-like T cell subsets. Moreover, this TCR-independent activation was found to be tightly regulated by mTOR signalling, primarily through the mTORC1 complex. IFN-γ production induced by IL-12 and IL-18 in CD8$^+$ T cells was also shown to be metabolically skewed toward greater dependence on glycolysis. However, it remains unclear whether these functional and metabolic features are consistent across different subsets of CD8$^+$ T cells.

It has been well established that CD8$^+$ T cells are not a homogeneous population but are composed of multiple subsets with distinct phenotypes and functional features. And these subpopulations exhibit significant differences in migration, tissue distribution, cytokine production capacity, and metabolic state (Billerbeck, Kang et al. 2010, Fernández-García, Franco et al. 2022, Koh, Lee et al. 2023, Koh, Kim et al. 2023). Investigating the metabolic and signalling features of these subpopulations under cytokine stimulation can provide deeper insights into the factors that shape immune heterogeneity.

Among the numerous T cell subpopulations, MAIT cells and certain CD8⁺ T cells expressing natural killer-like molecules (such as NKG2A and CD161) have recently been identified as representative of cells with innate-like characteristics (Ussher, Bilton et al. 2014, Kurioka, Klenerman et al. 2018, Koh, Kim et al. 2023). Similar to innate lymphocytes, these cells are capable of rapidly responding to cytokines such as IL-12 and IL-18, producing effector molecules such as IFN-γ within hours of stimulation (Gorski, Waller et al. 2006, Fergusson, Smith et al. 2014, Ussher, Bilton et al. 2014, Provine, Binder et al. 2018).

Although early studies suggested that MAIT cells have high metabolic activity at rest, recent evidence indicates that MAIT cells are in a metabolic resting state and only rapidly activate metabolic programmes after stimulation, relying on glycogen reserves to support early effector responses (Zinser, Highton et al. 2018, Cassidy, Kedia-Mehta et al. 2023). This feature suggests that these cells are metabolically in a pre-activated state rather than continuously metabolically active state.

Unlike MAIT cells, the activation of other CD8⁺ T cell subsets in response to cytokine stimulation remains less well understood. In particular, the differences in metabolic and signalling pathway dependencies across these subpopulations under TCR-independent activation conditions have yet to be fully elucidated. The mTOR signalling pathway is a key regulatory axis for T cell metabolic reprogramming and functional activation, with mTORC1 regulating glycolysis, mitochondrial function, and effector protein translation, while mTORC2 is more closely associated with cell survival and cytoskeletal regulation (Laplante and Sabatini 2012, Morita, Gravel et al. 2013, Dai and Thomson 2019, Szwed, Kim et al. 2021, Sun, Wang et al. 2023). How it functions in different CD8$^+$ T cell subsets remain unclear.

Therefore, this chapter was designed to systematically compare the features of CD8$^+$NKG2A$^+$CD161$^+$ T cells, MAIT cells and conventional CD8$^+$ T cells under IL-12+IL-18 stimulation, specifically including their function, mTOR activation levels and dependence on glycolysis and oxidative phosphorylation pathways.

4.2 Aims

1. Identify the primary cellular sources of IFN-γ production within CD8$^+$ T cells under IL-12+IL-18 stimulation

Through functional analysis of MAIT cells, CD8$^+$NKG2A$^+$CD161$^+$ T cells, and conventional CD8$^+$ T cells, this aim seeks to determine which CD8$^+$ T cell subsets serve as the predominant sources of IFN-γ following cytokine stimulation.

2. Compare the dependence of different subpopulations on mTORC1 and mTORC2 signalling

Using chemical inhibitors (Rapamycin and OSI-027) combined with pS6/IFN-γ dual labelling detection, this aim seeks to assess the activation of mTOR signalling and its regulation by mTORC1 specific and mTORC1/2 dual inhibitors.

3. Evaluate differences in the contribution of cellular metabolic pathways to IFN-γ expression in different subsets

Through glycolytic and mitochondrial chemical inhibition experiments and the analysis of transcriptional data, the aim seeks to compare metabolic dependencies between NKG2A$^+$CD161$^+$ and MAIT cells.

4.3 Results

4.3.1 IFN-γ production in CD8$^+$ T cell subsets is mainly concentrated in NKG2A$^+$CD161$^+$ and MAIT populations.

To investigate the functional responses of different CD8$^+$ T cell subsets under cytokine stimulation, the relative contribution of each subset to IFN-γ production was analysed. To this end, a flow cytometry-based assay was used for the detailed analysis of IFN-γ expression and CD8$^+$ T cell subpopulation distribution (Figure 4.1A).

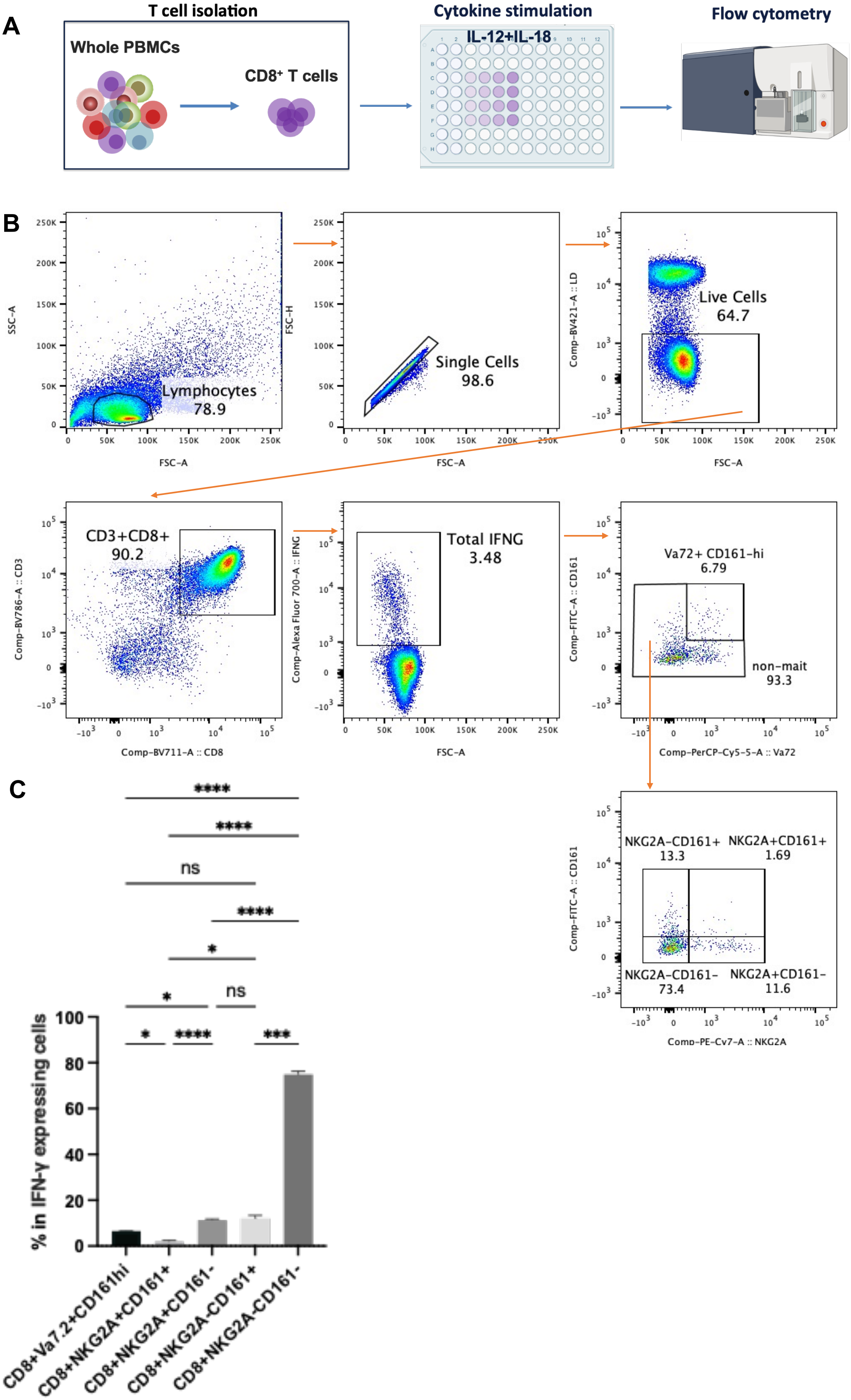
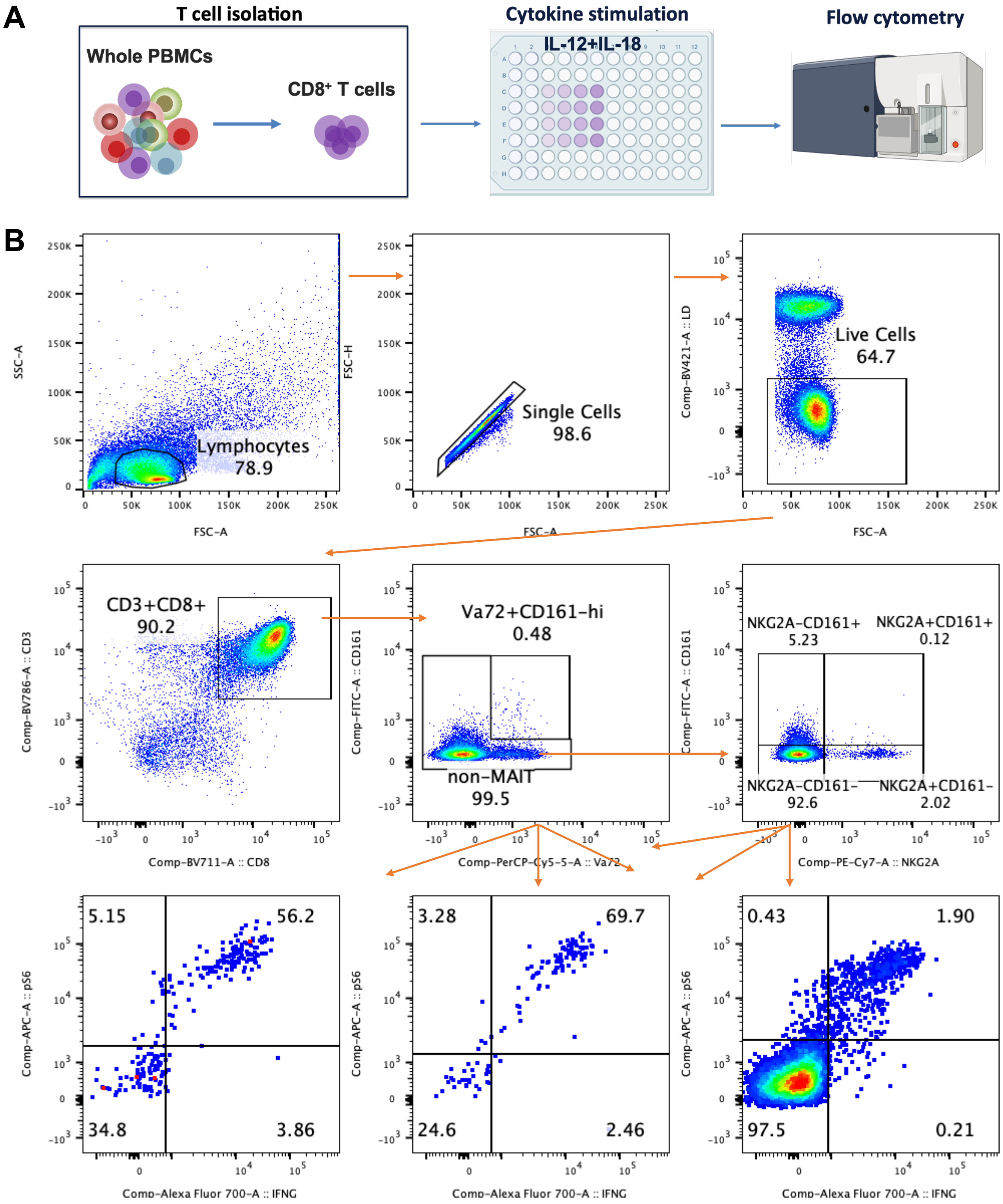

A
T cell isolation
Whole PBMCs
CD8+ T cells
Cytokine stimulation
IL-12+IL-18
Flow cytometry
B
SSC-A
FSC-A
Lymphocytes
78.9
FSC-H
Single Cells
98.6
Comp-BV421-A :: LD
Live Cells
64.7
Comp-BV786-A :: CD3
Comp-BV711-A :: CD8
CD3+CD8+
90.2
Comp-Alexa Fluor 700-A :: IFNG
Total IFNG
3.48
Comp-FITC-A :: CD161
Comp-PerCP-Cy5-5-A :: Va72
Va72+ CD161-hi
6.79
non-mait
93.3
NKG2A-CD161+
13.3
NKG2A+CD161+
1.69
NKG2A-CD161-
73.4
NKG2A+CD161-
11.6
Comp-PE-Cy7-A :: NKG2A
C
% in IFN-γ expressing cells
0
20
40
60
80
100
****
****
ns
****
*
*
ns
*
****
***
CD8+Va7.2+CD161hi
CD8+NKG2A+CD161+
CD8+NKG2A+CD161-
CD8+NKG2A-CD161+
CD8+NKG2A-CD161-

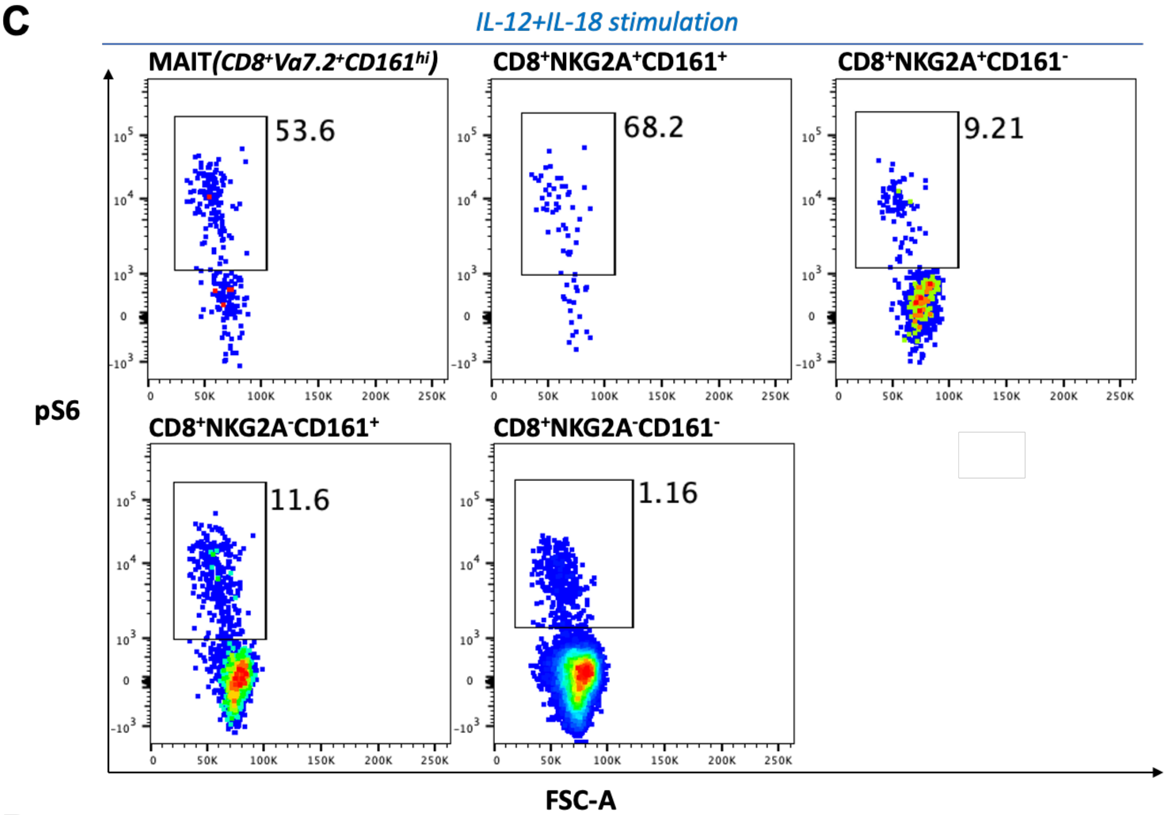
Figure 4.1. Experimental workflow and gating strategy for identifying the source of IFN-γ expression in cytokine-activated CD8$^+$ T cells.

(A) The experimental design of Figure 4.2-4.3. Human CD8$^+$ T cells were isolated from fresh or frozen whole PBMCs, followed by IL-12+IL-18 stimulation, the phenotype of CD8$^+$ T cell subsets were then measured by flow cytometry. (B) The gating strategy of the following experiment. (C) The summarised data from 3 biological replicates. Data are represented as mean ± SEM. Statistical analysis was performed using the One-way ANOVA analysis followed by Tukey's multiple comparisons test. ns=not significant; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

Human primary $CD8^+$ T cells were isolated by magnetic bead–based negative selection from PBMCs derived from fresh or frozen samples. This method effectively removes cell populations such as $CD4^+$ T cells, B cells, monocytes, and NK cells, and retains $CD8^+$ T cells in their inactivated state to avoid interference with experimental results by nonspecific activation. Human primary $CD8^+$ T cells were then cultured with IL-12+IL-18 for 7 hours (Figure 4.1A). After excluding debris and doublets, live $CD3^+CD8^+$ T cells were identified using a strict flow cytometry gating strategy, and their total intracellular IFN-γ expression was assessed (Figure 4.1B).

Within the IFN-γ-producing $CD8^+$ T cell population, MAIT cells ($CD8^+V\alpha7.2^+CD161^{hi}$) and four major subpopulations within non-MAIT cells were identified: $NKG2A^-CD161^-$, $NKG2A^-CD161^+$, $NKG2A^+CD161^-$, and $NKG2A^+CD161^+$. Further analysis revealed that IFN-γ-producing $CD8^+$ T cells were not evenly distributed but were concentrated in certain specific subpopulations (Figure 4.1C). It was also found that there were significant differences in the composition ratios of different subsets among $CD8^+$ T cells that produced IFN-γ after stimulation with IL-12+IL-18. The $CD8^+NKG2A^-CD161^-$ cell subpopulation accounted for approximately 75% of the total IFN-γ^+ cells. In contrast, other subsets including $CD8^+Va72^+CD161^{hi}$, $CD8^+NKG2A^+CD161^+$, $CD8^+NKG2A^-CD161^+$ and $CD8^+NKG2A^+CD161^-$ T cells accounted for a relatively small proportion of IFN-γ-producing cells, which were all below 20%. This distribution suggests that $CD8^+NKG2A^-CD161^-$ T cells numerically dominate the overall IFN-γ response, though their single-cell effector potential in this context warrants further investigation.


A
T cell isolation
Whole PBMCs
CD8⁺ T cells
Cytokine stimulation
IL-12+IL-18
Flow cytometry
B
SSC-A
FSC-A
Lymphocytes
78.9
FSC-H
Single Cells
98.6
Comp-BV421-A :: LD
Live Cells
64.7
Comp-BV786-A :: CD3
Comp-BV711-A :: CD8
CD3+CD8+
90.2
Comp-FITC-A :: CD161
Comp-PerCP-Cy5-5-A :: Va72
Va72+CD161-hi
0.48
non-MAIT
99.5
Comp-PE-Cy7-A :: NKG2A
NKG2A-CD161+
5.23
NKG2A+CD161+
0.12
NKG2A-CD161-
92.6
NKG2A+CD161-
2.02
Comp-APC-A :: pS6
Comp-Alexa Fluor 700-A :: IFNG
5.15
56.2
34.8
3.86
3.28
69.7
24.6
2.46
0.43
1.90
97.5
0.21


C
IL-12+IL-18 stimulation
MAIT(CD8+Va7.2+CD161hi)
53.6
CD8+NKG2A+CD161+
68.2
CD8+NKG2A+CD161-
9.21
CD8+NKG2A-CD161+
11.6
CD8+NKG2A-CD161-
1.16
pS6
FSC-A
10^5
10^4
10^3
0
-10^3
0
50K
100K
150K
200K
250K

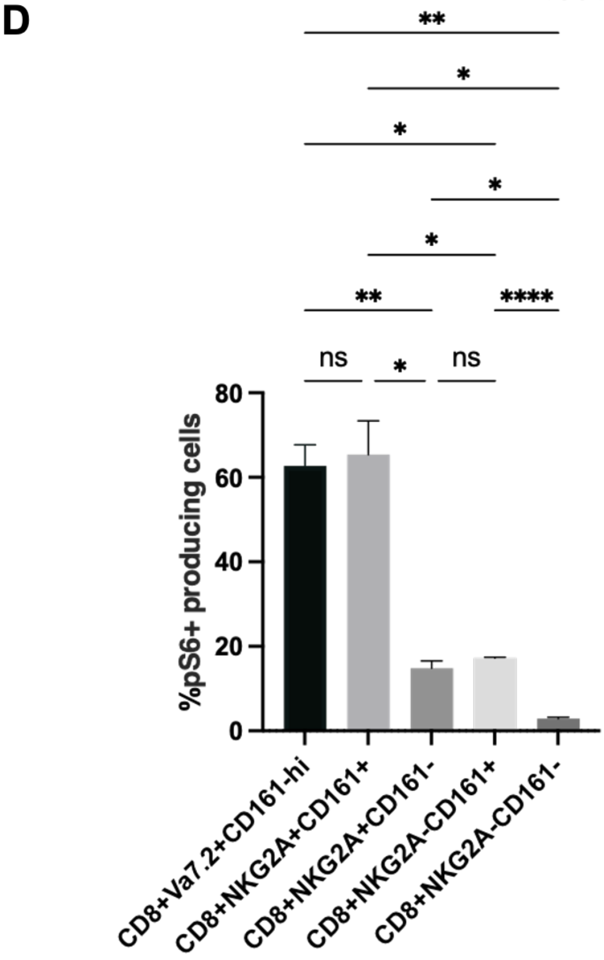

D
**
*
*
*
*
**
****
ns
*
ns
%pS6+ producing cells
80
60
40
20
0
CD8+Va7.2+CD161-hi
CD8+NKG2A+CD161+
CD8+NKG2A+CD161-
CD8+NKG2A-CD161+
CD8+NKG2A-CD161-

Figure 4.2 IFN-γ expression in $CD8^+$ T cell subsets when activated by IL-12+IL-18.

(A) The experimental workflow. Human $CD8^+$ T cells were isolated from fresh or frozen whole PBMCs, followed by IL-12+IL-18 stimulation, the phenotype of $CD8^+$ T cell subsets were then measured by flow cytometry. (B) The gating strategy of the following experiment in this chapter. (C) Representative plot data of IFN-γ production in MAIT and non-MAIT cells following cytokine stimulation. (D) The summarised data of 4 biological replicates. Data are represented as mean ± SEM. Statistical analysis was performed using the One-way ANOVA analysis followed by Tukey's multiple comparisons test. ns=not significant; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

IFN-γ expression in response to IL-12+IL-18 stimulation was then assessed across different $CD8^+$ T-cell subsets. (Figure 4.2A). Similar with previous experiments, a standardised gating strategy based on the expression of TCR Va7.2, CD161 and NKG2A was used to identify MAIT cells and four non-MAIT $CD8^+$ T cell populations (Figure 4.2B). IFN-γ production was then assessed in each subset after stimulation with IL-12 and IL-18 to evaluate their functional responses. All analyses were based on unstimulated control settings, ensuring that subpopulations were well defined and quantified accurately.

Representative analysis revealed that MAIT cells and $CD8^+NKG2A^+CD161^+$ subpopulations exhibited highest frequencies of IFN-γ^+ cells, with positive rates of 53.1% and 63.3% respectively (Figure 4.2C), showing strong cytokine responsiveness. In contrast, $CD8^+NKG2A^+CD161^-$, $CD8^+NKG2A^-CD161^+$ and especially $CD8^+NKG2A^-CD161^-$ subsets had lower IFN-γ expression levels. This result further revealed the differences in capacity to response to IL-12+IL-18 signalling in different $CD8^+$ subsets at a functional level.

