

The role of Interferon-gamma-mediated T cell collectivity on CD8⁺ T cell responses

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Abstract

The role of Interferon-gamma-mediated T cell collectivity on CD8⁺ T cell responses

by Lion F. K. Uhl

*Thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy,
University of Oxford, Michaelmas Term 2021*

Immune responses towards intracellular pathogens and tumours rely on CD8⁺ T lymphocytes, whose function it is to kill infected or cancer cells, while sparing healthy tissue. CD8⁺ T cell immune responses are characterized by a remarkable degree of poly-clonality and poly-functionality. However, their overall population response is consistent and robust. Much research has focused on individual T cell activation, but how individual, heterogenous T cell responses are integrated at the population-level remains incompletely understood. Upon encountering their cognate antigen in secondary lymphoid organs, CD8⁺ T cells arrest for prolonged period of times around antigen-presenting cells forming dense cell clusters, which can function as communication hubs enabling T cells to directly communicate with each other and therefore directly modulate each other's behaviour.

Here we investigated how CD8⁺ T cells regulate their response through direct T cell-T cell (T-T) communication, with a particular focus on the cytokine interferon-gamma (IFN- γ) and its effect on T cell responses. We found that specific deletion of the IFN- γ receptor on CD8⁺ T cells results in augmented expansion of antigen-specific effector T cells and in a skewing of the average functional avidity of the T cell repertoire, thus modulating multiple aspects of the response. In addition, we discovered that activating CD8⁺ T cells preferentially receive IFN- γ

from other CD8⁺ T cells and furthermore that this IFN- γ -mediated communication between T cells is restricted to a specific time window during the activation phase of the response. Finally, we identified a specific CD8⁺ T cell subpopulation, so-called virtual-memory T cells, to function as the primary source of this early wave of IFN- γ . Altogether, this study highlights an underappreciated facet of the regulatory role of IFN- γ and virtual memory T cells on CD8⁺ T cell responses against infection.

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For their incredible support, love, and generosity,
I dedicate this thesis to my family,
Katharina, Phoebe, Christoph,
Karin, Sigrid, Gundolf and Edith

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Life is not what is promised,
But what is sought.
Our lesson, all we have taken
& all we have brought
- "Life" by Amanda Gorman

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“Sing us a song you’re the piano man

Sing us a song tonight

Well, we’re all in the mood for a melody

And you got us feeling alright”

Excerpt of “Piano Man” by Billy Joel, 1973

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Abbreviations

AP-1	Activator protein 1
APC	Antigen-presenting cell
ActA	Actin-assembly-inducing protein
Bcl-2	B-cell lymphoma 2
Bcl-6	B-cell lymphoma 6
Bim	Bcl-2 interacting mediator of cell death
BM	Bone marrow
BMDCs	Bone marrow-derived dendritic cells
CCR7	CC-chemokine receptor 7
CD	Cluster of differentiation
CDR	Complementary-determining region
c-Jun	Jun oncogene
CSFE	Carboxyfluorescein succinimidyl ester
cSMAC	Central supramolecular activation cluster
CTL	Cytotoxic T lymphocyte
CXCR3	CXC-chemokine receptor 3
DC	Dendritic cell
Eomes	Eomesodermin
ERK	Extracellular signal-regulated kinase
eYFP	Enhanced yellow fluorescent protein
FOXO1	Forkhead box O1
FOXP3	Forkhead box P3

GAF	Gamma-activated factor
GAS	Gamma-activated site
GFP	Green fluorescent protein
GM-CSF	Granulocyte-monocyte colony-stimulating factor
HEV	High endothelial venule
HIV	Human Immunodeficiency virus
ICAM-1	Intercellular adhesion molecule 1
IFA	Incomplete Freund Adjuvant
IFN	Interferon
Ig:	Immunoglobulin
IL	Interleukin
iNKT	Invariant natural killer T cell
iNOS	Nitric oxide synthase
IP ₃	Inositol triphosphate
ITAM	Immunoreceptor tyrosine-based activation motif
IRF	Interferon-regulatory factor 1
IRES	Internal ribosome entry site
IS	Immunological Synapse
ISG	Interferon-stimulated gene
ISRE	Interferon stimulated response element
JAK	Janus kinase
KLRG1	Killer cell lectin-like receptor G1
LAT	Linker for activation of T cells
LAMP-1	Lysosome-associated membrane protein-1

LCMV	Lymphocytic choriomeningitis virus
LFA-1	Lymphocyte function-associated antigen 1
LLO	Listeriolysin O
LM	<i>Listeria monocytogenes</i>
LN	Lymph node
LPS	Lipopolysaccharide
LT α	Lymphotoxin- α
MACS	Magnetic-activated cell sorting
MAIT	Mucosal-associated invariant T cell
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MPEC	Memory precursor effector cell
mTOR	Mammalian target of rapamycin
NFAT	Nuclear factor of activated T cells
NF- κ B	Nuclear factor-kappa B
NK	Natural killer
NKG2D	Natural killer group 2D
NO	Nitric oxide
OVA	Ovalbumin
PBS	Phosphate-buffered saline
PI	PMA/Ionomycin
PKC	Protein kinase C
pMHC	Peptide-MHC
PTK	Protein tyrosine kinases

PRR	Pattern recognition receptor
PS	Phosphatidylserine
pSMAC	Peripheral supramolecular activation cluster
RAG	Recombination activating gene
SH2	Src Homology 2
SLEC	Short-lived effector cell
SLO	Secondary lymphoid organ
SPF	Specific pathogen-free
SPR	Surface plasmon resonance
SOCS	Suppressor of cytokine signalling
STAT	Signal transducer and activators of transcription
T _{CM}	Central memory T cell
TCR	T cell receptor
T _{EM}	Effector memory T cell
Tg	Transgenic
TGF- β	Transforming growth factor β
T _H	Helper T cell
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNT	Tunnelling nanotubes
TRAIL	TNF-related apoptosis-inducing ligand
T _{RM}	Tissue-resident memory T cell
T _{VM}	Virtual memory T cell
ZAP-70	Zeta chain of TCR-associated protein

General Introduction

1.1. T lymphocytes

The immune response to infection involves two components: an innate response, which acts rapidly in an antigen non-specific manner, and a delayed, adaptive response, which recognizes specific antigens of the invading pathogen and additionally also confers long-term protection against secondary infection through the formation of memory (**Figure 1**).

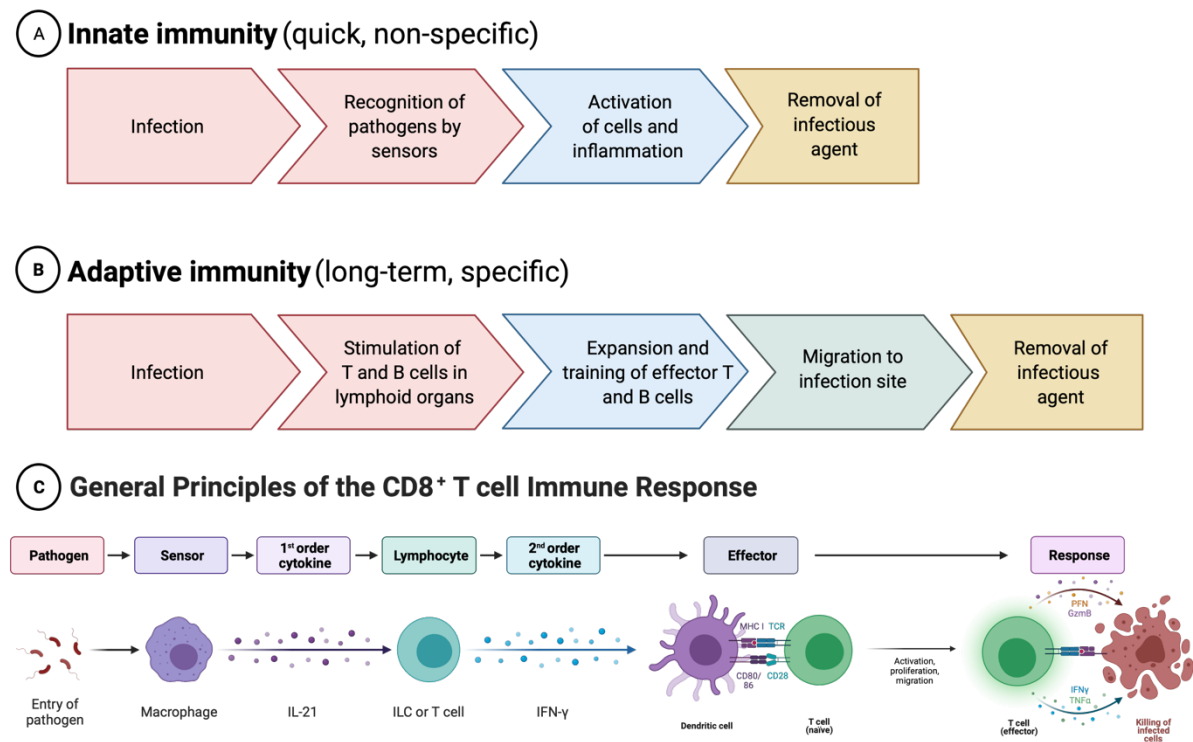


Figure 1. General principle of CD8⁺ T cell immune responses. The immune response to infection can be subdivided into two different components, which fulfil specific roles and are characterized by distinct features: An innate immune response (**A**), which acts quickly and non-specifically, and an adaptive immune response (**B**), which lags the innate immune response, but responds more specifically, whilst also conferring long-term protection against secondary infection through the generation of memory. (**C**) CD8⁺ T cell responses to infection involve multiple cell types and stages. Invading pathogens are sensed by specific immune cells, such as macrophages or dendritic cells, which triggers activation and recruitment of lymphocytes through expression of pro-inflammatory cytokines and chemokines. T cells are activated upon encountering their cognate antigen in secondary lymphoid organs (SLOs), which leads to the acquisition of effector functions and the subsequent killing of infected cells [Created with BioRender.com].

Antigen-specific lymphocytes, comprising T and B lymphocytes, which are both capable of responding to a vast array of immunogenic antigens, constitute the adaptive arm of the

immune system. Recognition of a wide range of antigens is made possible by the presence of highly variable antigen receptors on the surface of these cells, such as the T cell antigen receptor (TCR) expressed by T cells (see also [p.19](#)). Each mature T cell carries a unique TCR variant capable of recognizing and binding specific antigenic epitopes¹, which ensures that the resulting population of peripheral lymphocytes displays a broad repertoire of highly diverse receptors. The TCR enables T cells to inspect the proteome for anomalies by sampling peptide antigens presented by either major histocompatibility complex (MHC) class I or II molecules at the cell surface of antigen-presenting cells (APCs)^{2,3}.

T-Cell Development in Thymus

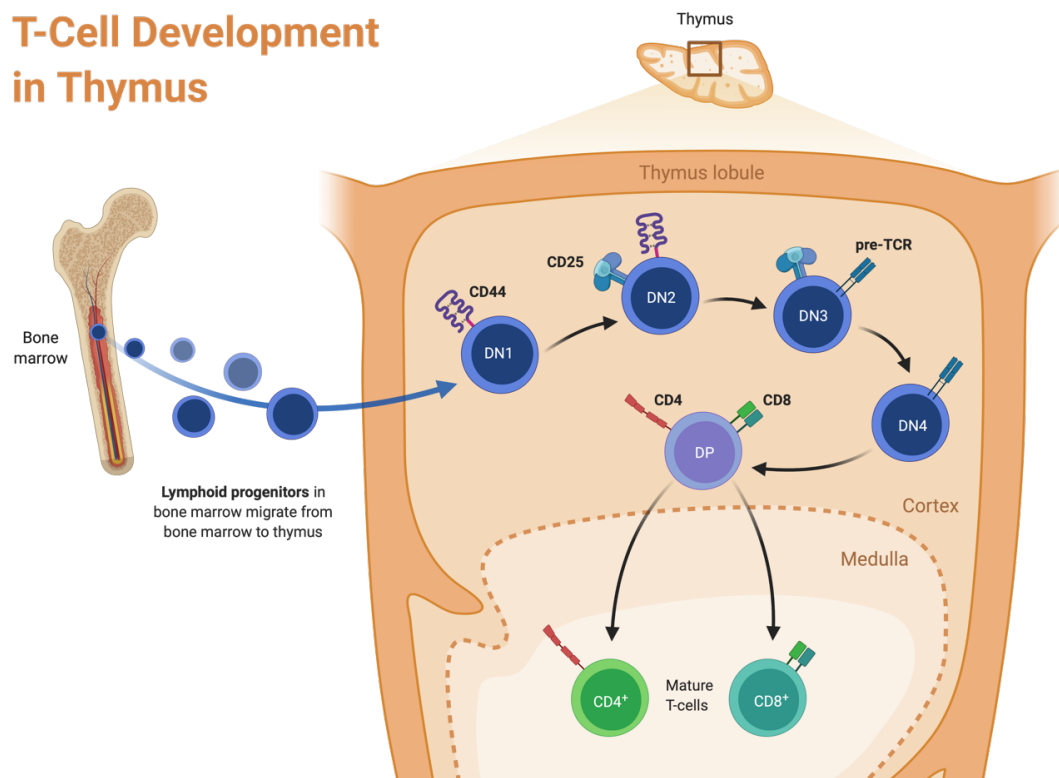


Figure 2. T cell development. T cells develop from common lymphoid progenitors or lymphoid-primed multipotent progenitors derived from the bone marrow (BM) or foetal liver and progress through different stages before becoming mature and fully functional T lymphocytes⁴. The four double-negative (DN1-4) stages are distinguished by their expression of the receptors CD44 and CD25. At the DN3 stage, a pre-TCR complex that consists of a pre-TCR α chain and a mature β chain first appears. The mature β chain in this receptor complex is a product of somatic DNA rearrangement by recombination activating gene 1/2 (RAG 1/2). T cells with a functional pre-TCR are positively selected and undergo massive proliferation while rearrangement of the *TCR α* gene is induced. At the double-positive (DP) stage, both the CD4 and CD8 coreceptors are expressed (CD4⁺ CD8⁺)^{4,5}, and the mature $\alpha\beta$ TCR is formed by replacing the pre-TCR α chain with the rearranged TCR α chain. Before being released into the periphery T cell encounter a few checkpoints: DP T cells expressing $\alpha\beta$ TCRs which recognize their MHC molecules, and are thus capable of productively generating a TCR signal, are positively selected, whilst self-reactive (also called autoreactive) T cells, bearing a TCR with very high-affinity for self, are either negatively selected and die or are agonist-selected to become specialised T cell subsets (i.e. regulatory T cells, CD8 $\alpha\alpha$ intraepithelial lymphocytes, or

invariant natural killer T cells)⁶. Additionally, DP T cells with dysfunctional TCRs that cannot receive or transduce TCR-mediated signals undergo apoptosis. The successfully selected cells further develop into CD4⁺ or CD8⁺ SP cells and exit the thymus to populate the periphery^{4,5} [Created with [BioRender.com](https://www.biorender.com)].

T lymphocytes come in different flavours and the peripheral pool of T cells can be subdivided on the basis of whether they express the surface receptors CD8 or CD4, which recognize class I and class II MHC molecules, respectively², and are either called cytotoxic (CD8⁺) or helper (CD4⁺) T cells. CD4⁺ T cells are referred to as “*helper*” T cells due to their capability to produce regulatory cytokines and chemokines, which either activate functions of other cells such as macrophages and neutrophils and/or mediate autocrine or paracrine control of T cell differentiation and/or regulate the differentiation of B cells^{2,7}. T cells develop from thymus-migrant hematopoietic lineage cells, particularly common lymphoid progenitors or lymphoid-primed multipotent progenitors derived from the bone marrow or the foetal liver^{8,9}. Developing T cells in the thymus progress through different stages (**Figure 2**), which ensures their full functionality, while in addition preventing self-reactive T cells to enter the periphery.

1.1.1. TCR structure and signalling

The T cell receptor is a multiprotein complex comprising subunits containing highly variable antigen-binding sites associated noncovalently with invariant signal transduction subunits, whose structural component have been revealed in the 1980s^{2,10}. The T cell receptor is composed of two transmembrane protein chains, the TCR α and β chains (**Figure 3A and C**). Each chain contains a variable and a constant region, creating together a single antigen-binding site^{2,11} (**Figure 3C**). Rearrangements of the DNA sequences encoding the antigen-binding region, a process known as VDJ recombination (**Figure 3B**), create a vast diversity in antigen-receptor structures², with three so-called complementary-determining regions

(CDRs) defining the antigen specificity of each receptor. TCR $\alpha\beta$ heterodimers are coupled to invariant CD3 subunits, which are essential for trafficking and stability of the $\alpha\beta$ subunits at the plasma membrane^{2,12}.

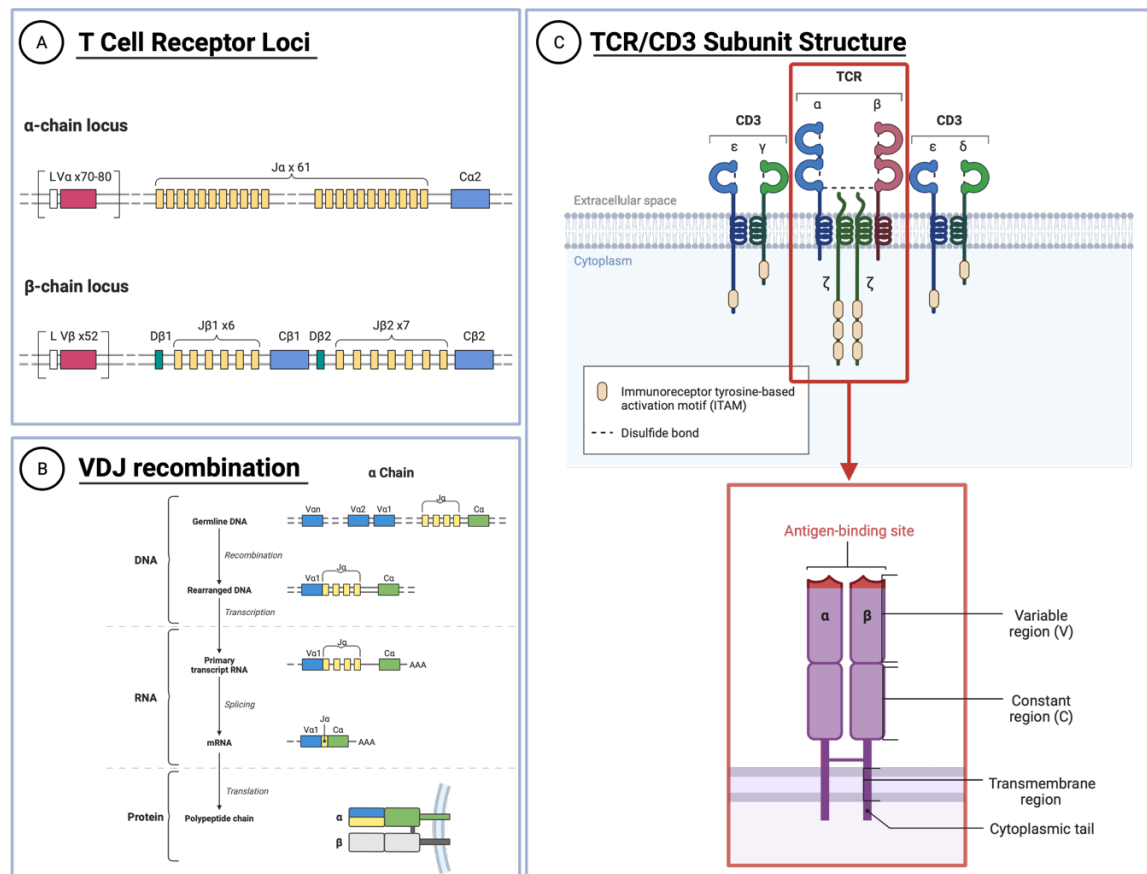


Figure 3. T cell receptor. (A) The TCR α chain locus is encoded on chromosome 14 and consists of variable (V) and joining (J) genes, while the TCR β chain is located on chromosome 7 and consists of V, J, and diversity (D) gene segments. (B) A vast repertoire of TCRs is generated by gene rearrangement between exons of the variable domains of the V-J segments of the α chain locus and V-D-J segments of the β chain locus, a process known as VDJ recombination (only the recombination sequence for the α chain locus is depicted as a representative example). Additional junctional diversity (V-N-J/V-N-D/D-N-J) is introduced by random inserts or deletions at these regions, with the total diversity being expressed in the complementary determining regions (CDRs) of the TCR, which make up the antigen-recognition site of the receptor^{13,14}. (C) The complete TCR complex consists of TCR $\alpha\beta$ chains bonded with CD3 subunits. [Created with [BioRender.com](https://www.biorender.com)].

While not being directly involved in antigen recognition, CD3 transmits the TCR-triggered signal through immunoreceptor tyrosine-based activation motifs (ITAMs) in its cytoplasmic tail¹⁵, which couple the TCR to intracellular tyrosine kinases^{2,16}. ITAM phosphorylation is induced upon TCR engagement by protein tyrosine kinases (PTKs), including Src family PTKs such as Lck and Fyn and the Syk family PTK zeta chain of TCR-associated protein (ZAP-70), which enables downstream effector molecules to interact with the TCR complex (Figure 4)^{5,17}.

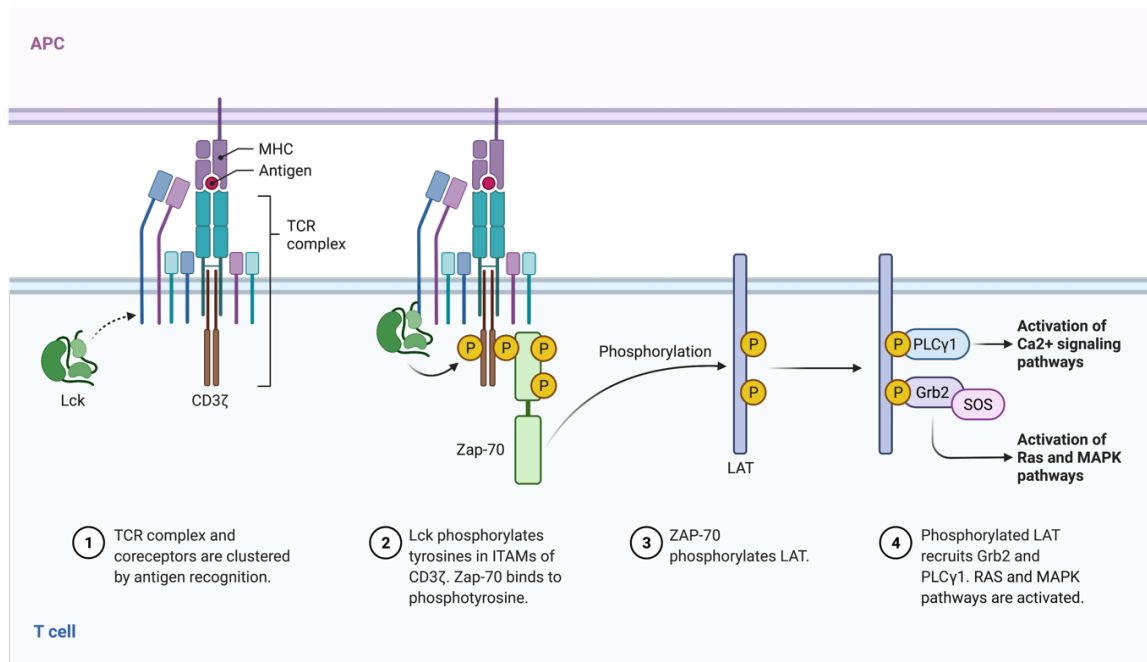


Figure 4. TCR signalling. Upon T cell receptor (TCR) binding to peptide-MHC (pMHC), the kinase Lck is recruited to the TCR complex by the colocalization of CD4 or CD8 coreceptors to pMHC molecules, where Lck can then phosphorylate ITAM signalling motifs. ITAM motifs each contain two tyrosine residues that, when phosphorylated, create binding sites for the tandem Src Homology 2 (SH2) domains of the Zap70 kinase. By binding to phosphorylated ITAMs, Zap70 is recruited to the plasma membrane, which disrupts its steady state (autoinhibited) conformation and facilitates further phosphorylation of its active conformation by Lck. Once recruited and activated, Zap70 is then able to propagate signalling events from the TCR through phosphorylation of the linker for activation of T cells (LAT), which serves as a signalling hub for further downstream signalling effectors¹⁸ [Created with [BioRender.com](https://www.biorender.com)].

1.1.2. T cell activation

Naïve T cells are initially activated through their TCR by binding to cognate peptide-MHC (pMHC) complexes presented by APCs. Early experiments revealed that productive TCR signalling (signal 1), followed by co-stimulatory signals (signal 2), are required for complete T cell activation^{19,20}. Successful T cell activation results in the activation of a network of signalling cascades – involving the Ras-extracellular signal-regulated kinase (ERK)-activator protein (AP)-1 pathway, the inositol triphosphate (IP₃)-Ca²⁺-nuclear factor of activated T cells (NFAT) pathway, the protein kinase C (PKC)θ-IκB kinase (IKK)-nuclear factor (NF)-κB pathway, and the tuberous sclerosis complex (TSC)1/2-mammalian target of rapamycin (mTOR) pathway – which collectively mediate T cell differentiation, proliferation, and survival (recently reviewed here^{2,5,21}). Notably, T cells have been discovered to bind in general with

comparatively low-affinity to cognate antigen, and the precise molecular mechanisms ensuring sensitivity and specificity of TCR activation, and thus successful discrimination between self and foreign antigens, are a matter of ongoing research and debate (recently reviewed here^{22,23}).

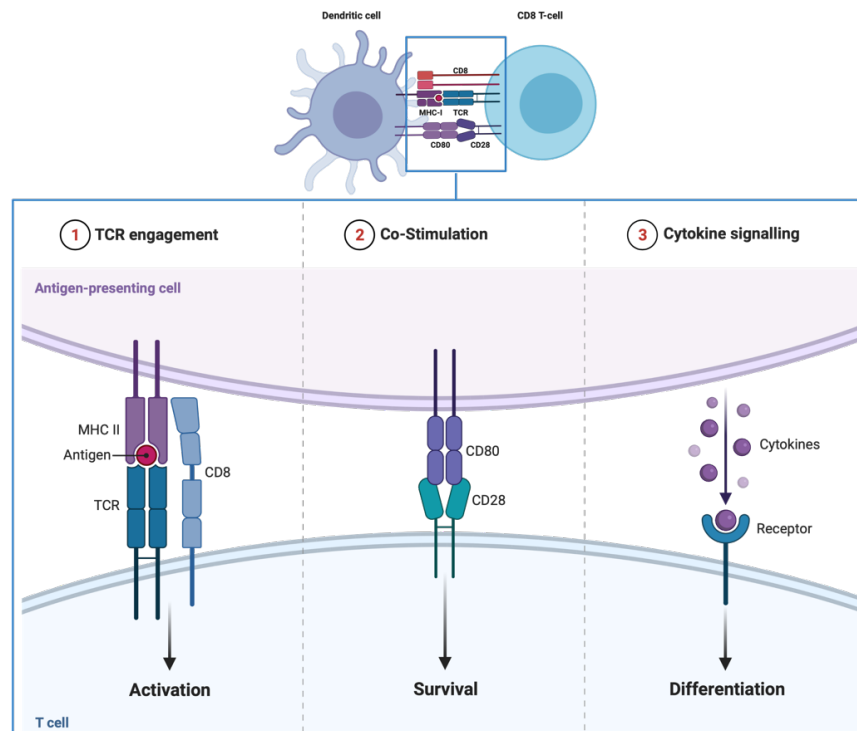


Figure 5. T cell activation. Three signals are required to initiate a productive T cell response: **(1)** Binding of TCR to its cognate antigen embedded in MHC molecules on the surface of APCs (signal 1), **(2)** co-stimulation (signal 2) and **(3)** cytokine signalling (signal 3) [Created with [BioRender.com](https://www.biorender.com/)].

TCR and co-stimulatory signals are sufficient to initiate activation and proliferation of naïve T cells. However, in the absence of a specific cytokine signal cells fail to develop optimal effector functions, survive poorly, and do not form a responsive memory population²⁴. Because this third cytokine signal is therefore required for a productive response^{25–30}, along with TCR (signal 1) and costimulatory signals (signal 2), it has been termed “*signal 3*” (**Figure 5**), which provides a means for CD8⁺ T cells that encounter antigen to determine if there is danger present and to initiate appropriate responses^{24,28}.

1.2. Experimental tools to study CD8⁺ T cell responses to infection

1.2.1. TCR transgenics as model systems to study CD8⁺ T cell biology

Much of our knowledge about T cell biology is based on the study of murine TCR transgenic (Tg) T cells, which are genetically modified to express a single TCR of defined specificity. The term “*transgenic*” refers to a genetically modified genome, and the basic principle behind generating TCR Tg mice is based on mouse germline transformation methods, which involves generation of a T cell clone expressing specific TCR α and β chains of interest and the subsequent cloning of the rearranged TCR chains into a genomic construct, which is then microinjected into mice with the genetic background of interest³¹. An alternative technology of generating TCR Tg mice was introduced in 2006 by Holst and co-workers³², which was termed “*retrogenic*” (“*retro*” from retrovirus and “*genic*” from transgenic) and uses retrovirus-mediated stem cell gene transfer. Additionally, CRISPR-mediated TCR replacement techniques have recently become popular tools to generate TCR Tg mice more efficiently³¹.

T cells expressing already rearranged TCR chains should in theory not express non-Tg V α and V β TCRs⁴. However, it was discovered that T cells may express dual TCRs recognizing unique antigens and are thus capable of responding to distinct stimuli³³. These additional and distinct antigen receptors generally result from a single β chain that dimerizes with different α chains, and it is estimated that up to 15% of murine $\alpha\beta$ T cells express two functional α chains at the cell surface^{34–36}. While bearing two different TCRs significantly expands the repertoire for foreign antigen^{37,38}, it has also been implicated to lead to autoimmunity^{39–41}. Thus, recombination may still occur in a TCR Tg context, giving rise to a T cell co-expressing Tg and endogenous TCR receptors³¹.

One major strength of the TCR transgenic adoptive transfer approach is the ability to limit clonal variability by focusing specifically on a population of monoclonal T cells carrying a TCR of defined specificity. In addition, the use of congenic markers enables straightforward identification of adoptively transferred cells in host animals. Based on the reported experimental design used in the initial adoptive transfer study⁴², many follow-up studies transferred 10^6 or more TCR transgenic T cells into naïve mice, which was considered a strength of this experimental system as it facilitated identification of transferred cells at all times. However, as pointed out by Harty and colleagues⁴³, this experimental design creates a situation in which antigen-specific T cells are present at 1000- to 100.000-fold higher numbers than the corresponding endogenous naïve precursor T cell frequency, thus inhibiting the endogenous T cell response to the same epitope^{43,44}, which was revealed to not just alter several features of the T cell response⁴⁵⁻⁴⁸, but to influence in essence all facets of the CD8⁺ T cell response, from proliferation to phenotype and function⁴⁴. However, CD8⁺ T cells responding to infection or immunization are known to proliferate robustly, which allows for the transfer of very low numbers of TCR-transgenic T cells⁴⁴, thus creating an experimental context that approximates normal physiology that furthermore enables direct comparison with the endogenous T cell response of the host. This experimental approach was utilised in subsequent years to great effect to interrogate T cell behaviour at the single-cell level^{49,50}. In summary, while the potential caveats must be kept in mind, the combined advantages of the TCR-transgenic adoptive transfer system ensure its continued relevance as an invaluable experimental tool to study T cell behaviour.