After combining the data from multiple biological replicates, the statistical analysis results further validated the above observation (Figure 4.2D). Although the $CD8^+NKG2A^-CD161^-$ subset constituted the highest percentage of total IFN-γ^+ cells, its ability to respond at the single cell level was much lower than that of $CD8^+NKG2A^+CD161^+$ and MAIT cells. In other words, these results suggest that $CD8^+$ T cells subsets with an innate-like phenotype, including the $CD8^+NKG2A^+CD161^+$ and MAIT subpopulations, have higher capacity in IFN-γ production in response to IL-12+IL-18 stimulation. This rapid response may not depend on traditional TCR-engaged recognition mechanisms, but rather due to certain functional preparations that are already in place in the resting state, such as a higher activation potential or the ability to respond rapidly.

Taken together, these results revealed the composition of IFN-γ-producing CD8$^+$ T cells in response to IL-12+IL-18 stimulation and emphasise the greater response of innate-like CD8$^+$NKG2A$^+$CD161$^+$ and MAIT cell subsets in this context.

4.3.2 Cytokine stimulation strongly activates mTORC1 signalling in CD8$^+$ T cell subsets

Based on the findings described in the previous chapter, it was next examined whether this rapid functional response is driven by its distinct signalling activation profile. It has been shown that the mTOR pathway is a central regulator linking cellular metabolic state to effector function and plays a key role especially in fast effector T cells (Waickman and Powell 2012, Pollizzi, Patel et al. 2015). Consistent with this, Chapter 3 data indicated that mTOR activity is essential for cytokine-induced IFN-γ production in CD8$^+$ T cells, highlighting its contribution to rapid effector responses. To investigate the mechanism of rapid functional response of CD8$^+$ T cell subsets under IL-12+IL-18 stimulation, the activation status of metabolic and signalling pathways was assesses, with a particular focus on mTOR signalling.

Building on the flow panel established for different CD8$^+$ subsets, an assay was further developed to measure pS6, a key marker of mTORC1 activation (Figure 4.3A). After IL-12+IL-18 stimulation, pS6 levels were significantly increased in CD8$^+$NKG2A$^+$CD161$^+$ cells and MAIT cells, indicating strong mTORC1 activation in these subgroups. In contrast, CD8$^+$NKG2A$^-$CD161$^-$ cells showed only a modest increase in pS6 levels after stimulation, indicating a weaker activation of the mTORC1 pathway (Figure 4.3B). Statistical analysis further confirmed the significance of these differences (Figure 4.3C). This result was consistent with my previous functional assays (Figures 4.1-4.2) and supported the idea that CD8$^+$NKG2A$^+$CD161$^+$ and MAIT cells were at a state to respond rapidly to cytokine stimulation, which potentially enabled stronger IFN-γ generation.

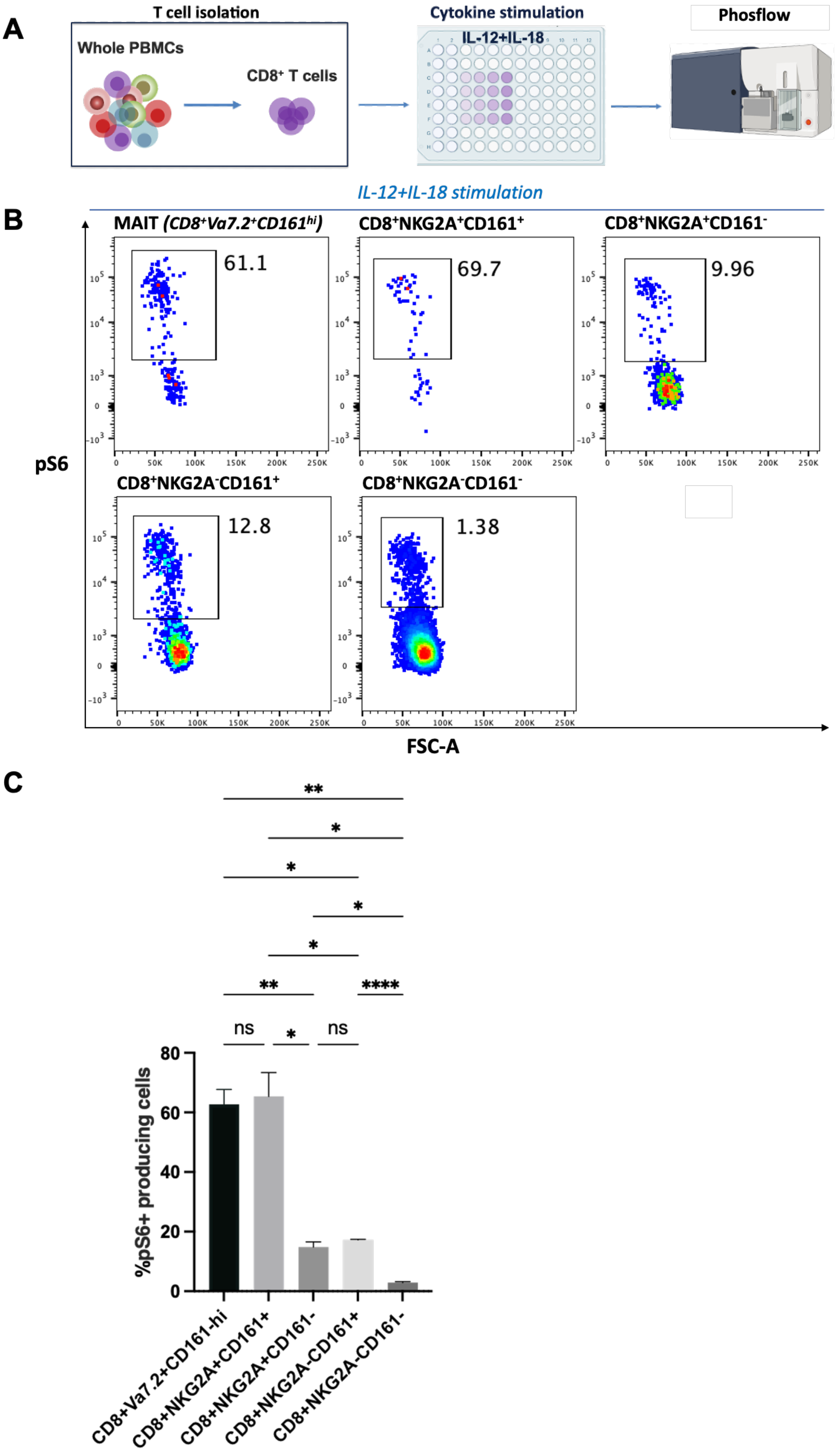

A
T cell isolation
Whole PBMCs
CD8⁺ T cells
Cytokine stimulation
IL-12+IL-18
Phosflow
B
IL-12+IL-18 stimulation
MAIT (CD8⁺Va7.2⁺CD161^hi)
61.1
CD8⁺NKG2A⁺CD161⁺
69.7
CD8⁺NKG2A⁺CD161⁻
9.96
CD8⁺NKG2A⁻CD161⁺
12.8
CD8⁺NKG2A⁻CD161⁻
1.38
pS6
FSC-A
C
**
*
*
*
*
**
****
ns
*
ns
%pS6+ producing cells
80
60
40
20
0
CD8+Va7.2+CD161-hi
CD8+NKG2A+CD161+
CD8+NKG2A+CD161-
CD8+NKG2A-CD161+
CD8+NKG2A-CD161-

Figure 4.3 mTORC1 pathway is activated in cytokine-stimulated CD8$^+$ T cell subsets.

(A) The experimental workflow. Human CD8$^+$ T cells were isolated from fresh or frozen whole PBMCs, followed by chemical inhibition of mTOR and IL-12+IL-18 stimulation, the phenotype of CD8$^+$ T cell subsets were then measured by flow cytometry. (B) Representative plot data of pS6 expression in MAIT and non-MAIT cells following cytokine stimulation. (C) The summarised pS6 expression data from 4 biological replicates. Data are represented as mean ± SEM. Statistical analysis was performed using the One-way ANOVA analysis followed by Tukey's multiple comparisons test. ns=not significant; $^*p < 0.05$, $^{**}p < 0.01$.

In line with Chapter 3, the dual mTORC1/2 inhibitor OSI-027 suppressed cytokine-induced IFN-γ production more strongly than mTORC1 inhibition alone, supporting a contribution of mTORC2 signalling in cytokine-induced effector responses. To further assess mTORC2 signalling pathway activity, AKT phosphorylation at Ser473 (pAKT), which is often used as a downstream readout of mTORC2 activation, was analysed (Figure 3.2). However, in MAIT and all other non-MAIT subpopulations, stimulation induced minimal changes in pAKT and no consistent pattern of activation was observed (Figure 4.4).

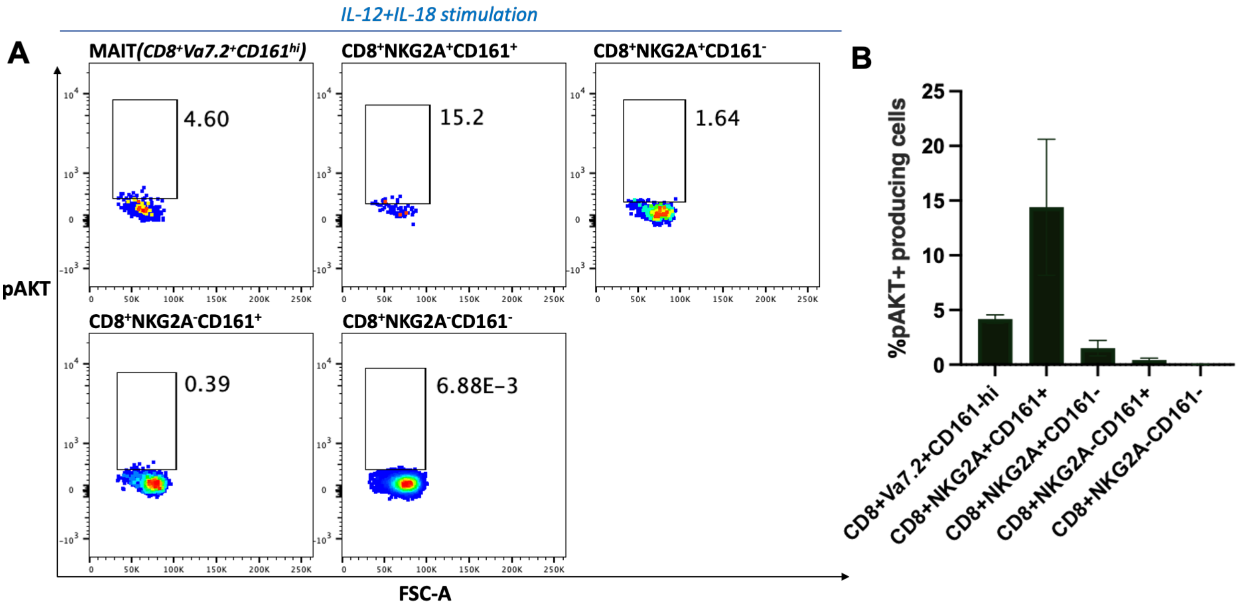


Figure 4.4 The activation of mTORC2 pathway is difficult to detect by Phosflow.

(A) Representative plot data of pAKT expression in MAIT and non-MAIT cells following cytokine stimulation. (B) The summarised pAKT expression data. n=4 biological replicates. Data are represented as mean ± SEM.

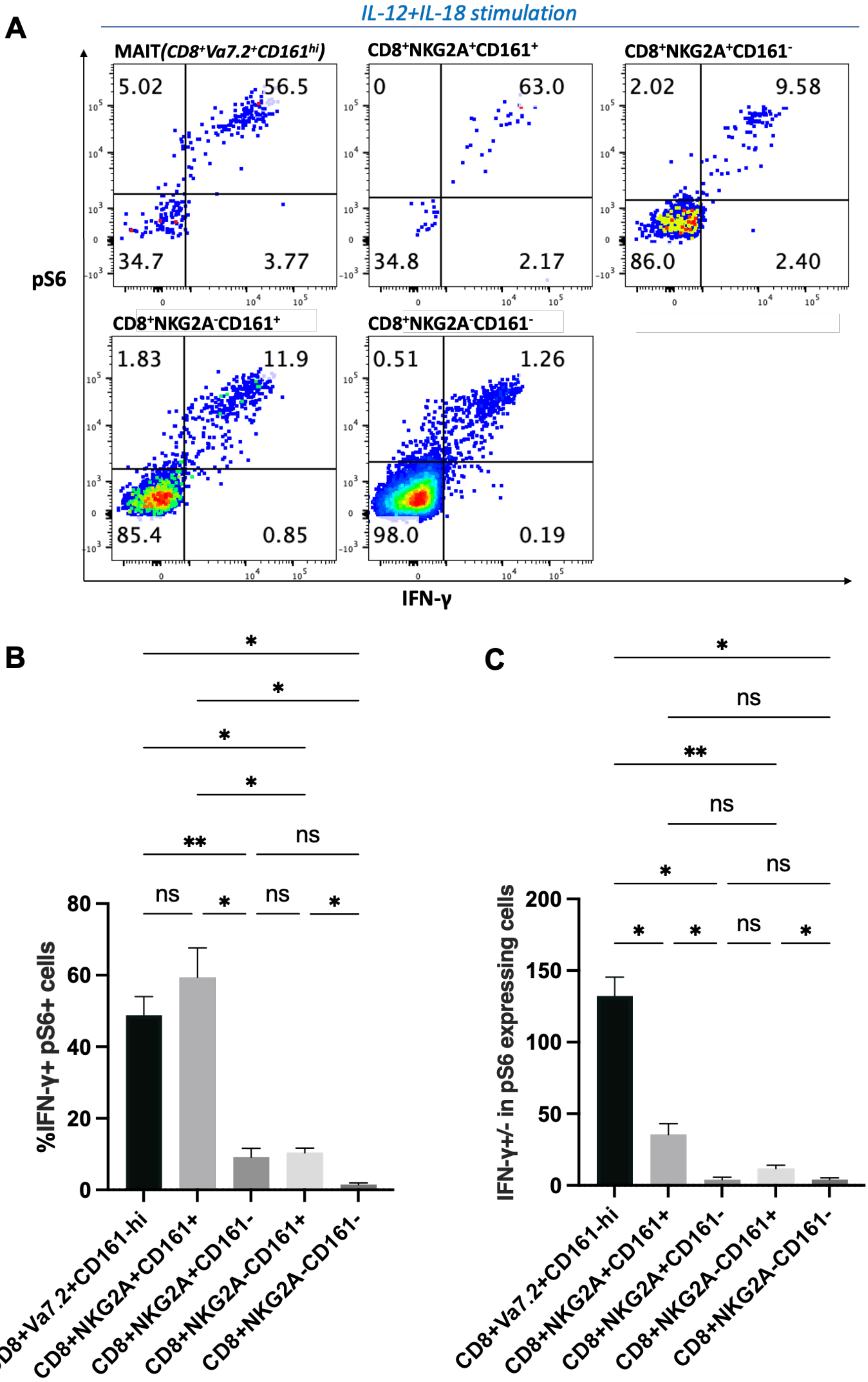
Western blot was considered to confirm these findings, but the limited yield of primary CD8⁺ T cells made this impractical. Given the evidence for mTORC2 involvement presented in Chapter 3, the lack of a clear pAKT signal here is more likely due to technical limitation rather to a true absence of mTORC2 activity. Previous studies have also indicated that mTORC2 activation is more transient and spatially restricted, potentially occurring only in specific membrane regions or at early time points (Yang, Murashige et al. 2015, Ebner, Sinkovics et al. 2017), which is difficult to capture by traditional Phosflow detection.

In summary, the strongest IL-12+IL-18 responsiveness was observed in specific CD8$^+$ T cell subsets, primarily CD8$^+$NKG2A$^+$CD161$^+$ T cells and MAIT cells. In these subsets, pS6 expression was significantly upregulated compared to other subpopulations, suggesting that activation of the mTORC1 pathway plays a key role in this process. In contrast, the expression of pAKT did not show significant changes under the conditions tested. Given that mTORC2 activation typically exhibits transient and spatially limited characteristics, this result is more likely to reflect limitations of the detection method rather than a complete absence of mTORC2 functional involvement.

These findings highlighted the close relationship between enhanced mTORC1 activation and innate-like CD8$^+$ T cell subsets, suggesting that these cells may have a signalling advantage in TCR-independent immune responses.

4.3.3 IL-12 and IL-18 induce the concurrent upregulation of pS6 and IFN-γ in human CD8$^+$ T cells

Given the induction of IFN-γ and pS6 by IL-12 and IL-18, the next objective was to determine if this upregulation occurred concomitantly within individual cells. Using Phosflow to detect the co-expression of pS6 and IFN-γ, it was found that IFN-γ expression in all CD8$^+$ T cell subsets was significantly associated with the phosphorylation level of pS6, a downstream indicator of mTORC1 activity (Figure 4.5A). This result indicates that the association between mTORC1 signal activation and effector function following cytokine stimulation is shared across different CD8 subsets. Notably, the proportion of IFN-γ$^+$pS6$^+$ cells was highest in CD8$^+$NKG2A$^+$CD161$^+$ cells and MAIT cells (Figures 4.5B and 4.5C). In contrast, although CD8$^+$NKG2A$^-$CD161$^-$ T cells were the most abundant subset among CD8$^+$ T cells, they exhibited the lowest levels of IFN-γ and pS6 co-expression, suggesting lower responsiveness to IL-12 and IL-18 stimulation.


A
IL-12+IL-18 stimulation
MAIT(CD8+Va7.2+CD161hi)
CD8+NKG2A+CD161+
CD8+NKG2A+CD161-
CD8+NKG2A-CD161+
CD8+NKG2A-CD161-
5.02
56.5
34.7
3.77
0
63.0
34.8
2.17
2.02
9.58
86.0
2.40
1.83
11.9
85.4
0.85
0.51
1.26
98.0
0.19
pS6
IFN-γ
B
%IFN-γ+ pS6+ cells
0
20
40
60
80
*
*
*
*
**
ns
ns
*
ns
*
CD8+Va7.2+CD161-hi
CD8+NKG2A+CD161+
CD8+NKG2A+CD161-
CD8+NKG2A-CD161+
CD8+NKG2A-CD161-
C
IFN-γ+/- in pS6 expressing cells
0
50
100
150
200
*
ns
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ns
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ns
*
*
ns
*
CD8+Va7.2+CD161-hi
CD8+NKG2A+CD161+
CD8+NKG2A+CD161-
CD8+NKG2A-CD161+
CD8+NKG2A-CD161-

Figure 4.5 MAIT and CD8$^+$ NKG2A$^+$CD161$^+$ T cells show more frequent IFN-γ and pS6 co-expression than other CD8$^+$ subsets when stimulated by IL-12+IL-18.

Representative plot data of pS6 and IFN-γ co-expression in MAIT and non-MAIT cells following cytokine stimulation (B) Summarised data of IFN-γ production level in different CD8$^+$ T cell subsets. n=4 biological replicates. (C) Percentages of IFN-γ-producing and non-producing cells in pS6$^+$ cells in different CD8$^+$ T cell subsets. n=4 biological replicates. Data are represented as mean ± SEM. Statistical analysis was performed using the One-way ANOVA analysis followed by Tukey's multiple comparisons test. ns=not significant; $^*p < 0.05$, $^{**}p < 0.01$.

4.3.4 mTORC1 and mTORC2 jointly participate in cytokine-driven activation of multiple CD8$^+$ T cell subsets

To further investigate the role of mTOR signalling in IL-12+IL-18-mediated activation of CD8$^+$ T cells, total CD8$^+$ T cells were pretreated with the mTORC1 inhibitor rapamycin or the dual mTORC1/2 inhibitor OSI-027 prior to IL-12+IL-18 stimulation. The analysis initially focused on the CD8$^+$NKG2A$^+$CD161$^+$ T cells and MAIT cells for the analysis of IFN-γ and pS6 expression following stimulation (Figure 4.6A). As expected, both subpopulations exhibited minimal IFN-γ expression and no significant pS6 signalling in the unstimulated condition. In the vehicle control, both subpopulations exhibited a strong response to IL-12+IL-18.

After treatment of Rapamycin, the expression of pS6 and IFN-γ was significantly reduced in the MAIT and NKG2A$^+$CD161$^+$ subsets, indicating that mTORC1 activity is key for cytokine-induced activation (Figure 4.6B). It is worth noting that the dual mTORC1/2 inhibitor OSI-027 exhibits a stronger inhibitory effect on IFN-γ expression compared to Rapamycin. This data indicates that although mTORC1 is the main driver of cytokine-induced activation, mTORC2 may provide additional support, particularly in maintaining IFN-γ production.

Statistical analysis further supported this finding. Compared with vehicle control, rapamycin significantly reduced the frequency of pS6$^+$IFN-γ$^+$ cells in CD8$^+$NKG2A$^+$CD161$^+$ T cells and MAIT cells (Figures 4.6C-D), suggesting that mTORC1 activity is critical for this response. Notably, dual inhibition of mTORC1 and mTORC2 with OSI-027 further decreased pS6 and IFN-γ expression in both cell types, suggesting that mTORC2 also play a supportive role in this response, although to a lesser extent than mTORC1.

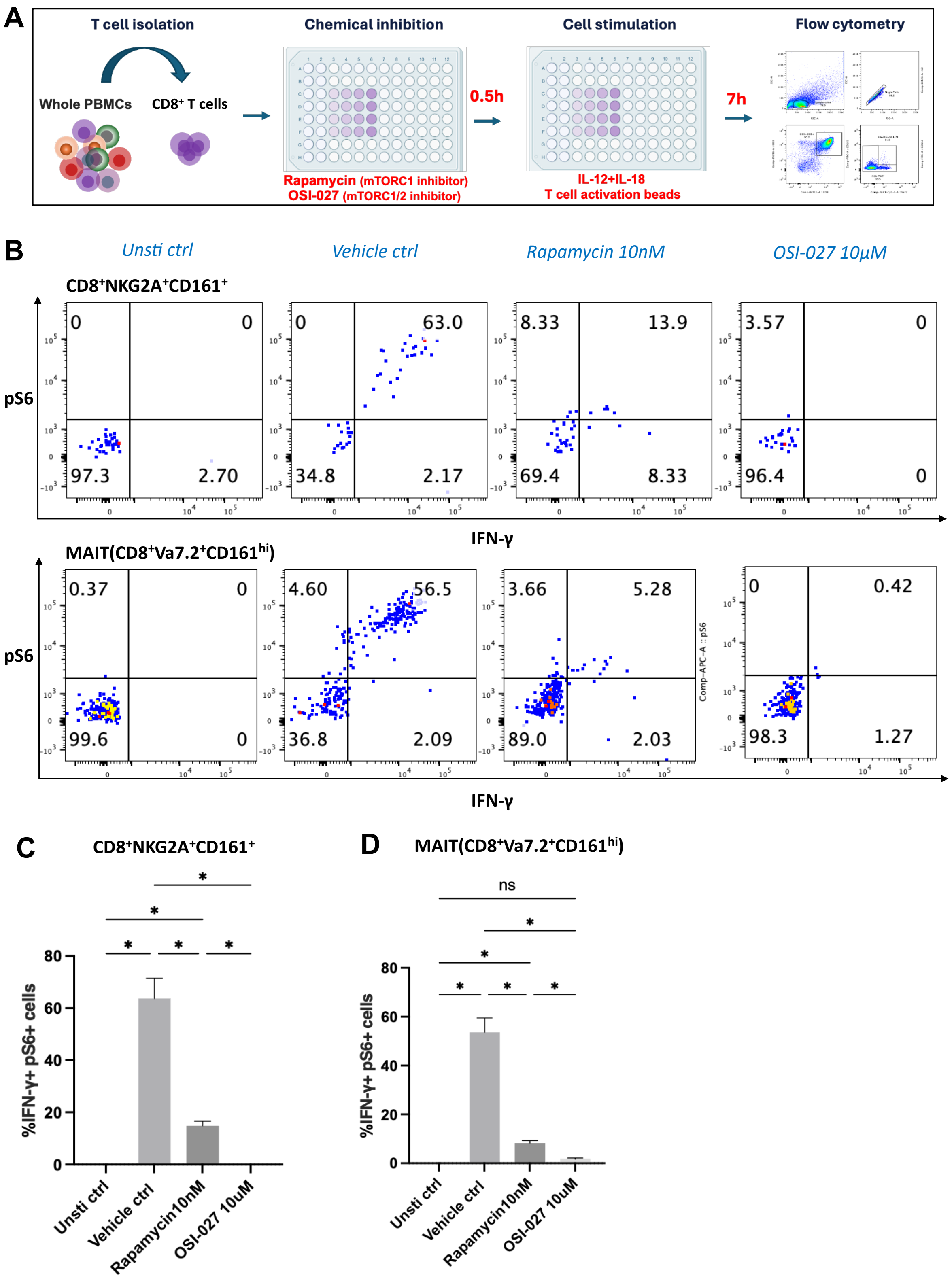
A
T cell isolation
Whole PBMCs
CD8⁺ T cells
Chemical inhibition
Rapamycin (mTORC1 inhibitor)
OSI-027 (mTORC1/2 inhibitor)
0.5h
Cell stimulation
IL-12+IL-18
T cell activation beads
7h
Flow cytometry
B
Unsti ctrl
Vehicle ctrl
Rapamycin 10nM
OSI-027 10µM
CD8⁺NKG2A⁺CD161⁺
pS6
0
0
97.3
2.70
0
63.0
34.8
2.17
8.33
13.9
69.4
8.33
3.57
0
96.4
0
IFN-γ
MAIT(CD8⁺Va7.2⁺CD161hi)
pS6
0.37
0
99.6
0
4.60
56.5
36.8
2.09
3.66
5.28
89.0
2.03
0
0.42
98.3
1.27
Comp-APC-A :: pS6
IFN-γ
C
CD8⁺NKG2A⁺CD161⁺
%IFN-γ+ pS6+ cells
0
20
40
60
80
*
*
*
*
*
Unsti ctrl
Vehicle ctrl
Rapamycin10nM
OSI-027 10uM
D
MAIT(CD8⁺Va7.2⁺CD161hi)
%IFN-γ+ pS6+ cells
0
20
40
60
80
ns
*
*
*
*
*
Unsti ctrl
Vehicle ctrl
Rapamycin10nM
OSI-027 10uM

Figure 4.6 Both mTORC1 and mTORC2 are involved in cytokine-driven activation of MAIT and CD8$^+$ NKG2A$^+$CD161$^+$ T cells.