1.2.2. Ovalbumin as a model antigen to study CD8⁺ T cell biology

Ovalbumin (OVA) is the major protein component of avian egg-white, a 45 kDa glycoprotein, which is part of the SERPIN (SERine Protease Inhibitor) protein family. Being one of the first proteins purified in its crystalline form⁵¹ and readily available, OVA has been used extensively in early immunological studies, which helped lay the groundwork for our current understanding of antibody-protein interactions and antibody specificity^{52–56}.

The generation of TCR transgenic mice with a CD8⁺ T cell repertoire specific for the OVA peptide of amino acids 257-264⁵⁷ (SIINFEKL, also known as N4 peptide) in the context of H-2K^b, has facilitated the use of OVA as a model antigen to study various aspects of CD8⁺ T cell responses. N4-specific CD8⁺ T cells, commonly referred to as OT-I T cells, exhibit a TCR that consists of α -chain variable region 2 (V α 2) and β -chain variable region 5 (V β 5), which are inherited via a single transgene. OT-I T cells have been widely used for studies of thymic selection⁵⁸, autoimmunity and tolerance⁵⁹, and vaccine- and infection-induced T cell responses^{43,60,61}. Of note, it was demonstrated that for the OT-I mouse model, transfer of fewer than 500 T cells was required to permit a concurrent robust endogenous CD8⁺ T cell response and to allow TCR-transgenic T cells to mimic endogenous CD8⁺ T cell behaviour to *L. monocytogenes* infection⁴⁴.

The OVA system has additionally been used to generate a series of so-called altered peptide ligands (APLs), which are peptide variants derived from the original antigenic peptide with substitutions of particular amino acid residues. The generated APLs can be grouped into different categories depending on their binding affinity to the TCR⁶². OT-I TCR transgenic T cells exhibit a wide range in affinities to modified versions of the original peptide N4. All of these different peptides bind equally well to H2-K^b but differ in their potency to simulate OT-

I cells, which has been utilised to great effect in a number of studies probing how the strength of the TCR stimulus shapes the subsequent fate and response of T cell clones (see also [p.52](#))⁶³. In addition, the availability of APLs makes it possible to mimic and investigate how clones of various TCR specificity behave during the responses. This system has furthermore facilitated studies of T cell responses to re-infection with pathogens carrying point mutations in immunodominant epitopes⁶⁴.

1.2.3. *Listeria monocytogenes* as a model infection system to study CD8⁺ T cell biology

Listeria monocytogenes (LM), first described in 1926⁶⁵, is a gram-positive food-borne bacterium that thrives in several distinct environments⁶⁶. Ingesting food products contaminated by this pathogen can lead to the disease listeriosis, which manifests as gastroenteritis, meningitis, encephalitis, mother-to-foetus infections, and septicaemia and mainly affects immunocompromised individuals, pregnant women, and newborns. Following ingestion, *L. monocytogenes* invades the intestinal epithelial, which allows crossing of the intestinal barrier and gaining access to internal organs. Infection by this pathogen has been a useful model for investigating cellular interactions that are critical for the initiation of the host T-cell response⁶⁶, and furthermore established itself as a model organism to study every phase of the CD8⁺ T cell response to intracellular bacterial infection⁶⁷.

Listeria Cell Cycle

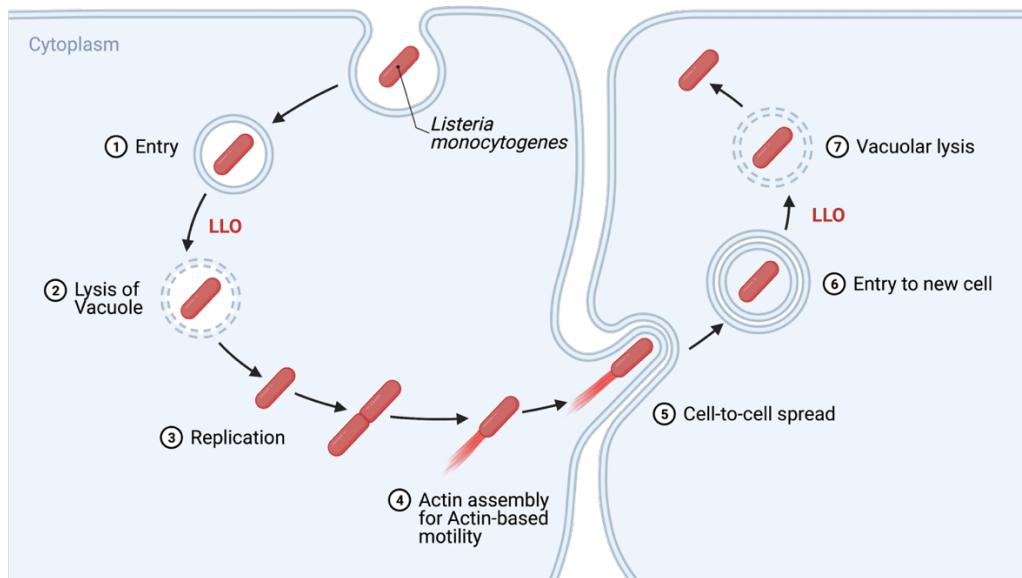


Figure 6. The life cycle of *Listeria monocytogenes* (LM). *Listeria* can trigger uptake into cells, which normally lack phagocytic capacities, and thus infect several different cell types. It escapes the phagosome through secretion of listeriolysin O (LLO), which breaks open the phagosomal membrane. Actin assembly enables Actin-based motility within the cytosol, which also mediates cell-to-cell spread.

L. monocytogenes can infect different cell types, due to its capacity to trigger uptake into cells which normally lack phagocytic capacities. Once inside the host cell, it escapes the phagosome by secreting a pore forming toxin known as listeriolysin O (LLO). Thus, gaining access to the host cytosol, bacterial replication ensues. Mobility within the cytosol is achieved by polar expression of the bacterial nucleating actin protein known as actin-assembly-inducing protein (ActA), which provides *L. monocytogenes* with a scaffold of polymerized F-actin to propel itself through the cytoplasm. Once it reached the plasma membrane, long protrusions containing bacteria are formed, which can be invaginated by neighbouring cells and thus enable infection of new host cells⁶⁷. This mode of direct cell-to-cell spread allows *L. monocytogenes* to largely avoid the extracellular milieu and escape neutralising antibodies.

The innate response to LM involves multiple cell types, cytokines, and bactericidal effector mechanisms⁶⁸, while the adaptive immune response to LM is mostly T cell-mediated, which is required for complete pathogen clearance⁶⁹. Circulating monocytes and resident

macrophages, such as Kupffer cells, play an important role in innate immunity by ingesting and destroying LM⁶⁸. Additionally, secretion of cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), Tumour Necrosis Factor (TNF) and interferon-gamma (IFN- γ) by multiple immune cells play an important role in the innate response⁷⁰. IL-1, produced by LM-infected macrophages, facilitates recruitment and activation of both neutrophils and macrophages⁷¹. Another cytokine critical for efficient neutrophil recruitment and rapidly secreted after LM infection is IL-6^{72,73}. Increased LM susceptibility has also been reported in lymphotoxin β receptor-deficient mice, which caused impaired nitric oxide production by macrophages and thus diminished their bactericidal effector mechanisms⁷⁴.

Four crucial cytokines in the immune response to LM infection are TNF⁷⁵, interleukin-12 (IL-12), interleukin-18 (IL-18) and IFN- γ . TNF on its own is sufficient to activate macrophages⁷⁶, and furthermore induces the production of IFN- γ ⁷⁷. IL-12 and IL-18 are produced mainly by activated macrophages and are both critical mediators in the coordination of the immune response against LM. Both additionally act as inducers of IFN- γ secretion from responding immune cells^{76–79}. The essential role of IFN- γ in the response to LM infection has been demonstrated in multiple studies^{76,80–82}, and mice lacking the IFN- γ receptor have been reported to exhibit increased susceptibility to LM infection due to impaired macrophage activity⁸³. Different mechanisms (reviewed here⁶⁶) enable LM to manipulate infected host cells and evade detection. However, its presence within the cytosol is usually still recognized by the immune system, which leads to the induction of a potent CD8⁺ T cell response.

Intravenous inoculation of *L. monocytogenes* into laboratory mice has been utilised as a model system to study intracellular bacterial infections since the 1960s, and has helped to demonstrate the central role of cellular immunity in controlling intracellular infections^{84,85}. These studies led to murine listeriolysis being established as perhaps the most utilised animal

model to study the immune response against intracellular bacterial pathogens⁶⁷. Murine listeriolysis represents a unique tool to investigate all aspects and all phases of CD8⁺ T cell responses to infection with intracellular pathogens. Moreover, an epitope hierarchy has also been established, with the peptide LLO₉₁₋₉₉ discovered to serve as the immunodominant epitope, while the peptide p60₂₁₇₋₂₂₅ is subdominant⁶⁷.

LM has been successfully engineered to express model antigens, such as the well-defined ovalbumin epitope (LM-OVA) that permits studies using the OVA-specific TCR transgenic T cell (OT-I)⁵⁷. Several additional recombinant LM strains suitable for other transgenic models have also been generated, such as recombinant *L. monocytogenes* strains expressing *Lymphocytic choriomeningitis virus* (LCMV) glycoprotein₃₃₋₄₁ (gp₃₃₋₄₁)⁸⁶, to which P14 TCR transgenic T cells are specific⁸⁷. Lastly, the generation of *L. monocytogenes* with attenuated pathogenicity has facilitated studies in immunocompromised mice⁸² and duration of infection can furthermore be modulated through the use of antibiotic treatment, providing another important advantage this infection model offers⁶⁷.

1.2.4. Detection of antigen specific CD8⁺ T cells by Tetramer staining

Analysis of antigen-specific T-cell populations by flow cytometry is made possible by peptide-MHC multimer staining, which was developed in 1996⁸⁸. pMHC multimers are commonly linked to fluorochromes, which facilitates detection of antigen-specific T cells by conventional flow cytometry. Alternatively, linkage to rare metal ions allows detection by mass spectrometry (MS)⁸⁹. While MS-based detection offers several advantages, most notably allowing in theory to separate cells in over 100 dimensions, it comes with the major disadvantage of cell incineration, which makes subsequent cell collection impossible. It

furthermore also does not yet allow cell sorting on the light-based properties of forward and side scatter¹¹.

As mentioned previously, the binding affinity between TCR and pMHC is comparatively weak, with interactions typically only lasting a few seconds. Multimerization of soluble pMHC can considerably extend the half-life of these interactions due to an added avidity effect, which produces reagents that stably adhere to the T cell surface bearing a cognate TCR¹¹. Combination of pMHC multimer staining with a cocktail of antibodies directed against other cell surface proteins furthermore enables the co-staining of antigen-specific T cells and segregation into various phenotypic populations by flow cytometry.

It should also be emphasised that pMHC multimer staining has several limitations. For example, the TCR-pMHC affinity required for pMHC tetramer binding exceeds that required for T cell activation⁹⁰, which means that conventional pMHC tetramer staining can fail to detect functional T cells with low-affinity TCRs, including self-specific T cell populations⁹¹. Some of these issues can be addressed by increasing the number of pMHC monomers and using higher-order multimers such as pentamers or dextramers⁹². In addition, several tricks, from staining with anti-coreceptor antibody after staining with pMHC multimer, to signal boosting with an anti-multimer antibody, can be employed to improve staining efficiency (described in more detail here¹¹).

1.3. CD8⁺ T cell responses to infection

DC recognition of pathogen-derived products through Toll-like receptors (TLRs) and other innate pattern recognition receptors (PRRs) creates immunogenic DCs that bear increased amounts of co-stimulatory molecules and antigen-MHC complexes^{93,94}. Activation of innate immune cells through PRRs also triggers the production of several pro-inflammatory cytokines, such as type I interferons (IFN α/β), IL-12 and IFN- γ ⁹⁵. Antigen encounter on immunogenic APCs induces CD8⁺ T cell activation, proliferation and differentiation into fully functional effector cells⁹⁶. The third signal provided by inflammatory cytokines helps to shape the ensuing effector and memory cell formation²⁸.

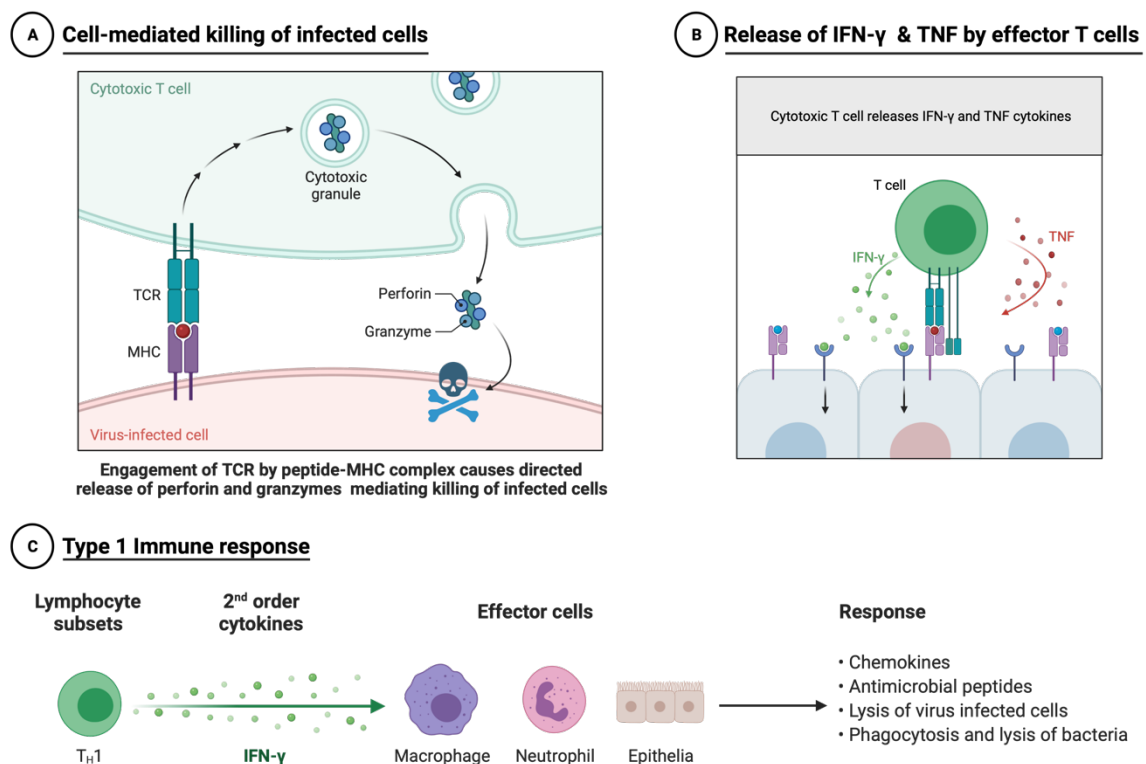


Figure 7. Type 1 Immunity. The immune system has tailored its effector functions to optimally respond to distinct species of microbes. Type I cell-mediated effector immunity provides an effective response against intracellular microbes, such as bacteria, protozoa, and some viruses, and it comprises T helper 1 cells (T_H1), as well as cytotoxic lymphocytes such as CD8⁺ T cells and natural killer (NK) cells. (A) Activation of CD8⁺ T cells results in cell-mediated killing of infected cells (B) and secretion of IFN- γ and TNF upon binding to cognate pMHC⁹⁷ (C) IFN- γ secretion by T_H1 cells in response to detection of intracellular pathogens leads to activation and conversion of macrophages and neutrophils into potent effector cells. [Created with BioRender.com].

Infections with viruses or intracellular bacteria induce so-called type 1 immune responses (Figure 7). These responses promote the differentiation of CD8⁺ T cells into cytotoxic T lymphocytes (CTLs), which kill antigen-bearing infected cells through effector molecules, such as granzyme and perforin and secrete cytokines such as IFN- γ and TNF⁹⁸. In parallel, CD4⁺ T cells are driven towards differentiation into T_H1 cells, which also produce IFN- γ , TNF and interleukin-2 (IL-2). T_H1 cells are critically involved in the coordination of the antiviral immune response by sustaining CD8⁺ T cell responses leading to the clearance of virus-infected cells⁹⁹. In parallel, T follicular helper T (T_{FH}) cells form, which primarily migrate into germinal centres following immunization to regulate the development of antigen-specific B cell immune responses. Of note, although these pools of effector CD4⁺ and CD8⁺ T cells formed during type I responses are dominated by particular effector traits (such as IFN- γ production and cytotoxicity), these cells do not constitute uniform populations and technological advances have revealed a plethora of specialised subsets within these distinct cell populations^{100–102}.

1.3.1. Primary CD8⁺ T cell responses

T cells need to balance effective protection against pathogenic microorganisms with the risk of potentially overreacting against self and thus causing immunopathology. Hence, to avoid immunologically driven tissue damage, T cells need to scale their clonal expansion and effector response according to the dose and strength of the antigenic trigger, which is accomplished by the temporal integration of TCR signalling with several additional regulatory signals^{103,104}. An initiated T cell response to an acute infection can be subdivided into three distinct phases: clonal expansion, contraction of the T cell population and the maintenance of a memory pool (Figure 8).

Stages of CD8⁺ T cell Immune Responses

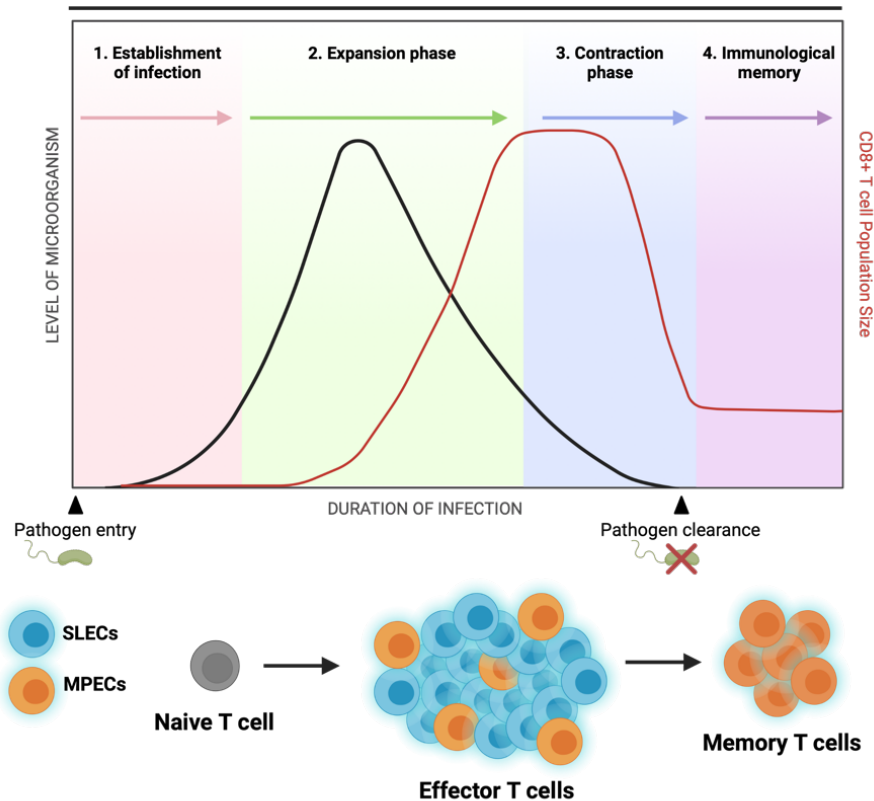


Figure 8. Kinetics of CD8⁺ T cell immune responses. During an acute viral infection, antigen-specific T cells rapidly proliferate during the expansion phase and differentiate into cytotoxic T lymphocytes (CTLs) that mediate viral clearance. Most of these cells die over the next several weeks during the so-called contraction phase of the response, and only a small percentage of effector T cells (5-10%) survive and further develop into mature long-lived memory CD8⁺ T cells. The pool of effector cells can be separated into multiple distinct subsets based on differences in gene and protein expression, effector functions, migratory patterns, proliferative capacity, and long-term fate. Not all effector T cells have equal potential to form memory T cells and cell-surface markers can be used to distinguish between short-lived terminal effector T cells (SLECs; shown in blue) and long-lived memory and memory precursor cells (MPECs; shown in orange) [Created with [BioRender.com](https://www.biorender.com/); adapted from Kaech and Cui 2012⁹⁹].

Primary T cell immune responses originate from a pool of antigenically naïve T cells harbouring a highly diverse repertoire of distinct TCRs. Most immune responses to defined peptide epitopes emerge from considerably more than just a single T cell, with multiple studies over the years showing a certain level of redundancy and cross-reactivity¹⁰⁵. It has also been discovered that during systemic infection nearly all the naïve T cells specific to a defined epitope of a bacterial or viral pathogen are effectively recruited into the response¹⁰⁶. In summary, T cell immune responses to a given pathogen, which can display a remarkable

degree of phenotypical and functional diversity, likely emerge from multiple naive T cells specific or cross-reactive to the same pathogenic epitope⁵⁰.

However, both in mice and humans the frequency of T cells carrying a TCR specific for a given epitope is nevertheless considered to be generally quite low, ranging between 1 to 10 per million CD4⁺ T cells and 1 to 100 per million CD8⁺ T cells^{50,105,107}. Hence, to provide effective immune protection during an acute infection with rapidly proliferating pathogens, an antigen-specific T cell population is required to undergo massive numerical expansion. The selectivity of antigen receptors ensures that only those lymphocytes expressing TCRs specific for a given antigen are selected to participate in the ensuing response. These antigen-specific T cells can expand >10.000-fold following activation in SLOs^{108–110}. Frank Macfarlane Burnet was the first to introduce this important concept of adaptive immunity in 1957 as the clonal selection theory¹¹¹. In most models of infection or vaccination, clonal expansion of antigen-specific T cells, which is accompanied by the acquisition of potent effector functions, usually reaches its peak within 5-8 days after activation^{67,99}.

Following the effector phase of the response, during which invading pathogens are typically cleared, the majority of activated effector CD8⁺ T cells die⁴³ and only a small percentage (approximately 5-10%) survive this so-called contraction phase to stably persist in the absence of antigen and further mature into long-lived memory CD8⁺ T cells. The resulting 100- to 1000-fold increase, relative to the preimmune state, of epitope-specific T cells in the form of memory cells facilitates more rapid and potent recall responses in the event of antigen reencounter^{43,50,99,112}.

1.3.2. Regulatory mechanisms controlling CD8⁺ T cell expansion

Classically, CD8⁺ T cell expansion was believed to correlate with antigen persistence and to be maintained until T cells somehow sense the clearance of the invading pathogen. However, experiments modulating the duration of infection *in vivo* have demonstrated that CD8⁺ T cells are swiftly programmed to undergo many rounds of division following an initial period of stimulation^{109,113}. In addition, *in vitro* experiments have revealed that 2 hours of antigen stimulation are sufficient to program CD8⁺ T cells to undergo several rounds of division and to acquire effector functions^{86,114,115}. Altogether, it was concluded that a short period of antigen stimulation is sufficient to activate a program of T cell expansion which continues for multiple rounds of cell division independently of further antigenic stimulation⁶⁷. The extent of CD8⁺ T cell clonal expansion was revealed to be proportional to antigen abundance and/or TCR binding affinity^{63,86,116}.

However, additional signals still exert a critical influence on the expansion of responding antigen-specific T cells^{28,43,117–119}, and multiple experimental interventions have been discovered to dampen clonal expansion, such as curtailing the overall exposure to antigens and inflammation by shortening the duration of the infection, recruiting naive T cells at the tail-end of the infection, or increasing the precursor frequency of antigen-specific naive CD8⁺ T cells, which increases intra-clonal competition for cognate antigen and cytokines^{47,120–127}. Notably, interventions shortening clonal expansion^{47,120–126} have furthermore been shown to hasten the differentiation of memory precursor cells (see also [p.42](#)).

These findings revealed the local cytokine milieu to be a critical determinant of the magnitude of CD8⁺ T cell expansion and pro-inflammatory cytokine to be required to achieve optimal accumulation²⁸. Further evidence for the importance of signal 3 cytokines in

modulating the expansion of antigen-specific CD8⁺ T cells following infection was provided by studies demonstrating that T cells display an impaired ability to proliferate if they lack the capacity to respond to IL-12 in the context of LM infection¹²⁸, or to type I interferon in the case of LCMV infection^{25,26}, although it was later discovered that other pathogen-dependent cytokines can also serve as signal 3¹²⁹. *In vitro* studies suggested that both type I IFNs and IL-12 can enhance T cell survival^{27,130,131}. However, the precise mode of action of these cytokines in controlling T cell expansion *in vivo* is thought to be highly context-dependent and remains to be further elucidated for different types of infection.

Following activation, CD8⁺ T cells acquire the capacity to exert effector functions through the expression of molecules such as granzyme B and perforin and cytokines such as IFN- γ and TNF^{98,108}, which play a critical role in the clearance of invading pathogens^{75,132–135}. In addition, some of these effector molecules have also been reported to modulate CD8⁺ T cell homeostasis over the course of infection. For instance, perforin can shape T cell expansion independently of its role in bacterial clearance¹³³ and may thus serve an important regulatory role in preventing overexpansion of antigen-specific CD8⁺ T cells⁶⁷.

1.3.3. Regulatory mechanisms controlling CD8⁺ T cell contraction

The capability of T cells to sense the disappearance of antigen was originally thought to not only modulate expansion, but to furthermore trigger contraction. However, studies truncating infection have clearly shown the *in vivo* clearance of infection and the onset of contraction to be not directly linked¹⁰⁹. Additionally, the onset and initial kinetics of antigen-specific CD8⁺ T cell contraction were shown to be similar after acute virus infection to that following chronic or persistent infection^{109,136,137}, which indicated that contraction is

programmed, independently of pathogen or antigen clearance, into the responding CD8⁺ T cell population by signals received early after onset of infection¹⁰⁹.

Subsequent studies revealed that inflammation, and thus cytokine signalling, early during the response plays a critical role in programming contraction of T cells¹²⁷. Additionally supporting the idea that contraction is programmed early during antigenic stimulation was the discovery that contraction is typically programmed to initiate approximately 5 days post peak antigen presentation¹³⁸. The precise mechanisms through which inflammatory cytokines regulate contraction remain to be fully understood, but induction of pro-apoptotic molecules such as the B-cell lymphoma 2 (Bcl-2) family member Bim is thought to play a critical role^{139,140}. However, additional pathways likely contribute to the death of previously activated CD8⁺ T cells^{141–143}, possibly by modulating the balance and activation state of pro- and anti-apoptotic Bcl-2 family members⁴³ or by inducing extrinsic pathways of apoptosis¹⁴⁴. Lastly, the expression of cytotoxic products in CTLs, such as granzyme B, has also been implicated in the death of effector T cells^{145,146}. In summary, contraction appears to be programmed early after antigen encounter and to be largely controlled by the induction of systemic inflammation⁶⁷. However, there are still many unknowns in our understanding of the mechanisms which govern life and death of activated CD8⁺ T cells.

1.3.4. CD8⁺ T cell memory

Immunological memory is a hallmark of the adaptive immune system. It was originally proposed that memory CD8⁺ T cells are randomly selected¹⁴⁷. However, effector cells were later discovered to be not equipotent, meaning memory cell potential is not inherited equivalently by all effector cells. Rather, some CD8⁺ T cells are intrinsically better suited than others to persist and differentiate into memory CD8⁺ T cells⁹⁹.

The traditional linear differentiation model of memory T cell development (see also [p.46](#)) proposes that the ontological decision to diversify into short- or long-lived T cells happens at the onset of contraction, after population expansion has reached its maximum^{147,148}. This idea has been challenged by various recent discoveries. A small subset of effector T cells with increased expression of interleukin-7 (IL-7) receptor subunit- α (IL-7R α , also known as CD127), and decreased expression of killer cell lectin-like receptor G1 (KLRG1), has been identified as exhibiting a heightened propensity to form long-lived functional memory cells and are thus also called memory precursor cells (MPECs)^{120,121,149–151}. Within an activated effector T cell population, MPECs can be discerned from terminally differentiated, short-lived effector cells (SLECs) well before onset of contraction¹²⁰, as early as day four after infection with various pathogens^{120,149,152,153}. However, it is important to highlight that these phenotypic features are not exclusive criteria for forming memory T cells. While IL-7 mediated survival signals may likely be involved in allowing cells to survive contraction and differentiate into long-lived memory cells, cell death has also been observed in the IL-7R α^{hi} effector T cell compartment following infection^{120,154,155}. In addition, constitutive expression of IL-7R α by CD8⁺ T cells was shown to be insufficient in preventing contraction following *L. monocytogenes*¹⁵⁶ or LCMV infection¹⁵⁷, and increased IL-7 signalling was insufficient to increase memory in the context of LCMV infection¹⁵⁸. Finally, conditions resulting in low systemic inflammation, such as DC immunization or peptide vaccination, were revealed to give rise to a much greater proportion of CD127^{hi} T cells, while contraction was minimally affected^{122,159–161}, altogether suggesting that high expression of IL-7R α on its own is not sufficient to enforce survival during the contraction phase. Furthermore, and in contrast to conventional thought, KLRG1^{hi} cells have recently also been discovered to be capable of losing KLRG1 expression again and convert into memory¹⁶².

Nevertheless, while KLRG1 and CD127 are not definitive markers, they offer a means of determining the relative memory cell potential and lifespan of effector CD8⁺ T cells during primary infection and are thus still commonly used to subdivide the responding antigen-specific T cell population. CD62L and CCR7 are additional markers often used to characterize the changing phenotype of CD8⁺ T cells progressing from naïve (CD62L^{hi}CCR7^{hi}), to effector (CD62L^{lo}CCR7^{lo}), and memory (CD62L^{low/hi}CCR7^{low/hi})⁹⁵.

Once an acute infection is cleared, memory CD8⁺ T cells are maintained in an antigen-independent, cytokine-dependent manner mainly through the actions of two cytokines, IL-7 and Interleukin-15 (IL-15). Both cytokines promote memory CD8⁺ T cell survival and self-renewal through homeostatic (basal) proliferation^{151,163–166}. By contrast, persistent infections often cause substantial alterations in the phenotype and functionality of memory CD8⁺ T cells^{137,167–169}, which may either result in the deletion of CD8⁺ T cells of certain specificities or the exhaustion of their effector functions¹⁶⁹, which describes a dysfunction in their ability to mobilise effector functions, such as IFN- γ and TNF, IL-2 and cytotoxicity¹⁷⁰.

1.3.5. Diversity within the memory CD8⁺ T cell compartment

Memory CD8⁺ T cells are capable of rapidly proliferating and generating effector functions upon encounter with their cognate antigen, thus producing a burst of CTLs helping to contain secondary infection ([Figure 9](#)).