(A) The experimental workflow. (B) Representative plot data of IFN-γ and pS6 co-expression in NKG2A$^+$CD161$^+$CD8$^+$ T and MAIT cells following cytokine stimulation and chemical inhibition of mTOR complexes. (C), (D) Summarised data of (B). *n*=4 biological replicates. Data are represented as mean ± SEM. Statistical analysis was performed using the One-way ANOVA analysis followed by Tukey's multiple comparisons test. ns=not significant; $^*p < 0.05$.

It was next examined whether these observations also apply to other CD8$^+$ T-cell subpopulations. Compared to innate-like cells, these cells are known to exhibit lower metabolic activity under resting status, but it remains unclear whether their functional activation similarly depends on the mTOR pathway. To address this, the same experimental protocol was applied (Figure 4.7A). Cells were pretreated with the mTORC1 inhibitor Rapamycin or the mTORC1/2 dual inhibitor OSI-027 prior to IL-12+IL-18 stimulation and their effects on the co-expression of IFN-γ and pS6 were analysed.

As expected, flow cytometry analysis showed that IFN-γ expression and pS6 signal were minimal in unstimulated non-MAIT and non-NKG2A$^+$CD161$^+$ cells. In the stimulated condition, a small proportion of cells within this population exhibited moderate pS6 phosphorylation and IFN-γ expression (Figure 4.7B). This observation also confirms that while non-MAIT and non-CD8$^+$NKG2A$^+$CD161$^+$ T cells account for the largest proportion of the total population and exhibit a lower responsiveness to IL-12+IL-18 than MAIT and CD8$^+$NKG2A$^+$CD161$^+$ T cells.

Interestingly, although the activation proportion of these cells was lower than that of MAIT and other subpopulations after stimulation, they still exhibited a strong mTORC1 dependence (Figures 4.7C-D). After Rapamycin treatment, the proportions of pS6$^+$IFN-γ$^+$ cells decreased significantly, suggesting that the mTORC1 pathway still plays an important role in their response. Treatment of OSI-027 nearly completely suppressed IFN-γ expression, suggesting that mTORC2 may play a more critical supplementary or maintenance role in these low-responsiveness cells as well.

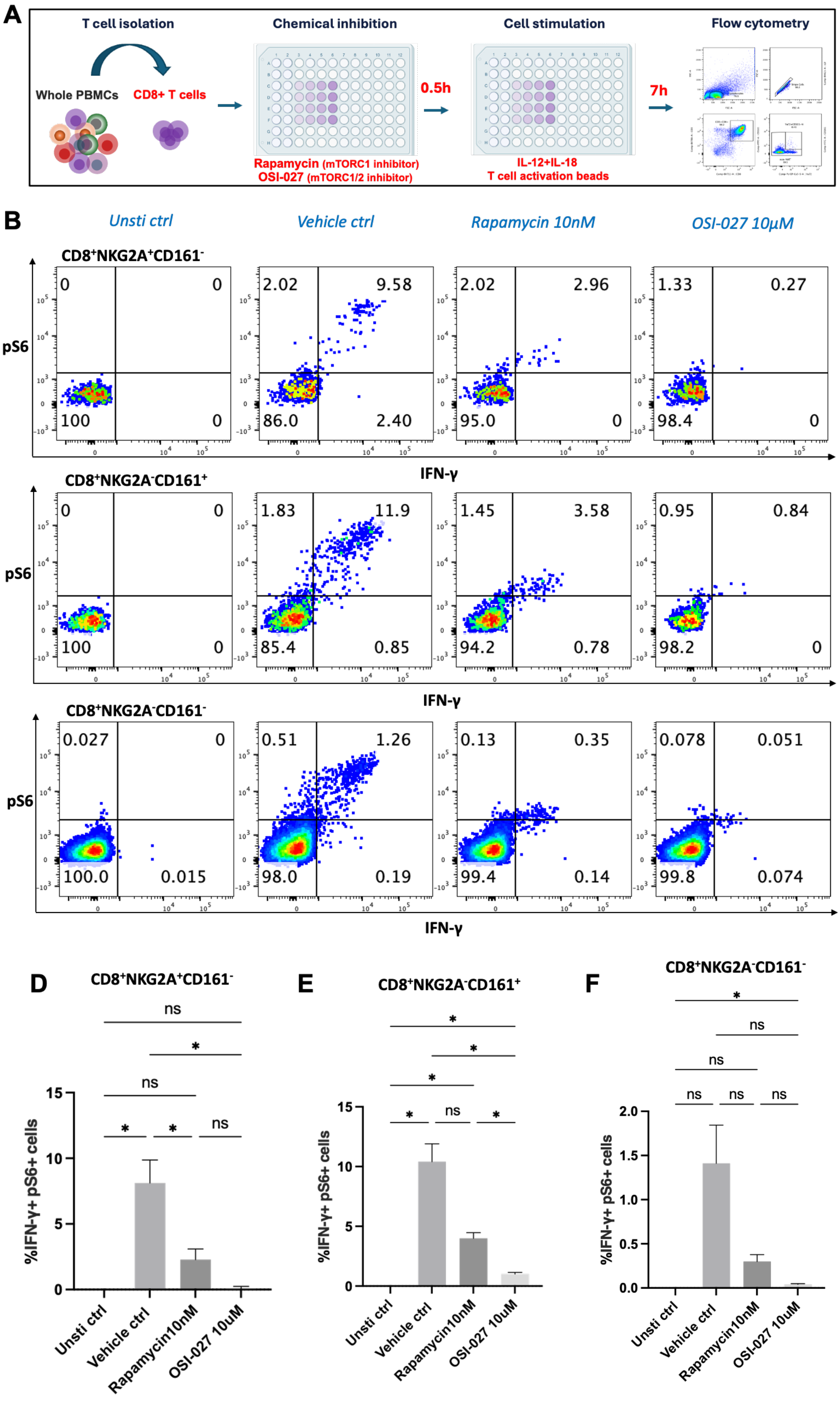
A
T cell isolation
Whole PBMCs
CD8+ T cells
Chemical inhibition
Rapamycin (mTORC1 inhibitor)
OSI-027 (mTORC1/2 inhibitor)
0.5h
Cell stimulation
IL-12+IL-18
T cell activation beads
7h
Flow cytometry
B
Unsti ctrl
Vehicle ctrl
Rapamycin 10nM
OSI-027 10μM
CD8+NKG2A+CD161-
pS6
IFN-γ
0 0 100 0
2.02 9.58 86.0 2.40
2.02 2.96 95.0 0
1.33 0.27 98.4 0
CD8+NKG2A-CD161+
0 0 100 0
1.83 11.9 85.4 0.85
1.45 3.58 94.2 0.78
0.95 0.84 98.2 0
CD8+NKG2A-CD161-
0.027 0 100.0 0.015
0.51 1.26 98.0 0.19
0.13 0.35 99.4 0.14
0.078 0.051 99.8 0.074
D
CD8+NKG2A+CD161-
%IFN-γ+ pS6+ cells
0
5
10
15
ns
*
ns
* * ns
Unsti ctrl
Vehicle ctrl
Rapamycin10nM
OSI-027 10uM
E
CD8+NKG2A-CD161+
%IFN-γ+ pS6+ cells
0
5
10
15
*
*
*
* ns *
Unsti ctrl
Vehicle ctrl
Rapamycin10nM
OSI-027 10uM
F
CD8+NKG2A-CD161-
%IFN-γ+ pS6+ cells
0.0
0.5
1.0
1.5
2.0
*
ns
ns
ns ns ns
Unsti ctrl
Vehicle ctrl
Rapamycin10nM
OSI-027 10uM

Figure 4.7 Cytokine-induced activation of non-MAIT and non-CD8$^+$NKG2A$^+$CD161$^+$ T cells involves both mTORC1 and mTORC2 signalling.

(A) The experimental workflow. (B) Representative plot data of IFN-γ and pS6 co-expression in non-MAIT and non-NKG2A$^+$CD161$^+$CD8$^+$ T cells following cytokine stimulation and chemical inhibition of mTOR complexes. (D), (E), (F) Summarised data of (B), n=4 biological replicates. Statistical analysis was performed using the One-way ANOVA analysis followed by Tukey's multiple comparisons test. ns=not significant; $^*p < 0.05$.

Taken together, these results suggest that mTORC1 signalling is critical in the early effector response to IL-12+IL-18 in all CD8$^+$ T cells. Among the analysed subsets, CD8$^+$NKG2A$^+$CD161$^+$ T cells and MAIT cells showed stronger expression of IFN-γ and pS6 after stimulation. Although subsets differ in their responses to IL-12 and IL-18, the requirement for mTORC1 in driving IFN-γ is shared across subsets. Thus, these data highlight functional differences between CD8$^+$ T cell subgroups in cytokines responsiveness while confirming a shared dependence on mTORC1 signalling.

4.3.5 $CD8^+NKG2A^+CD161^+$ T cells are less sensitive than MAIT cells to glycolytic inhibition during cytokine-driven IFN-γ expression

Although $CD8^+NKG2A^+CD161^+$ T cells and MAIT cells can produce IFN-γ under IL-12+IL-18 stimulation, it remains unclear whether they rely on the same metabolic pathways to support this response. To further explore their metabolic basis, particularly their dependence on glycolysis, a similar experimental setting described in Chapter 3 was used, applying the glycolysis inhibitor 2-DG and the mitochondrial respiration inhibitor Oligomycin to assess the metabolic sensitivity differences between the two cell types. MAIT cells was found to exhibit a strong IFN-γ response to IL-12+IL-18 in the vehicle control group, while $CD8^+NKG2A^+CD161^+$ T cells showed slightly lower IFN-γ expression. It is worth noting that the total frequency of IFN-γ^+ cells in this experiment was lower than that in previous experiments. This may be due to technical differences, as previous experiments used the Phosflow staining method, while this experiment used conventional intracellular cytokine staining, which may affect signal sensitivity.

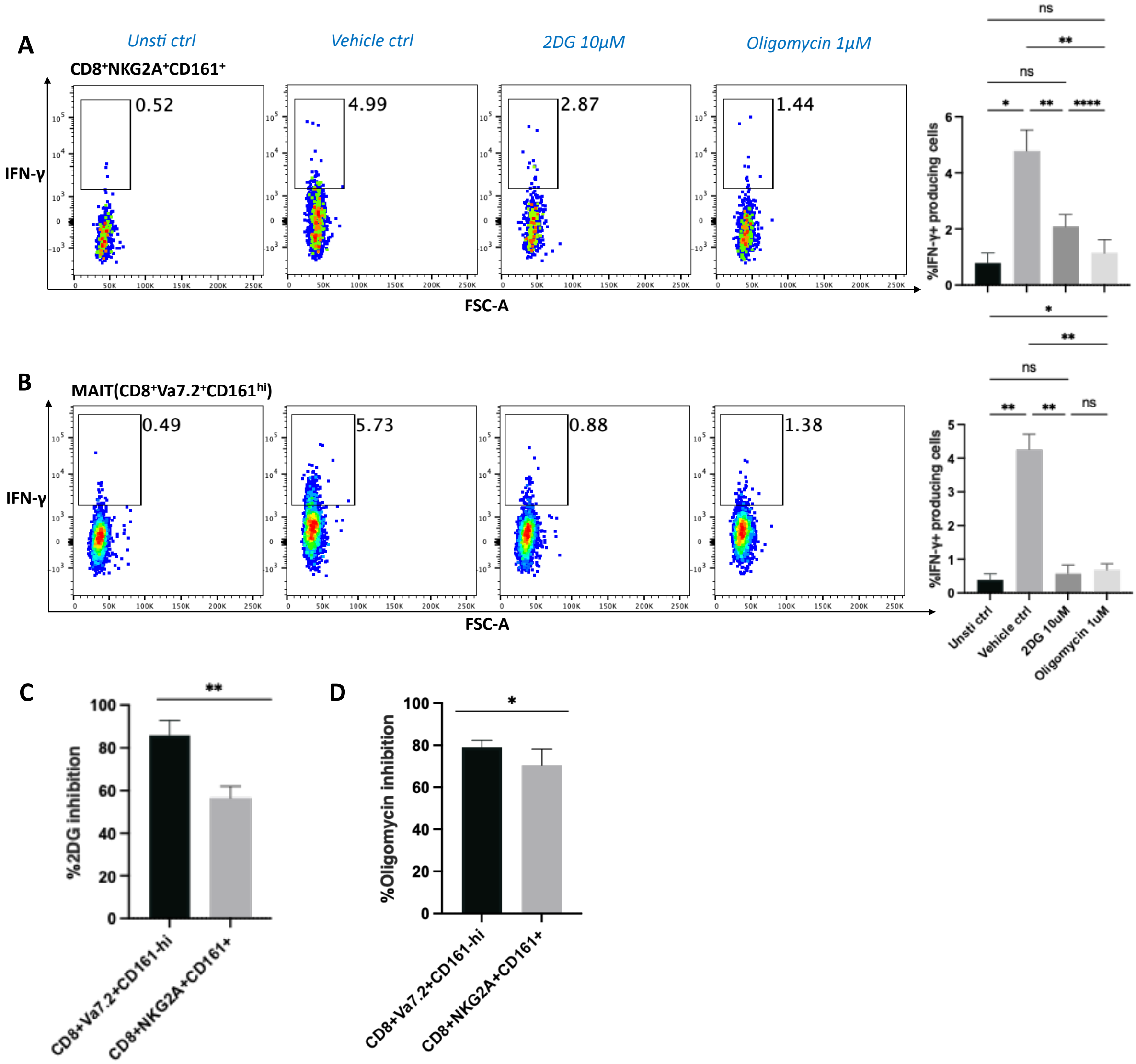

Figure 4.8 Sensitivity of cytokine-stimulated MAIT and CD8$^+$NKG2A$^+$CD161$^+$ T cells to glycolytic and mitochondrial inhibition.

(A) The right-hand side is representative FACS plots of IFN-γ expression in CD8$^+$NKG2A$^+$CD161$^+$ T cells following cytokine stimulation and chemical inhibition of 2-DG or Oligomycin; The left-hand side is the summarised data. n=4 biological replicates. (B) The right-hand side is representative FACS plots of IFN-γ expression in MAIT cells following cytokine stimulation and chemical inhibition of 2-DG or Oligomycin; The left-hand side is the summarised data. n=4 biological replicates. (C) The inhibition effect of 2-DG in MAIT and CD8$^+$NKG2A$^+$CD161$^+$ T cells. n=4 biological replicates. (D) The inhibition effect of Oligomycin in MAIT and CD8$^+$NKG2A$^+$CD161$^+$ T cells. n=4 biological replicates. Statistical analysis was performed using the One-way ANOVA analysis followed by Tukey's multiple comparisons test in A-B or paired t-test in C-D. ns=not significant; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

2-DG and Oligomycin significantly reduced IFN-γ production in MAIT cells and $CD8^+NKG2A^+CD161^+$ T cells, indicating that both glycolysis and mitochondrial respiration are involved in cytokine-induced responses (Figure 4.8A-B). Following 2-DG treatment, the proportion of IFN-γ^+ cells in MAIT cells decreased from around 6% to 1%, while in $CD8^+NKG2A^+CD161^+$ T cells, this proportion decreased from approximately 5% to 3%. Oligomycin treatment also reduced IFN-γ expression in both subpopulations. To further investigate whether $CD8^+NKG2A^+CD161^+$ T cells and MAIT cells rely on different energy sources in response to IL-12 and IL-18, data from Figure 4.8A-B were reanalysed and inhibitor-induced suppression of IFN-γ was expressed as an inhibition percentage (Figure 4.8C-D). The analysis revealed that IFN-γ production in $NKG2A^+CD161^+$ cells was significantly less affected by 2-DG than in MAIT cells, indicating lower dependence on glycolysis. Conversely, both cell types were sensitive to Oligomycin treatment, with MAIT cells showing a slightly greater reduction in IFN-γ production. Although the difference between the two groups was statistically significant, it was relatively small. These results suggest that while $CD8^+NKG2A^+CD161^+$ cells and MAIT cells share similar mTOR-dependent responsiveness to IL-12 and IL-18, they differ in their metabolic strategies to support effector function in this context.

These results further indicate that, even under similar cytokine stimulation conditions, metabolic dependencies vary significantly among T cell subpopulations, in particular, $CD8^+NKG2A^+CD161^+$ cells and MAIT cells have different preferences in metabolic pathways. While both subsets can respond rapidly to IL-12+IL-18, $CD8^+NKG2A^+CD161^+$ cells are less affected by glycolysis inhibition, indicating that they are less dependent on glycolysis than

MAIT cells. Although these two subgroups are also affected by mitochondrial inhibition, the small but significant differences between them indicate that there are subtle differences in how they utilise energy generated from the process of oxidative phosphorylation. These findings suggest that $CD8^+$ T cell subsets may employ different metabolic strategies to support effector functions, which may influence their performance in metabolically restricted environments such as low glucose and hypoxia.

4.3.6 $CD8^+NKG2A^+$ T cells exhibited metabolic-related transcriptional characteristics

To gain a more comprehensive understanding of the metabolic and functional characteristics of $CD8^+NKG2A^+CD161^+$ T cells, transcriptional differences between $NKG2A^+$ cells and naïve $CD8^+$ T cells were analysed in publicly available datasets (Choi, Koh et al. 2023) (Figure 4.9).

In the volcano plot (Figure 4.9A), each point represents a gene, with the x-axis showing the $\log_2$ fold change (logFC) and the y-axis showing the adjusted P-value ($-\log_{10}$(adj.P)). The analysis results showed that with Crocin-I as a stress-modulating perturbation, a great number of genes are significantly upregulated (red) or downregulated (blue) in $CD8^+NKG2A^+$ T cells compared to naïve $CD8^+$ T cells. The upregulated genes include immune and metabolism-related molecules such as SIRT1, CXCR3, GLIPR1, and ATP6V0C, which are associated with inflammatory responses, chemotaxis, and mitochondrial function. The downregulated genes include multiple ribosomal proteins and mitochondrial electron transport chain molecules, such as RPS8, RPL34, and NDUFS7, which may reflect the inhibition of specific metabolic pathways.

To systematically assess the functional pathways involved, GSEA (Gene Set Enrichment Analysis) was performed on the differentially expressed genes, focusing on immune and metabolism-related pathways in the Hallmark database (Figure 4.9B). It was found that CD8$^+$NKG2A$^+$ T cells were significantly enriched in multiple inflammation-related gene sets, including TNFA signalling via NF-κB, unfolded protein response, MTORC1 signalling, interferon gamma response and hypoxia, further supporting that this subpopulation possesses innate-like T cell characteristics and can rapidly respond to cytokine activation and enter an effector state under conditions without TCR stimulation.

This molecular characteristic is consistent with the functional results observed in this study. NKG2A$^+$CD161$^+$ T cells can effectively produce IFN-γ under IL-12+IL-18 stimulation, although they are not as dependent on glycolysis as MAIT cells, they mainly maintain their effector function through OXPHOS, demonstrating good metabolic adaptability. These findings suggest that NKG2A$^+$CD161$^+$ cells possess strong functional potential and environmental adaptability, potentially playing a significant role in TCR-independent immune responses.

Meanwhile, CD8$^+$NKG2A$^+$ T cells showed a significant downregulation trend in multiple metabolic and stem cell maintenance-related pathways, including oxidative phosphorylation, MYC target genes and WNT/β-catenin signalling pathways. This change may not represent a complete inhibition of mitochondrial metabolic function but rather reflect transcriptional level regulation under a highly activated state. Combining the functional experimental results in Figure 4.8, it can be observed that CD8$^+$NKG2A$^+$CD161$^+$ T cells exhibit a greater downregulation of IFN-γ expression under OXPHOS inhibition compared to glycolysis inhibition, suggesting that their effector functions remain largely dependent on the support of the mitochondrial oxidative phosphorylation pathway. Therefore, compared to MAIT cells that highly depend on glycolysis to maintain their function, NKG2A$^+$CD161$^+$ cells may apply a different metabolic programme, preferring to use OXPHOS to maintain their cytokine expression and immune effector function under cytokine stimulation.

In summary, transcriptomic data further support an innate-like programme with altered metabolic pathways in cytokine-activated CD8$^+$NKG2A$^+$ cells. This suggests that this subset occupies a unique position in T cell immune heterogeneity and provides new evidence for understanding how different T cell subpopulations regulate immune function through metabolic mechanisms.

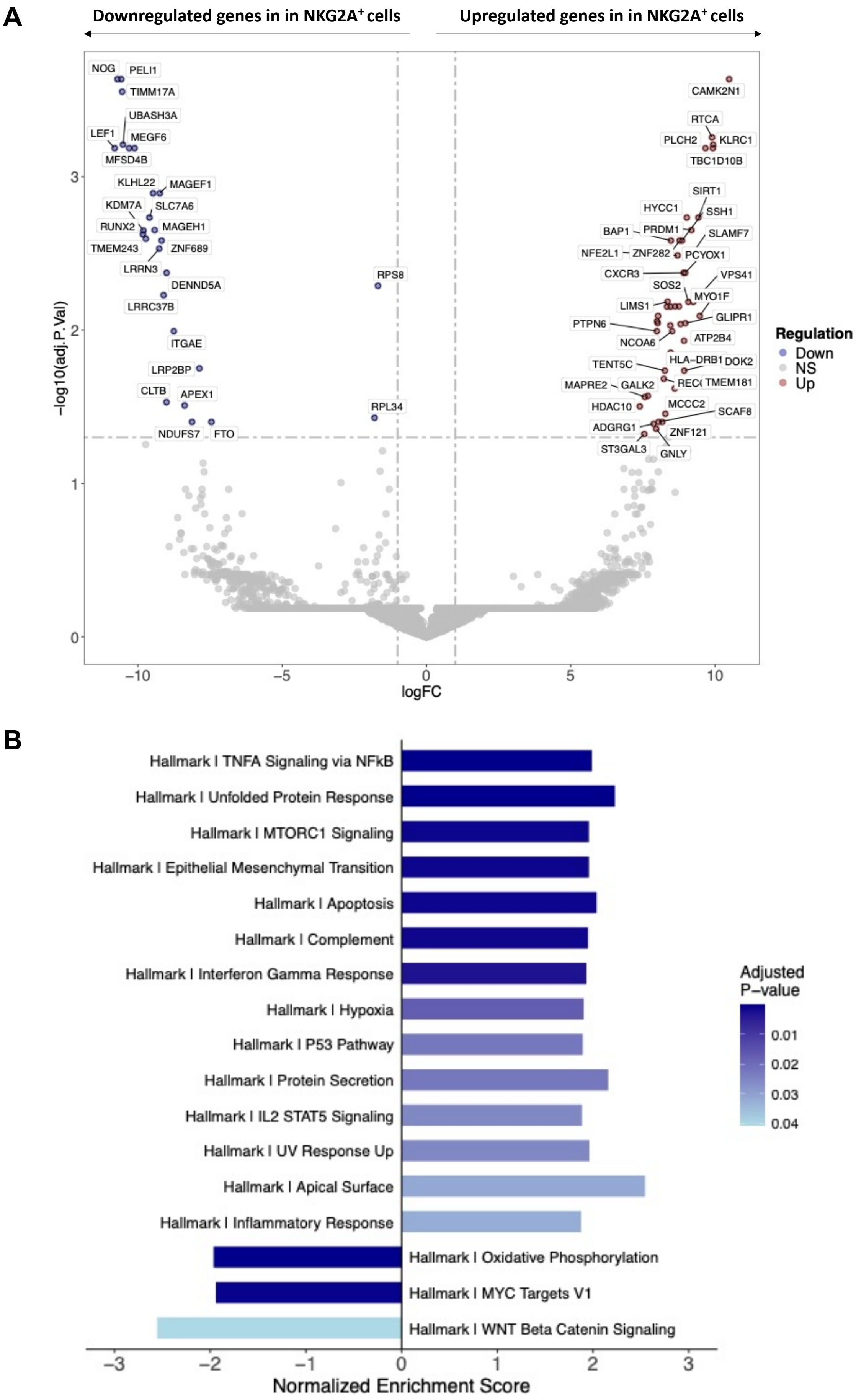
A
Downregulated genes in in NKG2A⁺ cells
Upregulated genes in in NKG2A⁺ cells
NOG
PELI1
TIMM17A
UBASH3A
LEF1
MEGF6
MFSD4B
KLHL22
MAGEF1
KDM7A
SLC7A6
RUNX2
MAGEH1
TMEM243
ZNF689
LRRN3
DENND5A
LRRC37B
ITGAE
LRP2BP
CLTB
APEX1
NDUFS7
FTO
RPS8
RPL34
CAMK2N1
RTCA
PLCH2
KLRC1
TBC1D10B
SIRT1
HYCC1
SSH1
BAP1
PRDM1
SLAMF7
NFE2L1
ZNF282
PCYOX1
CXCR3
VPS41
SOS2
MYO1F
LIMS1
GLIPR1
PTPN6
NCOA6
ATP2B4
TENT5C
HLA−DRB1
DOK2
MAPRE2
GALK2
REC(
TMEM181
HDAC10
MCCC2
SCAF8
ADGRG1
ZNF121
ST3GAL3
GNLY
−log10(adj.P.Val)
0
1
2
3
−10
−5
0
5
10
logFC
Regulation
Down
NS
Up
B
Hallmark I TNFA Signaling via NFkB
Hallmark I Unfolded Protein Response
Hallmark I MTORC1 Signaling
Hallmark I Epithelial Mesenchymal Transition
Hallmark I Apoptosis
Hallmark I Complement
Hallmark I Interferon Gamma Response
Hallmark I Hypoxia
Hallmark I P53 Pathway
Hallmark I Protein Secretion
Hallmark I IL2 STAT5 Signaling
Hallmark I UV Response Up
Hallmark I Apical Surface
Hallmark I Inflammatory Response
Hallmark I Oxidative Phosphorylation
Hallmark I MYC Targets V1
Hallmark I WNT Beta Catenin Signaling
−3
−2
−1
0
1
2
3
Normalized Enrichment Score
Adjusted P−value
0.01
0.02
0.03
0.04

Figure 4.9 Transcriptomic comparison of CD8$^+$ NKG2A$^+$ and naïve CD8$^+$ T cells.

(A) Volcano plots. Raw data downloaded from (Choi, Koh et al. 2023). The log FC indicates the mean expression level for each gene. Each dot represents one gene. After crocin-I administration, black dots represent no significant DEGs between CD8$^+$NKG2A$^+$ and naïve CD8$^+$ T cells, the blue dots represent down-regulated genes and red dots represent up-regulated genes. (B) Gene set enrichment analysis was performed to evaluate the enrichment of gene sets related to the metabolism and immune response in the Hallmark categories.

4.4 Discussion

Previous studies have shown that mTORC1 is a key pathway regulating T cell glycolysis, mitochondrial function, and effector protein translation (Chi 2012, Morita, Gravel et al. 2013, Salmond 2018). Therefore, the activation level of mTORC1 signalling may directly determine how strong CD8$^+$ T cells respond to cytokine stimulation. The results of this study further support that IL-12+IL-18 primarily activate the mTORC1 pathway to activate metabolically active subpopulations, such as MAIT and CD8$^+$NKG2A$^+$CD161$^+$ T cells.

Data from this chapter revealed significant subpopulation-level differences in CD8$^+$ T cell functional responses under IL-12+IL-18 stimulation. This heterogeneity is not only reflected in the production of IFN-γ, but also in differences in metabolic activity and dependence on the mTOR pathway. CD8$^+$NKG2A$^+$CD161$^+$ T cells and MAIT cells were found to be the most responsive subpopulations in this context, with their rapid IFN-γ expression capacity closely associated with significant upregulation of pS6, indicating strong mTORC1 involvement. In contrast, while traditional CD8$^+$ T cells were the dominant population in total CD8$^+$ T cells, they exhibited very limited functional response to the same stimuli. This suggests that the effector potential under cytokine stimulation is constrained by the internal signalling capacity of the subsets.

Although no significant change in pAKT levels was observed following stimulation (Figure 4.4), which are typically used as an indirect indicator of mTORC2 activity, this result should not be clarified as mTORC2 not being interpreted. As mentioned above, mTORC2 activation was shown to be more transient and spatially restricted, which exceeds the detection capability of a Phosflow technique. Therefore, the absence of pAKT signalling is more likely to reflect technical limitations rather than the absence of functional involvement of mTORC2.

Further functional inhibition experiments revealed that MAIT cells were highly sensitive to both glycolytic and OXPHOS inhibition. Following 2-DG treatment, their IFN-γ expression was downregulated by approximately 85%, and in the presence of Oligomycin, it decreased by approximately 80%. This suggests that the effector function of MAIT cells requires high metabolic throughput and shows strong inhibition by blockade of multiple pathways. This high inhibition sensitivity profile may facilitate their rapid response in nutrient-sufficient, highly activated environments, but may also limit their sustained function under metabolically restricted conditions.

In contrast, $CD8^+NKG2A^+CD161^+$ T cells exhibited relatively low sensitivity to inhibition overall. Under treatment with Oligomycin and 2-DG, their IFN-γ expression decreased by approximately 50% and 70%, respectively. This indicates that this subpopulation engages both glycolysis and OXPHOS yet is not fully constrained by either single pathway. This suggests that $NKG2A^+CD161^+$ cells may employ a more flexible or energy-efficient metabolic strategy to maintain their functional responses in different metabolic environments.

This characteristic is consistent with transcriptomic features observed in $CD8^+NKG2A^+$ cells, with enrichment of inflammatory and stress-related programmes such as TNF/NF-κB, mTORC1 pathway, UPR and hypoxia, and reduced expression of modules linked to oxidative phosphorylation, MYC targets, and protein translation. This transcriptional expression pattern supports their ability to maintain effector function under low metabolic supply or stress conditions.

In human $CD8^+$ T cells, mTOR signalling plays different roles in different subpopulations. Experimental results show that IFN-γ expression in $NKG2A^+CD161^+$ T cells, MAIT cells, and conventional $CD8^+$ T cells was highly dependent on mTORC1. Following treatment with Rapamycin, the proportion of IFN-$\gamma^+$$pS6^+$ cells in all subtypes decreased significantly, indicating that mTORC1 is the primary pathway supporting cytokine-driven effector functions.

Further treatment with the mTORC1/2 dual inhibitor OSI-027 resulted in a more pronounced inhibitory effect compared to Rapamycin, suggesting that mTORC2 also participates in regulating this process. Although its role is less prominent than that of mTORC1, it may contribute to maintaining cellular activity and basal metabolic state through downstream signals such as AKT and SGK1 (Patel, Heikamp et al. 2022, Ragupathi, Kim et al. 2024).

In summary, these results indicate that different $CD8^+$ T cell subsets exhibit differences in mTOR signalling, but mTORC1 is the primary driving pathway for rapid activation of effector functions, with mTORC2 playing a supportive role.

4.5 Limitations and Future Directions

Although this study systematically compared the signal responses and metabolic inhibition sensitivities of different CD8$^+$ T cell subsets under IL-12+IL-18 stimulation and revealed the unique functional and metabolic strategies of NKG2A$^+$CD161$^+$ and MAIT cells, there are still some aspects that require further optimisation and supplementation.

Firstly, this study primarily relied on functional observations under short-term stimulation, assessing the initial response capacity of T cells to IL-12+IL-18. However, mTORC1 and mTORC2 not only regulate early effector functions but also participate in subsequent cell survival, metabolic homeostasis, and memory formation(Waickman and Powell 2012, Saxton and Sabatini 2017, Zou, Tao et al. 2020, Szwed, Kim et al. 2021). This study mainly focuses on short-term responses and has not yet fully explored the role of these pathways in long-term functional maintenance. Future studies could test how each subpopulation survives and functions under metabolic stress over longer periods of time, using longer stimulation times, such as 24 to 72 hours.

Secondly, the detection of mTOR signalling primarily relies on pS6 and pAKT flow cytometry staining, which has been widely used in multiple studies (e.g. (Tao, Pan et al. 2021), but they still have limitations such as short temporal resolution and insufficient spatial localisation information. As mentioned above, mTORC2 activation has been shown to be transient and spatial specific, which makes it difficult to capture using conventional Phosflow. In the future, it would be helpful to combine time-resolved phosphorylation detection, subcellular localisation imaging, or dynamic observation of transcription factor activity (e.g. c-MYC) to more clearly assess signal activation states.

Thirdly, the metabolic assessment in this study primarily focused on functional inhibition and GSEA enrichment, without direct measurement of metabolic flux. Although 2-DG and Oligomycin have been widely used for assessment of glycolysis and OXPHOS inhibition effects (e.g. (Argüello, Combes et al. 2020), their results remain indirect inferences. To more accurately capture the metabolic characteristics of different T cell subpopulations during stimulation, future studies may consider applying metabolic flux analysis (e.g. Seahorse Real-Time Cell Metabolic Analysis) or isotope tracing techniques for direct measurement (e.g. SCENITH (Argüello, Combes et al. 2020). Additionally, metabolomics combined with single-cell transcriptomics (scRNA-seq) can provide higher-resolution data support (Lim, Park et al. 2024).

Finally, current subpopulation analysis is primarily based on dividing cells into NKG2A$^+$CD161$^+$, MAIT and conventional CD8$^+$ T cells. However, CD8$^+$ T cells have multiple different states and surface markers. These differences may vary depending on tissue type or stimulus type. This suggests that the current classification may be missing other important subpopulations. For example, CD161 is not limited to innate-like cells, its expression can be induced during viral infection (Billerbeck, Kang et al. 2010), which may complicate the identification of true innate-like subpopulations. To address this issue, future studies could combine TCR clonality analysis, chromatin accessibility (e.g. Assay for Transposase-Accessible Chromatin using sequencing

(ATAC-seq)), or metabolic reporter gene models to more precisely define functional subpopulations and their origins and development.

In summary, although the data in this chapter provide new insights into the functional differences and metabolic mechanisms of cytokine-activated CD8$^+$ T cell subpopulations, further optimisation is needed to improve signal detection, metabolic analysis methods, temporal extension, and subpopulation resolution. These improvements will help us gain a deeper understanding of the metabolic basis and regulatory mechanisms of T cell functional heterogeneity.

4.6 Conclusion

In conclusion, this study reveals how metabolism and signal transduction jointly shape the response of $CD8^+$ T cells during TCR-independent activation. Different subpopulations exhibit differences in their response readiness and signalling capacities. The ability to activate mTORC1 is a key factor in controlling the speed of cellular responses. Additionally, the pattern of inhibition sensitivity across glycolysis and OXPHOS influences cellular functional performance in various environments. These findings help explain the diversity of immune responses and may support future strategies targeting metabolic and signalling regulation to modulate T cell function.

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Chapter 5 Coordinated Regulation of Cytokine-Induced CD8$^+$ T Cell Responses by IRE1α/XBP1s and mTOR Signalling Pathways

5.1 Introduction

T cells are core components of the adaptive immune system, and it has traditionally been believed that their activation primarily depends on TCR recognition of antigens. In this process, the mTOR signalling pathway promotes cell proliferation and the formation of effector functions by regulating metabolism and protein synthesis (Powell, Pollizzi et al. 2012, Zeng and Chi 2017). However, recent studies have revealed that T cells can also be activated by certain cytokines without TCR recognition, leading to rapid production of IFN-γ and participation in early antiviral and antitumour immunity (Lertmemongkolchai, Cai et al. 2001, Munk, Sugiyama et al. 2011, Balint, Feng et al. 2024).

Findings from the last two chapters indicate that IL-12+IL-18 stimulation could rapidly induce CD8$^+$ T cells to produce IFN-γ in the absence of TCR signals and activate the downstream mTOR signalling pathway. Further analysis revealed that different CD8$^+$ T cell subsets exhibit significant differences in metabolic requirements and mTOR pathway dependency. Among these subpopulations, unconventional T cell subsets such as MAIT cells and CD8$^+$NKG2A$^+$CD161$^+$ T cells exert stronger mTORC1 activation capacity and are more metabolically dependent on oxidative phosphorylation and amino acid metabolism.

However, T cell activation is a complex process involving stress and signal reprogramming across multiple organelles, and it remains unclear whether other signalling pathways besides mTOR are involved in this regulation. The IRE1α/XBP1s pathway is a classic branch of the unfolded protein response (UPR). This pathway is activated by the accumulation of unfolded proteins. Once activated, IRE1α will go through dimerization and phosphorylation and gain RNase activity. This results in the cleavage of XBP1 mRNA and generating the functional transcription factor XBP1s, thereby regulating the expression of multiple target genes related to cellular homeostasis and immune responses (Bashir, Banday et al. 2021)(Figure 5.1). Previous studies have shown that this pathway not only regulates endoplasmic reticulum function but may also plays significant role in immune cell function, such as regulating the activation and differentiation of immune cells (Hetz and Papa 2018, Song, Sandoval et al. 2018).

Recent studies have indicated potential crosstalk between the IRE1α/XBP1s and mTOR signalling pathways in regulating immune cell function. It has been shown that mTOR signalling could enhance XBP1s expression and promote UPR pathway activity (Kato, Nakajima et al. 2012). Additionally, persistent inhibition of mTOR during ER stress recovery has been shown to delay the attenuation of IRE1α RNase activity, thereby prolonging the unfolded protein response independently of ER stress intensity (Sanchez-Alvarez, del Pozo et al. 2017). This regulation involves mTOR-dependent control of ER and mitochondrial contact sites, which are crucial for terminating IRE1α activity. This interaction suggests that IRE1α and mTOR may synergistically regulate immune cell function in stress and inflammatory contexts.

Based on this background, it was asked whether the IRE1α/XBP1s pathway also activated in the rapid effector response induced by IL-12+IL-18, and whether it co-regulate $CD8^+$ T cell function with the mTOR signalling pathway. This chapter will explore this question, clearly assessing the role of IRE1α signalling in cytokine-induced $CD8^+$ T cell responses through both chemical inhibition and genetic intervention to further investigate its functional relationship with the mTOR pathway in distinct subpopulations.

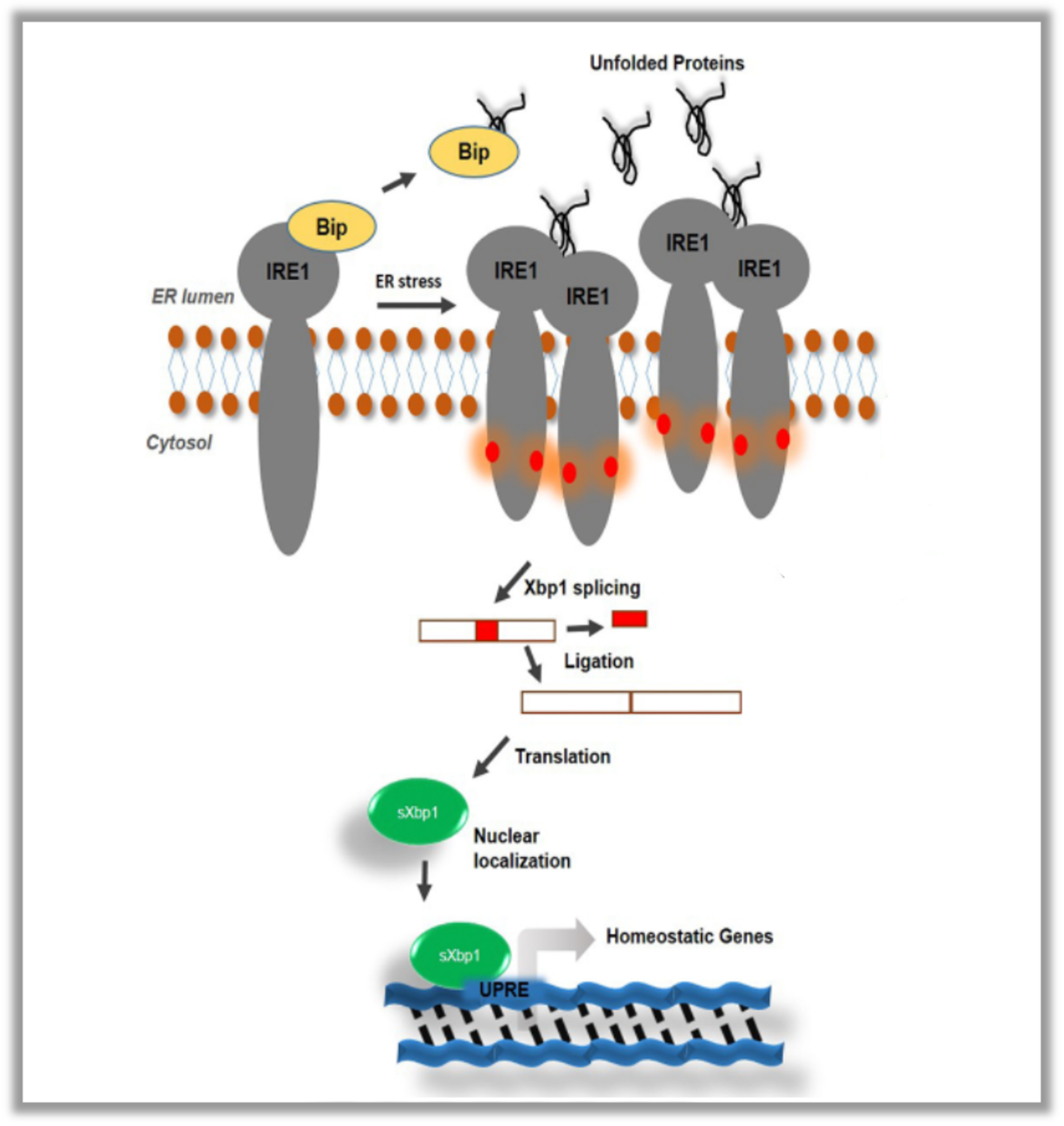


Figure 5.1 Scheme of IRE1α signalling pathway.

This figure was adapted from (Bashir, Banday et al. 2021). When unfolded proteins accumulate in the endoplasmic reticulum (ER), IRE1α undergoes dimerization and activation. This triggers its ribonuclease (RNase) activity, leading to the splicing of XBP1 mRNA. The spliced form (XBP1s) is translated and transported to the cell nucleus, where it drives the expression of genes involved in ER homeostasis.

5.2 Aims

This chapter aims to explore the functional role of the IRE1α/XBP1s pathway in the rapid effector response of human CD8$^+$ T cells induced by IL-12+IL-18, as well as its relationship with mTOR signalling. Previous chapters demonstrated that mTOR plays a crucial role in cytokine-mediated T cell activation. However, the activation of T cells is a complex process involving the cooperation of multiple pathways, and it is still unclear whether other signalling pathways are also involved in regulating this response. Given that the IRE1α/XBP1s signalling pathway is involved in maintaining endoplasmic reticulum homeostasis and has been shown to be closely associated with the activation and function of immune cells, this study will further investigate its role in the cytokine-induced activation process of CD8$^+$ T cells. It's also acknowledged that mTOR signalling and IRE1α/XBP1s pathway may interplay with each other in regulating immune cell function. Therefore, this chapter will focus on the following aspects:

Aim 1: Determine whether IL-12+IL-18 activates the IRE1α/XBP1s pathway in human T cells.

Aim 2: Assess whether this pathway is selectively activated in functional T cell subsets.

Aim 3: Using chemical inhibitors and genetic knockout methods, clarify whether IRE1α signalling participates in regulating cytokine-driven IFN-γ expression.

Aim 4: Examine the functional interactions between IRE1α and mTOR signalling during CD8$^+$ T cell activation.

5.3 Results

Although the previous chapters focused on the metabolic dependence of CD8⁺ T cells on cytokine stimulation, the regulation of T cell function depends not only on metabolic pathways but also on other stress and signalling mechanisms. To further elucidate these mechanisms, the regulatory role of the IRE1α pathway in the expression of effector molecules under IL-12+IL-18 stimulation was first validated in human primary T cells. Building on this, to examine the functional association between IRE1α and mTOR signalling, analyses were refocused on CD8⁺ T cell subsets, and their activation characteristics and pathway interactions across different subsets were assessed using the analytical method established in the previous chapters.

5.3.1 Identification of IRE1α/XBP1s activation in cytokine-stimulated T cell subsets

To investigate the role of the IRE1α pathway in T cell subsets under cytokine stimulation, an in vitro stimulation model was established, and a flow cytometry/ELISA detection workflow was used (Figure 5.2). Human PBMCs were isolated using isolation kit to obtain CD3⁺ T cells. After pre-treatment with IRE1α pathway inhibitors (4μ8C, MKC8866, or KIRA6) for 15 hours, the cells were co-stimulated with IL-12+IL-18 for 7 hours, followed by cell or supernatant collection for flow cytometry or ELISA analysis.

In flow cytometry analyses, CD3⁺CD8⁺ T cells were first gated through lymphocyte, single-cell, and live-cell gates, then distinguished from other CD161⁺ subpopulations based on Vα7.2 and

CD161 expression. Finally, IFN-γ and XBP1s expression was detected in each subpopulation to assess functional responses and IRE1α pathway activity.