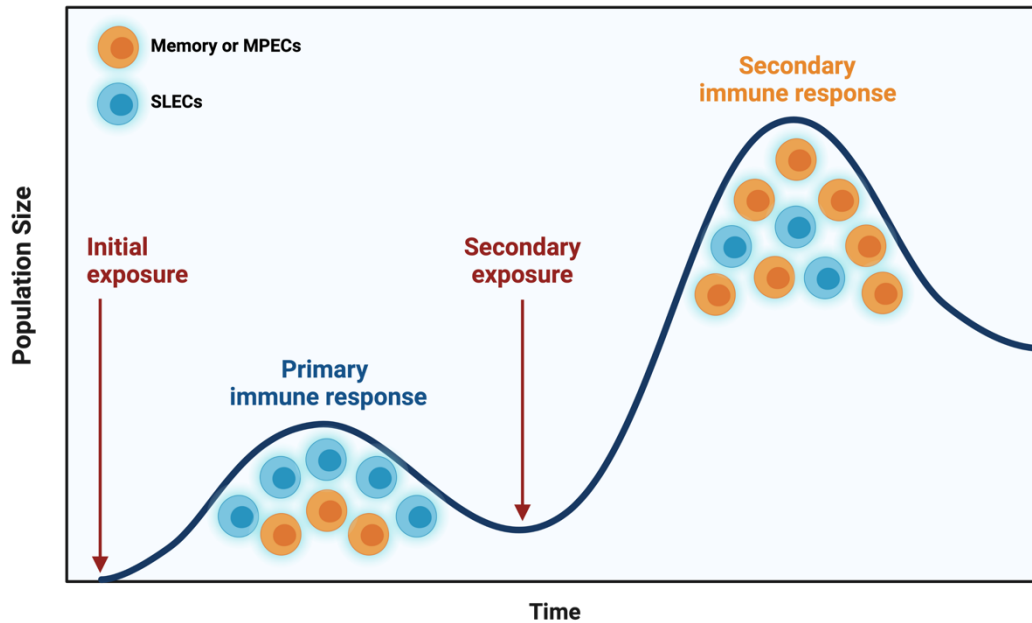


Figure 9. CD8⁺ T cell memory. Primary infection results in the activation and expansion of antigen-specific CD8⁺ T cells. While most responding cells are sent into apoptosis once infection is cleared, a small subset survives to become long-lived memory cells, which upon re-challenge are primed to respond more rapidly and potently [Created with BioRender.com].

Notably, substantial phenotypic and functional heterogeneity was revealed to exist even within a population of memory CD8⁺ T cells specific for a single epitope (**Figure 10**)¹⁷¹. Federica Sallusto and Antonio Lanzavecchia¹⁷² were the first to demonstrate that memory T cells can be subdivided by distinct expression patterns of adhesion molecules and chemokine receptors, that translate into migratory differences, which led to the characterization of central memory T cells (T_{CM}), which similar to naïve T cells continuously recirculate via the bloodstream to lymphoid organs, and effector memory T cells (T_{EM}), which preferentially migrate to nonlymphoid tissues¹⁷³. The more recent identification of tissue-resident memory T cells (T_{RM})^{174,175}, which may be further subdivided depending on the organ they reside in¹⁷⁶, adds to the complexity and diversity of the memory T cell compartment⁵⁰.

T_{CM} and T_{EM} cells are two memory populations residing in blood and spleen, which are traditionally defined by differential expression of the lymph node (LN) homing receptors CD62L and CCR7^{47,148,172}. T_{CM} cells have a higher proliferative capacity and are thought to

provide superior protection against re-infection than T_{EM} cells, at least under certain conditions. Like naïve cells, their expression of CD62L and CCR7 enables them to home to LNs and survey them for cognate antigen¹⁷⁷. In contrast, T_{EM} cells are more cytotoxic than T_{CM} cells, but are excluded from accessing LNs. Lastly, T_{RM} cells are a tissue-confined, non-migratory memory population, derived from effector cells that seed non-lymphoid tissues early after infection^{178–181}, although it was also suggested that T_{RM} cells may be progeny of T_{EM} cells¹⁸². Additionally, a few studies^{183–186} recently identified a naïve-like memory T cell subset, which due to its superior expansion capacity (compared to T_{CM} cells) was termed T memory stem cells (T_{SCM}). However, despite the name implying stemness, a stringent analysis of this subset’s multipotency and self-renewal capacity is still lacking and thus it is conceivable that this subset may only represent a transient cellular state in the differentiation path from naïve to stably maintained T_{CM} ⁵⁰.

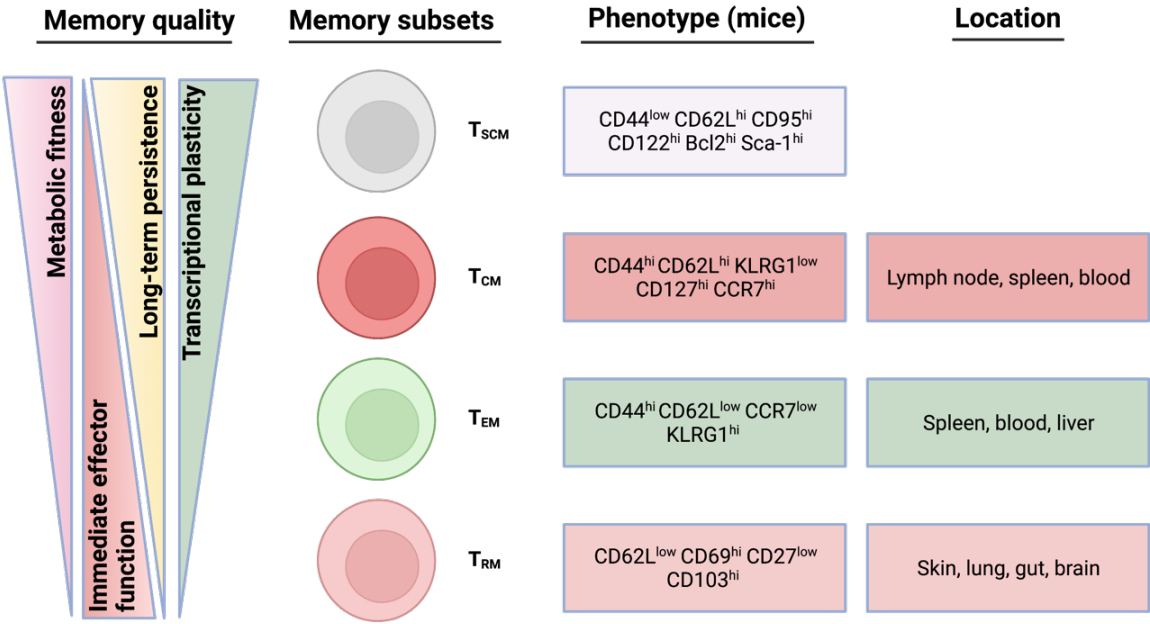


Figure 10. Memory CD8⁺ T cell subsets. Schematic representation showing the four main CD8⁺ memory T cell subsets (central panel), with their respective quality attributes (left panel) and the cell surface biomarkers used for their phenotypic characterization. T_{SCM} : stem cell like memory T cells; T_{CM} : central memory T cells; T_{EM} : effector memory T cells; T_{RM} : tissue-resident memory T cells [Created with [BioRender.com](https://www.biorender.com); adapted from Jandus et al. 2017¹⁸⁷].

1.4. CD8⁺ T cell differentiation and behaviour – Stochasticity at the single-cell level, robustness at the population-wide level

It has been appreciated for many years that epitope-specific CD8⁺ T cell populations exhibit substantial phenotypic and functional diversity, both during the effector and memory phase (Figure 11). Multivariate analysis on the phenotypic, functional, and transcriptional level have revealed even more complex patterns of diversification¹⁸⁸. Diversification of cellular progeny is a key feature of CD8⁺ T cell immunity, which provides specialised T cell subsets that can respond distinctively within shared environments⁵⁰. However, technological limitations made it previously difficult to follow the behaviour of a single T cell *in vivo*, and thus many models of T cell diversification had to be inferred from measuring population behaviour. However, more recent technologies advances, such as 2-Photon imaging^{189–192}, *in vivo* fate mapping through genetic cellular barcoding^{193,194} or the adoptive transfer of single T cells carrying distinct congenic markers^{195,196}, have revolutionised our understanding of T cell behaviour.

Collectively, these studies have demonstrated that individual T cells exhibit a remarkable degree of heterogeneity at the single-cell level and are in principle capable of generating a diverse offspring encompassing phenotypically and functionally distinct effector and memory T cells. These studies further indicated that early events create substantial variation in subsequent expansion and phenotype between individuals T cells, and it was suggested that the occurrence of these events on the single cell level should be regarded as stochastic in the sense that it cannot be predicted prior to the onset of the immune response which event or succession of events a single T cell will encounter⁵⁰. While many of these studies have been conducted with TCR Tg CD8⁺ T cells, adoptive transfer experiments of single CD4⁺ T cells¹⁹⁷ and CD8⁺ T cells¹⁹⁸ have confirmed that notable quantitative and qualitative variability of

single-cell-derived responses is also encountered within the endogenous polyclonal TCR repertoire.

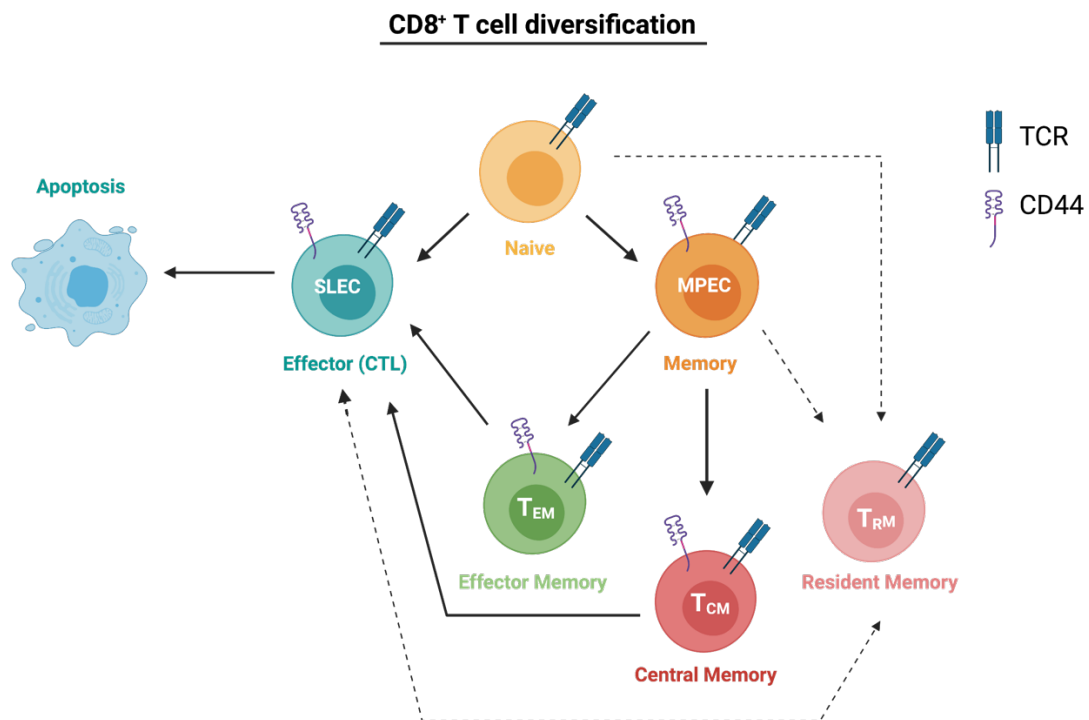


Figure 11. CD8⁺ T cell diversification. An activated CD8⁺ T cell can differentiate into several distinct subsets, based on the cumulative signal strength received during priming [Created with [BioRender.com](https://www.biorender.com)].

However, contrastingly, at the global level, immunological outputs, such as expansion and diversification, are tightly controlled, thus leading to reproducible and predictable response patterns to infection⁵⁰, which suggests that the immune system robustly integrates a stochastic pattern of extrinsic context-dependent signals and/or T cell-intrinsic factors through population averaging (**Figure 12**).

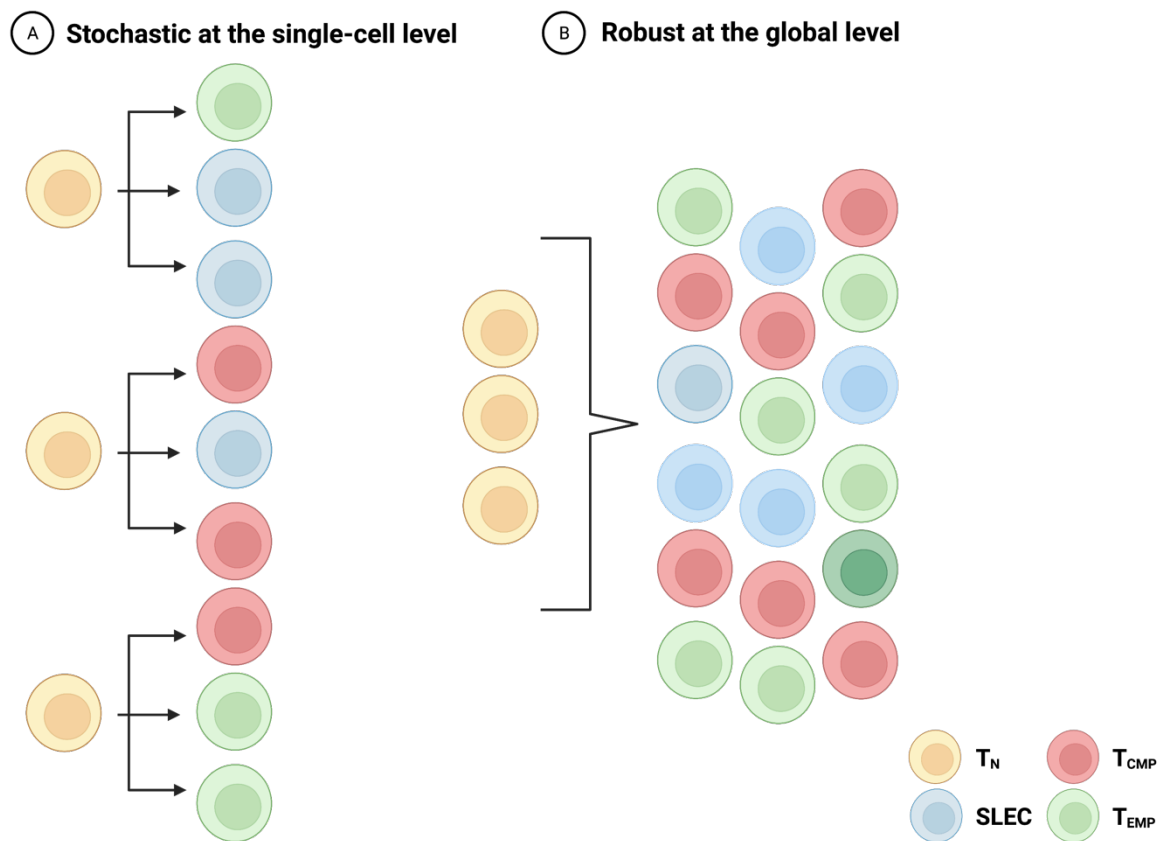


Figure 12. Robustness of T cell responses through population averaging. (A) During a T cell response, most naïve T cells specific for a defined epitope are recruited and induced to proliferate and differentiate. The combined progeny of these naïve precursors forms a phenotypically and functionally heterogeneous population of effector T cells, with some exhibiting a high propensity to become memory (MPECs), whereas others are destined to be short-lived (SLECs). (B) While displaying a remarkable degree of diversity at the single-cell level, epitope-specific T cell immune responses are robust and reproducible at the population-wide level. T_N: naïve T cell; T_{CMP}: central memory precursor; T_{EMP}: effector memory precursors; SLEC: short-lived effector cell [Created with BioRender.com; adapted from Buchholz et al. 2016⁵⁰].

Importantly, individual naïve T cells and memory T cells do not always realize their immense developmental potential. Single T cell–derived proliferation and differentiation is executed with massive variation, and recruitment of a sufficient number of naïve precursors is therefore necessary to provide a robust relationship between infectious input and immunological response^{196–199}. It was suggested that the notable redundancy of the TCR repertoire¹⁰⁷ and the high efficiency of recruiting T cells specific to a certain epitope into an immune response¹⁰⁶ may therefore be understood as an evolutionary adaptation to balance stochastic response variation at the single-cell level by population averaging⁵⁰.

Adoption of a certain T cell fate could either be determined autonomously, by cell-intrinsic development programs, or be mainly determined by cell-extrinsic cues provided by a certain tissue microenvironment. Studies examining population behaviour generally suggested a model of T cell diversification, in which antigen encounter synergises with environmental signals to drive CD8⁺ T cells down numerous differentiation states that vary both in effector function and survival capacity, and which need to be tightly coordinated to ensure effective responses to infection, while also maintaining peripheral tolerance⁹⁶.

Despite the stochastic plasticity observed at the single-cell level, several factors have been identified to promote either effector or memory differentiation, which help to explain how heterogeneous pools of effector and memory CD8⁺ T cells arise during infection⁹⁹. The three major classes of signals required for functional T cell activation – TCR triggering through binding to cognate antigen (signal 1), co-stimulation (signal 2) and cytokine signalling (signal 3) – all operate along gradients, whose integration determines subsequent T cell expansion and differentiation. It is important to highlight that the three signals required for functional T cell responses are not considered to be hierarchically equal. Generally, only upon TCR engagement are the other two signals allowed to contribute to T cell activation and fate determination. During infections, critical co-stimulatory receptors for CD8⁺ T cells include 4-1BB and CD27 (binding to CD70), while CD28 (binding to CD80 and CD86), CD40 and OX40 are also crucial in certain infectious settings^{20,98,200–206}. The main pro-inflammatory cytokines produced during viral and other intracellular infections are type I IFNs, IFN- γ , IL-2, IL-12, IL-27 and IL-33, which have been suggested to enhance the proliferation, differentiation and survival of CTLs through various mechanisms such as increasing the expression of cytokine and co-stimulatory receptors^{24,26,211,212,119,122,127,155,207–210}. These three types of signals are closely linked and inseparable *in vivo* during infection and the duration or relative amount of

these signals defines the cumulative signal intensity T cells receive^{213–215}, which determines the final number, phenotype, function, and long-term fate of activated CD8⁺ T cells⁹⁹ (see also [p.52](#)).

1.4.1. Models of CD8⁺ T cell diversification

Based on the available evidence, three models were put forth to explain T cell diversification ([Figure 13](#)). The decreasing-potential model proposes that repetitive stimulation of T cells with antigen and pro-inflammatory cytokines drives greater proliferation and terminal effector cell differentiation. Terminal differentiation is thought to be accompanied by a loss of memory cell properties, such as enhanced longevity, proliferative potential and IL-7R α expression. Such a linear progression model provides a mechanism for how the cumulative history of signals encountered during infection may generate a wide spectrum of effector T cells at various differentiation states^{99,147}. Such a model is supported by the observation that shortening the duration of antigen exposure and decreasing inflammation through antibiotic treatment or DC immunization accelerates memory T cell formation while simultaneously decreasing the fraction of SLECs observable at peak expansion, and also helps to explain why cells recruited at the tail-end of the response preferentially differentiate into T_{CM} cells^{120–123,127,216}. It has furthermore been shown that inflammatory cytokines, mainly IL-12 and type I interferons, selectively drive SLEC expansion by prolonging their responsiveness to IL-2²¹⁷, while depletion of inflammatory cytokines results in diminished SLEC numbers and reduced contraction^{122,216}.

The decreasing potential model ([Figure 13A](#)) thus postulates that memory formation is the default state of activated T cells, and that T_{EM} or SLEC differentiation is only possible if a certain level of cumulative signal intensity is surpassed^{99,218}. Conceptually, this model relies

on the initial priming event chiefly for triggering proliferation, whereas fate diversity is subsequently generated through instructive signals that drive the differentiation of expanding long-lived precursors into various shorter-lived descendants^{99,147,219}. Whether this activation level represents a binary threshold, or whether effector potential is gradually increased according to increasing signal strength is a matter of ongoing debate and may be dependent on the molecules that are used as surrogates for quantifying the threshold value^{220–225}.

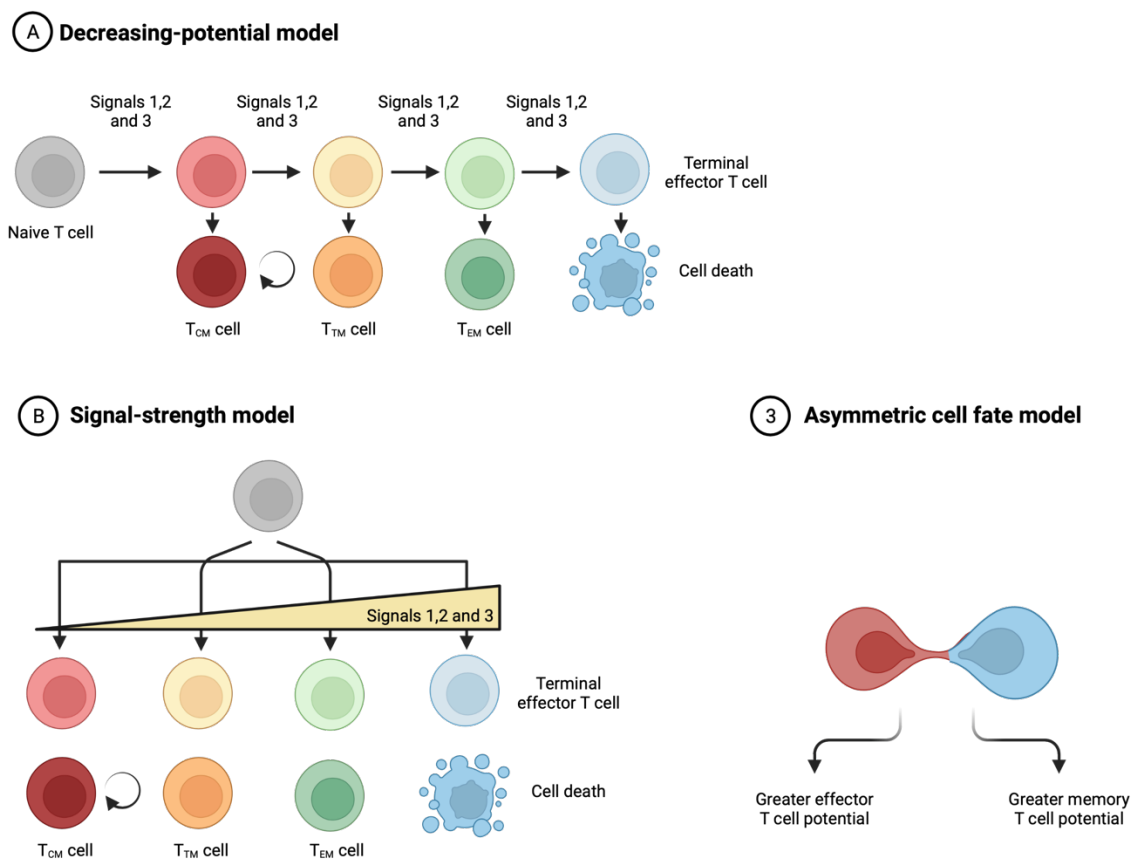


Figure 13. Models of CD8⁺ T cell diversification. (A) The decreasing-potential model suggests that effector T cells adopt various differentiation states according to the cumulative history of signals that they encounter during infection. (B) In the signal-strength model, the formation of heterogeneous effector cell populations is dependent on the overall strength of the signals that are encountered early during T cell priming. (C) The asymmetric cell fate model suggests that effector and memory T cell fates can arise from a single-precursor T cell through asymmetric cell division that occurs as early as the first cell division after antigenic stimulation. Signals 1, 2 and 3 denote antigen, co-stimulatory molecules, and cytokines, respectively. T_{CM}: central memory T cell; T_{EM}: effector memory T cell; T_{TM}: transitional memory T cell [Created with BioRender.com; adapted from Kaech and Cui 2012⁹⁹].

Available evidence also supports another diversification model, known as the signal-strength model (**Figure 13B**), which posits that the formation of heterogeneous effector cell populations is significantly dependent on the integrated strength of the cumulative signals

delivered by the antigen, co-stimulation and pro-inflammatory cytokines encountered early during T cell priming^{226–228}. Additionally, a strong signal is suggested to drive enhanced clonal expansion and to identify T cells that are competent to form memory cells, while driving terminal effector differentiation beyond a certain threshold⁹⁹. Importantly, this model suggests that different cell fates can be specified early during the response according to the cumulative signal intensity received, rather than in a linear, stepwise manner driven by successive rounds of stimulation, as proposed by the decreasing-potential model.

The final model, known as the asymmetric cell fate model ([Figure 13C](#)), suggests that asymmetric cell division may serve as a possible mechanism of T cell diversification, which is suggested to occur as activated cells clonally expand²²⁹. Asymmetric cell division is a process well-studied in yeast cells and produces two daughter cells with distinct cellular fates (in the context of T cell diversification memory and effector cells) from a single precursor cell, which is mediated by unequal inheritance of cell surface receptors and associated cytoplasmic proteins. This concept, which was first introduced into T cell biology in 2007²²⁹, proposes that asymmetric partitioning of signalling molecules and certain components of the intracellular machinery, such as the proteasome²³⁰, results in the generation of two distinct daughter cells from a single primed T cell, with one daughter cell destined for a short-lived effector fate, whilst the second daughter cell displays heightened memory potential. Asymmetric cell division has furthermore been proposed to occur after priming of T_{CM} cells²³¹ and is thought to be preferentially induced by high-affinity TCR binding²³². However, this model remains controversial^{50,185,233} and awaits further confirmation *in vivo*.

1.4.2. Transcriptional regulation of CD8⁺ T cell diversification through T-bet and Eomes

Much progress has been made over the last two decades in our understanding of the transcriptional circuitry underlying T cell diversification and several transcription factors that regulate effector and memory CD8⁺ T cell development have been identified (reviewed here⁹⁹). Notably, multiple of them function in pairs that form counterregulatory axes to regulate effector and memory cell potential. In the following section we will only briefly introduce one of these pairs most relevant for this thesis – the T-box transcription factor family T-bet and eomesodermin (Eomes), which are both critical to create CTLs^{120,209,234–237}.

T-bet expression is induced upon productive TCR engagement, which is subsequently amplified by IL-12-mediated signals and mTOR activity in effector CD8⁺ T cells^{120,238,239}. CD8⁺ T cells lacking T-bet are impaired in forming KLRG1^{hi}IL-7R α ^{low} terminal effector cells and, when overexpressed, T-bet is sufficient to induce the formation of these effector cells¹²⁰. It was thus suggested that accumulating amounts of T-bet in effector CD8⁺ T cells may drive them towards terminal differentiation¹²⁰.

Eomes is a homologue of T-bet with an identical DNA recognition motif^{240,241}, whose expression was correlated with memory potential^{120,238,242}. Notably, OT-I cell studies indicated that Eomes expression is high in cells activated with low-affinity ligands^{243,244}. Induction of Eomes expression was observed subsequently to that of T-bet in a Runx3-dependent manner and to be amplified by IL-2, while IL-12 and mTOR were shown to repress Eomes expression^{209,239,241,245}. Notably, Eomes-deficient effector CD8⁺ T cells have been demonstrated to efficiently generate MPECs but to be incapable of generating memory cells with normal expression of CD122, CD62L, CXCR3 and CXCR4, thus containing fewer T_{CM} cells and exhibiting impaired homeostatic turnover and long-term persistence²³⁵. In memory CD8⁺

T cells, T-bet and Eomes cooperate to sustain memory CD8⁺ T cell homeostasis through expression of IL-2R β (also known as CD122), which facilitates IL-15-mediated homeostatic proliferation of memory cells^{120,209,234,235}.

Importantly, the expression of T-bet and Eomes was discovered to be somewhat reciprocal, with T-bet expression reaching its highest level in early effector CD8⁺ T cells, while progressively declining during the effector to memory transition²⁴⁶. Eomes on the other hand displays the exact opposite expression dynamic^{209,235,246,247}. Hence, the ratio of T-bet to Eomes is highest at effector cell stages and lowest at memory cell stages. Altogether, it was concluded that the phenotype, function, and long-term fate of effector CD8⁺ T cells are highly sensitive to the relative T-bet/Eomes expression ratio (**Figure 14**)^{120,235,236,246}. Factors shown to regulate this ratio during infection include the balance of FOXO1 activity and exposure to pro-inflammatory cytokines (such as IL-12, type I IFNs and IL-2) and other cytokines (such as IL-4, IL-10 and IL-21)^{238,239,245,247,248}. However, how the T-bet/Eomes expression ratio precisely controls T cell diversification awaits further elucidation.

Other transcriptional pairs have also been discovered to be critically involved in the transcriptional programming of CD8⁺ T cell differentiation (reviewed here⁹⁹), and it was suggested that these competing sets of transcription factors act along opposing gradients of expression, and depending on the overall balance of these counter-regulating factors, cells are either driven towards a more terminally differentiated phenotype or memory fate⁹⁹. Such a gradient model equips T cells with a critical level of flexibility in controlling the quantity and quality of their response according to the unpredictable properties of a given infection, which can vary in intensity, tropism and duration. How these factors cooperate or impede each other's function and how they collectively program activating CD8⁺ T cells remains to be further elucidated.

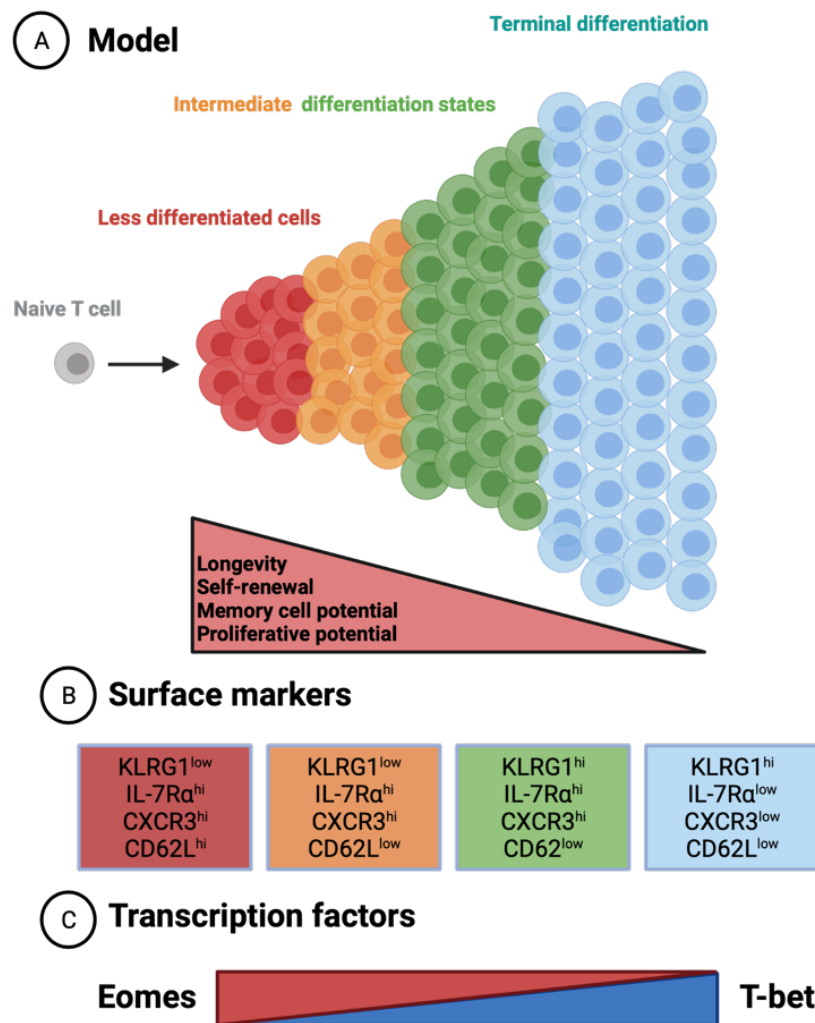


Figure 14. Graded activity of T-bet and Eomes control effector and memory T cell differentiation. (A) This model, suggested by Kaech and Cui⁹⁹, postulates that in response to different levels of signal input, the differentiation of antigen-specific effector CD8⁺ T cells occurs along a continuum, with cells exhibiting greater memory cell potential on one end of the spectrum, and terminally differentiated effector T cells present at the other end of the spectrum. (B) The diverse differentiation states of effector CD8⁺ T cells can be distinguished by the expression of several surface markers, most notably killer cell lectin-like receptor G1 (KLRG1), IL-7 receptor subunit- α (IL-7R α), CD27, CXC-chemokine receptor 3 (CXCR3) and CD62L. (C) Multiple studies suggest that the graded expression or activity of certain competing sets of transcription factors, such as T-bet and Eomes, control terminal effector cell differentiation and memory cell potential. As the relative expression or activity of T-bet increases and surpasses a given threshold during infection, effector T cells acquire a more terminally differentiated state that is associated with a reduced proliferative capacity and longevity. T-bet counter-regulates, and can also interact with, an opposing set of transcriptional regulators, including eomesodermin (Eomes), that prevent terminal differentiation of effector T cells and help to maintain memory cell properties [Created with BioRender.com; adapted from Kaech and Cui 2012⁹⁹].