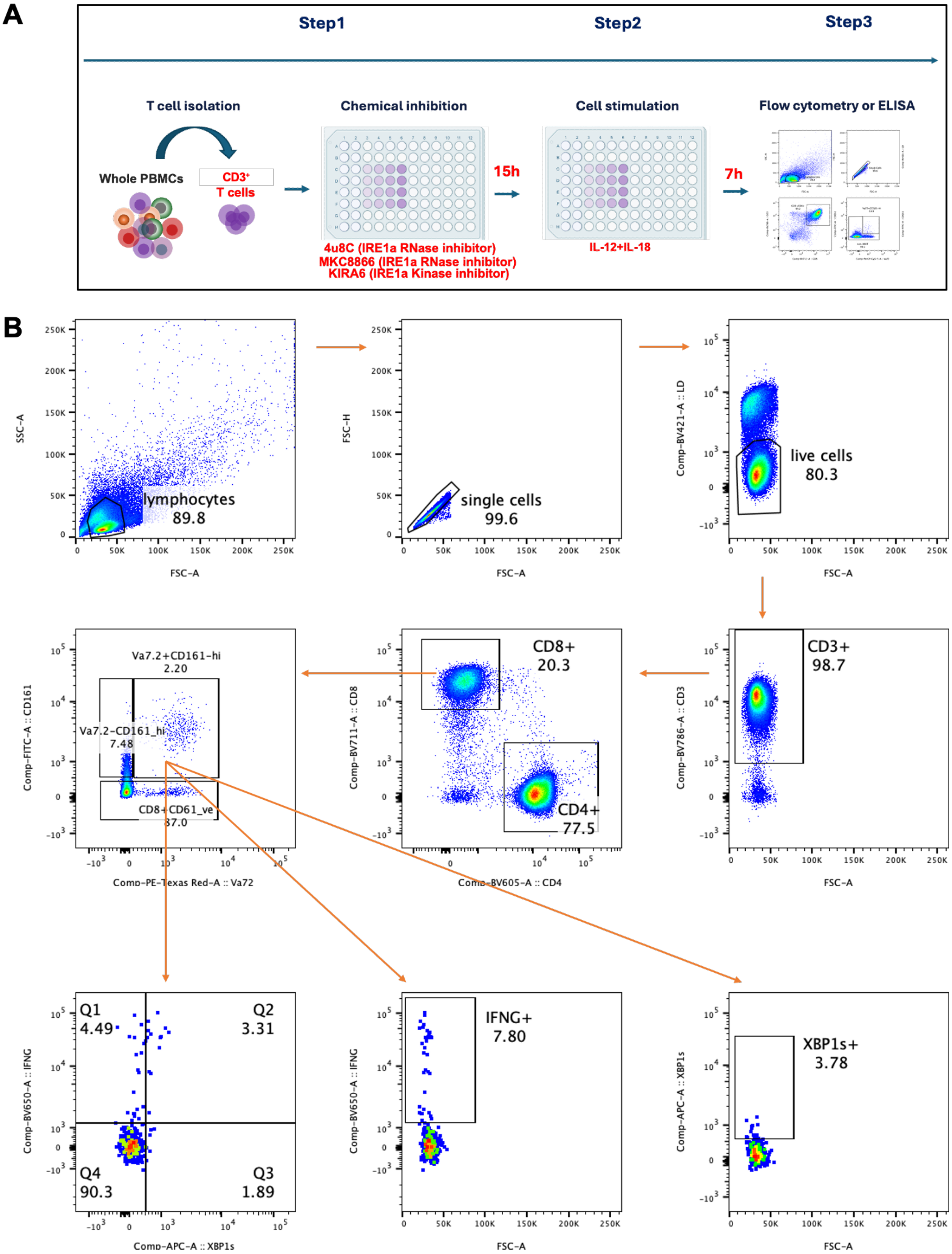


Figure 5.2 The experimental workflow and gating strategy.

(A) The experimental design of figure 5.3-5.8. Human CD3$^+$ T cells were isolated from fresh or frozen whole PBMCs, followed by IL-12+IL-18 stimulation, the phenotype of CD8$^+$ T cell subsets was then measured by flow cytometry. (B) The gating strategy of the following experiment.

To assess whether cytokine stimulation can induce activation of the IRE1α pathway, the functional status of different T cell subsets was analysed under IL-12+IL-18 stimulation (Figure 5.3). Under unstimulated conditions, none of the subsets expressed IFN-γ, and no obvious XBP1s splicing signals were observed. Following stimulation, MAIT cells (CD8$^+$Vα7.2$^+$CD161^{hi}) and CD8$^+$Vα7.2$^-$CD161^{hi} T cells exhibited significant IFN-γ expression, and other subpopulations such as γδ T cells and CD4$^+$ CD161^{hi} cells also showed high response (Figure 5.3A).

The summary results showed that the proportion of IFN-γ producing cells was significantly increased in MAIT cells and CD8$^+$Vα7.2$^-$CD161^{hi} T cells (Figure 5.3B). Additionally, XBP1s was induced in these cells, suggesting activation of the IRE1α pathway in this response (Figure 5.3C). Further analysis revealed that XBP1s was primarily present in IFN-γ$^+$ cells, indicating that the activation of the IRE1α pathway is closely associated with cellular effector functions.

These results suggest that, in the absence of TCR signalling, IL-12 and IL-18 can induce specific T cell subsets to produce IFN-γ, accompanied by activation of the IRE1α pathway, implying that this pathway may participate in regulating cytokine-induced innate-like immune responses.

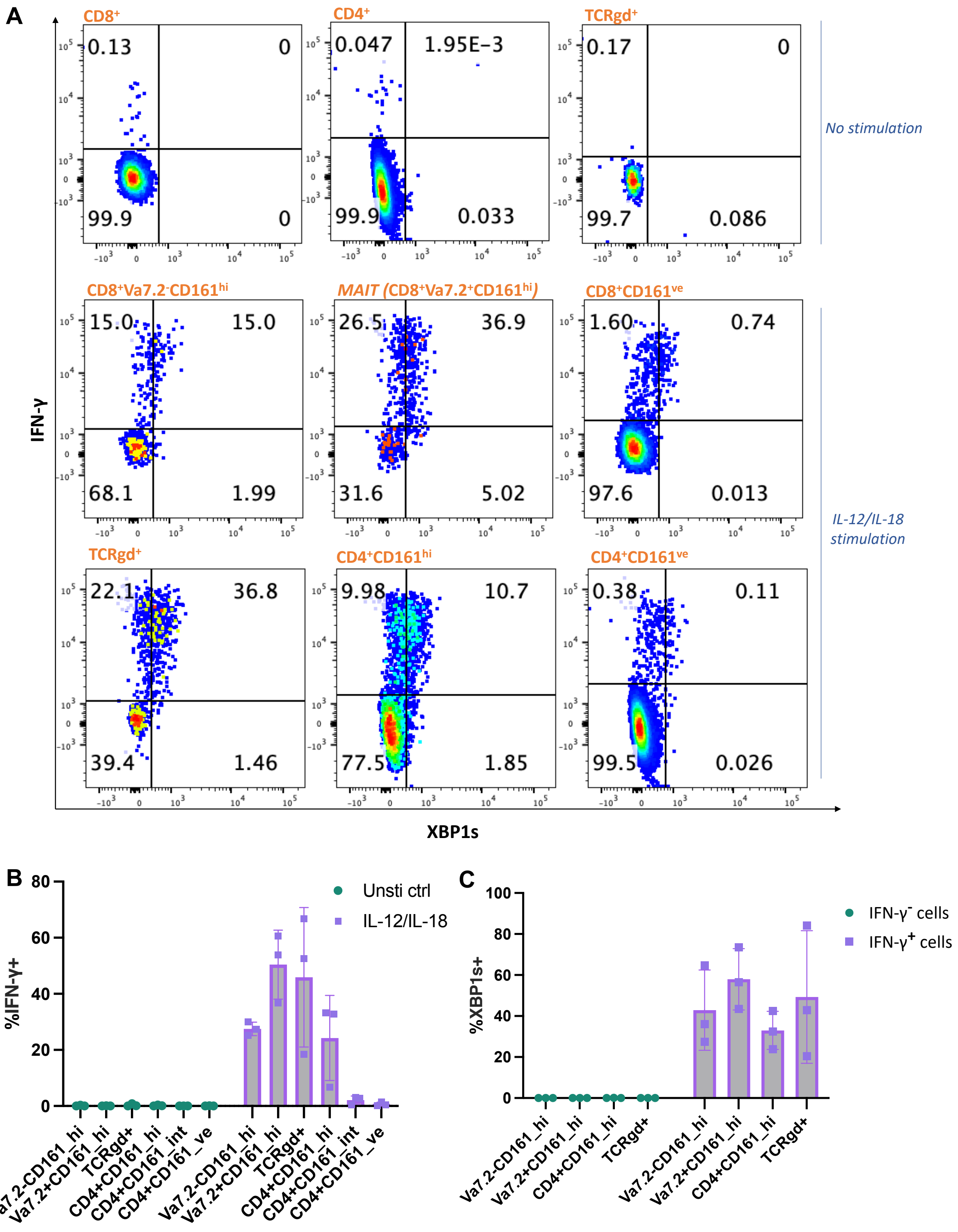


Figure 5.3 Enrichment of XBP1s expression in IFN-γ$^+$ cells is evident in all unconventional subsets following IL-12+IL-18 stimulation.

(A) Representative FACS plots of IFN-γ and XBP1s expression in T cell subsets following cytokine stimulation. (B) The summarised data of IFN-γ expression, n=3 biological replicates. (C) The summarised data of XBP1s expression in either IFN-γ$^-$ or IFN-γ$^+$ cells, n=3 biological replicates. Data are represented as mean ± SEM.

5.3.2 Functional role of IRE1α signalling in unconventional T cell effector responses

To investigate whether the IRE1α pathway participates in regulating cytokine-induced responses in T cells, three chemical inhibitors were used to block its different domains. MKC8866 (MKC) and 4μ8C mainly inhibit its RNase activity, while KIRA6 targets its kinase domain (Chaudhary, Ah Kioon et al. 2022, Chen, Wu et al. 2022, Gsottberger, Meier et al. 2023). Before functional analysis, the effects of these inhibitors on T cell activity and survival were first assessed across concentrations to exclude non-specific toxicity affecting cell survival.

Following treatment of total T cells with these three inhibitors, no significant decrease in cell numbers was observed in the total T cell subsets, including MAIT cells, $CD8^{+}V\alpha7.2^{-}CD161^{hi}$ T cells, $CD4^{+}CD161^{hi}$ T cells, γδ T cells, $CD4^{+}CD161^{-}$ T cells and $CD8^{+}CD161^{-}$ T cells (Figure 5.4A). Relative live cell counts remained essentially stable within the 0-8 μM concentration range, suggesting that MKC8866 has no significant effect on cell viability at this dose. Similarly, after treatment with KIRA6 (0-2 μM), the number of surviving cells in each T cell subset also showed no significant changes (Figure 5.4B).

Additionally, another IRE1α RNase inhibitor 4μ8C was validated across multiple T cell subpopulations. Results showed that normalised live cell counts were not detectably affected within the concentration range used in the experiment (Figure 5.4C). Overall, these results indicate that, under the dosage conditions employed in this study, IRE1α pathway inhibitors did not measurably impact T cell survival. This supports their use for subsequent experiments examining how IRE1α signalling regulates T cell function.

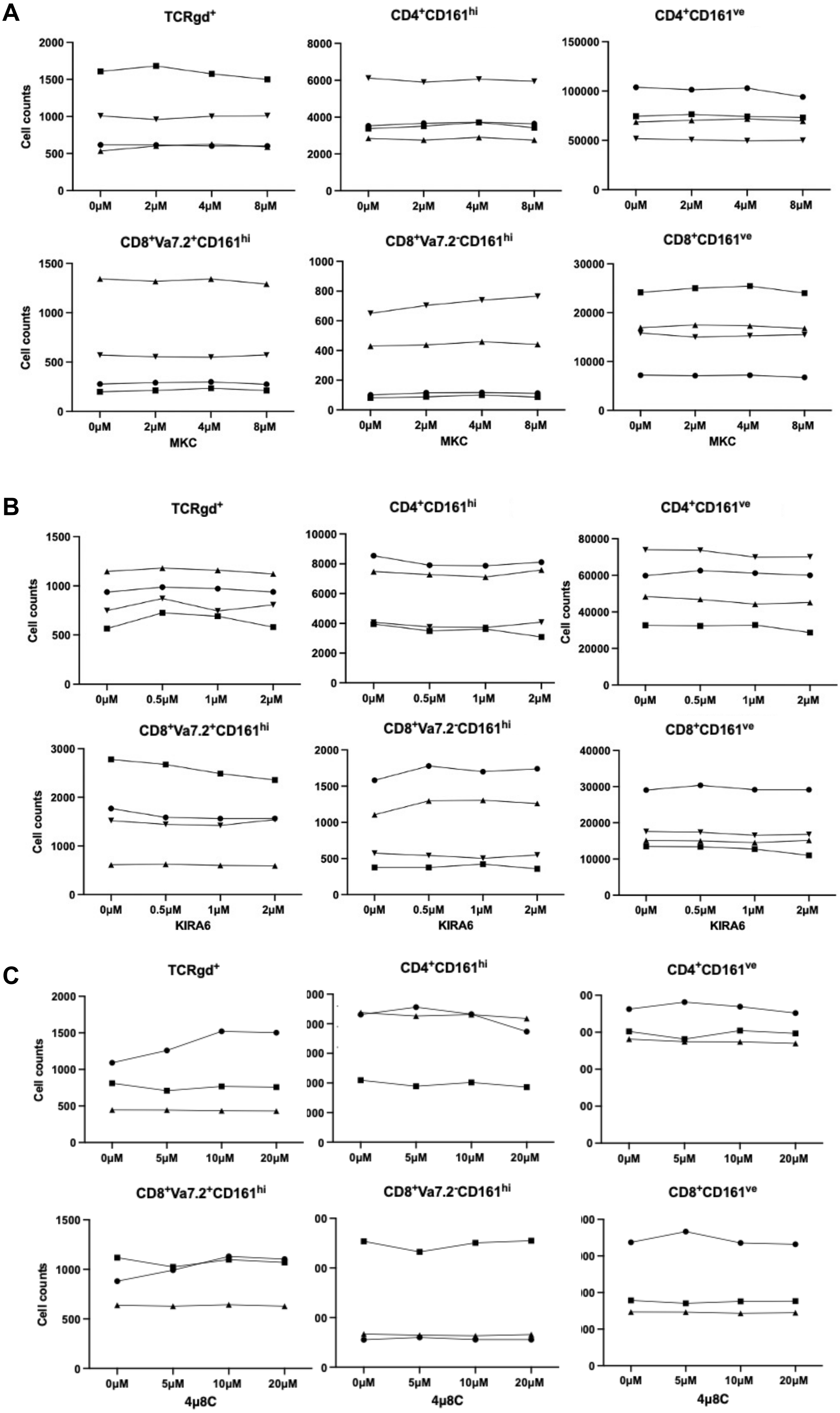

A
TCRgd+
CD4+CD161hi
CD4+CD161ve
CD8+Va7.2+CD161hi
CD8+Va7.2-CD161hi
CD8+CD161ve
Cell counts
0µM
2µM
4µM
8µM
MKC
B
TCRgd+
CD4+CD161hi
CD4+CD161ve
CD8+Va7.2+CD161hi
CD8+Va7.2-CD161hi
CD8+CD161ve
Cell counts
0µM
0.5µM
1µM
2µM
KIRA6
C
TCRgd+
CD4+CD161hi
CD4+CD161ve
CD8+Va7.2+CD161hi
CD8+Va7.2-CD161hi
CD8+CD161ve
Cell counts
0µM
5µM
10µM
20µM
4µ8C

Figure 5.4 MKC8866, KIRA6 and 4µ8C show no measurable impact on viability within the tested ranges.

(A-C) Cells were stained with a fixable LIVE/DEAD viability dye and analysed by flow cytometry. For each sample, acquisition was stopped after collecting 2×10^5 live $CD3^+$ events. Therefore, “live cell counts” represent the relative measure of subset abundance, rather than bead-derived absolute cell numbers. Individual biological replicates are shown. (A) Normalised subset event counts following MKC8866 treatment (0-8µM), n = 4. (B) Normalised subset event counts following KIRA6 treatment (0-2µM), n = 4. (C) Normalised subset event counts following 4µ8C treatment (0-20µM), n = 3.

To explore the role of this pathway in unconventional T cell effector functions, the IRE1α RNase inhibitor MKC was used to interfere with T cell activation under IL-12+IL-18 stimulation conditions. Flow cytometry analysis showed that IFN-γ expression in MAIT cells significantly decreased upon MKC treatment, with a concentration-dependent inhibitory effect (Figure 5.5A-B). In the untreated control group, approximately 64% of MAIT cells expressed IFN-γ, whereas this proportion decreased to approximately 44% after treatment with 8 μM MKC. This finding suggests that IRE1α RNase activity positively regulates the effector function of MAIT cells in the absence of TCR stimulation.

This inhibitory trend was also observed in other T cell subsets expressing CD161, including $CD8^{+}V\alpha7.2^{-}CD161^{hi}$ (Figure 5.5C), $CD4^{+}CD161^{hi}$ (Figure 5.5D), and γδ T cells (Figure 5.5E). These subpopulations are known for their strong responsiveness to cytokines and their innate properties. Their ability to rapidly respond may be partly due to transcription programmes regulated by the IRE1α/XBP1s pathway. Additionally, ELISA detection of IFN-γ levels in culture supernatants following stimulation revealed that MKC8866 treatment significantly reduced total T cell IFN-γ secretion (Figure 5.5F).

These findings suggest that the RNase activity of IRE1α is necessary to produce IFN-γ in unconventional T cells activated by IL-12+IL-18. This indicates that the IRE1α/XBP1s pathway may also contribute to cytokine-induced effector responses that occur without TCR signalling, extending its known role beyond adaptive immunity (Hetz and Papa 2018, Song, Sandoval et al. 2018).

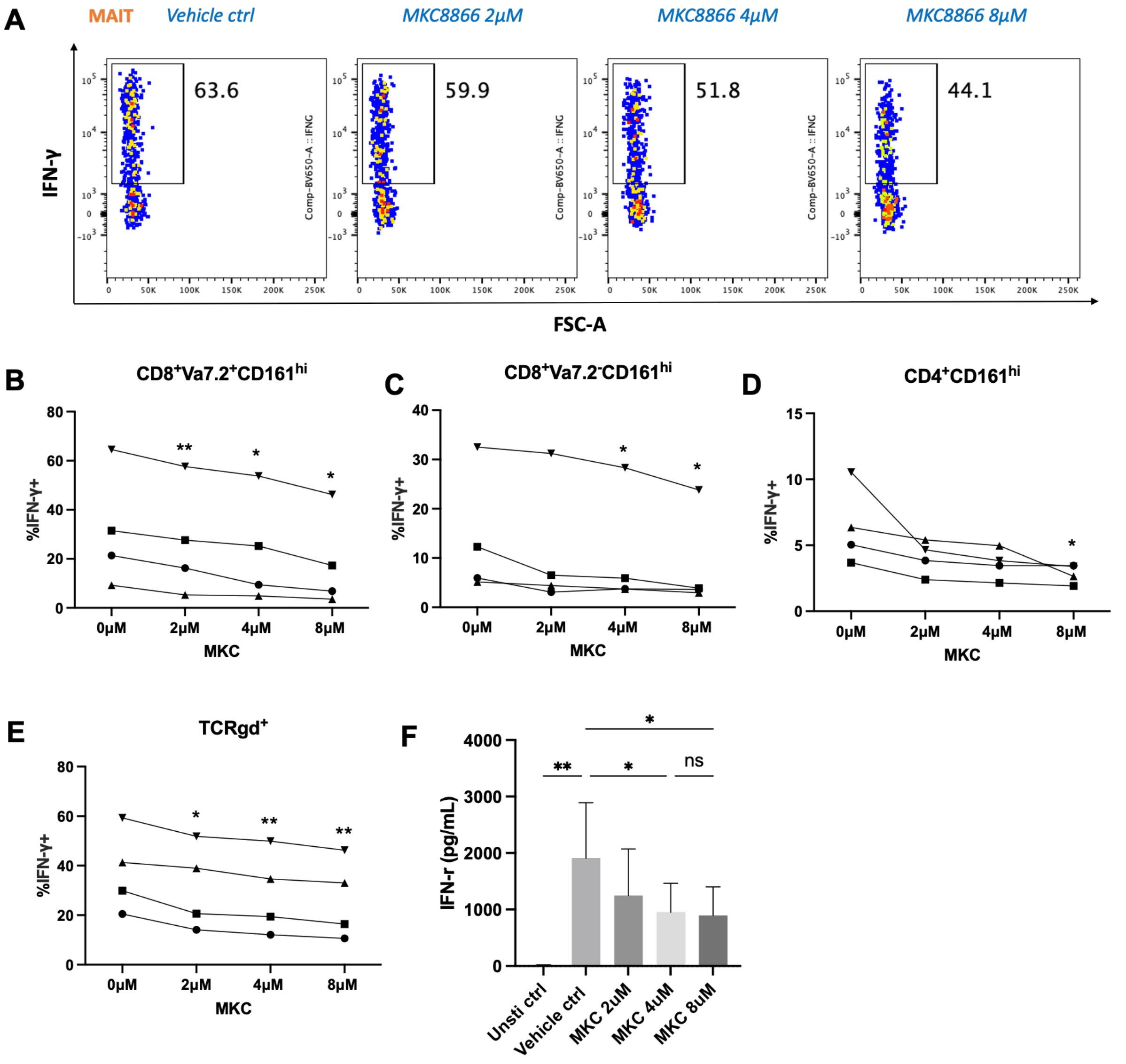


Figure 5.5 MKC8866 suppresses the expression of IFN-γ in unconventional T cells when activated by IL-12+IL-18.

(A) Representative FACS plots of IFN-γ expression in MAIT cells following chemical inhibition of IRE1α and cytokine stimulation. (B) The summarised data of IFN-γ expression in MAIT cells. n=4 biological replicates. (C) The summarised data of IFN-γ expression in CD8$^+$Va7.2$^-$CD161^{hi} T cells. n=4 biological replicates. (D) The summarised data of IFN-γ expression in CD4$^+$CD161^{hi} T cells. n=4 biological replicates. (E) The summarised data of IFN-γ expression in γδ T cells. n=4 biological replicates. (F) The ELISA data of IFN-γ expression in total T cells. n=4 biological replicates. Statistical analysis was performed using one-way ANOVA analysis followed by Dunnett's comparisons test in B-E or Tukey's multiple comparisons test in F. ns=not significant; $^*p < 0.05$, $^{**}p < 0.01$.

To further validate the inhibitory effect of MKC8866 on the IRE1α/XBP1s pathway, the expression of XBP1s was examined under IL-12+IL-18 stimulation. XBP1s is a direct downstream marker of IRE1α RNase activity, whose splicing depends on the mRNA cleavage reaction mediated by IRE1α and is widely used to assess the activation level of this pathway (Bashir, Banday et al. 2021).

In MAIT cells, IL-12+IL-18 stimulation induced significant XBP1s expression at approximately 28.1% level. With the addition of MKC8866, the proportion of XBP1s-producing cells decreased significantly with increasing dose, reached only 8.94% in the 2 µM treatment group, and further decreased to 5% and 1.29% under 4 µM and 8 µM conditions, respectively (Figure 5.6A-B). These results directly demonstrate that MKC8866 functionally blocks the RNase activity of IRE1α.

In other unconventional T cell subsets, including CD8$^+$Vα7.2$^-$CD161^{hi}, CD4$^+$CD161^{hi} and γδ T cells, MKC8866 similarly significantly inhibited XBP1s expression (Figure 5.6C-E). These cells show high XBP1s expression under stimulatory conditions, indicating that the IRE1α pathway is broadly involved in regulating their activation.

Combined with the IFN-γ expression patterns shown in Figure 5.5, it can be inferred that the IRE1α/XBP1s pathway not only participates in stress induction in unconventional T cells but is also closely associated with their cytokine effector functions. This result supports the possibility that IRE1α plays an important role in regulating the innate-like response of unconventional T cells, acting independently on the TCR signalling pathway.