1.4.3. TCR activation signal 1 and its modulation through signal 2 and 3

An important intrinsic factor that has been clearly demonstrated to control proliferation and differentiation of CD8⁺ T cells is the binding affinity of their TCR for a given peptide-MHC complex⁶³, which defines the strength of the TCR-derived signal. Based on the observation that only cells of high TCR affinity vigorously expand upon activation²⁴⁹, initial models of T cell activation proposed that successful activation and formation of effector and memory cells requires a cumulative signal of high overall strength²⁵⁰. However, this model was challenged by several subsequent studies, such as the previously discussed observation that even a brief 2 hours priming phase was sufficient to induce the complete diversity of effector and memory CD8⁺ T cell subsets^{86,113,114}. It was furthermore discovered that even very weak TCR-pMHC interactions are sufficient to activate naïve cells, induce proliferation and mediate the formation of both functional effector and memory T cells^{63,251,252}.

Nonetheless, the intensity of the TCR signal was shown to correlate positively with the potency to induce effector cell proliferation^{63,116,253}, which may serve an important role in preventing the exhaustion of limited amounts of available resources by clones of low specificity and efficiency. Notably, single-cell studies linked strong proliferative expansion to terminal differentiation and loss of memory capacity^{196,199}, even though the mechanisms underlying this variation remain unclear⁵⁰. While high-affinity clones display a clear proliferative advantage⁶³, activating low-affinity clones were shown to undergo negative selection based on a reduced capacity to access and thus outcompete other clones for limited resources²⁵⁴, such as the cytokine IL-2, which mediates T cell survival by triggering the PI3K signalling cascade and sustaining the pro-survival protein Mcl-1²⁵⁵. Moreover, activating T cells induce expression of the IL-2 receptor in an antigen-affinity dependent manner^{63,255}.

Hence, high-affinity effector T cell clones have a clear competitive survival advantage over low-affinity T cells in their ability to access IL-2, which narrows the clonal diversity of the responding effector pool²⁵⁵.

However, whereas TCR affinity is structurally fixed for T cells that carry the same TCR, other intrinsic factors, such as the amount of signalling molecules present in a given T cell, can vary between individual cells, which may create a range of antigen responsiveness even among the members of a monoclonal population²⁵⁶. Indeed, two studies mapping the fate of single-cell-derived responses of a monoclonal population of TCR transgenic T cells *in vivo*^{196,199} revealed that responses derived from single CD8⁺ T cells are characterized by immense variation in proliferative output. Despite harbouring the same TCR specificity, single naive OT-I T cells generated between 200 and 200,000 daughter cells at peak expansion after infection with *L. monocytogenes*. The median burst size was shown to be dependent on the type and dose of infection. However, variability of individual clonal family sizes remained consistently high, both in response to high- and low-affinity antigens^{196,199}.

A recent *in vitro* study examining transcriptional changes downstream of OT-I T cell activation by single-cell RNA-sequencing (scRNA-seq)²⁵⁷ has revealed that TCR stimulation strength modulates the rate with which cells initiate transcriptional activation, while the core transcriptional activation trajectory was largely shared between clones of high- and low-affinity. Notably, after accounting for the activation status of each cell, a small number of genes remained differentially expressed at the mRNA level between cells stimulated by strong or weak TCR ligands²⁵⁷.

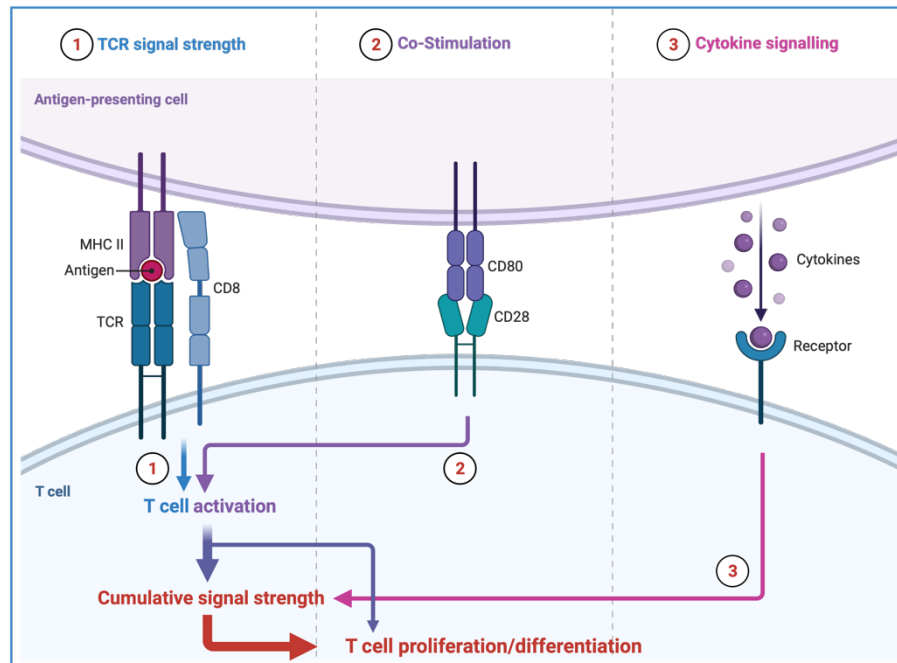


Figure 15. Cumulative signal strength. T cells receive 3 signals during priming, which are integrated to specify the subsequent expansion and differentiation of antigen-specific CD8⁺ T cells. Understanding how T cells integrate alternative signal combinations to decide the strength of the ensuing immune response or alternatively trigger tolerance mechanisms poses a considerable experimental challenge. It was suggested that antigen affinity, co-stimulation and cytokine inputs sum linearly to amplify T cell expansion²⁵⁸. T cell receptor (TCR) and costimulatory signals were furthermore shown to imprint an early, cell-intrinsic, division fate, which represents an autonomous program that can be extended by cytokines^{258,259} [Created with BioRender.com]

While the strength of the TCR signal is thus clearly an important factor defining activated T cell behaviour, T cell response size and subset composition are not solely modulated by T cell-intrinsic signals, but additionally also regulated by various extrinsic signals, which critically shape the immunological output (**Figure 15**)⁴³. These extrinsic signals can be envisioned as skewing the phenotypic and functional composition of a T cell response by two nonexclusive mechanisms: instruction of activated T cells to adopt a certain phenotype through differentiation, and/or selection of a certain signal-receptive T cell phenotype to survive, proliferate, or die⁵⁰.

Co-stimulation and cytokines greatly contribute to the cumulative activating signal intensity and therefore have a major impact on TCR affinity-mediated regulation of CD8⁺ T cell expansion and differentiation. Ligation of co-stimulatory receptors, including CD28, CD27

and CD2, can augment TCR signals and enhance activation^{258,260–262}, which may be essential for proper CD8⁺ T cell responses of cells receiving weak TCR signals²³. Conversely, high antigen doses and prolonged antigen stimulation can compensate for a lack of CD28 co-stimulation *in vivo*^{263,264}. CD27-driven co-stimulation promotes production of IL-2 in activated T cells²⁶⁵, which is generally expressed in a stimulation strength-dependent manner by CD8⁺ T cells^{266–268}. Animals deficient for CD27 therefore have reduced access to IL-2, resulting in a less clonally diverse effector response of increased overall affinity²⁶⁰. Notably, expression of CD70, the ligand of CD27, is also regulated by antigen avidity^{260,269–271}, but it is currently unknown how this affects the activation and recruitment of high- or low-affinity clones.

Cytokines are the third critical factor shaping cell fate decisions and clonal selection mechanisms. CD8⁺ T cells activated in the presence of high levels of IL-2 or IL-12 exhibit increased proliferation rates and superior effector functions^{63,122,127,209,221,250}. Moreover, exogenous addition of IL-2 is sufficient to rescue survival of low-affinity cells²⁵⁵, as well as translation and proliferation deficiencies observed in cells stimulated with low dose of antigen or low-affinity ligands^{221,272}. This effect of IL-2 on low-affinity cells is thought to be mediated by the transcription factor Myc, which controls division potential of activating cells and requires ongoing protein synthesis due to rapid turnover^{221,222,272,273}.

In summary, TCR signalling is not a binary on/off switch. Rather, it enables integration of signals with different intensities, such as those provided by cytokines and co-stimulatory molecules. How substantial the effect of TCR affinity is, relative to effects determined by the antigen and costimulatory makeup of the priming APC and the inflammatory signals provided by the microenvironment, remains to be further elucidated, even though a few models have been put forward (summarised in [Figure 16](#)).

Notably, even within a uniform, monoclonal T cell population carrying a TCR of high affinity for its cognate antigen, only a relatively small fraction of clones dominates the ensuing effector response^{196,199}, indicating that only a small number of cells reaches the cumulative signalling threshold required for productive T cell expansion. Jenkins and colleagues¹⁹⁷ have furthermore demonstrated that responses originating from smaller epitope-specific CD4⁺ endogenous T cell populations were substantially more variable in size and phenotypic composition than those originating from large ones. Collectively, these studies indicate that the robustness of T cell immune response quantity and quality at the systemic level requires recruitment and activation of a sufficient number of naive precursor T cells sharing the same epitope-specificity to average out fate-determining stochastic variations observed at the single cell level⁵⁰.

1.4.4. CD8⁺ T cell response poly-clonality

TCR-driven selection mechanisms during activation are particularly relevant to understand T cell behaviour at the endogenous level, in the context of a highly poly-clonal T cell repertoire. The endogenous naïve CD8⁺ T cell pool consists of millions of clones, each unique based on its specific antigen receptor, with each clone present at low frequency^{274–277}, ensuring recognition of a vast number of potential threats. Statistical probability dictates that for any given antigen, many more low- than high-affinity clones exist. Clonal selection based on antigen-specificity and TCR-affinity is thus a crucial factor in mounting an effective T cell response^{255,278}. As TCR affinity is an important determinant for the capacity of activating T cells to access critical co-stimulatory molecules, cytokines, and nutrients²⁵⁵, it was proposed to call this fourth factor “*competitive fitness*”²⁵⁴. Supporting the relevance of this fourth factor in shaping the ensuing response, the relative abundance of certain CD8⁺ memory clones was

shown to be critically determined by early TCR signals, and initial TCR signal strength to correlate positively with the likelihood that a certain clone enters the memory pool, with weak TCR signals associated with premature arrest of expansion and early onset of contraction⁶³. Notably, a similar competition between low- versus high-affinity clones was also shown to govern B cell response dynamics^{279,280}.

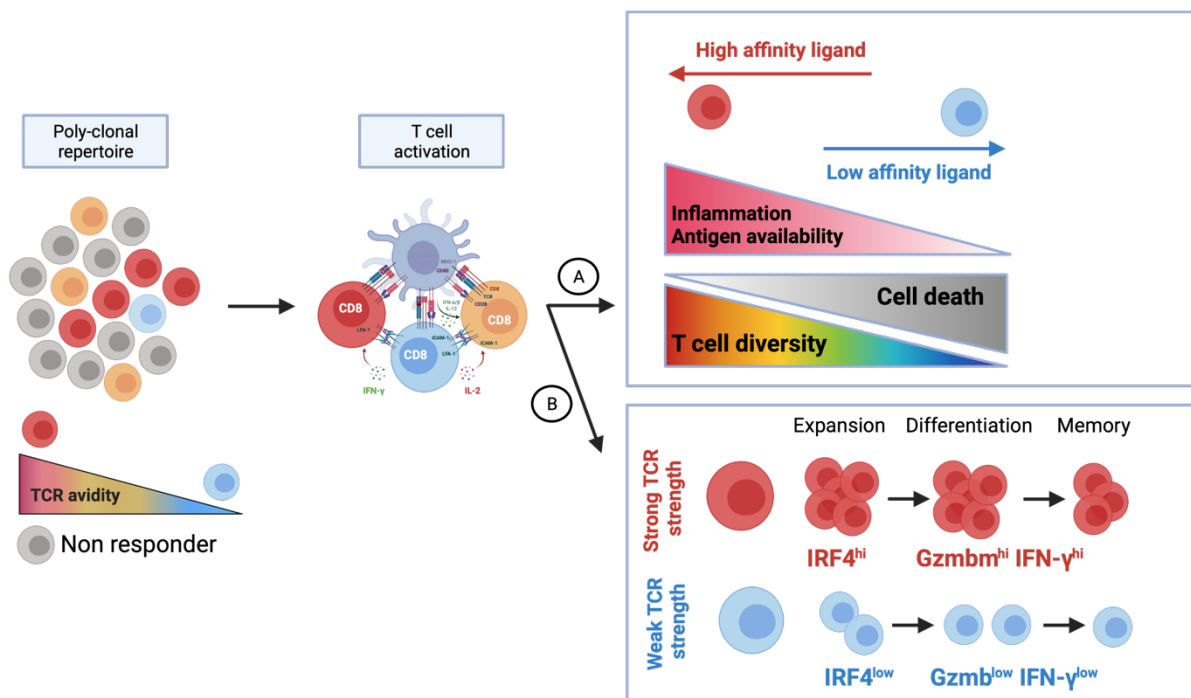


Figure 16. Low- vs high-affinity T cell responses. The poly-clonal T cell repertoire contains T cell clones capable of recognizing a given antigen with their TCRs with a range of different affinities. (A) Low-affinity stimulated T cells are thought to be more susceptible to Bim-mediated cell death. However, it was shown that this fate can be partially overcome by increasing inflammation²⁸¹. Increasing antigen availability in the context of inflammation can furthermore fully overcome the Bim-mediated cell death and preserve low-affinity T cell responses²⁸¹. (B) Interferon regulatory factor 4 (IRF4) was shown to be regulated by TCR signalling strength via mTOR²⁸². IRF4 is suggested to translate differential strength of TCR signalling into different quantitative and qualitative CD8⁺ T cell responses, with IRF4^{hi} cells exhibiting heightened expansion and effector differentiation [Created with [BioRender.com](https://www.biorender.com)]

Thus, upon pathogen encounter, a restricted number of antigen-specific CD8⁺ T cells are selected into the effector and memory pools. Their clonal diversity determines the range of antigens that the population as a whole is capable of recognizing and given that the immune system faces distinct challenges during the immune response, the selection of particular clones must fit those specific purposes. Effector cells are typically faced with an actively replicating pathogen and thus benefit most from selecting predominantly clones with the

highest antigen specificity, which guarantees the most potent and efficient response possible^{110,255}. Contrastingly, immunological memory must “*think*” long-term, and on the one hand ensure protection against re-infection with a previously encountered antigen, while also anticipating that pathogens may alter their antigenic composition as they move through their host populations due to immunological and selective pressures^{283–285}. To fulfil this task the memory compartment must therefore promote a higher degree of clonal diversity compared with the effector pool to increase its chance of being capable of recognizing mutated pathogens. Hence, selection of memory clones can be considered a trade-off between specificity and diversity. Too much specificity may restrict antigen-recognition, which precludes responsiveness against pathogens displaying mutated immunogenic epitopes, while too much heterogeneity, although increasing the probability of recognizing mutated pathogens, may impair efficiency of recall responses. Given these competing demands, it is conceivable, that effector and memory cells may be intrinsically different in their clonal selection strategies^{64,286}. Additionally, given that memory cells are maintained independently of antigen^{164,287}, and that differences in cytokine receptor expression between high- and low-affinity cells are lost at later time-points^{63,255}, the average avidity of the antigen-specific pool may still change in the first weeks after pathogen clearance, but eventually stabilises once long-lived effector cells undergo apoptosis²⁸⁸. Once the clonal composition of the memory compartment is established, it remains stable for month to years after initial infection, both in humans and mice^{289–292}, altogether highlighting that the generation of a memory pool of sufficient diversity is an important, long-term investment of the immune system to be able to counter viral mutants.

1.4.5. Regulatory mechanisms modulating CD8⁺ T cell poly-clonality

It is only partially understood how clonal selection within the effector and memory cell populations is regulated. Despite the great progress made in understanding the transcriptional programs driving effector and memory differentiation (see [p49](#); reviewed in more detail here⁹⁹), how these different programs are triggered and modulated is still a matter of ongoing debate. However, a few general patterns have become discernible.

The impact of TCR affinity on differentiation has been examined mostly by studying TCR transgenic T cells. These studies suggested that high-affinity clones have several selective advantages over clones with low-affinity TCRs. Selection of high-affinity clones into the effector pool was discovered to be dependent on both increased proliferation⁶³, and an enhanced ability to access prosurvival cytokines^{243,253,255}, as well as increased and prolonged accessibility to APCs²⁹³, resulting in prolonged retention in the lymph node²⁹⁴. Increased access to exogenous factors such as IL-2, IL-12 or CD28 co-stimulation adds to the cumulative activating signal, which promotes differentiation towards an effector phenotype up to a threshold level^{221,238}, while pushing T cells into exhaustion if a certain level of stimulation is exceeded^{295,296}. In contrast, even cells of very low-affinity are capable of forming functional memory, while also exhibiting a strongly reduced capacity to induce effector differentiation^{63,244} and it is thought that memory potential is typically associated with overall weaker signals²⁴⁴. Low affinity clones have also been reported to depend upon the co-stimulatory molecule CD27²⁶⁰ and displaying higher dependence on TNF receptor signalling during recall²⁹⁷.

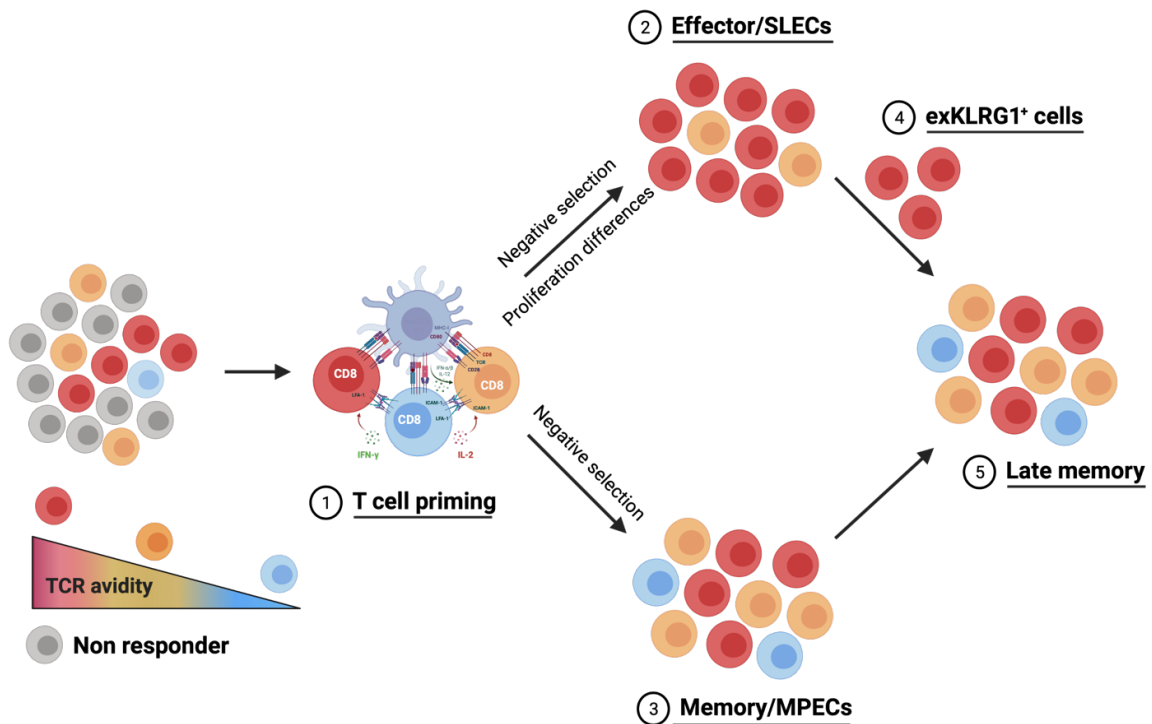


Figure 17. Model for affinity selection within the effector and memory pools. Antigen encounter will activate a small number of high-affinity cells and a much larger number of cells with lower affinity. The effector pool is thought to be stringently selected for cells of the highest affinity both through negative selection of low-affinity cells and through a proliferative advantage of high- over low-affinity cells. In contrast, affinity-based selection of early memory is less strong since differences in proliferation between high- and low-affinity cells are less pronounced. Selection of high-affinity cells will therefore primarily occur due to competition of survival factors such as CD27 co-stimulation and cytokines. Late memory is supplemented by exKLRG1 cells, which are predominantly of high-affinity, thus increasing the overall affinity of the memory pool [Created with [BioRender.com](https://www.biorender.com); adapted from Kavaczovic et al. 2018²⁸⁶].

Altogether, these findings indicate that low affinity memory precursors have increased dependence on factors that contribute to the cumulative activating signal, and thus may have an inherent survival disadvantage when competing for these factors²⁸⁶. However, given that a low cumulative signal strength appears to favour the formation of memory²⁴⁴ and is associated with reduced proliferation^{63,253}, it was suggested that low affinity clones may offset their competitive disadvantage over high affinity cells, at least partially, by preferential programming towards a memory fate (Figure 17).

In agreement with the observed competitive advantages of high-affinity clones, the effector pool against a given antigen is typically dominated by only a few greatly expanded clones that bind the antigen with very high affinity^{260,298,299}, while the memory compartment

in contrast displays a much more clonally diverse composition^{260,278,298,300}. However, clones that dominate the effector pool are also dominant in the memory population, albeit to a lesser extent²⁶⁰. Increased clonal diversity is only possible if affinity-based selection is less stringent, which was confirmed by a recent study⁶⁴ demonstrating that the responding CD8⁺ T cell effector pool against influenza virus is more stringently selected for clones of high-affinity than the memory pool directed against the same antigen. How clones of both high- and low-affinity for the activating antigen are selected into the memory pool remains unclear. The recent discovery that KLRG1⁺ cells can still form memory after pathogen clearance¹⁶², albeit at much lower frequency than MPECs, indicates that the high number of KLRG1⁺ cells generated during an immune response may allow “*exKLRG1*” cells to make a significant contribution (in absolute numbers) to the memory pool. Given that high-affinity cells preferentially differentiate into cells with a SLEC phenotype, whereas low-affinity cells are more prone to become MPECs²⁴⁴, it was hypothesised that low-affinity memory cells may be formed directly, at least predominantly, whereas high-affinity memory may be additionally derived from *exKLRG1*⁺ effector type cells²⁸⁶ (**Figure 17**). However, direct experimental evidence to support this hypothesis is still lacking.

In summary, the experimental evidence discussed so far suggests that the cumulative activating signal strength received upon activation is a key determining factor for effector versus memory cell-fate decisions¹⁹⁶, demonstrating that, on average, an increase or reduction of the cumulative signal intensity favours effector- or memory-cell formation, respectively^{120,125}. However, it remains unknown how this affinity-based model integrates the concept of clonal diversity in its selection criteria, and given that most of our current knowledge is inferred from studies of TCR Tg T cell clones responding to high- or low-affinity ligands⁶³, which largely negate the crucial aspect of clonal competition and cooperation, it

remains to be further elucidated how intrinsic and extrinsic cues combine to shape the poly-clonality of the endogenous T cell response. Of note, a very recent study⁶⁴ discovered that TCR strength-dependent interplay between T-bet and Eomes to play a critical role in shaping the poly-clonality of the response and in the persistence of low-affinity clones into memory.

1.5. Coordination of T cell responses through T cell collectivity

Considerable work has focused on understanding the various intrinsic and extrinsic cues regulating the activation and diversification of T cells⁵⁰, but an often-underappreciated aspect of T cell biology is that they are embedded and function within a highly networked cellular community. All the evidence discussed so far supports a model, in which competition between individual T cells for trophic survival and other limiting factors²⁵⁴ critically determines T cell activation, differentiation and expansion. However, the benefits of cooperative actions may often outweigh the advantages each cell derives from acting purely autonomously^{301–304}. The microenvironment is a key driver in modulating T cell differentiation, as demonstrated by studies linking early migratory patterns of activated T cells to subsequent cell fate commitments^{99,305–307}.

The fact that CD8⁺ T cells are capable of proliferating and differentiating autonomously after antigen stimulation^{86,114} furthermore suggests that cell-cell communication and interactions beyond T cell-APC interactions are crucial in specifying the exact outcome of the T cell response³⁰⁸. An important and well-studied component in ensuring effective CD8⁺ T cell responses is help provided by CD4⁺ T cells (reviewed recently here³⁰⁹), which is predominantly mediated indirectly through the regulation of antigen-presenting DCs. Less well studied, but previously shown to modulate the differentiation of T cells^{310–313}, is direct T cell-T cell (T-T) communication, which may provide an important regulatory mechanism that helps T cells to integrate individual T cell behaviour into a robust collective response.

T-T communication has been observed to occur either over longer distances through the secretion and diffusion of soluble mediators, such as cytokines or chemokines, or through direct cell-cell contact. These various modes of short- and long-range intercellular

communication likely act in concert to facilitate T cells to not solely act as individual responders, but as a collective through the coordination and integration of individual cell actions. We will first highlight the relevance of T cell collectivity in controlling T cell responses, before reviewing distinct modes of communication (recently reviewed by our group here¹⁰⁴)

1.5.1. Relevance of T cell collectivity in controlling T cell responses

1.5.1.1 Sensing of Population Size during Immune responses

Available evidence suggests that T cells may use mechanisms resembling so-called “*quorum sensing*” to regulate their population size. Quorum sensing is a type of cellular communication mediated by extracellular factors (so-called “*autoinducers*”) typically produced and detected by the same type of cells ([Figure 18](#)). The detection of autoinducers and the activation of downstream signalling pathways occurs upon reaching a sufficiently high autoinducer concentration within the local environment, which in turn requires a sufficiently high local cell population density of signal producers³¹⁴. While many of these discoveries may be specific for the microbial world³¹⁵, it is becoming increasingly evident that similar mechanisms may also be employed by immune cells to tune their responses^{316,317}.

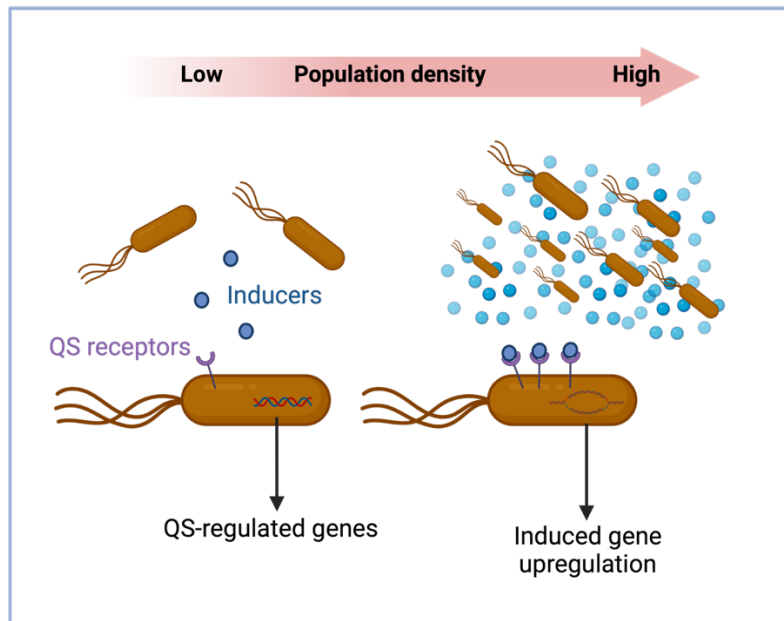


Figure 18. Quorum sensing. Bacteria use signalling molecules (so-called inducers) that are released into the environment to communicate with each other. The term “*quorum sensing*” describes the phenomenon through which signalling molecules enable a single cell to sense the number of bacteria in its environment (i.e., the local cell density) and to adjust its behaviour accordingly, helping to shape and maintain the response of the entire bacterial population [Created with [BioRender.com](https://www.biorender.com/)].

The cytokine IL-2 has been suggested to function as an autoinducer-like molecule between T cells^{303,318}. IL-2, discovered more than 30 years ago, acts as a potent stimulator of T cell growth^{319,320} and as an important regulator of T cell responses^{321,322}. It is produced mainly by T cells³²³ and promotes the maintenance of T_{reg} cells and the differentiation of activating CD4⁺ T cells. For CD8⁺ T cells, IL-2 signals have been demonstrated to optimize both effector T cell generation and memory differentiation^{324,325}. While not being an absolute requirement for T cell proliferation and expansion, it is evident that IL-2 has nevertheless a critical impact on the quality of the T cell response^{318,322}. Strong IL-2 signals drive both CD4⁺ and CD8⁺ T cells towards a terminally differentiated state, while low IL-2 levels drive responding CD4⁺ T cells towards the T_{FH} or T_{CM} state and promote survival of long-lived memory CD8⁺ T cells³²⁵.

Evidence of its potential quorum sensing-like functionality was provided by a few recent studies. A minimal number of IL-2 secreting effector CD4⁺ T cells is required *in vitro* to maintain critical levels of phosphorylated signal transducer and activator of transcription 5

(STAT5) in CD4⁺ T cell, which are necessary for T cell activation and subsequent expansion, alluding to a population density-dependent regulation³²⁶. Mathematical modelling³²⁷ furthermore demonstrated that a group of T cells can compete for the same global pool of secreted IL-2 by tuning their ‘*activation threshold*’ (i.e., the IL-2 concentration at which cells can switch on proliferation-promoting genes).

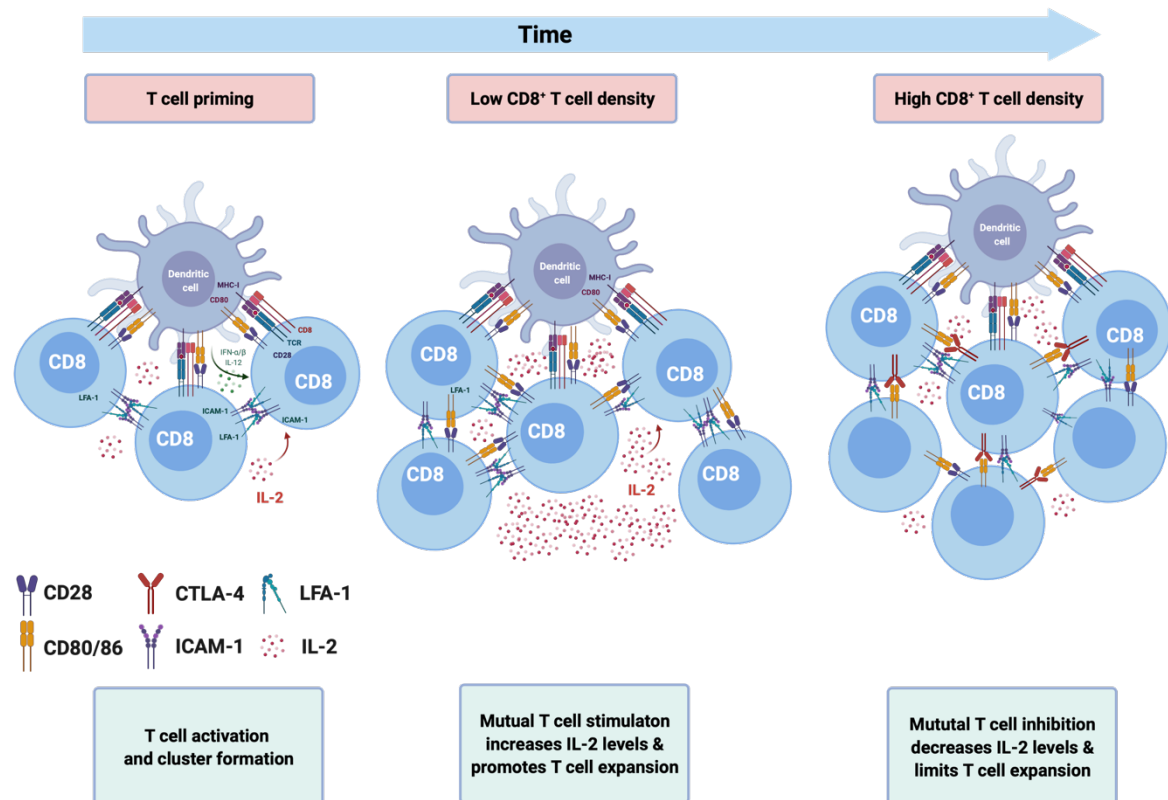


Figure 19. Quorum regulation via T cell expressed CD80/86. ICAM-1-mediated clustering enables CD8⁺ T cells to collectively regulate the balance between proliferation and apoptosis through nested antagonistic feedback circuits mediated by the receptors CD28 and CTLA-4 [Created with BioRender.com; adapted from Zenke et al. 2020³²⁸].

Similarly, antigen-specific CD8⁺ T cells have been observed to critically rely on autocrine IL-2 production by neighbouring activating CD8⁺ T cells^{325,329}. Recently, a paper by Zenke and colleagues³²⁸ revealed that nested antagonistic IL-2 and CD80/86-CTLA-4-mediated feedback mechanisms rely on local cell density as a “*rheostat*” to either promote or inhibit T cell expansion (Figure 19). These T cell population-intrinsic mechanisms are facilitated by ICAM-1-mediated T cell clustering, which enables collective self-regulation of proliferation and

apoptosis during expansion. Well-controlled *in vitro* experiments have furthermore shown that IL-2 exerts seemingly paradoxical effects depending on its local concentration. Above a critical local population density of IL-2-secreting cells it mainly promotes cell death, while conversely driving cell proliferation if cell numbers are below this threshold concentration³³⁰, indicating again that the cell-specific effects of IL-2 may be cell density-dependent.

Both IL-2 receptor and cytokine expression are controlled by several feedback mechanisms³²⁶. Under steady state, naïve CD4⁺ T cells do not express IL-2 and IL-2R α (also known as CD25) at significant levels, but production of both proteins is strongly induced upon activation. As IL-2R α levels remain low at the beginning of the immune response, and only increase around day 2 post priming, IL-2 can accumulate in the environment without immediately triggering cell signalling, which is another feature associated with autoinducer-like signalling factors. Once the receptor levels reach a critical threshold, IL-2 signalling in CD4⁺ T cells is induced, leading to the activation of two successive feedback loops – a positive feedback loop driving further IL-2R α expression and eventually a negative feedback loop inhibiting the production of more IL-2³³¹.

1.5.1.2 Regulation of T cell differentiation

T collective behaviour is emerging as an underappreciated parameter controlling differentiation at the population level. Polonsky and co-workers³³² have recently shown that the effector and memory differentiation of CD4⁺ T cells is regulated by local cell density, with increased cell density driving MPEC differentiation of activating CD4⁺ T cells ([Figure 20](#)). IL-2 and IL-6 were identified as the main drivers of collective CD4⁺ T cell memory differentiation, with IL-2 regulating the proliferation rate and IL-6 promoting the survival of responding T cells. An additional regulatory role was attributed to the cell surface molecule SLAMF6.

Several groups investigated how homotypic clusters mediated through ICAM-1/Leukocyte function-associated antigen-1 (LFA-1) interactions shape the differentiation of T cells. In CD4⁺ T cells, LFA-1/ICAM-1 ligation in human T cells was reported to promote T_H1 polarization³³³. T-T interactions have furthermore been shown to trigger CD4⁺ T cell activation, proliferation, and differentiation *in vitro*³³⁴, and to induce the generation of CD4⁺ T cells with a regulatory phenotype, which is characterised by the production of IL-4, IL-10 and TGF- β ³³⁵. In CD8⁺ T cells, LFA-1/ICAM-1 dependent clustering promotes their expansion^{310,312,328}. Mechanistically, cluster dependent-expansion has been associated with CTLA-4 and CD80 interactions, which mediate activation of the Hippo pathway, linking the magnitude of clonal expansion by activated cells to their commitment to terminal differentiation through the induction of Blimp1³³⁶. However, the precise effect of LFA-1/ICAM-1 interactions on CD8⁺ T cell differentiation and function remains unclear, as blocking CD8⁺ T cell clustering appears to increase effector differentiation and IFN- γ production *in vitro* and following Incomplete Freund Adjuvant (IFA)-OVA priming³¹², while causing a reduction in effector differentiation^{310,328} and IFN- γ production *in vivo*³¹⁰. However, direct T-T cell interactions were also shown *in vitro* to enhance the functionality of T cells through amplification of cytokine and cytokine receptor expression³³⁷.

Although the reason for these discrepancies remains to be further investigated, it is tempting to speculate that T cell cluster formation through LFA-1/ICAM-1 interactions constitute a platform where different signals are integrated to tailor the overall immunological output to the context-specific type of infection or injury. Notably, available evidence suggests that T cell contacts also occur between distinct T cell subsets. For instance, direct contacts between antigen-experienced CD8⁺ memory T cells and naïve CD8⁺ T cells during priming were shown to promote the accelerated differentiation of naïve cells into T_{EM}

cells, at the expense of less differentiated stem cell memory and T_{CM} cells³³⁸. These effects are mediated by contact-dependent nonapoptotic Fas-FasL interactions. The extent of accelerated naïve CD8⁺ T cell differentiation was dependent on the density of the two subsets, alluding once more to a quorum sensing-like regulatory mechanism.

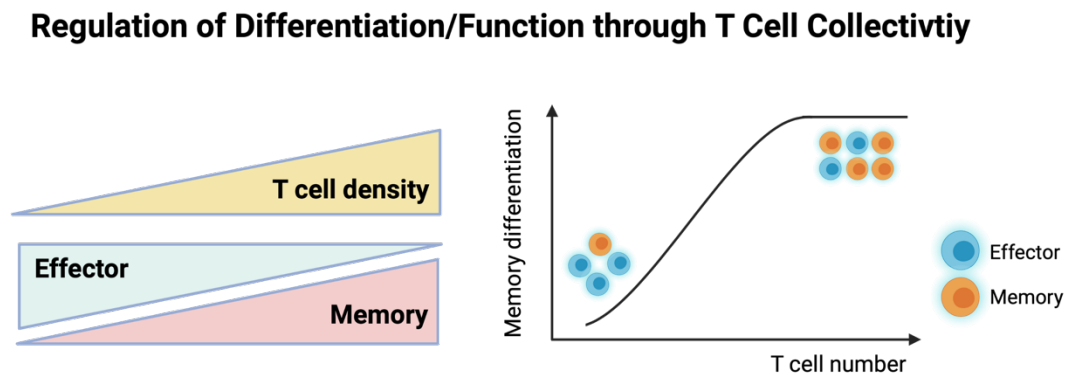


Figure 20. Current knowledge on T-T communication and associated functions. Following activation, T cells use a variety of communication channels to regulate their expansion and differentiation according to the injury. This results in a robust population response following infection, fine-tuned by T cell density [Created with BioRender.com].

1.5.2. Modes of communication between T cells

1.5.2.1 Contact-based T cell-T cell communication

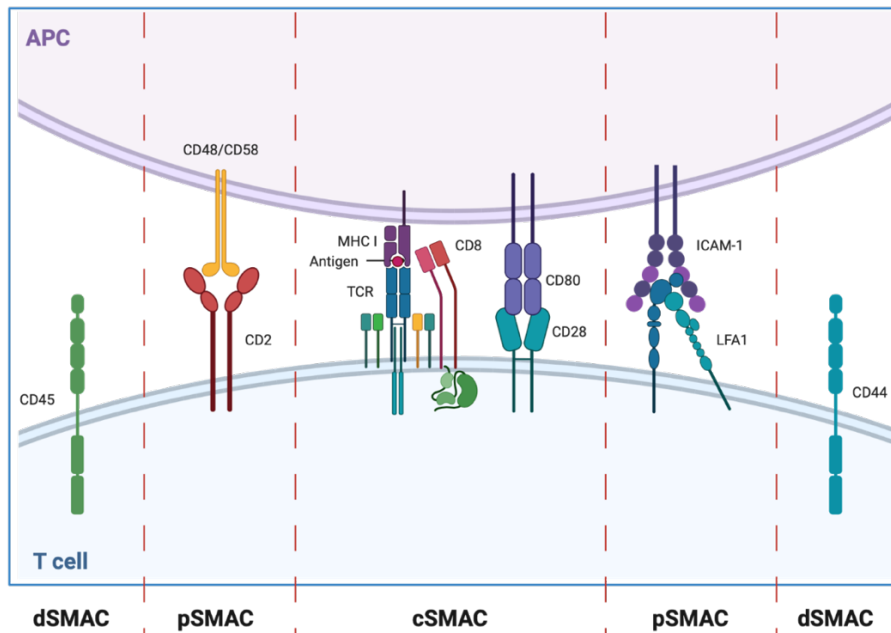
An important feature of an effective immune response is the specific activation of appropriate responders and the subsequent directed targeting of an invading pathogen while sparing self, which necessitates the exclusion of bystanders. In many cases this is accomplished by the formation of adhesive interfaces between the surfaces of interacting immune cells, with a synaptic space in between them. The immunological synapse (IS) formed between T cells and APCs is the best studied example of such a structure (**Figure 21A**)³³⁹, but other types of immune synapses, such as T-T synapses, have also been described^{310,311}.

Immunological synapses between antigen-specific T cells and APCs are sites of signal initiation and integration for T cell activation and subsequent programming^{340–342}, which is

characterised by the formation of a particular membrane structure, consisting of a central supramolecular activation cluster (cSMAC), surrounded by a peripheral supramolecular activation cluster (pSMAC). While the cSMAC consists mainly of clustering TCR, cross-junctionally interacting with pMHC, the pSMAC is rich in adhesion molecules such as LFA-1, which serve to stabilise long-term cell-cell interactions. Several other molecules, such as CD2, CD4, CD8, CD28, Lck and Fyn have also been identified to predominantly cluster in this peripheral region³⁴³. Of note, the formation of this structure was later discovered to not be a universal feature of all types of IS, such as those formed between T cells and APCs³⁴⁴.

Antigen-specific T cells also interact with themselves, forming clusters during priming in secondary lymphoid organs^{311,345–347}. T cell clusters are a hallmark of efficient T cell activation *in vitro*, and are mediated by the activated form of LFA-1, which binds to its ligands intercellular adhesion molecules (ICAMs), with the ligation to ICAM-1 (also known as CD54) being the best studied example^{348–351}. LFA-1, ICAM-1 and ICAM-3 are constitutively expressed in T cells, irrespective of their phenotype, and ICAM-1 expression is strongly upregulated upon T cell activation^{335,352,353}. Before being able to bind its ligands, LFA-1 needs to transition from a closed to an active conformation, which is induced by TCR triggering. As a result, only primed T cells engage in T-T contacts^{348,352}. Electron microscopy revealed that contacts between responding CD4⁺ T cells are organized in multifocal synapses³¹¹. Modest enrichment of CD86, MHC, TCR, CD4, and CD28 is found within the T-T synapse (**Figure 21B**), which contrasts the organization of the IS formed between T cells and APCs³¹¹.

A Immunological Synapse



B Current knowledge on T-T synapse topology

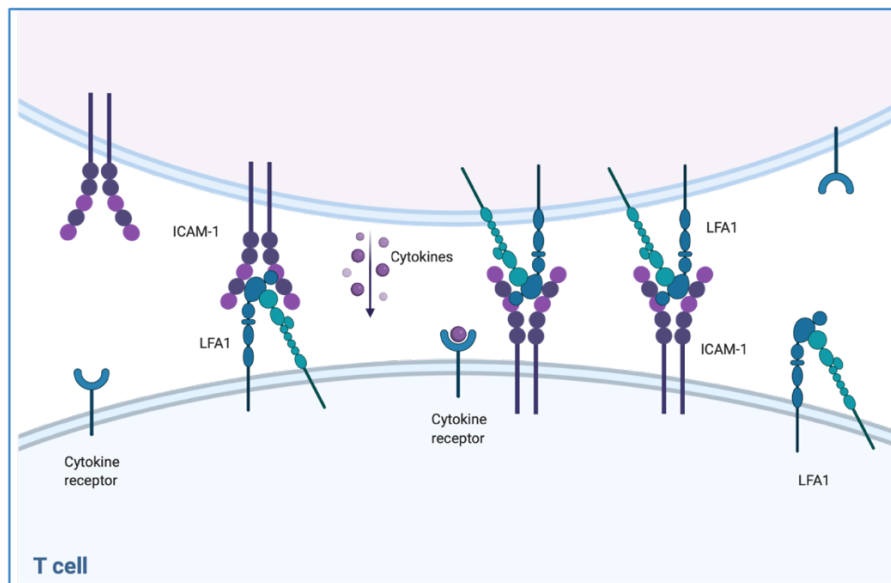


Figure 21. Topology of the immunological synapse (IS) and T-T synapses. Comparison between the immunological synapse formed between an antigen-specific T cell and antigen-presenting cell (APC) (A) and the T-T cell synapse (B). While the T-APC immunological synapse is characterized by a high level of membrane organisation, the T-T synapse does not show a clear structural organisation. Cytokines are secreted at the centre of T-T synapses, but it is unclear if they diffuse into the cleft between the cells or are immediately taken up by receptors, which have been observed to be enriched at contact sites, but without being organised into distinct clusters [Created with [BioRender.com](https://www.biorender.com)].

T-T interactions are quite dynamic, with dwell times of around 5–10 min and thus much shorter than the interaction times between T cells and APCs³¹⁰. Despite their transient nature, these structures facilitate the directional sharing of the cytokines IL-2 and IFN- γ between interacting T cells^{310,311}, thus providing a platform for concomitant signalling between different mediators. Evidence furthermore suggests that ICAM-1 and LFA-1 may not only function as adhesive structures, but also as co-stimulatory molecules at the T-T synapses³⁵⁴. This additional function was first highlighted by the observation that antibody-mediated ligation of ICAM-1 on human T cells has a costimulatory effect similar to anti-CD28 antibody-mediated co-stimulation, through enhancement of CD3-mediated T cell activation³⁵⁵. Of note, while LFA-1 is believed to be the primary adhesion molecule mediating T-T interactions, other adhesion molecules, such as members of the SLAM family, have also been implicated in T cell clustering³⁵⁶. While the precise role of T-T synapses awaits further experimental interrogation, it is intriguing to hypothesise that these transient interactions create critical platforms of integration for a diverse mix of signals shared exclusively between T cells.

Another contact-mediated mode of communication between T cells which is not restricted to immediately adjacent cells is created through the formation of so-called “*tunnelling nanotubes*” (TNTs)^{357,358}. These structures, consisting of long thin F-actin-based membranous channels, which can connect distant cells, have been described in numerous cell types including almost all immune cells³⁵⁹. While having been shown to enable the transfer of both soluble signals and cargo such as mitochondria or other organelles, it proved to date difficult to clearly elucidate the functional significance of these structures during an immune response, especially *in vivo*. Nevertheless, while many open questions remain, TNTs open up interesting possibilities for intercellular communication, by facilitating the sharing of a wide range of different biological material between cells³⁵⁷.

1.5.2.2 Long-range T cell-T cell communication

T cells produce and secrete soluble factors into the local environment, which enables them to translate and coordinate local interactions into tissue-wide global behaviour. Communication over long distances enables a population of immune cells to coordinate their individual behaviours and tune the global response towards an optimal outcome for the whole system. Cytokine penetration in tissues is dependent on the density of local cytokine consumers, which contributes to non-genetic cell-to-cell variability by creating variable cytokine niches in the reactive microenvironment³⁶⁰. The current dogma posits that cytokine penetration occurs when high cytokine levels overwhelm the consumption capabilities of local consumers and thus lead to systemic diffusion throughout the tissue. One of the best studied cytokines involved in T-T communication is IL-2, but multiple other inflammatory cytokines are also shared between T cells, including IFN- γ ³¹⁰ (reviewed in more detail in [Chapter 1.5.3](#)), interleukin-4 (IL-4)^{361,362}, IL-6^{363–365}, Transforming growth factor β (TGF- β)^{366,367} and interleukin-10 (IL-10)³⁶⁸ (recently reviewed by our group here¹⁰⁴).

T cells furthermore rely on chemokines for long-range communication. Chemokines are chemotactic cytokines orchestrating the migration and positioning of immune cells and are thus involved in diverse immune cell processes, such as fate determination or cell activation³⁶⁹. Activated CD4⁺ T cells clustering around DCs *in vivo* cause CD8⁺ T cell accumulation through CCL3/CCL4-CCR5 binding, which enhances CD8⁺ T cell/DC contact formation³⁷⁰, highlighting the crucial role of chemokines in allowing activating T cells to find each other and create a niche for themselves that serves as a platform for subsequent T-T communication. While this mechanism relies on another cellular intermediate, it is conceivable that T cells also directly attract each other via chemokines, given that they have been shown to upregulate multiple chemokine/chemokine receptor pairs upon activation³²⁸.

Immune cells are also known to communicate through the secretion of extracellular vesicles, in particular endosomally derived exosomes³⁵⁷. Exosomes can carry a number of different molecules, which makes them ideal conduits for the intercellular transfer of information. The transfer of genetic information in secreted exosomes such as messenger RNA (mRNA) and microRNA (miRNA) suggests that exosomes are also utilized to regulate the protein expression of target cells^{371–373}. Exosome secretion by T cells has been discovered by a number of studies to be involved in the regulation of T cell responses^{374–376}. However, most of the studies investigating exosome transfer between immune cells have been performed *in vitro* and the biological significance *in vivo* awaits further experimental elucidation.

1.5.3. T cell-T cell communication through the cytokine interferon-gamma

A recent computational analysis of publicly available datasets to pinpoint possible receptor/ligand pairs involved in early T-T communication, conducted by the group of Jan Rohr³²⁸, identified the IFN- γ /IFN- γ receptor (IFN- γ R) pair as their top cytokine hit. Earlier studies have already shown that CD8⁺ T cells secrete IFN- γ shortly after having been primed³⁷⁷, which was suggested to fulfil an important role in regulating the CD8⁺ T cell response^{133,378}. Multiple studies have furthermore implicated IFN- γ to be required for productive effector and memory CD8⁺ T cell differentiation^{133,378}, with some suggesting its involvement in direct T-T communication^{210,211,310,379}. While the precise function of IFN- γ on CD8⁺ T cells remains controversial^{210,211,379}, the available evidence to date suggests that IFN- γ may be an important mediator of T cell collectivity.

1.5.3.1 The structure of interferon-gamma

IFN- γ is a major component in immunological cell signalling and a critical regulatory protein for overall immune system function, which was first discovered in 1965, when leukocytes

were observed to secrete an antiviral molecule in response to phytohemagglutinin that was different to the previously described type I interferons³⁸⁰ (although the name IFN- γ was formally only introduced in 1980). IFN- γ is a pleiotropic cytokine produced by all major immune cell types and capable of eliciting diverse responses in both immune and non-immune cells. In the course of numerous infections, IFN- γ appears as a key cytokine produced by all subsets of T and NK lymphocytes and is often essential for effective protection^{381–383}. Its pleiotropic functions on the immune system will be reviewed in more detail in [Chapter 3.1.1](#).

The protein structure of IFN- γ comprises mainly of α -helices, with two molecules of IFN- γ forming a dimer in an antiparallel manner through non-covalent bonds³⁸⁴. The functional IFN- γ homodimer is immediately secreted and can be detected extracellularly as early as 6 hours after TCR activation, and its level typically peaks at 12-24 hours³⁸⁵. Notably, secreted mouse IFN- γ was shown to persist in the blood for much longer (0.94 hour) than other cytokines such as IL-2 (0.2 hour)^{386,387}, which is thought to be due to the expression of IFN- γ R by platelets, which may bind IFN- γ to facilitate systemic transport³⁸⁸.

1.5.3.2 Interferon-gamma receptor signalling

All these distinct and diverse responses are facilitated by the same signalling machinery driving expression of target genes via a conserved Janus kinase (JAK)/signal transducer and activator of transcription 1 (STAT1) axis, which is triggered upon binding of IFN- γ to the IFN- γ receptor ([Figure 22](#)). The IFN- γ receptor is composed of two ligand-binding IFN- γ R1 chains associated with two signal-transducing IFN- γ R2 chains, which are responsible for connecting the cytoplasmic transduction machinery, thus forming a tetramer complex to which the homodimer IFN- γ binds. The IFN- γ receptor lacks intrinsic kinase activity and requires the

adaptors JAK1 and JAK2 and the transcription factor STAT1 to mediate downstream signalling³⁸⁹. IFN- γ binding and receptor association triggers JAK2 autophosphorylation, transphosphorylation and activation, which uncovers docking sites for SH2 domain-containing signal transducers³⁹⁰, leading to STAT1 recruitment. Phosphorylation of STAT1 by JAK1/2 causes its translocation into the nucleus to bind GAS elements as a homodimer which leads to subsequent expression of target genes^{391,392}.

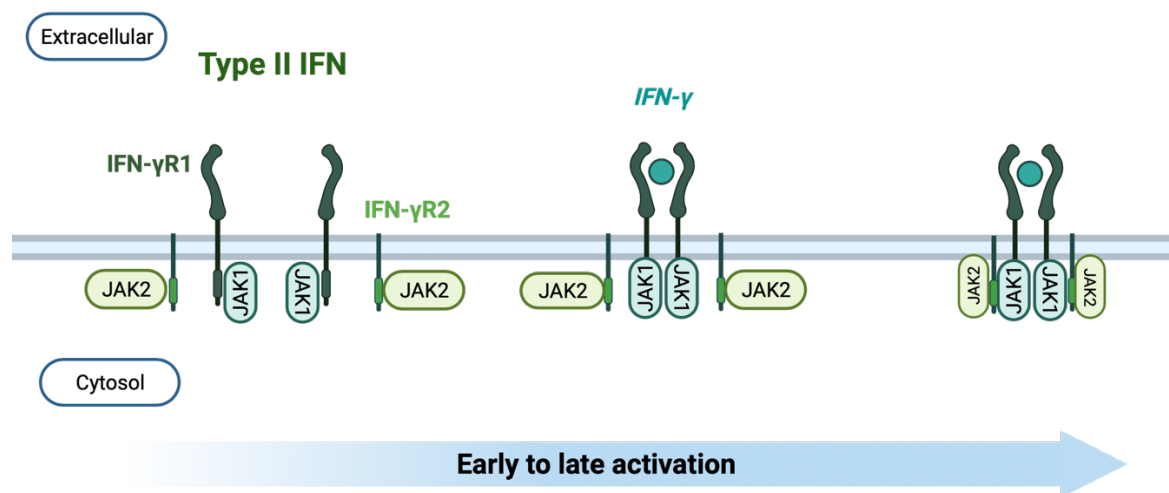


Figure 22. Interferon-gamma (IFN- γ) receptor. IFN- γ receptor (IFNGR) consists of IFN- γ R1 (α -subunit) and IFN- γ R2 (β -subunit)³⁸⁹. IFN- γ R1 is constitutively expressed by all nucleated cells at 200-25,000 molecules per cell, with its expression reaching its highest measured levels in non-lymphoid tissues such as the skin, nerves, placenta and syncytiotrophoblasts, alluding to a role for IFN- γ in embryonic development, tissue homeostasis and tolerance³⁹³. IFN- γ R1 mRNA expression levels by immune cells vary, with monocytes expressing the highest levels, followed by B cells, NK cells and lastly T cells^{394,395}. In contrast, the inducible expression of IFN- γ R2 by transcription factor SP1, activating protein 2 (AP-2) and nuclear factor κ B (NF- κ B) allows regulation of IFN- γ -induced signalling³⁹⁶. IFN- γ R1 has a major role in ligand binding, whereas IFN- γ R2 has a predominant role in signalling via Janus kinases (JAKs) and signal transducers and activators of transcription (STATs), albeit both receptor subunits are required³⁹⁷. Interestingly, only IFNGR1 has a nuclear localisation signal for translocation with STAT1^{385,398} [Created with [BioRender.com](https://www.biorender.com)].

The *Ifngr1* and *Ifngr2* genes are localised on chromosome 6 and 21, respectively, and their expression differs significantly. While IFN- γ R1 is constitutively expressed at moderate levels on the surface of almost all cells, IFN- γ R2 is constitutively expressed at low levels, and its expression is tightly regulated, according to the state of cellular differentiation or activation³⁹⁹. Thus, IFN- γ appears to regulate the expression of its own receptor on specific

cell types, representing a regulatory mechanism of cellular desensitization in response to cytokines present in the local microenvironment.

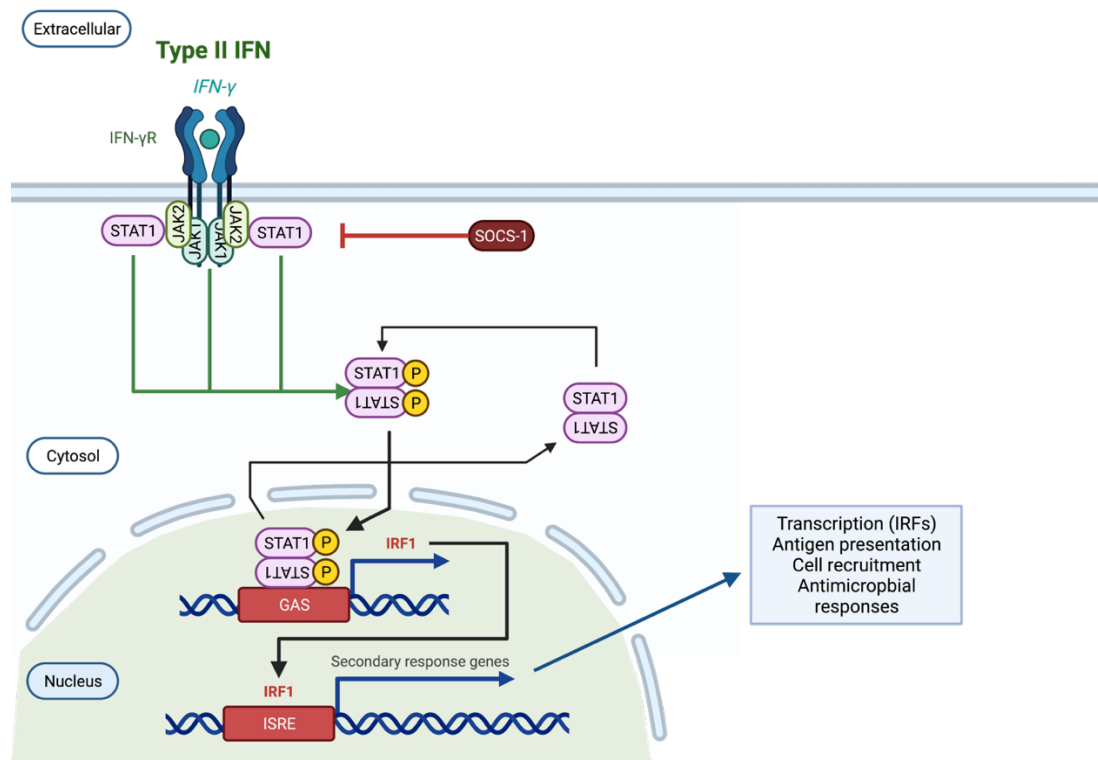


Figure 23. Interferon-gamma (IFN- γ) canonical signalling pathway. IFN- γ acts on its receptor to induce rapid and transient JAK-STAT signalling and interferon-stimulated gene induction. Initiated by the binding of IFN- γ , IFN- γ R1 and IFN- γ R2 oligomerize and transphosphorylate, activating Janus activated kinase (JAK) 1 and JAK2. These, in turn, phosphorylate IFN- γ R1, creating a docking site for the signal transducer and activator of transcription (STAT) 1. Phosphorylated STAT1 homodimerizes in an antiparallel configuration, forming a complex gamma-activated factor (GAF), which translocates to the nucleus and binds to gamma-activated site (GAS), located at the promoters of primary response genes, increasing their transcription. Upon induction, transcription factor interferon-regulatory factor 1 (IRF1) binds to interferon-stimulated response element (ISRE) and enhances the transcription of several secondary response genes responsible for several immunomodulatory functions. Suppressor of cytokine signalling (SOCS) proteins negatively regulate the IFN- γ pathway by inhibiting JAKs and STAT2 phosphorylation. Through dephosphorylation and deacetylation, the configuration of STAT1 homodimers reverts to parallel, triggering their exit from the nucleus [Created with [BioRender.com](https://www.biorender.com); adapted from Castro et al. 2018⁴⁰⁰].