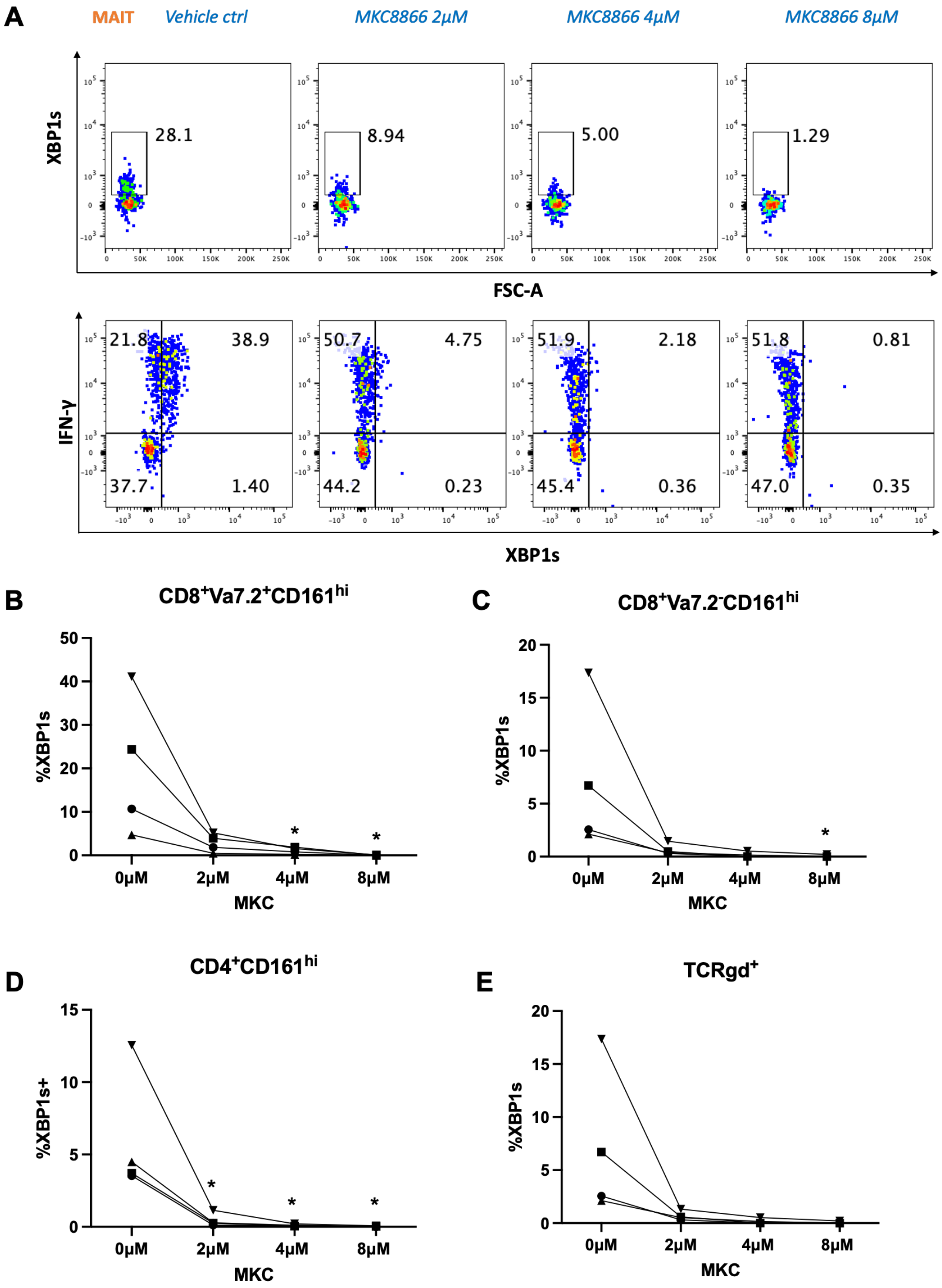


Figure 5.6 MKC8866 reduces XBP1s expression in unconventional T cells under IL-12+IL-18 stimulation.

(A) Representative FACS plots of XBP1 splicing in MAIT cells following chemical inhibition of IRE1α RNase activity and cytokine stimulation. (B) The summarised data of XBP1s expression in MAIT cells. n=4 biological replicates. (C) The summarised data of XBP1s expression in $CD8^+Va7.2^-CD161^{hi}$ T cells. n=4 biological replicates. (D) The summarised data of XBP1s expression in $CD4^+CD161^{hi}$ T cells. n=4 biological replicates. (E) The summarised data of XBP1s expression in γδ T cells. n=4 biological replicates. Data are represented as mean value. Statistical analysis was performed using one-way ANOVA analysis followed by Dunnett's comparisons test. ns=not significant; $^*p < 0.05$.

To further validate the functional role of IRE1α RNase activity under cytokine stimulation, another inhibitor 4μ8C was used to assess its regulatory effects in non-traditional T cell subsets. 4μ8C is a selective inhibitor of the RNase activity of IRE1α. It blocks the splicing of XBP1 mRNA and is widely used in research into immune pathways related to ER stress (Stewart, Estrada et al. 2017).

Under IL-12+IL-18 stimulation, flow cytometry analysis showed that the frequency of IFN-γ-expressing MAIT cells increased significantly, and this induction was inhibited following 4μ8C treatment in a dose-dependent manner (Figure 5.7A-B). At a concentration of 2 μM, the proportion of IFN-γ^+ cells in MAIT cells was reduced to nearly background levels. In the meanwhile, XBP1s expression was also effectively suppressed, suggesting that its downstream signalling activity was significantly disrupted (Figure 5.7C). In addition to MAIT cells, 4μ8C also significantly reduced XBP1s and IFN-γ expression in $CD8^+V\alpha7.2^-CD161^{hi}$ T cells, $CD4^+CD161^{hi}$ T cells and γδ T cells, further indicating that the IRE1α/XBP1s pathway exerts consistent regulatory effects across various non-traditional T cell subsets (Figure 5.7C).

These results are consistent with those from the MKC8866 experiments, further solidifying the central role of the IRE1α/XBP1s pathway in IL-12+IL-18-induced effector responses in unconventional T cells, providing a basis for future studies using inhibitors targeting different sites and gene knockout approaches.

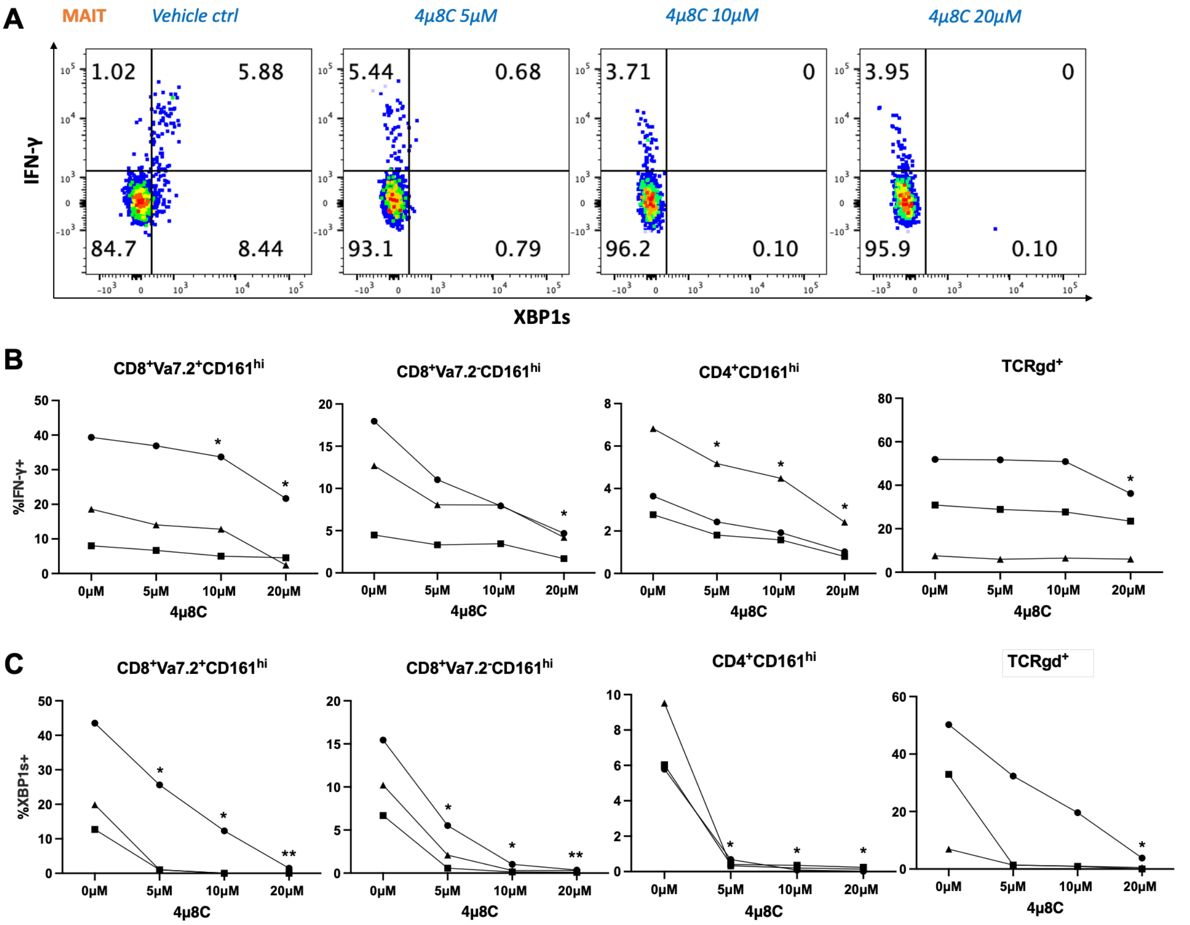


Figure 5.7 4μ8C decreases both IFN-γ and XBP1s expression in unconventional T cells when activated by IL-12+IL-18.

(A) Representative FACS plots of IFN-γ and XBP1s production in MAIT cells following chemical inhibition of IRE1α RNase activity and cytokine stimulation. (B) The summarised data of IFN-γ expression in unconventional T cell subsets. n=3 biological replicates. (C) The summarised data of XBP1s expression in unconventional T cell subsets. n=3 biological replicates. Data are represented as mean value. Statistical analysis was performed using one-way ANOVA analysis followed by Dunnett's comparisons test. ns=not significant; $^{*}p < 0.05$, $^{**}p < 0.01$.

Having observed that MKC8866 and 4μ8C, both targeting the RNase domain of IRE1α, reduced IFN-γ in cytokine-stimulated unconventional T cells, it was next tested whether blocking the kinase domain of IRE1α would produce a similar effect. To do this, another chemical inhibitor KIRA6 targeting IRE1α kinase domain was used. KIRA6 indirectly blocks IRE1α RNase activity by inhibiting the autophosphorylation of its kinase domain (Ghosh, Wang et al. 2014). This mechanism differs from the direct inhibition of RNase activity by MKC8866 and 4μ8C, providing a complementary approach to validate the regulatory function of IRE1α.

Consistent with previous observation, IL-12+IL-18 stimulation significantly upregulated IFN-γ and XBP1s expression in MAIT cells (Figure 5.8A). Upon addition of KIRA6, the proportion of IFN-γ^+ and XBP1s^+ cells decreased in a dose-dependent manner. At 0.5 μM KIRA6, XBP1s expression was already significantly suppressed. XBP1s^+ cells were almost completely absent when treated with 1 μM and 2 μM KIRA6. At the same time, IFN-γ expression was also significantly reduced, indicating that the IRE1α kinase domain regulates its effector function. Similar trends were observed in other unconventional T cell subsets, including $CD8^+V\alpha7.2^-CD161^{hi}$ T cells, $CD4^+CD161^{hi}$ T cells and γδ T cells (Figure 5.8B-C). This indicates that this mechanism is highly consistent across different cell types.

It is worth noting that at higher KIRA6 concentrations, XBP1s level and IFN-γ expression were almost completely supressed, with an apparent inhibitory effect greater than that observed with the RNase inhibitor 4μ8C. Given the extent of suppression observed, potential off-target effects should be considered, as KIRA6 has been reported to engage targets such as HSP60 or other ATP-binding proteins, which may affect various cellular functions (Rufo, Korovesis et al. 2022).

Therefore, while the experimental results of KIRA6 further support the critical regulatory role of IRE1α in unconventional T cell activation, due to the possibility of non-specific effects, the specificity of the conclusions still needs to be further verified through methods such as gene knockout to ensure their reliability.

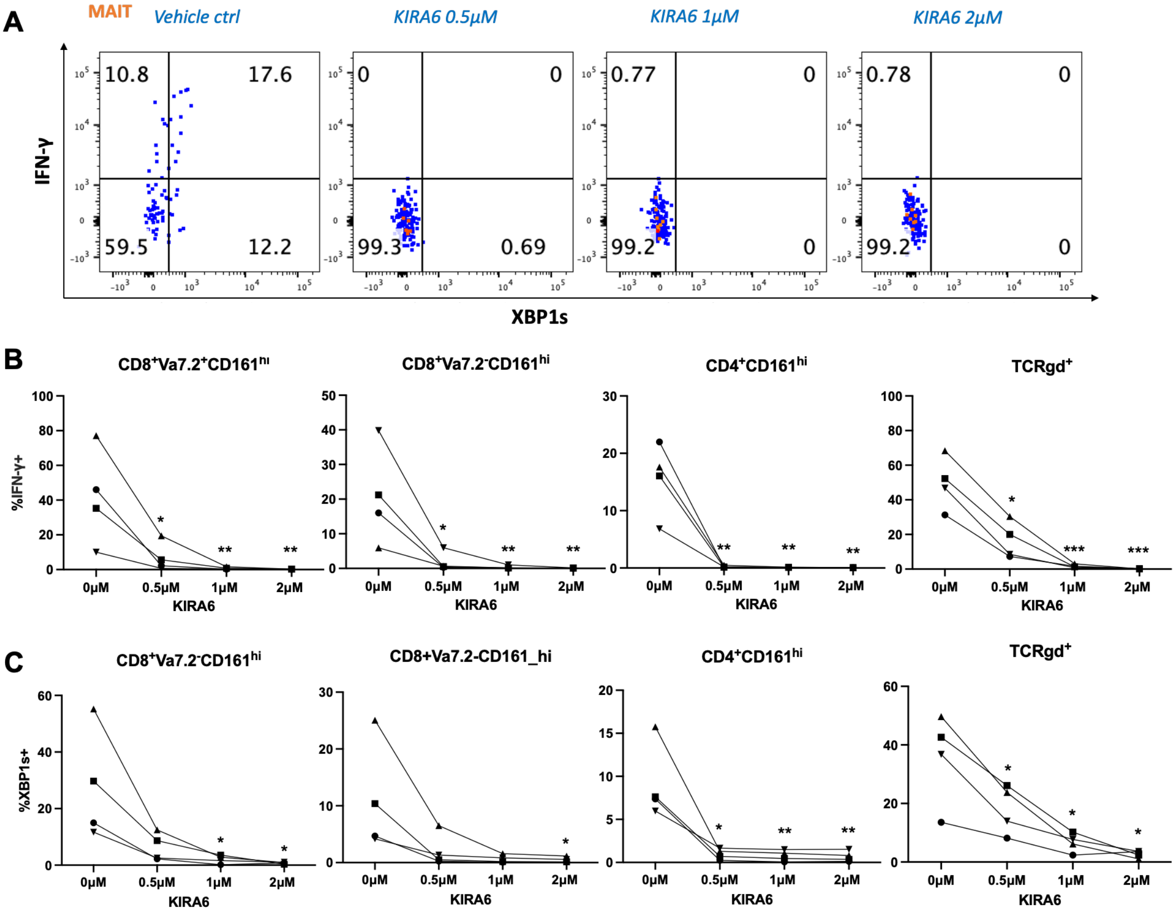


Figure 5.8 KIRA6 potently supressed both IFN-γ expression and XBP1 splicing in unconventional T cells when activated by IL-12+IL-18

(A) Representative FACS plots of IFN-γ expression and XBP1 splicing in MAIT cells following chemical inhibition of IRE1α Kinase activity and cytokine stimulation. (B) The summarised data of IFN-γ expression in unconventional T cell subsets, n=4 biological replicates. (C) The summarised data of XBP1s expression in unconventional T cell subsets, n=4 biological replicates. Statistical analysis was performed using one-way ANOVA analysis followed by Dunnett's comparisons test. ns=not significant; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

In summary, these findings indicate that the IRE1α pathway plays an important regulatory role in the cytokine response of non-traditional T cells. In these cells, inhibition of IRE1α RNase activity by MKC8866 and 4μ8C significantly reduced IFN-γ expression and XBP1s splicing levels under IL-12+IL-18 stimulation. KIRA6, which targets the kinase domain of IRE1α, showed an even stronger effect, but this may be partly due to off-target activity. Therefore, further validation using genetic knockout method is necessary. The overall results suggest that IRE1α exerts consistent functional regulatory effects across multiple CD161^{hi} T cell subpopulations, playing a critical role in regulating their cytokine responses.

5.3.3 Genetic inhibition of IRE1α reduced cytokine-induced effector responses in unconventional T cells

The above results indicate that chemically inhibiting the RNase activity of IRE1α can significantly reduce the expression of IFN-γ in MAIT cells, CD8$^+$Vα7.2$^-$CD161^{hi} T cells, CD4$^+$ CD161^{hi} T cells and γδT cells under IL-12+IL-18 stimulation and inhibit the splicing of its downstream signalling molecule XBP1s. This suggests that the IRE1α/XBP1s pathway is widely involved in the regulation of effector responses in non-traditional T cell populations.

Based on the use of multiple chemical inhibitors to clarify the function of the IRE1α pathway, an IRE1α genetic knockout model was established to further validate the key role of this pathway in non-traditional CD8$^+$ T cell activation and to exclude interference from non-specific drug inhibition. Under IL-12+IL-18 stimulation conditions, genetic inhibition of IRE1α enabled direct assessment of downstream XBP1s splicing and IFN-γ expression, thereby validating the regulatory role of the IRE1α signalling pathway in functional immune responses.

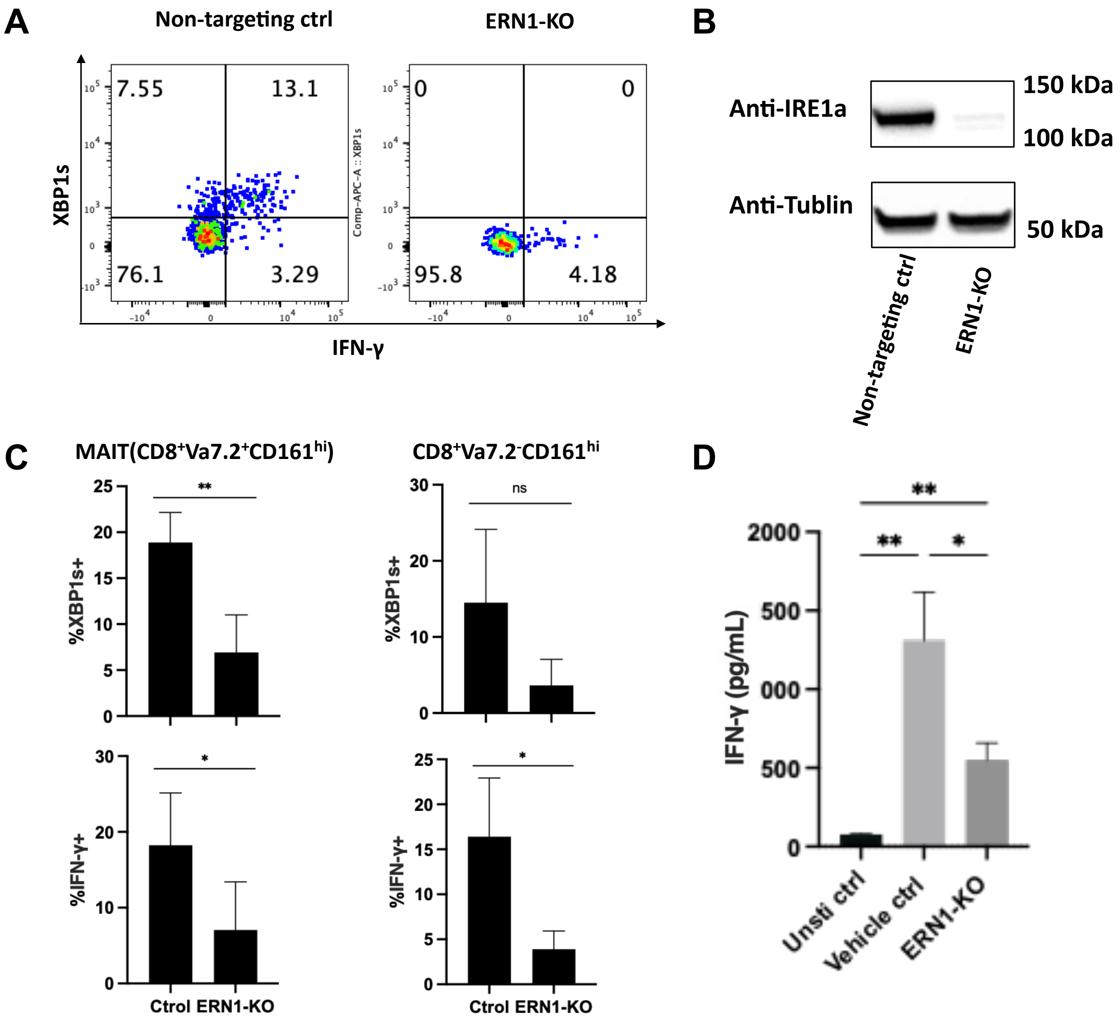


Figure 5.9 Genetic knockout of IRE1α reduces the production of both IFN-γ and XBP1s.

(A) Representative FACS plots of XBP1s expression in MAIT cells following genetic knockout of IRE1α and cytokine stimulation. (B) Representative western blot data of IRE1α production in CD8$^+$ T cells following genetic knockout of IRE1α and cytokine stimulation. (C) XBP1s and IFN-γ expression in CD8$^+$ CD161^{hi} T cell subsets following genetic knockout of ERN1 and cytokine stimulation. n=3 biological replicates. (D) ELISA data of IFN-γ expression in IL-12+IL-18-activated CD8$^+$ T cells. n=3 biological replicates. Statistical analysis was performed using paired two tailed t-test in C or one-way Anova analysis followed by Tukey's multiple comparisons test in D. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

To further validate the critical role of IRE1α in cytokine-induced T cell effector functions at the genetic level, a CRISPR/Cas9-mediated gene editing strategy was employed to target and knockout the ERN1 gene encoding IRE1α. The knockout efficiency was confirmed by Western blot analysis (Figure 5.9B), showing that IRE1α protein expression was significantly downregulated in ERN1-KO CD8⁺ T cells, confirming the success of the knockout.

Under IL-12+IL-18 stimulation conditions, IRE1α deficiency resulted in complete loss of XBP1s expression in MAIT cells (Figure 5.9A), consistent with the previously observed chemical inhibition results. Further analysis revealed that the proportion of XBP1s⁺ cells was significantly reduced in both MAIT cells and CD8⁺Vα7.2⁻CD161^{hi} T cells (Figure 5.9C, top row), while the proportion of IFN-γ⁺ cells also decreased at the same time (Figure 5.9C, bottom row). In MAIT cells, the IFN-γ⁺ proportion decreased from 16% in the control group to approximately 4%; a similar trend was observed in the CD8⁺Vα7.2⁻CD161^{hi} subpopulation, suggesting that IRE1α exerts a stable positive regulatory effect on the expression of effector molecules Induced by IL-12+IL-18.

This phenomenon was also validated at the overall cellular functional level. ELISA results showed that CD8⁺ T cells lacking ERN1 secreted significantly lower levels of IFN-γ protein after cytokine stimulation compared to the control group (Figure 5.9D), further demonstrating the important role of IRE1α in the functional response of CD8⁺ T cell subpopulations.

In summary, genetic knockout of IRE1α not only blocks the splicing of its downstream signalling molecule XBP1s but also significantly inhibits IFN-γ production, thereby genetically validating the central regulatory role of this pathway in unconventional T cell effector functions. However, these data do not exclude off-target effects of KIRA6. Taken together, my findings support a specific regulatory role of IRE1α signalling in cytokine-induced immune responses and provide a basis for future mechanistic and therapeutic research.

5.3.4 IRE1α and mTOR signalling worked together to regulate cytokine-induced effector responses in CD8$^+$ T cells subsets

In the previous sections, the regulatory role of IRE1α signalling in IL-12+IL-18-induced effector function was demonstrated using chemical inhibitors and gene knockout approaches. Notably, this pathway not only has a consistent function in traditionally defined innate-like T cells such as MAIT and γδT cells but also shows a clear activity and function correlation in the CD8$^+$Vα7.2$^-$ CD161^{hi} subpopulation. These results suggest that IRE1α signalling may play a broader role in CD8$^+$ T cell populations.

mTORC1 signalling has been identified as a primary driver of TCR-independent CD8$^+$ T cell activation, and previous studies suggest a possible functional interaction between mTOR and the IRE1α pathway (Kato, Nakajima et al. 2012, Sanchez-Alvarez, del Pozo et al. 2017). To explore whether mTOR synergistically regulates cytokine-induced CD8$^+$ T cell function with the IRE1α pathway, XBP1s expression was assessed under mTOR inhibition conditions and downstream functional readouts were evaluated accordingly. The co-expression of XBP1s and pS6 in CD8$^+$ T-cell subsets was analysed to determine whether IRE1α/XBP1s and mTOR signalling are functionally coordinated.