IFN- γ signalling (**Figure 23**) has conventionally been defined as a cascade of tyrosine phosphorylation events, which results in the initiation of interferon-stimulated gene (ISG) transcription. JAKs activation induces phosphorylation of tyrosine residues in the IFN- γ R complex cytoplasmic domain, creating recruitment and binding sites for STAT1⁴⁰¹. Each molecule of the STAT1-STAT1 antiparallel homodimer is phosphorylated at Tyr701 by JAK1 and/or JAK2, resulting in immediate receptors dissociation. Maximum transcriptional activity

requires additional STAT1 phosphorylation at Ser727 via a PI3K- and AKT-dependent mechanism. Tyrosine phosphorylation of STAT1 by JAK promotes the formation of STAT1 homodimer complexes, known as gamma-activated factors (GAFs), which translocate to the nucleus and regulate gene expression through binding to gamma-activated site (GAS) elements in the promoters of interferon-stimulated genes (ISGs)⁴⁰². In addition to inducing STAT1 dimers that bind GAS elements and can cooperate with IFN- γ -induced interferon regulatory factors (IRFs), IFN- γ can also activate non-canonical transcriptional complexes that are similar to the interferon-stimulated gene factor 3 (ISGF3) complexes induced by type I interferons in that they contain IRF9 and bind to interferon-stimulated response elements (ISREs).

Studies of *Stat1*-knockout (KO) cells have also revealed STAT1-STAT1-independent IFN- γ signalling via STAT3 (non-canonical IFNGR signalling)^{403,404}. STAT3 and STAT1 are structurally similar and compete for binding to the phosphorylated Tyr419 of IFNGR. However, STAT1 was shown to display higher affinity for Tyr419⁴⁰⁵. STAT3 forms either a homodimer or a heterodimer with STAT1, which regulates GAS3 and GAS2, respectively. As IL-6 and IL-10 both signal via STAT3, the expression of cytokine receptors dictates which signalling pathway dominates^{385,406}.

One of the major primary response genes induced by STAT1 signalling is the transcription factor interferon-regulatory factor 1 (IRF1), a member of the IFN regulatory transcription factor family⁴⁰⁷. IRF1 functions as a transcription activator of ISRE sites, leading to the transcription of multiple secondary response genes³⁸². The transcriptional potency of STAT1 is furthermore modulated by post-translational modifications, most notably phosphorylation of the transcription activation domain^{382,408}.

1.5.3.3 Regulation of interferon-gamma receptor signalling

JAK-STAT signalling pathways are amongst the most central signalling pathways in effector T cell differentiation and in the survival and homeostasis of memory CD8⁺ T cells. The STAT family in mammals comprises seven members (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6), which are mainly known for their functions as transcription factors, although non-nuclear STAT functions have also been described^{409–411}. Although most JAK-STAT-associated cytokine receptors function through a dominant STAT protein, multiple STATs can be activated downstream of a single receptor. Understanding how different cytokines regulate gene expression in effector CD8⁺ T cells through distinct and cooperative mechanisms is thus often challenging. For example, STAT1 phosphorylation is known to not only reflect the magnitude of the IFN- γ response, as its phosphorylation may also be triggered by other cytokines, such as IL-21, IL-27 and IL-35⁴⁰⁰.

The JAK/STAT signalling pathway is regulated at several levels by positive and negative mechanisms. One of the most important negative regulators of this pathway is the suppressor of cytokine signalling (SOCS) proteins, which expression is increased in response to IFN- γ signalling through IRF1^{412,413}. SOCS proteins are an important set of inhibitors that refine the quality and quantity of JAK-STAT signalling in T cells⁴¹⁴. SOCS blocks the activity of JAKs by a negative feedback loop, but also modulates the downstream signalling of other cytokines. SOCS proteins can directly bind to phosphorylated tyrosine residues of activated JAKs, inhibiting both the recruitment of signal transducer adaptors, such as STATs, and JAK activity⁴¹². In addition, SOCS can trigger ubiquitination and proteasome degradation of JAK/STAT signalling components^{415,416}. SOCS1 can even prevent T_{reg} cells from producing IFN- γ via STAT1 suppression, preventing their conversion into effector cells⁴¹⁷.

Alternatively, the transcriptional activity of STAT1 can also be positively regulated by other signalling cascades triggered by IFN- γ binding, such as the mitogen-activated protein kinase (MAPK) pathway, protein kinase C, and PI3K/AKT, which induce STAT1 phosphorylation in its transactivation domain⁴¹⁸. Adding to the complexity, IFN- γ has also been shown to be capable of activating STAT1-independent pathways through other transcription factors, namely STAT3⁴¹⁹, STAT5⁴²⁰, NF- κ B⁴²¹, and AP-1⁴²².

In summary, given its complex signalling networks and the ability to cooperate with other signalling pathways, it is not surprising that IFN- γ exerts highly diverse context-dependent functions on immune cells, which helps to explain why different studies attributed opposing effects to the function of IFN- γ on CD8⁺ T cells. However, it is intriguing to hypothesise, that the pleiotropic nature of IFN- γ may also provide an ideal means for CD8⁺ T cells to self-regulate their response collectively according to the specific context they find themselves in.

1.6. Thesis hypothesis and objectives

The most effective CD8⁺ T cell responses to intracellular pathogens comprise a breadth of distinct T cell clones (clonal diversity), a diversity of effector T cell types (poly-functionality) and long-lived memory cells. Despite a remarkable degree of plasticity and functional heterogeneity in their individual response, the overall T cell response to infection is reproducible and robust. This implies a coordinated response on a global level to facilitate pathogen control whilst simultaneously maintaining self-tolerance. Much research has focused on individual T cell activation and differentiation, but how the stochastic individual responses of T cells are integrated into a collective response remains to be further investigated. Coordination on a system-wide level necessitates a critical level of intercellular communication and recent research has highlighted the importance of activating T cell-T cell (T-T) crosstalk in regulating the ensuing T cell response. Previous studies demonstrated that those direct T-T interactions create an ideal conduit for targeted cytokine sharing and may thus serve an important role in the collective regulation of T cell responses.

Cytokines are critical mediators in immune cell communication and the cytokine IFN- γ is a major component in immunological cell signalling and a critical regulatory protein for overall immune system function, including the control of T cell response behaviour. While its function in the effector phase of the CD8⁺ T cell response is well-studied, its role during the priming phase, when activating CD8⁺ T cells receive a mix of signals guiding their subsequent expansion and fate commitment, is much less well established. We hypothesised that IFN- γ -mediated T-T communication during the early phase of the response plays a critical role in regulating the ensuing CD8⁺ T cell response and that IFN- γ may serve as a critical modulator in enabling T cells to tune their response to the specific infection context.

To test this, we aimed to address the following objectives in chapters 3-5, respectively:

- 3) Establish models to study the direct effect of IFN- γ on CD8⁺ T cells and investigate its functional role in regulating CD8⁺ T cell responses to infection
- 4) Assess the cellular source of IFN- γ , its involvement in direct T-T communication and the temporal context of IFN- γ signalling in activating CD8⁺ T cells
- 5) Characterize in detail IFN- γ -secreting CD8⁺ T cell subsets and determine the stimuli that trigger IFN- γ expression in a specific subset of CD8⁺ T cells

The results presented in this thesis suggest that IFN- γ modulates CD8⁺ T cell response behaviour on multiple levels, both by shaping the expansion and functional avidity of the response. Mechanistically, IFN- γ communication is required to occur within a specific temporal context to successfully skew responding T cell behaviour. Moreover, productive IFN- γ signalling is dependent on CD8⁺ T cells communicating directly with each other. IFN- γ expression during the priming phase is furthermore restricted to a specific CD8⁺ T cell subpopulation, so-called virtual memory CD8⁺ T cells. Overall, this thesis demonstrates a novel autoregulatory role of IFN- γ and virtual memory T cells in controlling CD8⁺ T cell responses to infection.

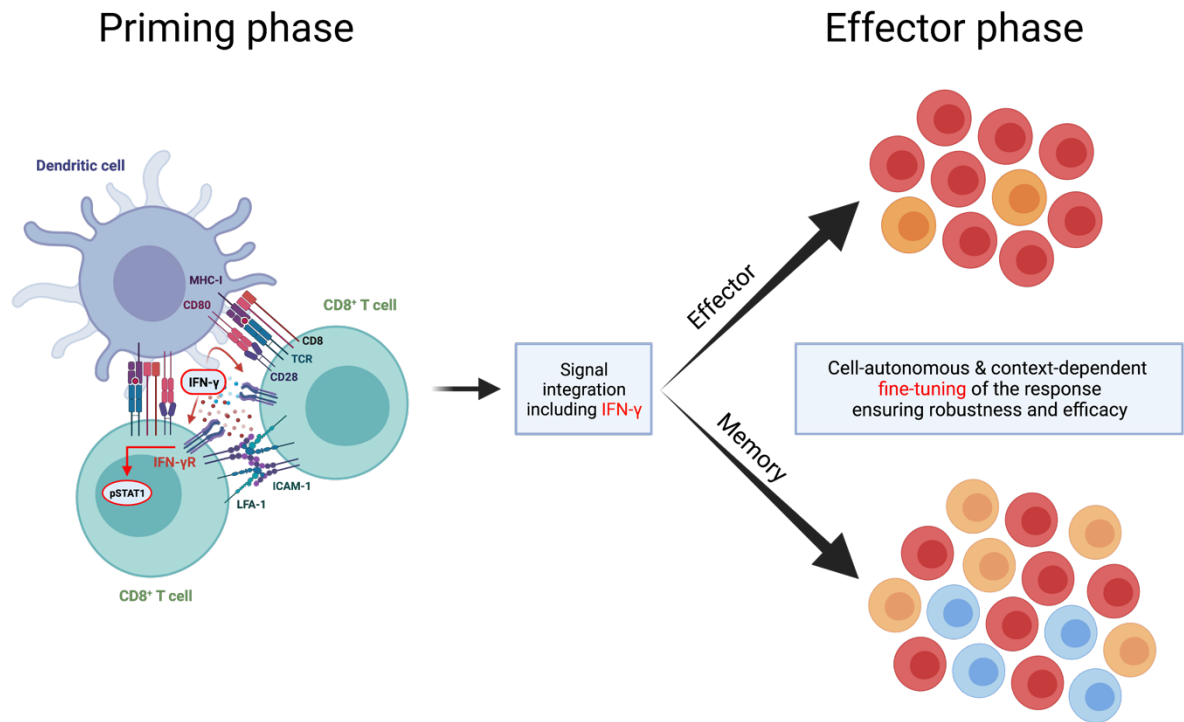


Figure 24. Graphical presentation of the central hypothesis of the thesis. CD8⁺ T cell activation results in the formation of multicellular clusters around antigen-presenting cells (APCs) and the expression of multiple cytokines, such as IFN-γ. Robust and efficient CD8⁺ T cell responses require coordination of individual T cell behaviours and fine-tuning of the quality and quantity of the ensuing response to the specific inflammatory context. We hypothesise that IFN-γ-mediated T cell-T cell communication may be ideally suited to fulfil this autoregulatory role [Created with BioRender.com].

2. Materials and Methods

2.1. Animals

CD8a-Cre^{GFP} (JAX stock no.: 008766), IFN- γ R floxed (JAX stock no.: 025394), IFN- γ -GREAT^{YFP} (JAX stock no.: 017580), P14 (JAX stock no.: 004694), Nur77-GFP (JAX stock no.: 018974), CD8a KO (JAX stock no.: 002665) and OT-I (JAX stock no.: 003831) were purchased from Jackson Laboratory (<https://www.jax.org>). 5-weeks-old wild-type C57BL/6J males were purchased from Charles River, UK (JAX stock number: 000664) and housed for 1-2 weeks prior to experimental start. To generate CD8 IFN- γ R KO mice *CD8a*-Cre^{GFP} mice were crossed with IFN- γ R floxed mice, which were subsequently crossed to Rosa26-tdTomato mice (kind gift from the group of Tal Arnon). Mice were bred, fed and housed under specific pathogen-free (SPF) conditions in the Biological Services Unit at the Kennedy Institute of Rheumatology, University of Oxford. All experiments involving mice were conducted in agreement with the United Kingdom Animal (Scientific Procedures) Act of 1986 and performed in accordance to approved experimental procedures by the Home Office and the Local Ethics Reviews Committee (University of Oxford) under UK project license (PPL P4BEAEBB5).

2.2. Generation of mixed bone marrow chimeric mice

Recipient mice were irradiated with 4.25 Gy per cycle for two irradiation cycles, 4 hours apart. Bone marrow from IFN- γ KO, CD8a KO or C57BL/6J mice were prepared from femur and tibia. Cells were counted using a haemocytometer and diluted to a concentration of 1.25×10^6 cells/mL in PBS. Cell suspension was filtered through a 40 μ M strainer shortly prior to intravenous injection of 100 μ L (equivalent to 1.25×10^5 cells/mouse). Mice were kept for two-week post-transplantation on antibiotics (Enrofloxacin) in drinking water to avoid infection.

2.3. Genotyping

Ear clips were lysed in 75 μ L lysis buffer (50 mM NaOH and 0.5 M EDTA in ddH₂O) lysis solution for 30 min at 95°C. Lysis was quenched by adding 75 μ L neutralisation buffer (Tris-HCl in ddH₂O). Polymerase chain reaction (PCR) was performed for each individual transgene in 20 μ L reactions using my-TaqTM Red Mix (Bioline, #BIO-25044). All primers were used at a concentration of 100 μ M.

Volume [μ L]	<i>CD8a</i> -Cre	IFN- γ R ^{f/f}
Per primer (100 μ M)	1	1
2x MyTaq RedMix	10	10
RNase/DNase-free water	2	2
DNA sample	4	4

Table 1. Reaction mix used for genotyping by Polymerase chain reaction

Primer sequences:

CD8a-Cre: CAA TGG AAG GAA GTC GTG GT
TGG GAT TTA CAG GGC ATA CTG
CAC ACA TGC AAG TCT AAA TCA GG

IFN- γ R^{f/f} TTG TTT GAT CCA TTC TTT AAA TTG
GCT TCT TTG AAG GGC TGG A

Cycling conditions:

Step	<i>CD8a</i> -Cre	IFN- γ R ^{f/f}
1	94	94
2	94	94
3	65	65
4	68	68
5	Repeat steps 2-4 for 10 cycles	Repeat steps 2-4 for 10 cycles
6	94	94

7	60	60
8	72	72
9	Repeat steps 6-8 for 28 cycles	Repeat steps 6-8 for 28 cycles
10	72	72
11	Hold	Hold

Table 2. Cycling conditions used for genotyping by Polymerase chain reaction

PCR products were separated on a 2 % agarose (Invitrogen, #16500-500) gel, prepared from TAE buffer (40 mM Tris-Acetate, 1mM EDTA) with SYPR® Safe DNA Stain (Invitrogen, #S33102, dilution 1:10.000), and were run at 120 V for 25 minutes until separated. Bands were visualised via G-Box (Syngene).

2.1. Treatment of mice

For indicated experiments, mice received a single intraperitoneal (i.p.) injection 16-24 hours post infection of 75 µg of isotype matched control antibody (rat IgG1, BioXCell, #BE0088) or anti-Interferon-gamma (BioXCell, clone: XMG1.2).

2.2. Infections

Mice were given an intravenous (i.v.) injection of 20×10^3 colony-forming units (cfu) of *Listeria monocytogenes* (LM) expressing either a secreted form of OVA (LM-OVA) or one of the cognate peptides (LM-N4, LM-V4, LM-T4 or LM-gp33). LM strains were provided by Dietmar Zehn (TU Munich). Frozen down LM aliquots were expanded in Brain Heart Infusion (BHI) broth (Sigma, #53286-100G) and LM suspensions were plated on BHI agar plates (Sigma, #70138-500G). Aliquots were frozen down at -80°C in freezing media (20% Glycerol [Fisher Scientific, #BP229-1] in BHI broth). Splenocytes were subsequently isolated from spleens of infected mice at the indicated time points for further analysis.

2.3. Isolation of CD8⁺ T cells

CD8⁺ T cells were isolated through negative separation from lymph nodes and spleens of 6- to 12-week-old naïve mice, using the MojoSort™ CD8⁺ T cell isolation kit (Biolegend, #480008). Harvested organs were meshed in isolation buffer (1x PBS (Gibco, #20012-068) supplemented with 2% FCS (Sigma, #F9665-500ML) and 10 mM EDTA (Santa Cruz, #sc-286969) through a 70 µM strainer to collect the single cell suspension in a 50 mL falcon tube, which was counted and resuspended to obtain a final concentration of 10⁸ cells/mL. Resuspended cells were then sequentially mixed and incubated with Biotin-Antibody cocktail and nanobeads for 15 minutes on ice, with each being added to reach 10-fold dilution. Following incubation, 2.5 mL isolation buffer was added to the mixture, which was then transferred to collection tubes for placement in MojoSort™ magnets (Biolegend, #480019) for 5 minutes at room temperature. The liquid containing CD8⁺ T cells was collected in a 15 mL falcon tube and was resuspended in complete RPMI (RPMI 1640 [Gibco, #21870-076] supplemented with 2% FCS and 100x Penicillin-Streptomycin [Gibco, #10378-016]) to reach a concentration of 10⁶/mL for seeding.

2.1. Isolation of splenocytes

Spleens were harvested from mice at indicated time points and collected in 3 mL PBS in 6-well plates on ice. Spleens were then mashed in 1x PBS and filtered through 70 µM filter paper for the collection of the splenocyte suspension in a 15 mL falcon tube. The splenocytes were resuspended in 3 mL red lysis buffer (155 mM NH₄Cl, 12 mM NaHCO₃ and 0.1 mM EDTA in ddH₂O) and incubated on ice for 5 minutes, before being washed with 1x PBS twice and counted using the CASY® cell counter (OMNI Life Science).

2.1. Adoptive naïve T cell transfer

CD8⁺ T cells ($2-3 \times 10^6$ cells or 500.000 cells for 24 hours or d7-10 experiments, respectively), isolated as described above, were transferred into recipient, gender-matched mice by i.v. injection. Mice were infected 16-24 hours later. Depending on the time point of analysis, splenocytes were subsequently isolated from spleens of adoptively transferred animals either 24 hours or 7-10 days post-infection for downstream processing of isolated cells. Adoptively transferred CD8⁺ T cells were distinguished from host splenocytes by staining for congenic markers (CD45.1⁺ and/or CD45.2⁺). Efficiency of adoptively transferred cell recovery was estimated to average 80-90%.

2.2. *In vitro* T cell stimulation assay

For *in vitro* re-stimulation experiments, cells were activated at low density either with cognate peptide or phorbol 12-myristate 13-acetate (PMA) (2 ng/mL) and ionomycin (20 ng/mL). For *in vitro* stimulation experiments, 96-well U-bottom plates were seeded with isolated CD8⁺ T cells resuspended in complete RPMI, at a concentration of 10^5 /well and either stimulated with bone marrow-derived dendritic cells (BMDCs) loaded with cognate peptides at 10 ng/mL or antibodies. Peptides were either purchased (SIINFEKL, Sigma, #S7951) or kindly provided by Dietmar Zehn (TU Munich).

For experimental conditions including TCR stimulation, wells were pre-coated with anti-CD3 ϵ (Biolegend, #145-2C11) prepared in PBS at 1 μ g/mL for 2-3 hours at 37°C before being emptied for seeding. For ICAM-1 stimulation, wells were pre-coated with 5 μ g/mL ICAM-1 (Biolegend, #553006) for 2-3 hours at 37°C before being emptied for seeding. Following coating with anti-CD3 ϵ and/or ICAM-1, cytokine preparations were added to the wells

according to the specified experimental conditions, which included different combinations of IL-12 (Biolegend, #577004), IL-18 (Biolegend, #767004), IL-15 (Biolegend, #566302), TNF (PeproTech, #315-01A-20uG), IL-33 (Biolegend, #580504), IFN- α (Biolegend, #752804), IFN- β (Biolegend, #581304), IL-2 (Biolegend, #575404), IL-1 β (Biolegend, #575102), and IL-7 (PeproTech, #217-17). For co-stimulation, 1 μ g/mL anti-CD28 (Biolegend, #37.51) was also added to wells pre-coated with anti-CD3 ϵ . The cells were incubated overnight under the specified conditions at 37°C in 5% CO₂ before being subjected to staining and flow cytometry analysis.

2.3. *In vivo* cytotoxicity assay

Isolated and washed splenocytes were resuspended in complete RPMI and divided into two 15 mL falcon tubes, each having 1 mL splenocyte suspension. OVA peptide SIINFEKL (N4) was added to one splenocyte suspension to a final concentration of 10 μ g/mL (peptide-loaded fraction). Both the peptide-treated and untreated splenocyte suspensions were incubated at 37°C for 30 minutes and shaken half-way. The cells in both tubes were counted and resuspended to obtain 1 mL suspensions containing 10×10^6 cells. For the initial experiment, peptide-loaded and non-loaded splenocyte suspensions were stained with CFSE prepared in 1x PBS at 5 μ M and 0.5 μ M, respectively, for 25 minutes at room temperature in darkness. In the optimized protocol, the peptide-loaded and non-loaded cell suspensions were incubated with 1 μ M eFluor 670 and 2 μ M CFSE in 1x PBS, respectively. The splenocyte suspensions were mixed with their respective dye solutions at a 1:1 ratio. The dye-stained cell suspensions were centrifuged and resuspended in 1x PBS, before being mixed at a 1:1 ratio for injection into LM-OVA-infected recipient mice, as well as a naïve mouse used for control. Splenocytes were isolated from the spleens of infected and naïve mice 24 hours following injection, and

cytotoxic killing by OVA-specific CD8⁺ T cells was assessed through flow cytometry analysis. The degree of cytotoxicity is given as the percentage of target cell lysis relative to the naive mouse, calculated by the following formula: $100-100 \times (R_{\text{infected}}/R_{\text{naive}})$, where the R values are equal to the % peptide-loaded population/% non-loaded population ratios in the infected and naive mice (the formula was adapted from here⁴²³).

2.4. Flow Cytometry

Single-cell suspensions obtained from spleen or cultured CD8⁺ T cells were used for flow cytometry analysis. Single cell suspensions were stained in V-bottom 96-well plates in a total staining volume of 50 μ L flow cytometry buffer (2% FCS, 2 mM EDTA, and 0.02% sodium azide in 1x PBS). First, live dead staining and surface staining was performed using Zombie NIR Fixable Viability Kit (Biolegend, #423106/423105), TruStain FcX[™] (anti-mouse CD16/32, Biolegend, #101319) and fluorochrome-conjugated primary antibodies (Biolegend, Cell Signaling Technology or BD Biosciences), respectively. For experiments that included tetramer staining, cells were incubated with either Alexa Fluor 647- or BV421-conjugated, N4-specific MHC I tetramers (obtained from the National Institutes of Health Tetramer Core Facility [Emory University, Atlanta]) diluted 1:500 in flow cytometry buffer for 30 minutes at room temperature, prior to surface staining. If not otherwise indicated, all cells were fixed either in 4% paraformaldehyde (PFA) for 10 minutes at room temperature or using eBioscience[™] Foxp3/Transcription Factor Staining Set (Invitrogen, #00-5523-00) for 30 minutes at 4°C. Intracellular transcription factor staining was performed after fixation and permeabilization using fluorescently labelled primary antibodies. Intracellular cytokine staining was performed after 15 minutes fixation using BD Cytofix/Cytoperm[™] Fixation/Permeabilisation Kit (BD Biosciences, #554714). If cells were counted by beads on

flow cytometer, 50.000 Polybead Polystyrene Black Dyed Microspheres were added per sample before sample acquisition (Polyscience, #24293). Bead compensation controls were prepared using UltraComp beads (eBioscience, #01-2222-42). Flow cytometry data were recorded on BD LSRII or FortessaX20 using DIVA software and analysed using FlowJo™ software (Tree Star). For staining of intracellular cytokines, cultured cells were treated with 7 µg/mL brefeldin A (BFA, ChemCruz, #sc-200861A) 30 minutes post-stimulation and incubated for 4.5 hours at 37°C in 5% CO₂ before further processing and animals were injected i.p. with 250 µg BFA 6 hours before being culled at the indicated time after infection. Surface markers used for flow cytometry analysis included: anti-CD8 (Biolegend, clone: 53-6.7), anti-CD4 (Biolegend, clone: RM4-5), anti-CD69 (Biolegend, clone: H1.2F3), anti-CD44 (Biolegend, clone: IM7), anti-CD49d (Biolegend, clone: R1-2), anti-NK1.1 (Biolegend, clone: PK137), anti-KLRG1 (Biolegend, clone: 2F1/KLRF1). Antibodies used for intracellular cytokine staining included anti-IFN-gamma (Biolegend, clone: XMG1.2) and anti-TNF (Biolegend, clone: MP6-XT22).

2.5. Fluorescence-Activated Cell Sorting (FACS)

Isolated CD8⁺ T cells were stained and filtered through a 70 µM strainer before sorting commenced. Single-cell sorting was performed on FACS Aria (BD Biosciences). After doublet exclusion, isolated single cells were sorted into 5 mL FACS tubes containing 1 mL of collection media (20% FCS in 1x PBS). Sorted cells were stored on ice until further processing.

2.6. Single-cell RNA sequencing

Isolated CD8⁺ T cells were cryopreserved in cryopreservation media (20% FBS and 10% DMSO in RPMI) which were further processed by Single Cell Discoveries, Netherlands. In brief, frozen down samples were thawed and centrifuged at 150 rcf for 3 min before the

supernatant was removed and cells were resuspended in 1 mL thawing media (0.04% BSA in 1x PBS). Resuspended cells were centrifuged again, supernatant discarded, and cells resuspended again in 1 mL thawing media, which was used for cell counting using a Countess II Automated Cell Counter or a haemocytometer to obtain a final cell concentration of 700-1200 cells/uL. Samples were further processed in accordance with 10x Genomics single cell protocols. Single cell libraries were prepared using the Chromium 3' v2 platform (10x Genomics, Pleasanton, CA) following the manufacturer's protocol. In brief, single cells were encapsulated into gel beads in emulsions (GEMs) in the GemCode instrument followed by cell lysis and barcoded reverse transcription of RNA, amplification, shearing and 3' adaptor and sample index attachment. Approximately, 5000 to 7000 cells were recovered. Libraries were sequenced on the Illumina NovaSeq 6000. Read mapping, alignment to GRCm38 and quantitation of sample count matrices was performed with the 10x Genomics Cell Ranger pipeline (v 4.0.0). Datasets were analysed using Seurat version 4.0.5 (Hao et al., 2021). Samples were merged on a single Seurat object. We filtered out cells having less than 600 and more than 5,000 detected genes, cells in which mitochondrial protein-coding genes represented more than 10% of UMI. Cells were then further filtered based on the expression of *Cd2*, *Cd8a* and *Cd8b1*. Samples were normalized with the scTransform function of Seurat and variation associated with mitochondrial and ribosomal UMI percentage were regressed out. Principal components were calculated using the top 3,000 variable features. These genes were used as input for principal component analysis (PCA), and significant PCs (n = 30) identified using Seurat ("JackStraw" test and "Elbowplot"). Clustering was performed with the Louvain algorithm (n = 30 PCs, resolution = 0.3). For differential expression analysis, NormalizeData and ScaleData were run on the RNA assay of the integrated data. Significant differentially expressed genes between clusters were identified using the "FindAllMarkers"

function, Wilcoxon test and selecting markers expressed in at least 25% of cells. Significant differentially expressed genes between stimulation within clusters were identified using the “FindMarkers” function, Wilcoxon test and selecting markers expressed in at least 25% of cells. Pathway analysis was performed with Fast gene set enrichment analysis (fgsea), using the Gene Ontology or the Reactome pathway repositories and with the R package gsfisher.

2.7. Statistical Analysis

One-way ANOVA was selected for pairwise comparisons across multiple experimental conditions and unpaired student's *t*-tests was used to compare two conditions for statistical significance. Data were considered statistically significant when $p < 0.05$. Data are presented as mean $\pm$ SEM or $\pm$ SD. Statistical analysis was performed using GraphPad Prism 7 software. Ns=non-significant, * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0002$ and **** $P < 0.0001$.

3. Modulation of CD8⁺ T cell responses to infection through the cytokine interferon-gamma

3.1. Introduction

This chapter will focus on the creation and validation of new experimental tools and their subsequent utilisation as *in vivo* models to study the effect of IFN- γ on mature CD8⁺ T cells. While much is known about the function of IFN- γ during the effector phase of the response, the direct modulatory function of IFN- γ on CD8⁺ T cells remains controversial and to a large extent unclear. In the following introduction, I highlight the current understanding of IFN- γ in shaping immune cell behaviour and the CD8⁺ T cell response, as well as strategies and experimental tools used to study CD8⁺ T cell behaviour *in vivo*.

3.1.1. Interferon-gamma as a modulator of immune cell behaviour and CD8⁺ T cell responses

IFN- γ modulates immune cell behaviour in a highly complex manner. Its pleiotropic nature and the fact that all nucleated cells are theoretically capable of responding to this cytokine signal complicates the dissection of its specific effect on CD8⁺ T cells considerably.

It is well established that IFN- γ stimulates the expression of many different genes. Notable IFN- γ -regulated genes include: **(1)** genes encoding for proteins involved in antigen presentation such as MHC Class I molecules and the costimulatory molecules CD80 and CD86; **(2)** genes orchestrating cell proliferation such as *Myc*, *Rb1* (encoding for Retinoblastoma-associated protein), as well as *Cdkn1a* and *Cdkn1b* (encoding for the Cyclin-dependent kinase inhibitor 1A or 1B, respectively); **(3)** genes involved in apoptotic processes such as *Fas* and

*Fas*l; **(4)** immunosuppressive genes encoding for *Cd274* (Programmed cell death 1 ligand 1 [PD-L1]) and *Pdcd1lg2* (PD-L2), as well as *Ido1* (Indoleamine 2,3-dioxygenase 1, which catalyses the breakdown of tryptophan into kynurenines, resulting in T cell apoptosis induction via caspase 8 activation and mitochondrial cytochrome c release⁴²⁴); **(5)** genes involved in T cell effector functions such as *Tbx21* (encoding for the transcription factor T-bet), *Serpinb9* (encoding for Serpin B9, which inactivates granzyme B) and *Itk* (encoding for the IL-2 inducible T cell kinase involved in T cell activation and T_H2 differentiation); **(6)** migration-related genes such as *Cxcr3*, *Cxcl9*, *Cxcl20*, *Cxcl11*, *Ccl2*, *Ccl5* and *Icam1*; and **(7)** genes encoding additional transcription factors such as *Irf1* (Interferon regulatory factor 1), *Bcl6* (B cell lymphoma 6), *Nfkb1* (NF-κB), *Creb1* (cAMP-responsive element-binding protein 1), *Jun* (Activator protein 1 subunit JUN) and *Fos* (Cellular Oncogene FOS)^{385,425,426}.

Of particular relevance in the context of studying CD8⁺ T cell responses is the effect IFN-γ has on MHC class I expression and the induction of chemokine receptor and integrin expression^{427,428}. Before focusing on the function IFN-γ exerts specifically on CD8⁺ T cell responses, insights about the immunomodulatory role of IFN-γ gained from studying a variety of other immune cells will be briefly summarised.