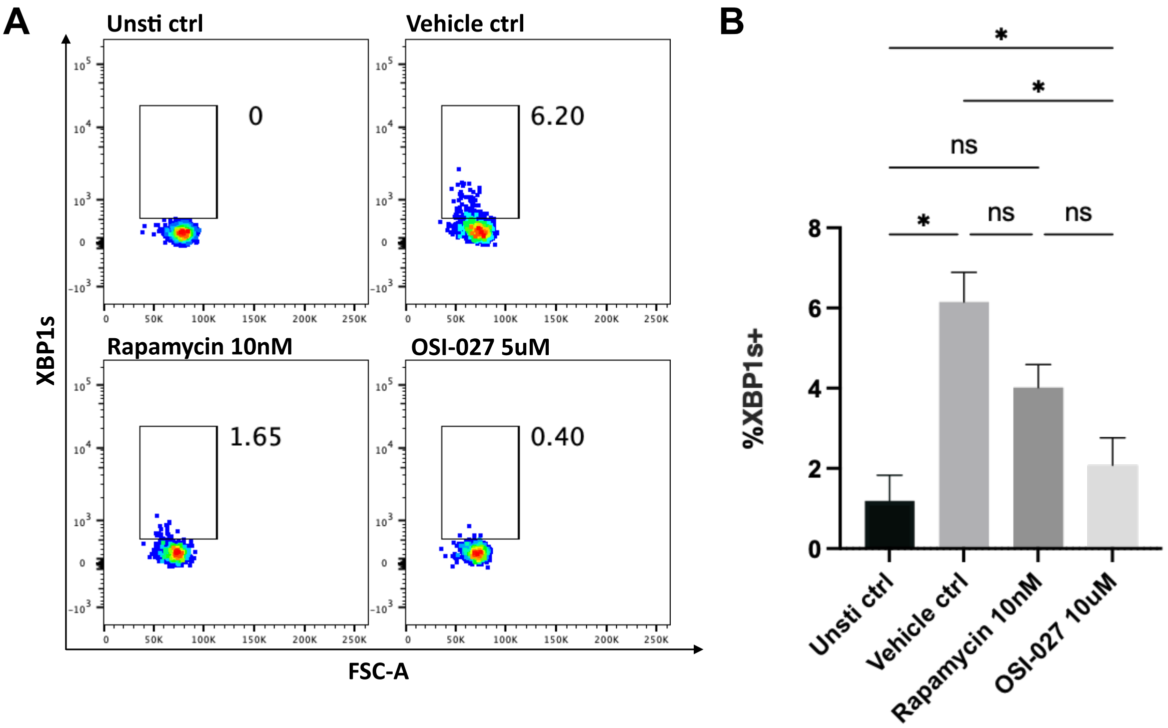


Figure 5.10 mTOR inhibition reduces cytokine-induced XBP1s in CD8$^+$ T cells.

(A) Representative plot data of XBP1s expression in CD8$^+$ T cells following cytokine stimulation and chemical inhibition of mTOR complexes. (B) XBP1s expression in CD8$^+$ T cells following cytokine stimulation and chemical inhibition of mTOR complexes. n=3 biological replicates. Data are represented as mean ± SEM. Statistical analysis was performed using one-way Anova analysis followed by Tukey's multiple comparisons test. ns=no significance, $^*p < 0.05$.

To investigate whether mTOR is involved in regulating the IRE1α/XBP1s pathway, CD8$^+$ T cells were pretreated using two mTOR inhibitors (Rapamycin and OSI-027) before stimulation with IL-12+IL-18. XBP1s expression was then measured as a downstream indicator of IRE1α pathway activation.

Compared to the unstimulated group, cytokine stimulation significantly induced XBP1s expression from 0% to 6.2%. Following rapamycin treatment, the proportion of XBP1s$^+$ cells decreased to around 2%, while OSI-027 treatment further inhibited it to levels close to baseline (Figure 5.10A). Statistical analysis further revealed that this inhibitory effect exhibited significant differences across multiple replicates (Figure 5.10B).

These results suggest that the mTOR pathway plays a regulatory role in the activation of the IRE1α/XBP1s signal. Compared with the inhibition of mTORC1, the stronger inhibitory effect of dual inhibition of mTORC1/2 indicates that overall mTOR activity may be upstream of and key for the effective activation of the IRE1α pathway. This finding links metabolic regulatory mechanisms with ER stress signals, further supporting the possibility of their synergistic role in cytokine-induced CD8$^+$ T cell activation.

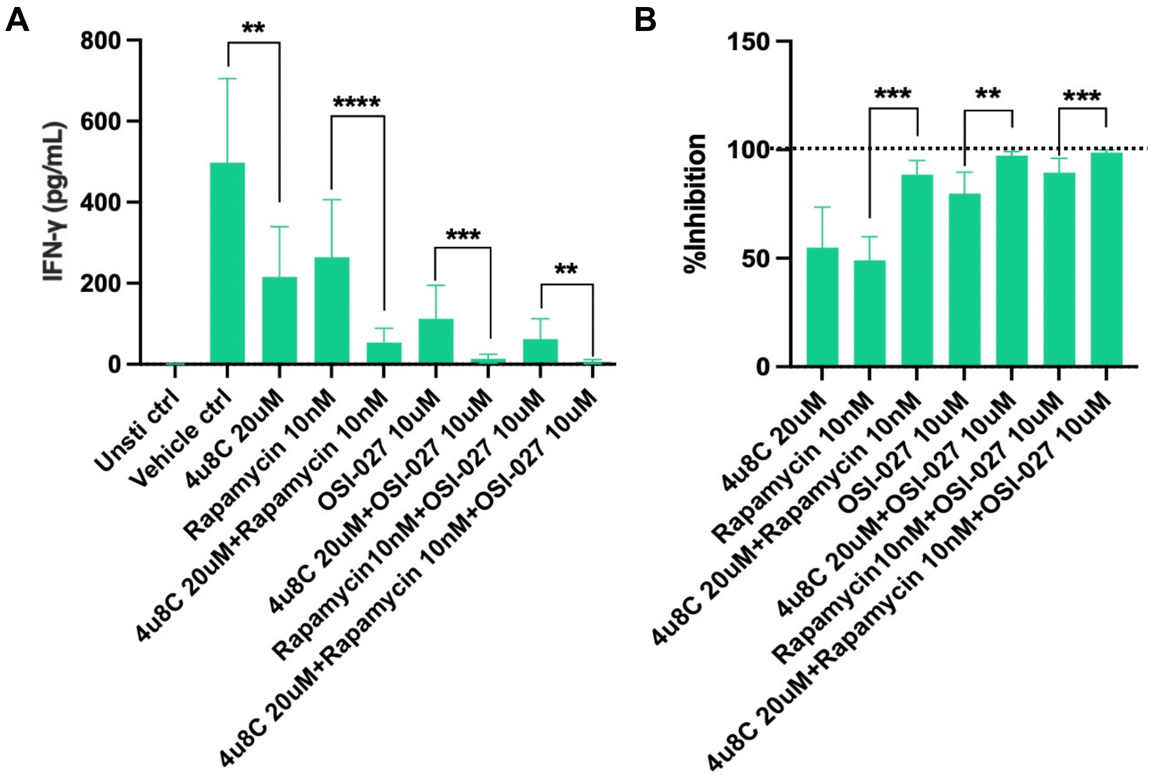


Figure 5.11 IRE1α and mTOR pathways exert an additive effect on cytokine-induced IFN-γ production in CD8^{+} T cells.

(A) IFN-γ expression in CD8^{+} T cells following IL-12+IL-18 stimulation and chemical inhibition of IRE1α or mTOR complexes. n=4 biological replicates. (D) Percentage of IFN-γ reduction in CD8^{+} T cells following cytokine stimulation and chemical inhibition of IRE1α or mTOR complexes. n=4 biological replicates. Data are represented as mean ± SEM. Statistical analysis was performed using one-way Anova analysis followed by Tukey's multiple comparisons test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

To further clarify the regulatory relationship between IRE1α and mTOR in mediating cytokine production in cytokine-activated CD8^{+} T cells, IFN-γ expression induced by IL-12+IL-18 was assessed under conditions of single or combined pathway inhibition (Figure 5.11A).

When stimulated by IL-12+IL-18, CD8^{+} T cells produced high levels of IFN-γ, with concentrations reaching approximately 500 pg/mL in the control group. However, after treatment with the IRE1α RNase inhibitor 4μ8C (20 μM) or the mTORC1 inhibitor Rapamycin

(10 nM), IFN-γ levels decreased significantly. The mTORC1/2 dual inhibitor OSI-027 (10 µM) caused an even stronger IFN-γ reduction (Figure 5.11A). These findings validate the Chapter 3 mTOR results and extend the analysis to the IRE1α pathway in this chapter.

Notably, combining of 4µ8C with Rapamycin or OSI-027 further reduced IFN-γ expression compared to 4µ8C treatment alone, and the reduction was significantly greater than that observed with either single-agent treatment (Figure 5.11A). This additive effect was also reflected in the inhibition efficiency, which approached or exceeded 90% under combination treatment, compared to 50-70% with single agent treatments (Figure 5.11B).

These results indicate that the IRE1α and mTOR signalling pathways play both overlapped and independent regulatory roles in the cytokine response of CD8$^+$ T cells and exert functionally additive effects. This also suggests that the two pathways may exhibit a certain degree of separation in their downstream targets or regulatory mechanisms.

5.3.5 IRE1α and mTOR pathways are simultaneously activated and functionally linked in cytokine-activated unconventional CD8$^+$ T cells

Although the above results suggest that the IRE1α and mTOR pathways function in parallel and partially independently of each other to regulate cytokine-induced effector responses in total CD8$^+$ T cells, it remains unclear whether these pathways are simultaneously activated within specific T cell subsets and whether their activation correlates at the level of individual cells.

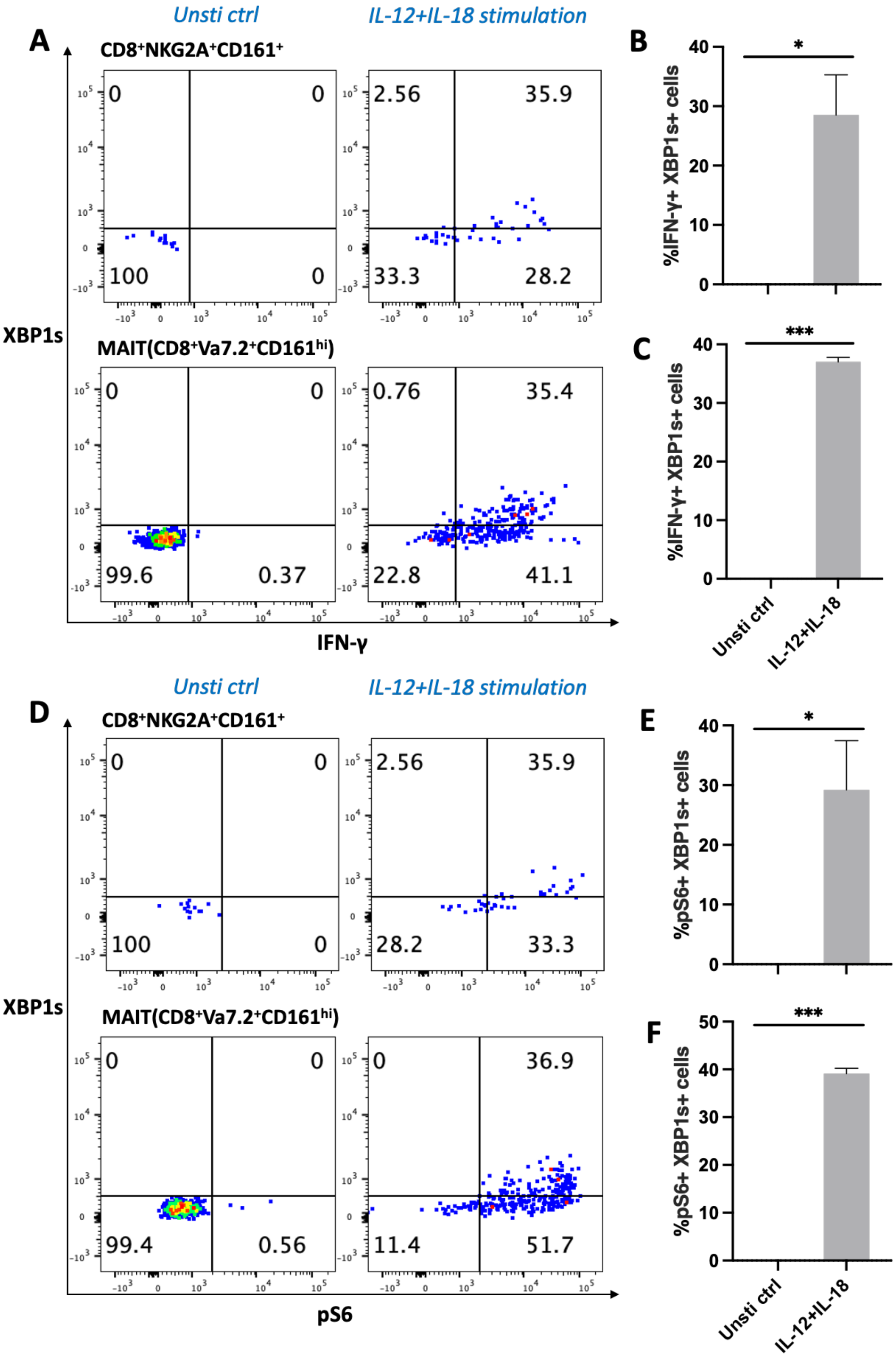
Since $CD8^+NKG2A^+CD161^+$ T cells and MAIT cells are the most responsive IFN-γ-producing populations under IL-12+IL-18 stimulation, the extent of simultaneous activation of the IRE1α/XBP1s and mTOR pathways was next examined in these subsets. Furthermore, to determine whether the two pathways are functionally linked at the subpopulation level, the impact of mTOR inhibition on XBP1s expression and associated IFN-γ expression was evaluated, and the effect of IRE1α inhibition on pS6 expression was evaluated together with its impact on IFN-γ production.

Following the observation that both mTOR and IRE1α pathways contributed to cytokine-induced IFN-γ production (Figure 5.11), it was next investigated whether these pathways could be co-activated within the same $CD8^+$ T cell subsets.

IL-12+IL-18 stimulation typically induced activation of the IRE1α/XBP1s pathway, parallel to downstream mTOR signalling. Following IL-12+IL-18 stimulation, flow cytometry results showed that the proportion of $IFN\text{-}\gamma^+XBP1s^+$ double positive cells potently increased in $CD8^+NKG2A^+CD161^+$ T cells and MAIT cells (Figure 5.12A). Quantitative analysis further confirmed that cytokine stimulation significantly increased the frequency of $IFN\text{-}\gamma^+XBP1s^+$ double positive cells compared to the unstimulated condition (Figure 5.12B-C), suggesting that XBP1s splicing is closely associated with effector molecule expression.

Next, it was examined whether the IRE1α pathway and mTOR signalling were co-activated in the same cells. IL-12+IL-18 stimulation significantly increased the frequency of $XBP1s^+pS6^+$ double positive cells in both $CD8^+NKG2A^+CD161^+$ T cells and MAIT cells (Figure 5.12D). Consistent with this, statistical analysis showed that a significant proportion of $XBP1s^+$ cells also expressed pS6 (Figure 5.12E-F), further supporting the co-activation of these two pathways in functional $CD8^+$ T cell subsets.

In summary, these results indicate that IL-12+IL-18 stimulation leads to simultaneous activation of the IRE1α/XBP1s and mTOR pathways in non-traditional $CD8^+$ T cell subsets. This suggests that the two pathways may work together to regulate functional responses in these cells.


Unsti ctrl
IL-12+IL-18 stimulation
A
CD8+NKG2A+CD161+
0
0
100
0
2.56
35.9
33.3
28.2
XBP1s
MAIT(CD8+Va7.2+CD161hi)
0
0
99.6
0.37
0.76
35.4
22.8
41.1
IFN-γ
B
%IFN-γ+ XBP1s+ cells
*
C
%IFN-γ+ XBP1s+ cells
***
Unsti ctrl
IL-12+IL-18
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CD8+NKG2A+CD161+
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Unsti ctrl
IL-12+IL-18

Figure 5.12 IRE1α/XBP1s pathway activation accompanies mTOR activation in IL-12/IL-18 activated CD8$^+$ T cells.

(A) Representative FACS plots of XBP1s and IFN-γ co-expression in CD8$^+$NKG2A$^+$CD161$^+$ T and MAIT cells following cytokine stimulation. (B) Summarised data of XBP1s and IFN-γ co-expression in NKG2A$^+$CD161$^+$CD8$^+$ T cells, n=3 biological replicates. (C) Summarised data of XBP1s and IFN-γ co-expression in MAIT cells, n=3 biological replicates. (D) Representative FACS plots and summarised data of XBP1s and pS6 co-expression in NKG2A$^+$CD161$^+$CD8$^+$ T and MAIT cells following cytokine stimulation. (E) Summarised data of XBP1s and pS6 co-expression in NKG2A$^+$CD161$^+$CD8$^+$ T cells, n=3 biological replicates. (F) Summarised data of XBP1s and pS6 co-expression in MAIT cells, n=3 biological replicates. Data are represented as mean ± SEM. Statistical analysis was performed using the student paired t-test. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Following the results from the previous section showing that cytokine-induced mTOR signalling and the IRE1α/XBP1s pathway could be co-expressed in the same $CD8^+$ T cell subset, it was next assessed whether inhibiting mTOR would affect the activation of the IRE1α pathway to determine whether there is a regulatory synergistic effect between the two.

In $CD8^+NKG2A^+CD161^+$ T cells, inhibition of mTORC1/2 using OSI-027 significantly reduced the frequency of $XBP1s^+pS6^+$ double positive cells. Treatment of IRE1α RNase inhibitor 4μ8C alone also effectively reduced the proportion of double positive cells. When the two inhibitors were used in combination, $XBP1s^+pS6^+$ population was almost eliminated (Figure 5.13A), demonstrating the interdependent contribution of the two pathways to this activation state.

The effect of these inhibitory conditions on IFN-γ expression was then assessed (Figure 5.13B). Inhibition of either IRE1α or mTOR alone decreased the frequency of IFN-γ^+ cells, whereas combined inhibition of both pathways further decreased IFN-γ^+ cells compared with either single treatment. These data suggest that IRE1α and mTOR may play a complementary or synergistic role in regulating cytokine production.

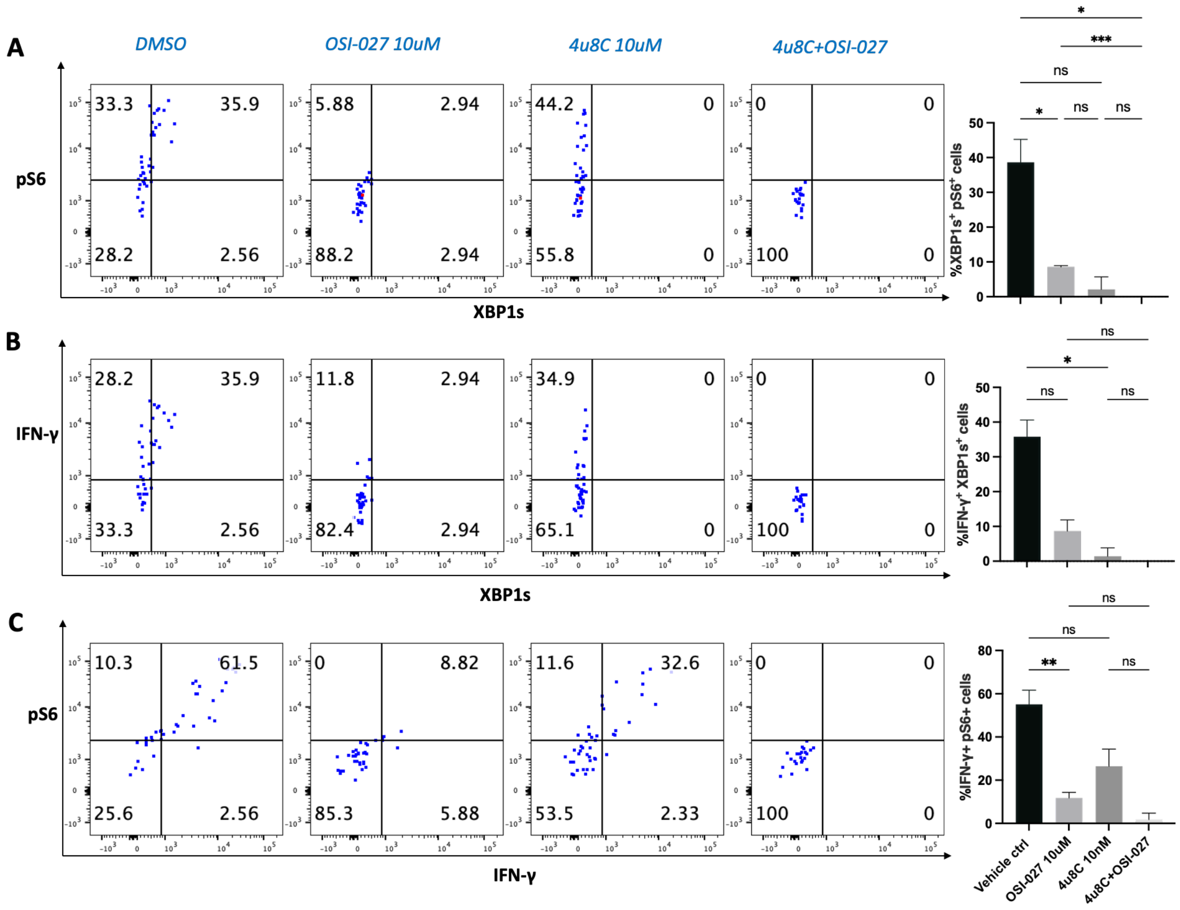

Figure 5.13 Inter-regulation of IRE1α/XBP1s and mTOR pathways in cytokine-stimulated CD8$^+$ T cells.

(A) Representative FACS plots and summarised data of XBP1s and pS6 co-expression in NKG2A$^+$CD161$^+$CD8$^+$ T cells following cytokine stimulation and chemical inhibition of mTOR complexes. n=4 biological replicates. (B) Representative FACS plots and summarised data of IFN-γ and XBP1s expression in CD8$^+$NKG2A$^+$CD161$^+$ T cells following cytokine stimulation and chemical inhibition of mTOR complexes. n=4 biological replicates. (C) Representative FACS plots and summarised data of IFN-γ and pS6 expression in CD8$^+$NKG2A$^+$CD161$^+$ T cells following cytokine stimulation and chemical inhibition of mTOR complexes. n=4 biological replicates. Statistical analysis was performed using one-way Anova analysis followed by Tukey's multiple comparisons test. ns=not significant; $^{*}p < 0.05$, $^{**}p < 0.01$.

Taken together, these results demonstrate that the IRE1α/XBP1s and mTOR pathways are both active in the same cells during IL-12+IL-18 stimulation. They may also work together to regulate T cell effector responses. This finding provides experimental evidence for a functional link between these two pathways in regulating non-conventional T cell immune functions.

5.4 Discussion

In T cell immune responses, metabolism and stress regulation are important for maintaining effector function and environmental adaptability. The mTOR signalling pathway plays a central role in regulating glucose metabolism, protein synthesis and cytokine production in CD8$^+$ T cells (Chi 2012, Chapman and Chi 2014, Huang, Long et al. 2020). In addition, the IRE1α/XBP1s pathway plays a central role in endoplasmic reticulum stress adaptation and has been shown to directly regulate lipid biosynthesis and endoplasmic reticulum membrane expansion to meet increased protein folding demands (Sriburi, Jackowski et al. 2004, Lee and Glimcher 2009). In this chapter, activation of the IRE1α/XBP1s pathway under IL-12+IL-18 was assessed and its functional roles was investigated, and the functional interplay between mTOR activity and IRE1α/XBP1s signalling during cytokine-driven activation was tested.

To explore the mechanism, small-molecule inhibitors and CRISPR/Cas9 knockout were used. IRE1α RNase inhibitors such as MKC8866 directly block XBP1s splicing and RIDD (Logue, McGrath et al. 2018). By contrast, IRE1α kinase inhibitors such as KIRA6 can indirectly reduce RNase activity by inhibiting IRE1α autophosphorylation and may exhibit off-target effects (Rufo, Korovesis et al. 2022). Because KIRA6 has reported off targets, key findings were validated using direct IRE1α RNase inhibitors including MKC8866 and 4μ8C, and further confirmed genetically by ERN1 knockout.

More importantly, a functional synergistic relationship between the IRE1α signalling pathway and mTOR signalling was observed. Inhibition of mTORC1/2 not only significantly reduced pS6 levels but was also accompanied by a decrease in XBP1s expression. This effect may be due to the role of mTOR in regulating protein synthesis, which increases the demand for protein folding in the endoplasmic reticulum, indirectly activating the unfolded protein response and promoting the activation of IRE1α.

High co-expression of XBP1s and pS6 was observed in CD8$^+$NKG2A$^+$CD161$^+$ T cells and MAIT cells, suggesting that the mTOR and IRE1α/XBP1s pathways can be activated simultaneously in individual cells under cytokine stimulation. Combined inhibition of mTOR and IRE1α pathways led to a marked decrease in IFN-γ expression more than either inhibitor alone, accompanied by a significant reduction in XBP1s$^+$pS6$^+$ cells, consistent with complementary roles of the two pathways in regulating cytokine-induced effector responses. These findings suggest that in the absence of TCR stimulation, mTOR and the UPR may cooperate to support rapid effector responses in unconventional T cell subsets.

It is also important to consider the differences between subpopulations. Co-activation of the IRE1α and mTOR pathways was primarily concentrated within the CD161^{hi} subpopulation, while the response in conventional CD161$^-$ T cells was relatively weak. This may reflect differences in how T cell subpopulations respond to stress and regulate metabolism, which could be related to their developmental origins and tissue distribution. Future work could combine single-cell transcriptomics and metabolic phenotyping to better understand how each subpopulation is programmed to manage stress and energy demands.

In summary, this study demonstrates that the IRE1α/XBP1s pathway plays an important role in the cytokine-driven activation of $CD8^+$ T cells. It also shows for the first time that this pathway has a synergistic relationship with mTOR signalling to regulate TCR-independent immune response in unconventional T cells. These findings offer new insights into antigen-independent immune activation and may support future strategies for modulating T cell activity in cancer, infection, or chronic inflammatory diseases.

5.5 Limitations and Future Directions

This chapter reveals that the IRE1α/XBP1s pathway promotes T cell activation under cytokine stimulation, particularly the MAIT and CD161$^+$ subpopulations. Further research investigates the synergistic regulatory mechanism between this pathway and mTOR signalling. Although these findings provide new mechanistic support for the regulation of TCR-independent T cell functions, this study still has certain limitations that need to be further refined in future research.

Firstly, although multiple small molecule inhibitors (MKC8866, 4μ8C, KIRA6) and CRISPR/Cas9-based knockout approaches were used in this study to modulate the IRE1α pathway, potential off-target effects of the inhibitors cannot be ruled out. For example, KIRA6 has been reported to inhibit not only IRE1α but also several SRC family kinases such as LYN and FYN, as well as NF-κB pathways (Rufo, Korovesis et al. 2022, Wunderle, Wilhelm et al. 2025). Although the main findings of this study were supported by both chemical and genetic approaches targeting IRE1α, this study utilized only a single gRNA, meaning off-target effects remain a potential confounding variable. Future experiments should incorporate multiple gRNAs targeting the same sequence to definitively validate the findings. Further confirmation using in vivo models or more selective chemo genetic tools such as PROTACs (Zhang, Yan et al. 2023) would help further improve specificity and strengthen conclusions.

Secondly, the function of the IRE1α/XBP1s pathway is typically linked to the level of endoplasmic reticulum activity and cellular stress conditions (Fu and Doroudgar 2022, Chen, Shi et al. 2023). However, under the current experimental conditions, it remains unclear whether other branches of the UPR, such as PERK/eIF2α or ATF6, are also activated. Given the potential crosstalk or functional compensation between these pathways, future studies could employ measurement of pPERK and activity-specific probes (e.g. ATF6 reporters) to comprehensively assess the activation patterns of the UPR and their coordinated role in cytokine-driven T cell responses.

Thirdly, this study is mainly based on analysis of T cells from peripheral blood. Although cytokine-stimulated CD161$^+$ T cells show strong co-activation of IRE1α/mTOR in vitro, their behaviour in the microenvironment of tissues such as the liver, lungs or intestinal mucosa remains to be investigated. Previous research has shown that T cells present in tissues differ significantly from peripheral blood cells in terms of stress, metabolism and functional response (Kumar, Connors et al. 2018). Therefore, tissue explant or organoids could be used in the future to verify the conclusions of this study in a more physiological context.

Furthermore, the synergistic mechanism between mTOR and UPR has not yet been fully elucidated. mTOR can activate the UPR by promoting protein synthesis and increasing the functional demand on the endoplasmic reticulum.(Appenzeller-Herzog and Hall 2012). Although the IRE1α/XBP1s pathway has been shown to play a key role in lipid synthesis and endoplasmic reticulum membrane expansion (Sriburi, Jackowski et al. 2004), there is currently no direct evidence that this pathway can reversely regulate mTOR activity. Therefore, even though these two pathways may synergistically regulate cellular stress adaptation, their specific interaction mechanisms in CD8$^+$ T cells remain to be further clarified.

In the future, methods such as mass spectrometry and integrated analysis of multiple systems may help identify key molecules such as Rheb, Raptor and SREBP1c that connect the functions of IRE1α and mTOR.

In summary, although this chapter has established a new model for the synergistic regulation of $CD8^+$ T cell function by IRE1α/mTOR, the completeness and tissue specificity of the regulatory network requires further investigation. In the future, multi-omics, dynamic imaging, and in vivo models should be used to gain a complete understanding of the role of this signalling axis in different physiological and pathological environments and to provide theoretical support for the design of relevant immunotherapies.

5.6 Conclusion

In conclusion, this chapter shows that the IRE1α/XBP1s signalling pathway is engaged during cytokine-induced activation of unconventional CD8$^+$ T cell subsets and contributes to their effector function. Using chemical inhibitors and IRE1α gene knockout techniques, reduced XBP1s and IFN-γ expression was observed, indicating a functional role for this pathway under IL-12+IL-18 stimulation. Additionally, this study reveals the co-activation of IRE1α/XBP1s and mTOR signalling pathways at the cellular level. Combined inhibition of both pathways reduced IFN-γ expression more than either inhibitor alone, indicating non-redundant and complementary contributions. This discovery not only expands our understanding of the mechanisms underlying antigen-independent T cell activation but also provides a theoretical foundation and potential targets for future therapeutic strategies targeting abnormally activated T cells.

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Chapter 6 Discussion and Conclusion

In this study, the interaction between mTOR and IRE1α/XBP1s pathways during cytokine-induced activation of human CD8⁺ T cells was investigated, with a particular focus on innate-like subsets. In addition, the functional impact of glycolysis and oxidative phosphorylation inhibition was assessed using 2-DG and Oligomycin. By comparing the functional responses and their sensitivity to glycolysis or OXPHOS inhibition under TCR-dependent and cytokine-driven activation conditions, distinct activation features of CD8⁺ T cells were defined. It reveals the rapid response capacity of CD8⁺ T cells under cytokine-driven activation and their underlying signalling regulatory networks which include mTOR and IRE1α/XBP1s pathways. Together, these findings provide preliminary evidence for understanding the molecular basis of innate-like immune activation. This provides a new perspective on how the functional programme of innate-like CD8⁺ T cell subsets is established.

6.1 Role of mTOR signalling in cytokine-induced CD8⁺ T cell activation

6.1.1 mTORC1 is a major regulator of functional response in nonclassical activation

The mTOR signalling pathway plays a key regulatory role in the activation and function of CD8⁺ T cells. In mice, mTORC1 promotes translation and anabolic metabolism, supporting CD8⁺ T cell growth (Pollizzi, Patel et al. 2015, Hukelmann, Anderson et al. 2016), while mTORC2 regulates AKT and contributes to CD8⁺ T cell differentiation (Zhang, Tschumi et al. 2016). In human memory CD8⁺ T cells, mTOR activity was also found to sustain IFN-γ production and proliferation under TCR stimulation (Setoguchi, Matsui et al. 2015). Although previous studies have mainly focused on the functional roles of the mTOR pathway under TCR-dependent activation conditions, how mTOR functions in cytokine-induced activation modes remain less clear.

To address this, the role of mTOR in CD8$^+$ T cells was compared under IL-12+IL-18 stimulation versus TCR-engaging stimulation by T cell activation beads. IL-12+IL-18 stimulation induced enhanced mTORC1 activity, evidenced by increased pS6 expression. mTOR inhibitor assays further revealed that the mTORC1-specific inhibitor rapamycin significantly reduced cytokine-induced IFN-γ expression in CD8$^+$ T cells, whereas the dual mTORC1/2 inhibitor OSI-027 did not lead to significantly increased inhibition. In contrast, under TCR-induced activation conditions, the inhibitory effect of OSI-027 on IFN-γ expression was significantly stronger than that of rapamycin. These results suggest that mTORC1 plays a dominant role in regulating rapid cytokine-induced functional response of CD8$^+$ T cells, with a relatively larger contribution of mTORC2 when TCR is engaged.

These results suggest that under nonclassical activation conditions, mTORC1 is a major regulator supporting the rapid effector response of human CD8$^+$ T cells. This is consistent with previous findings that γ-chain cytokines such as IL-2 and IL-7 can activate mTORC1 in T cells (Rollings, Sinclair et al. 2018, Xu, Leary et al. 2022). In addition, IL-2-STAT5 signalling can enhance mTORC1 activity by upregulating RHEB and SLC7A5/CD98, indicating a route not solely dependent on PI3K-AKT signalling (Villarino, Laurence et al. 2022). Taken together, these findings identify mTORC1 as a key node for integrating cytokine signals and a priority target for mechanistic studies and potential interventions in cytokine-driven activation.

6.1.2 Functional sensitivity to glycolysis and OXPHOS inhibitors, and to amino acid availability

mTORC1 supports T cell activation and effector functions by promoting translation initiation, lipid synthesis and glycolytic activity (Pollizzi, Patel et al. 2015, Angela, Endo et al. 2016, Hukelmann, Anderson et al. 2016). In TCR-dependent activation, mTORC1 regulates transporter proteins such as GLUT1 and SLC7A5 to promote glucose and amino acid uptake (Sinclair, Rolf et al. 2013, Siska, van der Windt et al. 2016). However, direct evidence for its metabolic dependence in IL-12+IL-18-induced activation is still lacking.

Using chemical inhibition assays, it was observed that both the glycolysis inhibitor 2-DG and the mitochondrial inhibitor Oligomycin reduced IL-12+IL-18-induced IFN-γ expression, and CD8$^+$ T cells were more sensitive to glycolysis inhibition. This was distinct from TAB stimulation condition. In addition, amino acid supplementation restored IFN-γ production when cultured in R0 media. No such effect was seen with TAB stimulation. These data suggest that cytokine-induced rapid responses in CD8$^+$ T cells are sensitive to both energy supply and amino acid availability.

Under amino acid-sufficient condition, rapamycin significantly reduced IFN-γ in cytokine-activated human CD8$^+$ T cells. OSI-027 produced a greater suppression, but the additional effect was limited. Together with the previous observations, this is more consistent with a working model in which mTORC1 is dominant with a measurable mTORC2 contribution. Amino acid availability may enhance mTORC1 activity through upstream nutrient sensing and transport pathways, thereby amplifying the translational and synthetic demands imposed by the cytokine, while the contribution of mTORC2 in this model maybe relatively modest.

However, this study provides primarily functional evidence and has not yet performed dynamic measurements of metabolic fluxes and mitochondrial function, nor has it directly interfered with amino acid transporters. Additionally, the conclusions regarding mTOR signalling are largely based on pharmacological inhibition, without genetic validation of Raptor and Rictor via knockdown or knockout. In future work, targeted restriction of defined amino acids or inhibition of specific amino acid transporters such as SLC7A5 should be applied in this system. And genetic intervention of Raptor and Rictor should also be done to validate these findings.

6.2 Functional heterogeneity of CD8$^+$ T cell subsets under cytokine stimulation

Although MAIT cells are a well-defined innate-like population, CD161 and NKG2A expression also marks broader innate-biased CD8$^+$ T cell compartments that are not necessarily MAIT. CD161 (KLRB1) is enriched on tissue-imprinted and innate-like CD8$^+$ T cells and is associated with rapid cytokine responsiveness (Duurland, Santegoets et al. 2022), whereas NKG2A (KLRC1) is an inhibitory receptor whose expression can be shaped by inflammatory cues or repeated activation(Rapaport, Schriewer et al. 2015). Thus, CD161$^+$NKG2A$^+$ and related non-MAIT subsets may reflect heterogeneous developmental histories such as tissue conditioning and activation exposure, rather than a single lineage, and their precise origins remain incompletely resolved in humans.

6.2.1 Innate-like CD8$^+$ T subsets show stronger mTORC1-linked IFN-γ responses to IL-12+IL-18

CD8$^+$ T cell subsets differ significantly in their metabolic and signalling dependencies (van der Windt, Everts et al. 2012, Sinclair, Rolf et al. 2013, O'Sullivan, van der Windt et al. 2014), but subset-specific metabolic responses under cytokine-driven activation conditions remain incompletely characterized. NKG2A- and CD161-expressing CD8$^+$ T cells display innate-like characteristics (Fergusson, Smith et al. 2014, Choi, Koh et al. 2023), and may employ distinct metabolic strategies from conventional CD8$^+$ subsets (Kedia-Mehta, Pisarska et al. 2023). Most mechanistic evidence to date derives from antigen-dependent activation and may not fully generalize to IL-12+IL-18 contexts.

Within the same donors, MAIT, NKG2A$^+$CD161$^+$, NKG2A$^-$CD161$^-$ and single-positive subpopulations were compared in parallel under IL-12+IL-18 or TAB stimuli, with pS6 and IFN-γ measured by flow cytometry. mTOR activity was modulated with rapamycin and OSI-027, and metabolic inhibition was performed with 2-DG and Oligomycin. The results showed that under IL-12+IL-18 conditions, MAIT and NKG2A$^+$CD161$^+$ T cells showed a higher frequency of pS6$^+$IFN-γ$^+$ cells than other CD8$^+$ T cell subsets. This response was significantly reduced by rapamycin, with only modest additional inhibition by OSI-027, indicating the dominant role of mTORC1 in this context. Additionally, NKG2A$^+$CD161$^+$ T cells were more sensitive to OXHPHOS blockade than glycolysis inhibition, while MAIT cells were sensitive equally to both, suggesting different modes of energy utilisation.

Functionally, these findings support subset-specific regulation of mTOR activity and energy supply, particularly in inflammatory or infectious environments that require rapid IFN-γ production. This can be further validated at the single-cell level by combining flux metabolism with the transcriptomic analysis.

6.2.2 Engagement of the IRE1α/XBP1s pathway during cytokine-driven activation

Cytokine-driven activation rapidly increases the secretory output of CD8$^+$ T cells (Fergusson, Smith et al. 2014, Ge, Monk et al. 2019). The IRE1α/XBP1s branch of the UPR may be engaged to meet this demand (Wu, Zhang et al. 2024), but its behaviour under IL-12+IL-18 stimulation has not been well defined at the subset and single-cell levels.

To test this, CD8$^+$ T cells were stimulated with IL-12+IL-18 and intracellular XBP1s and IFN-γ were measured in CD8$^+$ T cell subsets, including MAIT, NKG2A$^+$CD161$^+$, NKG2A$^-$CD161$^-$, NKG2A$^+$CD161$^-$ and NKG2A$^-$CD161$^+$ cells. Compared with unstimulated controls, IL-12+IL-18 stimuli increased XBP1s expression, most potently in MAIT and NKG2A$^+$CD161$^+$ subsets, and was accompanied by increased IFN-γ production. Chemical inhibition of IRE1α lowered IFN-γ without major loss of viability and CRISPR knockout of ERN1 produced a similar reduction, indicating a functional contribution of the IRE1α/XBP1s pathway in this context.

Together, these data indicate that IRE1α/XBP1s is engaged during rapid cytokine-driven responses. A simple model might be that XBP1s expands ER protein folding capacity and secretion to sustain rapid effector output. Although off-target effects of small molecules cannot be ruled out and knockout efficiencies may vary, the pharmacological and genetic results are consistent and support an important contribution of the IRE1α/XBP1s pathway.

6.3 Functional interplay between mTORC1 and IRE1α/XBP1s pathways

6.3.1 Co-activation of mTORC1 and IRE1α/XBP1s in innate-like $CD8^+$ T cell subsets

In this study, activation of both mTORC1 and the IRE1α/XBP1s pathway was observed under IL-12+IL-18 stimulation. However, this is not necessarily implying intracellular co-activation, therefore it remains uncertain whether they are mobilised simultaneously within the same cell and whether this is enriched in specific subsets.

To test this, intracellular XBP1s and pS6 were quantified in MAIT, $NKG2A^+CD161^+$, $NKG2A^-CD161^-$, $NKG2A^+CD161^-$ and $NKG2A^-CD161^+$ subsets after short IL-12+IL-18 stimulation, with IFN-γ expression measured as the functional output. Relative to unstimulated cells, the frequency of $pS6^+XBP1s^+$ double-positive cells increased, especially in MAIT and $NKG2A^+CD161^+$ subsets, indicating XBP1s and mTORC1 activity co-occur within cytokine-responsive cells, particularly among $IFN\text{-}\gamma^+$ cells.

Functionally, identifying the co-activation of these two pathways suggests a potential model in which mTORC1 sustains the translational programme, while XBP1s supports ER protein folding and secretion for regulating rapid $CD8^+$ T cell effector responses. Further analysis of their functional interplay is presented in Section 6.3.2.

6.3.2 Interplay between mTORC1 and IRE1α/XBP1s in CD8$^+$ T cells

It's reported that in non-T-cell models of ER stress, mTORC1 acts upstream of IRE1 and selectively enhances IRE1-XBP1s/JNK pathway (Kato, Nakajima et al. 2012). During recovery from ER stress, AKT-mTOR has been found to promote reformation of ER-mitochondria contacts and thereby attenuates IRE1 RNase activity in epithelial cells (Sanchez-Alvarez, del Pozo et al. 2017). Under IL-12+IL-18 conditions, co-activation of mTORC1 and the IRE1α/XBP1s pathway was observed in human primary CD8$^+$ T cells. However, it remains unclear whether their concurrent activation reflects a functional interaction, whether the roles of the two pathways are distinct rather than overlapping, and whether the strength is consistent across different subsets.

To test this, the effect of dual pathway inhibition versus single pathway blockade were compared in IL-12+IL-18-activated CD8$^+$ subsets. Combined inhibition of mTOR and IRE1α/XBP1s reduced IFN-γ production more strongly than either inhibition alone. Additionally, mTOR inhibition reduced XBP1 splicing level, IRE1α blockade downregulated pS6 expression, and dual inhibition further reduced the frequency of XBP1s$^+$pS6$^+$ double-positive cells. These effects were most pronounced in NKG2A$^+$CD161$^+$ T cells and MAIT cells.

The data suggest an additive effect of the two pathways on cytokine induced rapid IFN-γ responses in CD8$^+$ T cells, which is particularly evident in the MAIT and NKG2A$^+$CD161$^+$ subsets. mTORC1 is likely to set the rate of synthesis, while XBP1s may determine the upper limit of folding and secretion. This model provides a practical basis for subsequent combined intervention strategies. However, as this study relied only on pharmacological inhibition of mTOR pathways, off-target effects cannot be ruled out, genetic perturbation will be needed for further validation.

6.4 Limitations and perspectives

Together, this study linked mTORC1 and IRE1α/XBP1s pathways to IL-12+IL-18 induced IFN-γ responses in CD8$^+$ T cells. The two pathways contribute additively to this process, with the highest involvement in NKG2A$^+$CD161$^+$ and MAIT subsets. Nevertheless, there are still some unanswered questions.

As this study relied heavily on pharmacological inhibitors, complementary approaches would strengthen the findings. For mTOR signalling, genetic perturbation of mTORC1 and mTORC2 components (Raptor/Rictor) or CRISPR-based suppression could be used alongside phospho-flow readouts. For amino acid control, targeting transport checkpoints such as SLC7A5 can help to modulate nutrient sensing upstream of mTORC1. Additionally, there is a lack of direct evidence of metabolic fluxes. Extracellular flux assays such as Seahorse XF record oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in living cells, allowing estimates of ATP made by glycolysis and by mitochondrial respiration (Kong, Lee et al. 2022). This analysis would provide direct measurements of pathway usage, while UPR engagement could be validated by parallel quantification of XBP1 splicing and additional branch-specific markers.

Mechanistically, the specific interaction between mTOR and XBP1s is unclear. It may act by regulating translational load or membrane lipid synthesis, and this needs further molecular mechanistic investigation. Evidence from non-T cells suggests that mTORC1 drives the IRE1-

XBP1/JNK branch during ER stress (Kato, Nakajima et al. 2012). During recovery, AKT and mTOR restore the connection between the endoplasmic reticulum and mitochondria and inhibit IRE1 RNase activity (Sanchez-Alvarez, del Pozo et al. 2017). mTORC1 also promotes lipogenesis (Lee, Zheng et al. 2017), and lipid bilayer stress can activate IRE1 (Halbleib, Pesek et al. 2017). Taken together, these clues outline a testable link between mTORC1 and IRE1α/XBP1s in CD8$^+$ T cells. In the future, genetic and time-course testing under IL-12+IL-18 should be used to determine timing and necessity.

In parallel, single-cell profiling at higher resolution would help to determine whether cytokine responsiveness reflects stable subset identity or inducible activation states within heterogeneous CD8$^+$ T cell populations. To resolve cytokine-responsive heterogeneity at higher resolution, future work could combine scRNA-seq with protein quantification (CITE-seq) to capture both transcriptional states and surface markers such as CD161, NKG2A and TCRVα7.2. Pooling donors using sample-barcoding antibodies in the same run would improve cross-donor comparability and increase statistical power. Paired TCR sequencing would help distinguish clonally expanded, antigen-experienced cells from bystander-activated cells. Profiling several time points after IL-12+IL-18 stimulation would further separate early transcriptional responses from later metabolic and secretory programmes.

Most samples in this study came from peripheral blood CD8$^+$ T cells. To test dynamic changes in the tissue environment, future work should include both mouse and human systems. For example, use in vivo mouse models of inflammation and analyse human inflammatory tissue biopsies with paired blood and tissue samples, including spatial profiling and longitudinal follow up. If the NKG2A$^+$CD161$^+$ and MAIT programmes observed in vitro are confirmed in vivo, then the mTORC1 and IRE1α/XBP1s pathways, which are both interacting and parallel, may provide precise subset-specific targets of intervention for functional modulation when antigen presentation is limited and cytokine cues dominate.

More broadly, this study focused on inflammatory cytokines IL-12 and IL-18, but in the context of tumours, chronic infections or autoimmune diseases, the stimulating environment in which T cells reside is much more complex. Particularly in metabolic stress situations such as hypoxia, lactate accumulation and nutritional deficiencies (Scharping, Rivadeneira et al. 2021). Beyond IL-12 and IL-18, cytokines that frequently contribute to bystander activation of CD8$^+$ T cells include IL-15 and type I interferons, which can enhance effector readiness and IFN-γ responses in infection-like settings (Kolumam, Thomas et al. 2005, Chu, Tyznik et al. 2013). Other cytokines worth testing to broaden the model include IL-1β, IL-21 and IL-27 (Tominaga, Yoshimoto et al. 2000, Cui, Liu et al. 2011, Schneider, Yaneva et al. 2011), either alone or in defined combinations with IL-12/IL-18. It is worth further investigating whether IRE1α and mTOR still maintain similar activation patterns and functional roles in these contexts. Understanding this could help explain mechanisms of T cell dysfunction in disease and provide new directions for therapeutic regulation of local immune responses.

Overall, these results suggest that mTOR signalling and IRE1α-XBP1s pathway are practical targets for regulating rapid, cytokine-driven IFN-γ responses, especially in innate-like CD8$^+$ T cell subsets. However, several compounds used here such as oligomycin are best regarded as mechanistic tools rather than translational candidates. Clinically, mTOR inhibitors are already used to modulate immune responses (Augustine, Bodziak et al. 2007), while IRE1-targeting small molecules are still mainly at preclinical stage (Grandjean and Wiseman 2020, Wiese, Siwecka et al. 2022), with limited clinical translation so far (Unal, Kuzu et al. 2024); in addition, some compounds (notably KIRA6) have documented off-target activities (Rufo, Korovesis et al. 2022). Future work should therefore rely more on target-specific perturbations and in vivo validation, and focus on defining dose and timing that reduce bystander inflammation without broadly suppressing protective immunity.

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