3.1.1.1 CD4⁺ T cells

Well-established is the role of IFN-γ in inducing T_H1-immune-response-related genes and robust T_H1 cell polarization via a positive feedback loop maintaining T-bet expression through STAT1-mediated signalling (**Figure 25**)^{429–432}. STAT-1-dependent upregulation of T-bet⁴³³ in turn induces IL-12R expression, and IL-12 can then signal in a STAT4-dependent manner to further increase T-bet and IFN-γ expression⁴³⁴. In addition, IFN-γ-secreting T_H1 cells increase their resistance for the apoptosis-inducing effect of IFN-γ signalling by decreasing *Ifngr2*

expression following differentiation. By contrast, T helper 2 (T_H2) cells that do not produce IFN- γ express higher levels of the IFN- γ R2 subunit, rendering them particularly susceptible to the inhibitory effects of IFN- γ ^{435–437}. However, IFN- γ R2 downregulation may also be induced in T_H2 cells when they are exposed to IFN- γ ⁴³⁶. Thus, IFN- γ appears to modulate its functional outcome through cellular desensitization by regulating the expression of its own receptor on specific cell types in response to its local microenvironment presence. As a result, IFN- γ R2 expression can be a limiting factor in IFN- γ responsiveness that can modulate the subsequent immune response mediated through the downstream signalling components and help to dictate the ensuing functional outcome, such as the T_H1–T_H2 phenotype switch⁴⁰⁰. Additionally, IFN- γ prevents T_H2 polarization via SOCS1 and T-bet, which inhibit Interleukin-4 receptor (IL-4R) signalling and GATA3 expression and function, respectively^{438,439}.

T helper 17 (T_H17) cell polarization is also actively repressed and T_H1 cell commitment reinforced via T-bet and inhibition of RUNX1⁴⁴⁰. It was also discovered that upon TCR stimulation in CD4⁺ T_H cell precursors, IFN- γ R1 becomes localised at the immunological synapse along with STAT1, which primes the cells to rapidly mediate T_H1 cell signalling upon TCR triggering. This co-recruitment process is inhibited by IL-4R expression in T_H2 cells. Notably, it was shown that IFN- γ R2 is ultimately downregulated in T_H1 cells, which indicates that the continuation of the T_H2/T_H17 cell antagonism may be reinforced by T-bet, rather than STAT1³⁸⁵. IFN- γ furthermore promotes apoptosis via downregulation of Bcl-2 expression, upregulation of *FAS* and *FASL*, and creation of a detrimental oxidative environment^{441–443}. Of note, in models of autoimmune disease and bacterial infection, T_{reg} cells have been reported to adopt a T_H-like (T-bet⁺IFN- γ ⁺) effector phenotype to better suppress the appropriate effector responses⁴⁴⁴, but how IFN- γ precisely regulates T_{reg} cells remains unclear³⁸⁵.

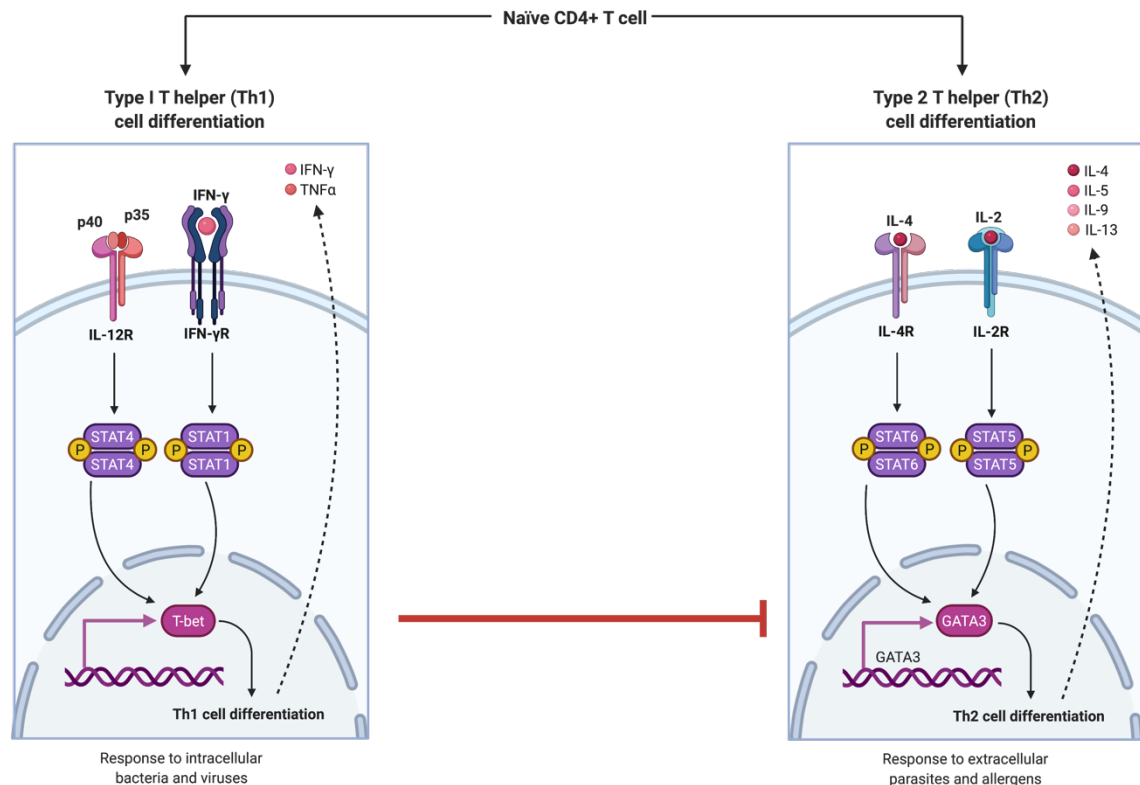


Figure 25. T helper cell (T_H) polarization through interferon-gamma (IFN-γ). CD4⁺ T cells can polarize into different cell subtypes in response to distinct cytokines. IFN-γR signalling promotes T_H1 differentiation through signal transducer and activator of transcription proteins 1 (STAT1)-mediated signalling, which furthermore prevents T_H2 polarization through suppressor of cytokine signalling 1 (SOCS1) and Tbet induction that inhibit interleukin-4 receptor (IL-4R) signalling and GATA3 expression and function, respectively [Created with BioRender.com].

3.1.1.2 Natural killer cells and dendritic cells

IFN-γ is an important effector cytokine secreted by natural killer (NK) cells, and additionally thought to also promote the maturation and activation of NK cells through STAT1⁴⁴⁵.

A key immune function of IFN-γ is the induced expression of MHC I and II molecules by APCs, such as DCs, macrophages and B cells⁴⁴⁶. MHC II upregulation is mediated through the binding of STAT1 and IRF1 to promoter IV of the MHC class II transactivator (CIITA) that is the non-DNA-binding master regulator of MHC class II transcription⁴⁴⁷, while MHC I expression is induced via IRF1 binding to the promoter NLRC5, which is an important transcriptional regulator of MHC class I⁴⁴⁸. IFN-γ furthermore drives expression of the co-stimulatory

molecules CD80 and CD86 by APCs, which promote T cell activation via CD28 engagement, and thus counteracts immunosuppression via competitive binding of CTLA4 to CD28⁴⁴⁹.

In addition, IFN- γ also promotes DC differentiation into conventional type I DCs (cDC1s) through induced expression of CD80, CD86, MHC class I, CD40, CD54 and CC-chemokine receptor 7 (CCR7), and the production of IL-1 β and IL-12, which promote T_H1 cell differentiation and CD8⁺ T cell activation⁴⁵⁰. DCs also respond to IFN- γ by expressing IDO1, which promotes immunosuppression through metabolic disruption⁴⁵¹. IDO1 catalyses the breakdown of tryptophan into kynurenines, which leads to the induction of TGF- β secretion in DCs, which then promotes T_{reg} cell differentiation and immunosuppression⁴⁵².

3.1.1.3 B cells and macrophages

In the context of B cell immunity, IFN- γ has been shown to induce, in combination with B cell receptor and CD40, activating signals and expression of the germinal centre master transcription factor B cell lymphoma 6 (Bcl-6). Hence, IFN- γ was suggested to be required for spontaneous development of germinal centres and T follicular helper cells⁴⁵³ and also works in concert with IL-12 to promote antibody class switching from IgM to IgG2a, thus generating higher-affinity and specialised antibodies⁴⁵⁴.

IFN- γ was initially named “*macrophage activation factor*”, alluding to its role in driving classical activation of macrophages. IFN- γ signalling primes macrophages for activation by Toll-like receptor (TLR)-induced inflammatory responses, which drives macrophages towards a proinflammatory phenotype via the downregulation of microRNA (miR)-3473b, which suppresses polarization towards an anti-inflammatory phenotype^{455,456}. In addition, IFN- γ also promotes inducible nitric oxide synthase (iNOS) expression in myeloid cells, which catabolizes the essential amino acid L-arginine to the free radical nitric oxide (NO).

3.1.1.4 CD8⁺ T cells

IFN- γ is an important effector cytokine produced by cytotoxic T lymphocytes^{99,457} and is known to be critically involved in cell-mediated clearance of infected cells⁴⁵⁷. IFN- γ is known to promote apoptosis via TNF-related apoptosis-inducing ligand (TRAIL), expression of which is enhanced by IFN- γ -induced IRF1⁴⁵⁸.

In addition to its role as a crucial effector cytokine, IFN- γ has also been shown to modulate CD8⁺ T cell behaviour and differentiation in manifold ways. Pro-inflammatory cytokines are generally thought to intensify antigen-specific T cell expansion and to promote terminal differentiation and a shortened lifespan of affected CD8⁺ T cells⁹⁹. However, the exact function of IFN- γ on CD8⁺ T cell expansion and differentiation remains highly controversial due to several contrasting findings, with some studies reporting that IFN- γ signalling promotes expansion of activating CD8⁺ T cells^{211,379} while others demonstrated a limiting impact on expansion¹³³. Similar diverging findings have been reported for the effect of IFN- γ on CD8⁺ memory differentiation^{210,378}.

IFN- γ has furthermore been implicated in the regulation of the contraction phase of CTL responses via FAS-FASL-mediated and Bim-mediated apoptosis, both of which are induced by STAT1 signalling⁴⁵⁹. High levels of IFN- γ during expansion have also been shown to limit the size of the memory population by inhibiting or downregulating IL-7R α expression, thus likely reducing signalling by the prosurvival cytokine IL-7^{127,460,461}. The precise mechanism by which IFN- γ regulates IL-7R α expression remains elusive, but some evidence indicates the AKT-FOXO1 pathway to be involved^{462,463}. Of note, overexpression of *Ifngr2* on CTLs did not affect their development or proliferation but limited their cytotoxic activity in response to antigenic stimulation by an unknown mechanism⁴⁶⁴.

Besides its effect on CD8⁺ T cell expansion and differentiation, IFN- γ has also been implicated in regulating the immunodominance, and thus the clonality of the CD8⁺ T cell response, against LM- and LCMV-derived antigenic peptides^{133,465,466}, which was speculated to be potentially due to differences in antigen processing and epitope generation⁶⁷, incomplete pathogen clearance²¹⁰, or, alternatively, due to a selective advantage of fast-onset antigen-specific T cells, with the rapidity of IFN- γ production suggested to be a major criterion determining the cell's survival and future expansion⁴⁶⁶.

Lastly, IFN- γ has also been reported to upregulate the expression of PD-L1 and/or PD-L2 on many cell types and it was suggested that owing to the expression of both PD1 and PDL1 by T cells, self-inhibition may possibly occur in *trans*⁴⁶⁷. Lastly, IFN- γ is also critically involved in the orchestration of immune cell migration and is known to promote the recruitment of immune cells through transcriptional regulation of several chemokines, such as CXCL9, CXCL10 and CXCL11, and their cognate receptor CXCR3 on T cells, NK cells, monocytes and DCs⁴⁶⁸.

Considering the pleiotropic functions of IFN- γ on several distinct immune cell subtypes, it is also important to highlight that only a few of the aforementioned studies investigated the direct effect of IFN- γ on CD8⁺ T cells^{211,378,379,460,461}, while the remaining studies relied on either full IFN- γ knock-out models or experimental conditions that did not induce IFN- γ production^{122,127,133,459,469,470}. Notably, even the studies that investigated the direct effect of IFN- γ on CD8⁺ T cells reported disparate findings, with some showing that direct IFN- γ is dispensable for the regulation of CD8⁺ T cell expansion, contraction and memory formation^{378,461}, while others demonstrated a clear positive effect on CD8⁺ T cell expansion^{210,211}, and a direct involvement in the regulation of activated CD8⁺ T survival and contraction⁴⁶⁰, as well as memory formation⁴⁶⁰.

Given that the degree of IFN- γ induction and the specific cytokine pattern, in general, are pathogen-dependent¹²⁹, it is reasonable to assume that the relative impact of direct and indirect IFN- γ effects on CD8⁺ T cell response quantity and quality vary between experimental systems. The pleotropic and diverging effects of IFN- γ on CD8⁺ T cells and other immune cells thus make it clear that the specific context in which CD8⁺ T cells sense IFN- γ is critical in understanding and determining the specific signalling outcome of this cytokine on CD8⁺ T cells. It is also important to highlight that most studies discussed so far used transgenic, monoclonal T cell populations to investigate the direct function of IFN- γ on CD8⁺ T cells, leaving the functional contribution of this cytokine on the endogenous CD8⁺ T cell response mainly unelucidated.

The temporal context of this cytokine signalling event in CD8⁺ T cells also remains largely unexplored. The responsiveness of CD8⁺ T cells to IFN- γ is dynamically regulated. Naïve cells are responsive to IFN- γ during the priming phase of the response and downregulates IFN- γ R2 shortly after stimulation, which likely renders them unresponsive to IFN- γ for a certain period²⁰⁷. During this early time window in which the fate of CD8⁺ T cells has yet to be established, IFN- γ may exert its modularly function on the CD8⁺ T cell response and thus regulate CD8⁺ T cell behaviour. The homotypic T-T synapses formed between activating CD8⁺ T cells during the priming of the response have been shown to heighten the local sensitivities to cytokine-mediated signalling^{310,311}, which suggests that receiving IFN- γ signalling during this early phase of the response may have a particularly influential modulatory effect. In addition, activated IFN- γ ⁺ CTLs have been reported to not express IFN- γ R2 *in vitro* and display no upregulation of IFN- γ -inducible genes in response to IFN- γ ⁴⁶⁴, a mechanism which counteracts IFN- γ -induced apoptosis. Due to the reported decline in cytokine sensitivity within 24 hours after initial antigen encounter²⁰⁷, it was suggested that CD8⁺ T are required

to either receive IFN- γ early in the response or much later on, during the contraction phase, when they regain partial responsiveness to IFN- γ . However, the temporal context of direct IFN- γ signalling in CD8⁺ T cells has not been thoroughly investigated by the aforementioned studies and it thus remains unclear when CD8⁺ T cells need to sense this particular cytokine stimulus.

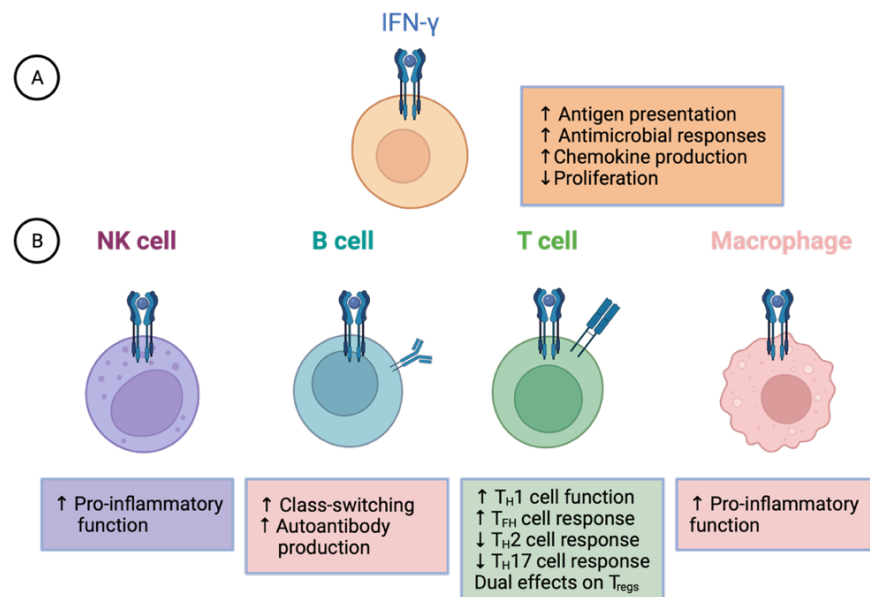


Figure 26. Effects of interferon-gamma (IFN- γ) on distinct immune cell subtypes. (A) IFN- γ has general effects on various immune cells, such as promoting antigen presentation. (B) Additionally, IFN- γ exerts specific effects on distinct immune cell populations, such as promoting class-switching in B cells. It is also important to highlight, that the effects of IFN- γ are highly context-dependent and can differ under homeostatic or disease conditions [Created with BioRender.com].

3.1.2. Context and aim of this chapter

Whilst the role of IFN- γ during the effector phase of CD8⁺ T cells responses is well-described^{99,457}, the regulatory function of IFN- γ on activating naïve CD8⁺ T cells remains largely elusive. The aim of this chapter was **(1)** to create and validate an appropriate experimental model to study the specific effect of IFN- γ on CD8⁺ T cells, **(2)** to investigate the immunomodulatory function of IFN- γ on the poly-clonality and poly-functionality of CD8⁺ T responses to infection and **(3)** to study the effect of IFN- γ on the CD8⁺ T cell memory compartment.

3.2. Results

3.2.1. Establishment and validation of a constitutive CD8⁺ T cell-specific interferon-gamma receptor knock-out mouse model

We sought to establish an animal model, which allows us to investigate the specific effect of IFN- γ on CD8⁺ T cells by targeted deletion of IFN γ -R1. Ensuring specific deletion in the CD8⁺ T cell compartment, while avoiding undesired off-target effects during T cell development in the thymus, required the use of a Cre recombinase system that is driven by a promoter sequence only active in peripheral CD8⁺ T cells. We identified the E8I-Cre system (*CD8 α -Cre*) reported by Maekawa and colleagues as the most suitable one for our purpose⁴⁷¹. The full E8I transgene comprises the 1.6-kilobase core of the E8I enhancer fragment, inserted in front of the *Cd8 α* promoter, as well as the Cre-encoding cDNA that also includes an internal ribosomal entry site (IRES)-GFP-poly(A) cassette, which was introduced after the *Cd8 α* promoter sequence. Importantly, Cre activity for this system has only been reported in CD8 α^+ CD8 β^+ $\alpha\beta$ T cells and CD8 α^+ CD8 β^- $\alpha\beta$ T cells but not in CD4⁺ CD8 α^- CD8 β^- $\alpha\beta$ T cells⁴⁷¹. We crossed this transgenic strain with IFN- γ R1^{flox/flox} (IFN- γ R KO) mice, to obtain a knock-out mouse model (Figure 27), in which the IFN- γ receptor is specifically deleted in CD8⁺ T cells (CD8 IFN- γ R KO). Cre activity in the E8I-Cre system was supposed to be indicated by concomitant green fluorescent protein (GFP) expression. However, in the mice we received from Jackson Laboratory we were unable to detect any GFP signal (data not shown).

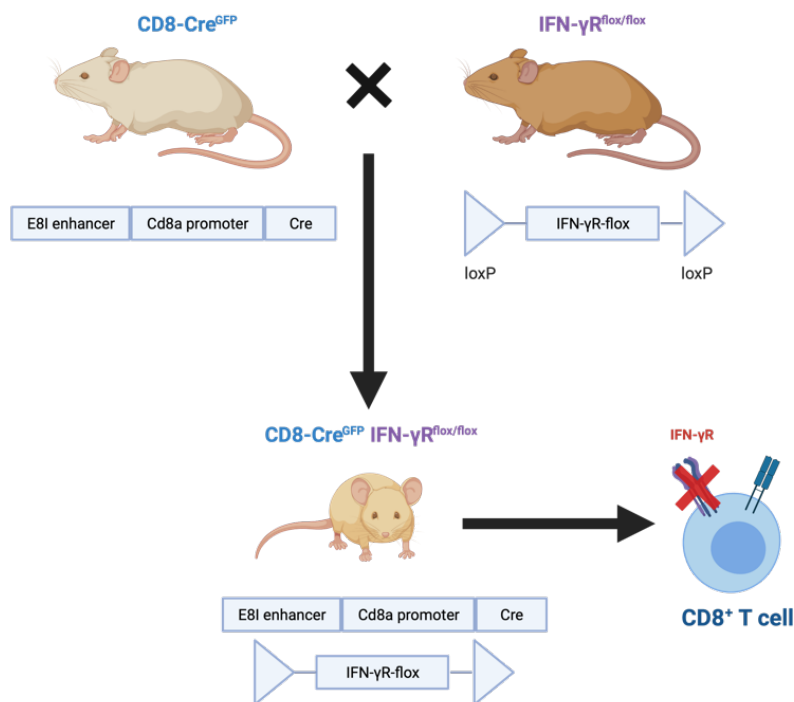


Figure 27. Generation of *CD8a-Cre IFN-γR1^{flox/flox}* mouse model. Crossing of *CD8a-Cre^{GFP}* (E8I) mice with *IFN-γR1^{flox/flox}* mice generated a knock-out (KO) mouse model, in which the IFN-γR1 is specifically deleted in CD8⁺ T cells [Created with [BioRender.com](https://www.biorender.com)].

To study the leakiness and specificity of the recombinase system *in vivo*, we next crossed the CD8 IFN-γR KO mouse with Rosa26-tdTomato reporter mice. In Rosa26-tdTomato mice, Cre-mediated excisional recombination leads to permanent expression of the red fluorescent protein tdTomato from the constitutively active *Rosa26* promoter⁴⁷², and thus facilitates detection of cells productively expressing Cre recombinase. This system also allowed us to focus in our subsequent studies on Cre-expressing CD8⁺ T cells, as we realised by RNA-sequencing that Cre expression in the CD8⁺ T cell compartment is not fully penetrant (data not shown). Focusing on the tdTomato⁺ CD8⁺ T cell population enabled us to circumvent this problem.

To validate the specificity of IFN-γR1 deletion in our CD8 IFN-γR KO mouse model we isolated spleens and lymph nodes of infected mice at d7-10 post-infection (p.i.) and analysed IFN-γR1 expression in different immune cell subtypes by flow cytometry (**Figure 28A and B**).

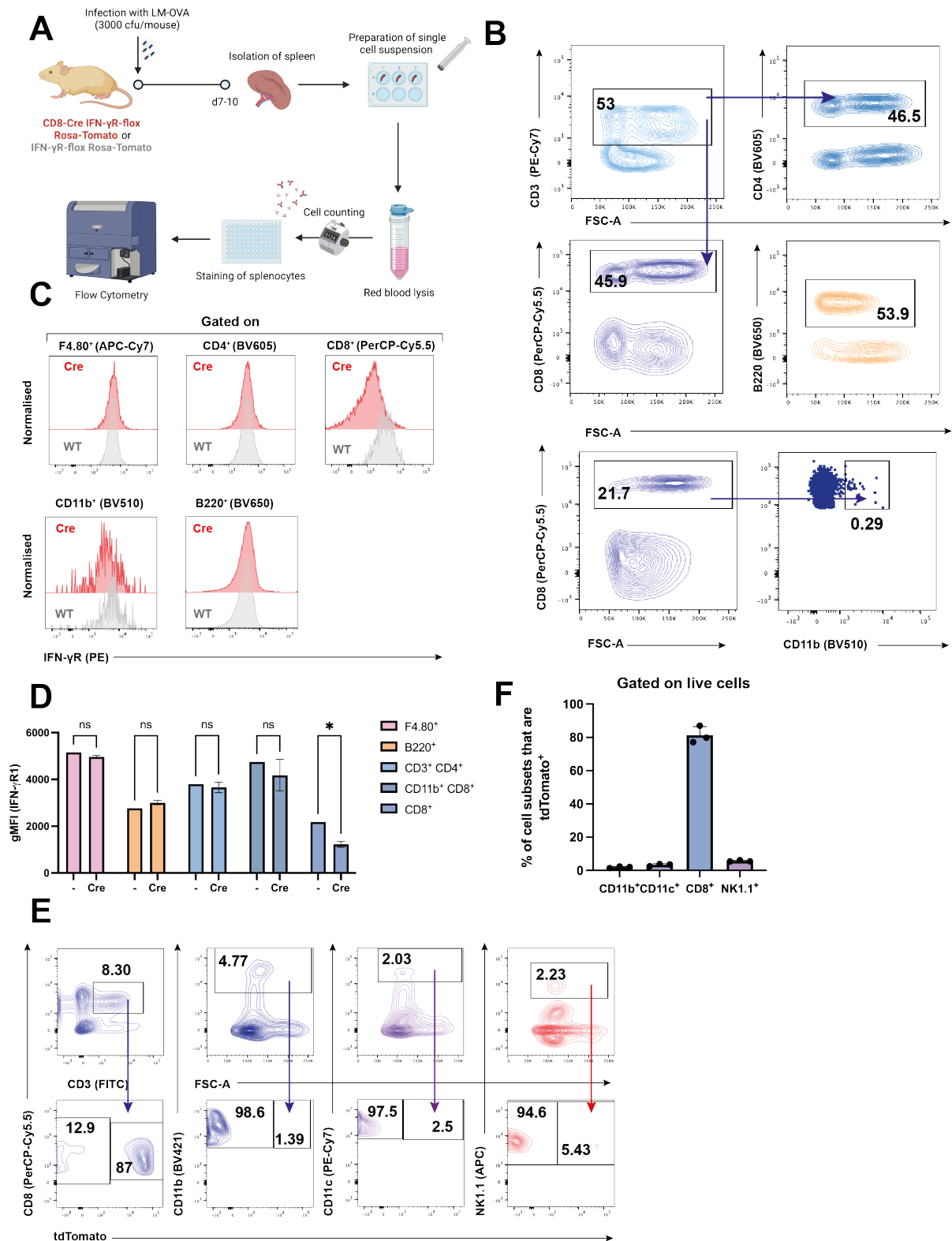


Figure 28. Characterization of the *CD8 α -Cre IFN- γ R1^{flox/flox}* mouse model. (A) CD8 IFN- γ R KO (KO) and *CD8 α -Cre* (WT) mice were infected with LM-OVA and splenocytes were isolated at the peak the response (d7-10). Cell-specific deletion was assessed either by flow cytometry analysis of IFN- γ R1 expression or measurement of tdTomato expression. (B) Representative dot plots of gating strategy for immune cell types. (C, D) Representative histograms (C) and quantification (D) of IFN- γ R1 expression in different immune cell types. (E, F) Representative dot plots (E) and quantification (F) of relative expression of tdTomato in different immune cell types. Data are representative of 2 (n = 3 x 3 technical replicates) independent experiments. Error bars indicate the mean $\pm$ s.d. Comparison between groups was calculated using the two-tailed unpaired Student's test. *P < 0.05; NS, not significant.

As shown in **Figure 28D**, only CD8⁺ T cell populations displayed effective receptor deletion. Once the Rosa26-tdTomato strain of our KO mouse model was successfully generated, CD8⁺-specific deletion of IFN- γ R1 was furthermore validated by flow cytometry analysis of tdTomato expression (**Figure 28E and F**). Overall, our data demonstrate the successful creation of a CD8⁺-specific IFN- γ R1 KO mouse model. In addition, we did not observe any off-target expression in other immune cell types, both by flow cytometry analysis of IFN- γ R1 expression and tdTomato expression.

3.2.2. Loss of interferon-gamma receptor 1 in CD8⁺ T cells results in a skewing of the functional avidity of the antigen-specific CD8⁺ T cell repertoire

If not otherwise indicated, the CD8 IFN- γ R KO mouse model (hereafter simply referred to as KO mice) was used in all subsequent experiments of this chapter, with *CD8 α -Cre Rosa26-tdTomato* mice used as corresponding controls (hereafter simply referred to as wild-type (WT) mice). To investigate the specific function of IFN- γ on CD8⁺ T cell responses in the context of infection, KO and WT mice were infected with LM-OVA. Splenocytes were subsequently isolated at the peak of the response (d7-10 p.i.) for further analysis (**Figure 29A**). We focused on the antigen-specific CD8⁺ T cell population by tetramer staining (SIINFEKL (N4) in the context of H-2K^b [N4-MHC-I]) (**Figure 29B**) and evaluated both the expansion and differentiation of effector CD8⁺ T cells. Notably, we observed a significant shift in tetramer binding towards higher mean-fluorescence intensity (MFI) in the KO population compared with the WT population (**Figure 29C, F and G**). As reported previously²⁶⁰, changes in tetramer binding may reflect differences in the clonality of the responding CD8⁺ T cell repertoire, or alternatively, may be caused by differential TCR expression. However, KO and WT CD8⁺ T cells did not differ in their expression levels of TCR and CD3 (**Figure 29H and I**). We furthermore

confirmed that KO and WT cells did not get differentially activated during priming by measuring expression of the activation marker CD69 24 hours post-infection ([Figure 29J](#)).

Additionally, we also measured a significantly higher relative frequency of antigen-specific (tetramer⁺) CD8⁺ T cells in KO as compared to WT mice ([Figure 29D](#)), and a trend towards higher absolute cell numbers ([Figure 29E](#)), suggesting that IFN- γ limits the expansion of responding CD8⁺ T cells. Those findings were also recapitulated on the monoclonal level, by investigating the effect of IFN- γ deletion on the expansion of adoptively transferred WT OT-I and IFN- γ R^{-/-} OT-I cells following LM-OVA expression. We found that the number of IFN- γ R^{-/-} OT-I cells at the peak of the effector response was up to 20-fold higher than that of their WT counterpart ([Figure 29K](#)), indicating again that direct IFN- γ signalling restricts CD8⁺ T cell expansion.

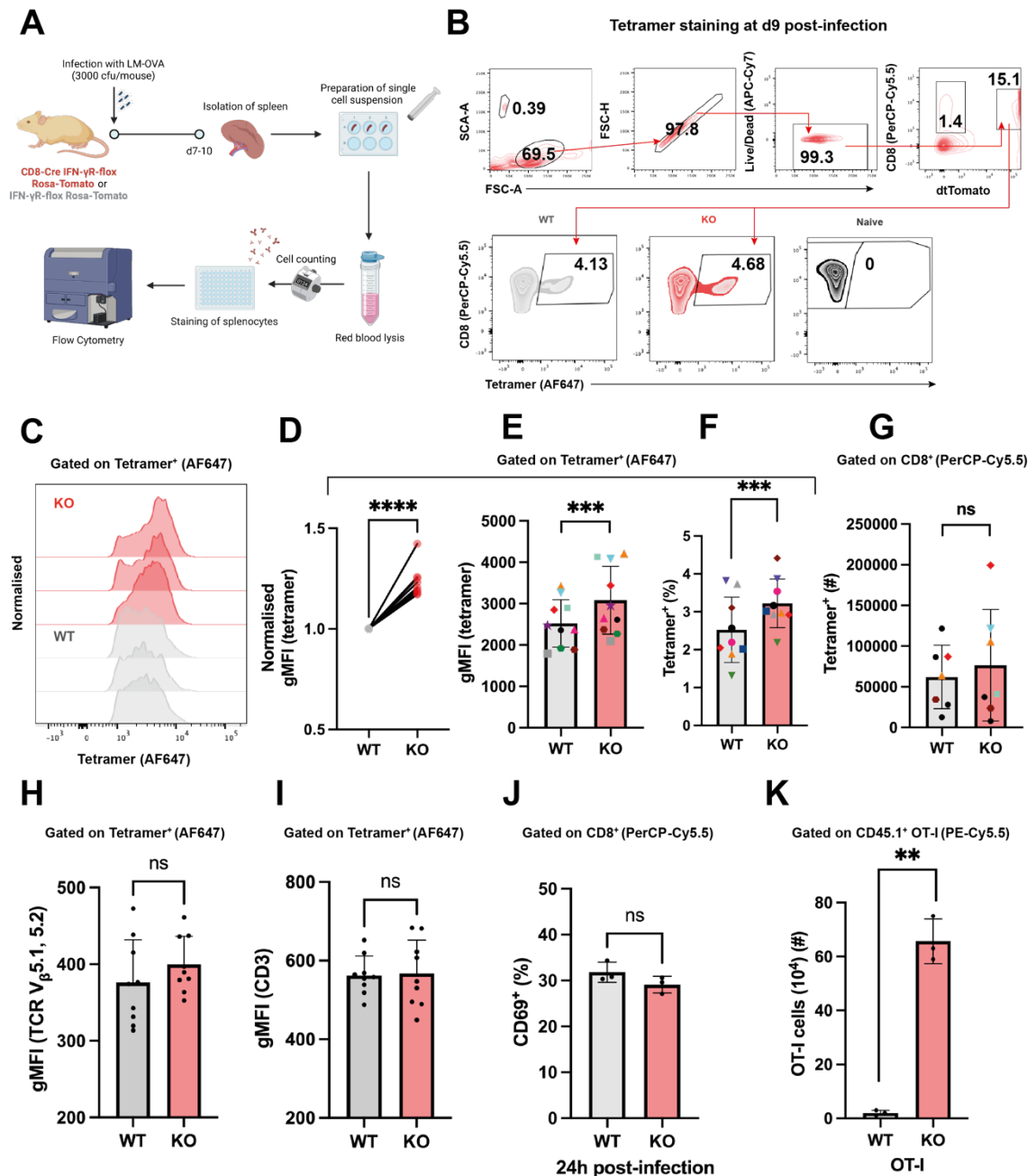


Figure 29. Loss of interferon-gamma receptor 1 (IFN- γ R1) signalling in CD8⁺ T cells skews the antigen-specific T cell receptor (TCR) repertoire towards increased expansion and higher average tetramer binding. (A) *CD8 α -Cre IFN- γ R1^{flx/flx} Rosa26-tdTomato* (KO) and *CD8 α -Cre Rosa26-tdTomato* (WT) mice were infected with LM-OVA and splenocytes were isolated at the peak the response (d7-10) and stained for flow cytometry analysis. Polybead Polystyrene Black Dyed Microspheres were added to all samples for quantification purposes. (B) Representative dot plots of gating strategy. (C, D, E, F, G) Representative histograms (C) of tetramer binding of KO (red) and WT (grey) CD8⁺ T cells and quantification (D, E) of tetramer-binding geometric mean fluorescence intensity (MFI) and relative abundance (F) and absolute numbers (G) of tetramer⁺ CD8⁺ T cells. (H, I) Quantification of TCR V β 5.1,5.2 (H) and CD3 (I) expression. (J) KO and WT were infected with LM-OVA and CD69 expression was assessed 24 hours post-infection by flow cytometry. (K) OT-I (WT) or OT-I IFN- γ R1^{-/-} (KO) CD8⁺ T were adoptively transferred into C57BL/6J host mice, which were infected with LM-OVA and OT-I cell numbers were evaluated at the peak of the response by flow cytometry. Data are representative of 10 (B, C, D, n = 3 x 3 technical replicates) or are pooled from 10 independent experiments (E, F, G, H, I). Error bars indicate the mean $\pm$ s.d. Tetramer-binding gMFI of KO samples was normalised to mean WT expression within each independent experiment. Comparison between groups was calculated using the two-tailed unpaired Student's t-test. *P<0.05; **P<0.01; ***P<0.001; NS, not significant.

To further examine whether variations in the functional avidity of the T cell repertoire caused the measured differences in tetramer binding, we performed *in vitro* peptide titrations on splenocytes isolated at the peak of the response. Isolated splenocytes were stimulated with different concentration of the SIINFEKL (N4) peptide for 5 hours prior to analysis of IFN- γ and TNF expression by flow cytometry (Figure 30A). IFN- γ responses of WT and KO were similar in magnitude upon restimulation with an optimal peptide dose (Figure 30B and E), but there was a significant difference in EC₅₀ of maximum IFN- γ secretion between OVA-specific KO and WT CD8⁺ T cells (Figure 30C and D), indicating a higher average avidity of the KO CD8⁺ T cell repertoire. TNF expression displayed a similar pattern (data not shown). Altogether, we concluded that IFN- γ modulates the clonal repertoire of the responding CD8⁺ T cell population, decreasing the overall antigen avidity of the epitope-specific CD8⁺ T cell population. One could imagine different mechanisms of how IFN- γ may regulate the avidity (and clonality) of CD8⁺ T cell responses. IFN- γ could either modulate the clonal recruitment into the response, or, alternatively, contribute to the preferential selection and expansion of specific clones. To answer this question, we sorted KO and WT tetramer⁺ CD8⁺ T cells at day 9 post-infection to characterize the responding T cell repertoire in more depth by single-cell RNA and TCR sequencing. Unfortunately, the analysis of the generated data was at the time of writing this thesis still pending and can therefore not be further discussed.

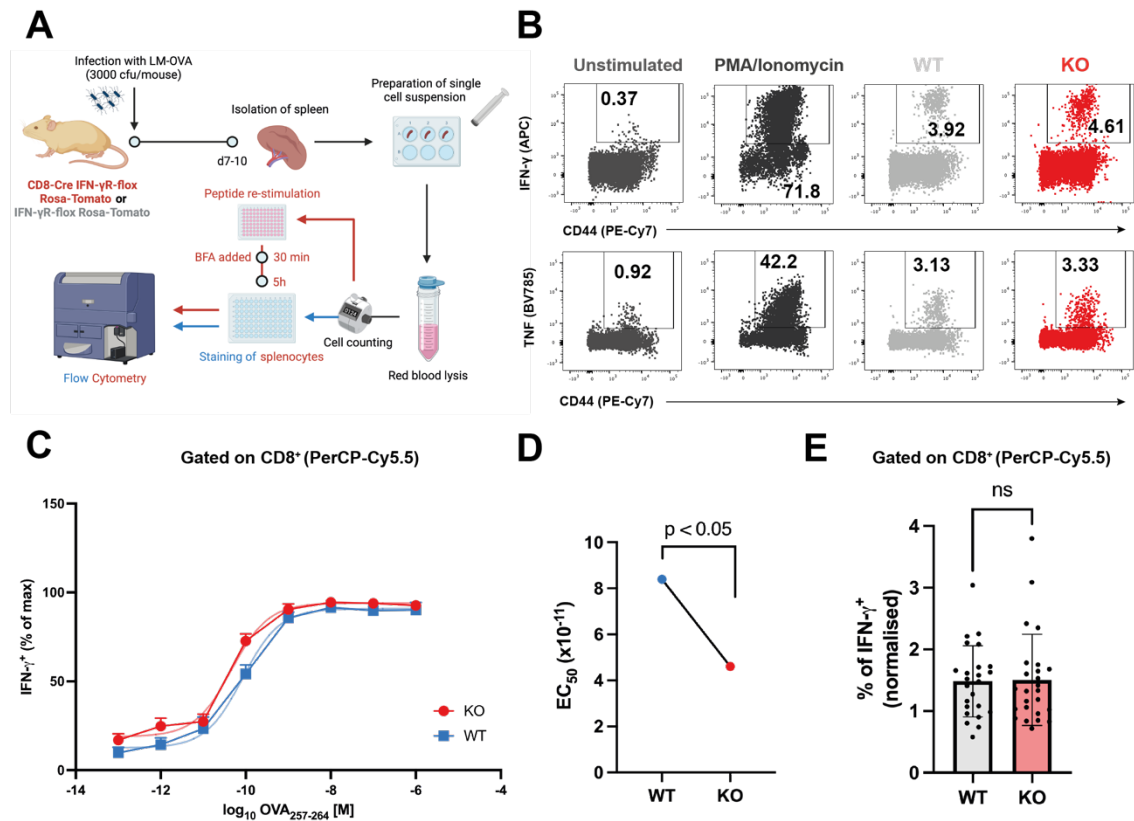


Figure 30. Absence of interferon-gamma receptor 1 (IFN-γR1) signalling skews the endogenous CD8⁺ TCR repertoire towards higher functional avidity. (A) *CD8α*-Cre IFN-γR1^{flx/flx} Rosa26-tdTomato (KO) or *CD8α*-Cre Rosa26-tdTomato (WT) mice were infected with LM-OVA and splenocytes were isolated at the peak of the response (d7-10) and re-stimulated with OVA₂₅₇₋₂₆₄ peptide (SIINFEKL [N4]) to quantify IFN-γ expression. (B, C, D) Representative dot plots of cytokine staining (B) and analysis (C) of relative IFN-γ expression and quantification (D) of EC₅₀. (E) Quantification of maximum IFN-γ expression at the optimal peptide concentration. Data are representative of 3 (C, D, E, n = 5 x 3 technical replicates) independent experiments. Error bars indicate the mean ± s.e.m. Comparison of relative IFN-γ expression and EC₅₀ between groups (C, D) was calculated by fitting a 3-parameter fixed-slope Hill function and confirming good fit by the R square function and visual inspection before performing a F-test to compare the model parameters. Comparison of maximum IFN-γ expression between groups (E) was calculated using the two-tailed unpaired Student's t-test. *P<0.05; **P<0.01; ***P<0.001; NS, not significant.

3.2.3. Regulation of CD8⁺ T cell differentiation through direct interferon-gamma

Following our observations that direct IFN-γ signalling regulates the expansion and avidity of the responding CD8⁺ T cell response, we next sought to investigate if IFN-γ signalling modulates the differentiation of effector CD8⁺ T cell cells. We again isolated splenocytes at the peak of the response and phenotyped effector CD8⁺ T by flow cytometry analysis of KLRG1 and CD127 expression but did not detect any differences between WT and KO in terms of relative frequency and absolute numbers of terminally differentiated effector (KLRG1^{hi}CD127^{low} SLEC) and memory precursor (KLRG1^{low}CD127^{high} MPEC) cells (Figure 31A, B,

C, D and E). However, when analysing tetramer binding of those two different effector subsets, a notable difference between KO and WT cells became apparent. While WT SLECs and MPECs did not differ in their average tetramer binding profiles, the SLEC compartment of KO CD8⁺ T cells displayed a significant shift towards higher average tetramer binding (Figure 31F and G), indicating that IFN- γ may limit the expansion of high-avidity clones and may therefore regulate differentiation at the clonal level.

Taken together, our data suggest that IFN- γ does not significantly impact the relative abundance of SLEC and MPEC subtypes at the endogenous population-wide level. However, given the observed differences in tetramer binding of KLRG1^{hi}CD127^{low} effector CD8⁺ T cells between the two experimental conditions, we concluded that a loss of direct IFN- γ signalling in CD8⁺ T cells causes a preferential selection of high-avidity clones into the terminally differentiated effector compartment, suggesting that IFN- γ may regulate effector differentiation at the clonal level. This would also explain why we observe significant differences in the expansion of IFN- γ R^{-/-} OT-I cells compared with WT OT-I, given that OT-I cells harbour a transgenic TCR of comparatively high-affinity for their cognate antigen. Based on these findings we hypothesise that, while the relative abundance of SLECs and MPECs appears to be largely unaffected by a loss of IFN- γ signalling in CD8⁺ T cells, the clonal composition may still be altered through IFN- γ . To explore if IFN- γ -mediated changes in the clonal composition of the CD8⁺ T cell repertoire at the peak of the response are translated into long-term changes in the resulting memory compartment, we next sought to study the memory response in more depth.

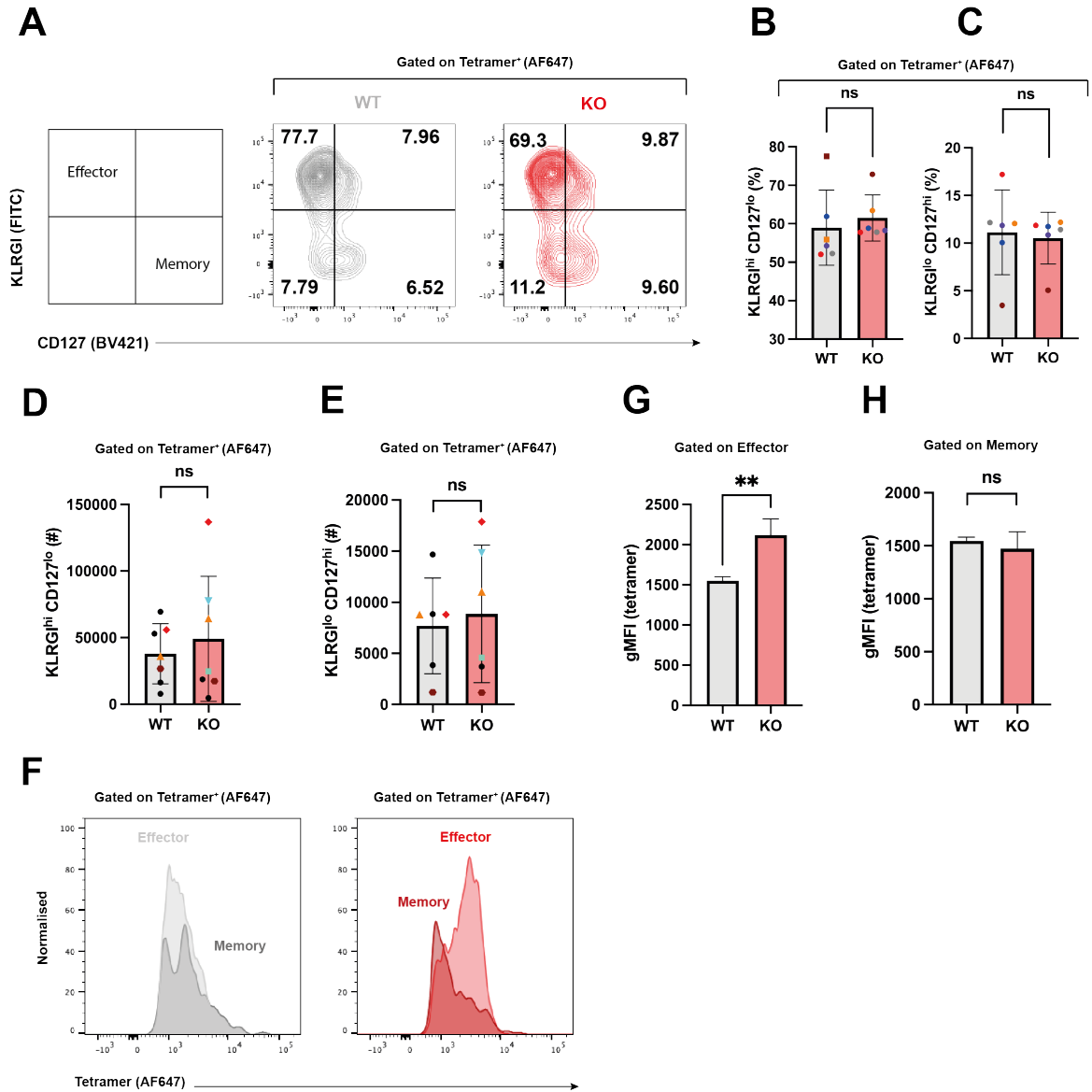


Figure 31. Regulation of endogenous CD8⁺ T cell differentiation through interferon-gamma (IFN-γ). *CD8α*-Cre IFN-γR1^{fllox/fllox} Rosa26-tdTomato (KO) and *CD8α*-Cre Rosa26-tdTomato (WT) mice were infected with LM-OVA and splenocytes were isolated at the peak of the response (d7-10) for flow cytometry analysis. **(A, B, C, D, E)** Representative flow plots **(A)** and quantification of relative abundance **(B, C)** and absolute numbers **(D, E)** of effector (KLRG1^{hi}CD127^{lo}) or memory (KLRG1^{lo}CD127^{hi}) CD8⁺ T cells, respectively. **(F, G, H)** Representative histograms **(F)** and quantification of effector **(G)** and memory cell **(H)** tetramer-binding. Data are representative of 10 (F, G, H, n = 3 x 3 technical replicates) or are pooled from 10 independent experiments (B, C, D, E). Error bars indicate the mean ± s.d. Comparison between groups was calculated using the two-tailed unpaired Student's t-test. *P<0.05; **P<0.01; ***P<0.001; NS, not significant.

3.2.4. Effect of interferon-gamma on the CD8⁺ T cell memory compartment

Our results obtained at the peak of the effector response to LM infection prompted us to investigate if the observed IFN-γ-mediated effect on KO MPECs translates into long-term changes of antigen-specific memory cells. At the peak of the response, we observed a

divergence in the tetramer binding profiles of MPEC and SLEC subpopulations in KO mice, with KO MPECs displaying lower average tetramer-binding whilst the tetramer binding of KO SLECs appeared skewed towards higher MFI. In contrast, the average tetramer binding profiles of WT MPECs and SLECs did not differ significantly. Based on these findings we hypothesised that the memory pool of WT and KO mice may display differential average functional avidity, which may affect their response to re-infection. We thus sought to investigate the function of direct IFN- γ signalling on the CD8⁺ T cell memory compartment, by infecting mice with LM-OVA and studying the memory compartment 60-90 days post-infection by tetramer staining.

Studying the antigen-specific memory population at day 60 post-infection requires enrichment of the tetramer⁺ CD8⁺ T cell population due to the expected low frequency of long-lived antigen-specific memory cells at that point in time ([Figure 32A](#)). Comparison of the memory populations of WT and KO mice at day 60 post-infection did not show differences in relative abundance ([Figure 32B and C](#)). However, we did observe a difference in the tetramer binding profiles of KO and WT memory cells, with KO memory CD8⁺ T cells displaying a decreased tetramer MFI ([Figure 32D and E](#)). This observation is in line with our hypothesis that loss of IFN- γ signalling in CD8⁺ T cells may modulate the clonal composition of MPECs towards lower average tetramer binding, plausibly indicating an average lower TCR avidity of the memory pool, while skewing the terminally differentiated effector population towards higher average tetramer binding.

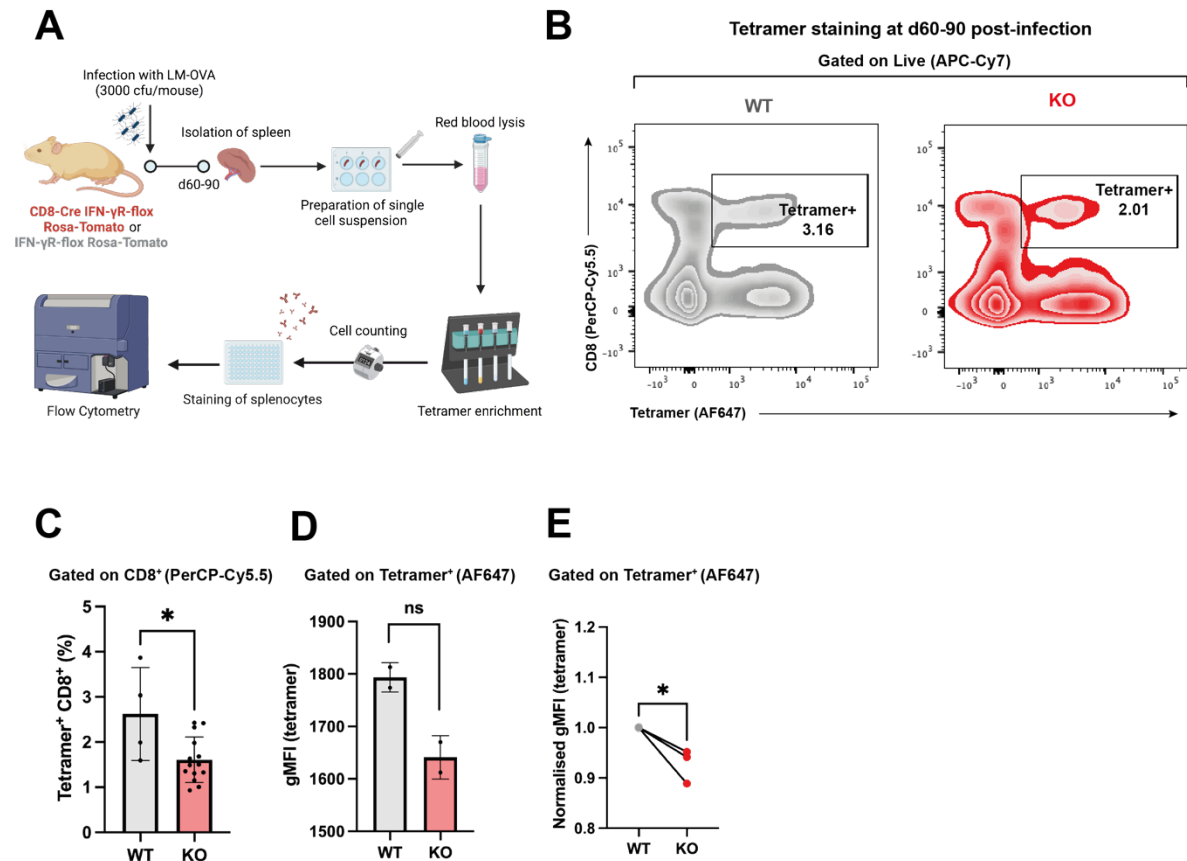


Figure 32. Absence of interferon-gamma (IFN- γ) signalling in CD8⁺ T cells leads to an altered tetramer binding profile in the memory compartment. (A) *CD8a-Cre IFN- γ R^{fllox/fllox} Rosa26-tdTomato* (KO) or *CD8a-Cre Rosa26-tdTomato* (WT) mice were infected with LM-OVA and splenocytes were isolated at day 60 post-infection and analysed by flow cytometry. (B, C, D) Representative dot plots of tetramer staining (B) and quantification of relative abundance (C) and mean fluorescence intensity (gMFI) of tetramer staining. (E) Tetramer-binding gMFI of KO samples was normalised to mean WT expression within each independent experiment. Data are representative of 3 (B, C, D, $n = 3 \times 3$ technical replicates) or are pooled from 3 (E, $n = 3 \times 2$ technical replicates) independent experiments. Error bars indicate the mean $\pm$ s.d. Comparison between groups was calculated using the two-tailed unpaired Student's t-test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS, not significant.

Enrichment for tetramer⁺ CD8⁺ T cells by positive selection allowed for efficient detection of the antigen-specific memory population. However, there is an associated risk that beads used for magnetic-activated cell sorting (MACS) remain bound to the tetramer, which could interfere with the subsequent detection and quantification of single-cell tetramer binding. Additionally, cells of lower avidity may be lost during the enrichment procedure, thus introducing an experimental bias. We therefore sought to optimise our experimental approach to not be dependent on prior enrichment and reasoned that re-challenging the mice with the same infectious dose as has been used for the primary infection should result in a rapidly expanding memory population at day 5 post-infection, which can then be analysed by

standard tetramer staining without the need for prior enrichment (Figure 33A). Upon re-challenge we observed again a skewing in the KO repertoire towards lower average tetramer binding (Figure 33E and F), while there was no difference in relative abundance of KO and WT memory cells (Figure 33B and C). CD3 expression in KO and WT tetramer⁺ CD8⁺ T cells was again assessed in parallel to evaluate TCR expression, but no differences in MFI were observed (Figure 33F), indicating again that the differences in tetramer binding are not due to differential receptor expression. Altogether, we concluded from these experiments that IFN- γ modulates the clonal composition of the CD8⁺ T cell repertoire at both the effector and memory stage of the CD8⁺ T cell response. We are currently in the process of confirming through *in vitro* re-stimulation assays if the changes in memory tetramer binding are again reflective of changes in functional avidity of the antigen-specific repertoire.

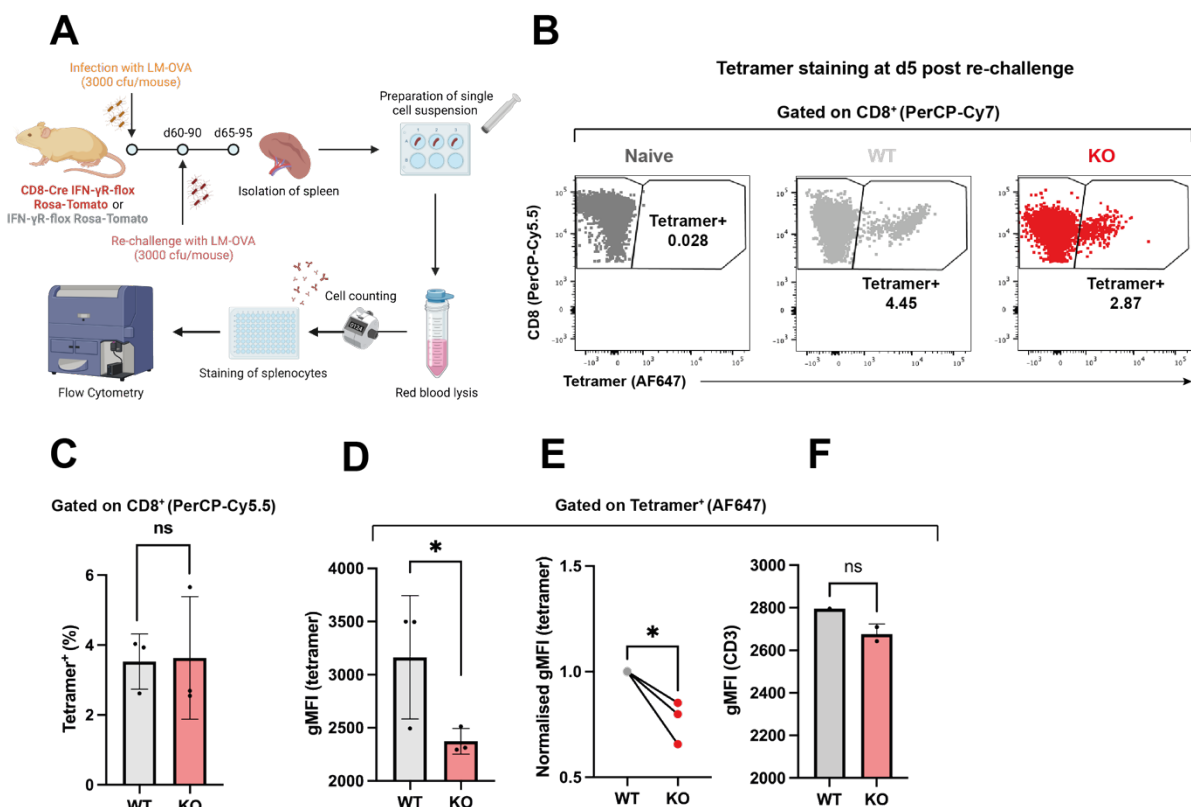


Figure 33. Absence of interferon-gamma (IFN- γ) signalling in CD8⁺ T cells leads to an altered tetramer binding profile in the memory compartment. (A) *CD8 α -Cre IFN- γ R1^{flox/flox} Rosa26-tdTomato* (KO) or *CD8 α -Cre Rosa26-tdTomato* (WT) mice were infected with LM-OVA and re-challenged at day 60 post-infection. Spleenocytes were isolated 5 days after re-challenge and analysed by flow cytometry. (B, C, D) Representative dot plots of tetramer staining (B) and quantification of relative abundance (C) and mean fluorescence intensity (gMFI) of tetramer staining. (E) Tetramer-binding gMFI of KO samples was

normalised to mean WT expression within each independent experiment. **(F)** Quantification of CD3 expression in CD8⁺T cells. Data are representative of 3 (B, C, D, n = 3 x 2 technical replicates) or are pooled from 3 (E, n = 3 x 3 technical replicates) independent experiments. Error bars indicate the mean $\pm$ s.d. Comparison between groups was calculated using the two-tailed unpaired Student's t-test. *P<0.05; **P<0.01; ***P<0.001; NS, not significant.

3.2.5. Relevance of direct interferon-gamma signalling for the functionality of the CD8⁺

T cell response

The observed skewing of the CD8⁺ T cell response at the peak of the response let us to hypothesise that IFN- γ may modulate the functionality of the responding T cell population. To evaluate the antigen-specific cytolytic capacity of endogenous CD8⁺ T cells, we implemented an *in vivo* assay, which involved the isolation of splenocytes from wildtype C57BL/6J (B6) mice carrying the congenic marker CD45.1. Isolated splenocytes were incubated with either OVA peptide ligand N4, or without peptide, and subsequently differentially labelled with either CFSE or eFluor670, respectively. Both differentially treated and labelled splenocytes populations were mixed and transferred into LM-OVA infected KO or WT mice on day 7 post-infection. The spleens of infected mice were harvested 24 hours post transfer and the cytolytic capacity of CD8⁺ T cells was quantified by calculating the ratio between peptide-loaded and non-loaded population, relative to a naïve control mouse, which had been transferred with the same splenocyte mixture ([Figure 34A](#)).

Using this optimised *in vivo* assay and gating on the CD45.1⁺ live cell population, the numbers of CFSE- and eFluor670-labelled splenocytes were quantified. To account for variable numbers of antigen-specific CD8⁺ T cells within the infected mice, quantification of antigen-specific T cells by tetramer staining was performed, which was used to normalise the calculated cytotoxicity. However, no significant difference was observed following normalisation ([Figure 34B, C and D](#)), suggesting that direct IFN- γ signalling was not

modulating the overall cytotoxic functionality of antigen-specific CD8⁺ T cells under the experimental conditions tested.

As an additional surrogate marker to compare the cytolytic capacity of KO and WT mice, we also measured surface expression of lysosome-associated membrane protein-1 (LAMP-1, also known as CD107a) in effector CD8⁺ T cells following *in vitro* re-stimulation with cognate peptide. LAMP family member proteins are highly glycosylated membrane proteins representing approximately 50% of the proteins in the lysosomal membrane^{473,474}. Cytotoxic granules share many characteristics with lysosomes, and their membranes have been revealed to be lined by LAMP-1^{475,476}. LAMP-1 expression on the cell surface has been described as a marker of cytotoxic CD8⁺ T cell degranulation⁴⁷⁷ and functional LAMP-1 was discovered to be required for efficient NK-cell cytotoxicity and perforin delivery to lytic granules in NK cells⁴⁷⁸ and to furthermore protect NK cells from degranulation-associated damage⁴⁷⁹. While its precise function in CD8⁺ T cells remains to be further investigated, several studies have demonstrated its validity as a sensitive candidate marker for the evaluation of cytotoxic activity^{480,481}.

To measure LAMP1 expression, we isolated CD8⁺ T cells at the peak of the response and re-stimulated them *in vitro* for 5 hours with cognate peptide to induce degranulation and analysed LAMP-1 expression by flow cytometry. We did not detect significant differences between WT and KO mice (**Figure 34E and F**). We concluded that absence of direct IFN- γ signalling in CD8⁺ T cells did not critically affect degranulation efficiency in effector cells.

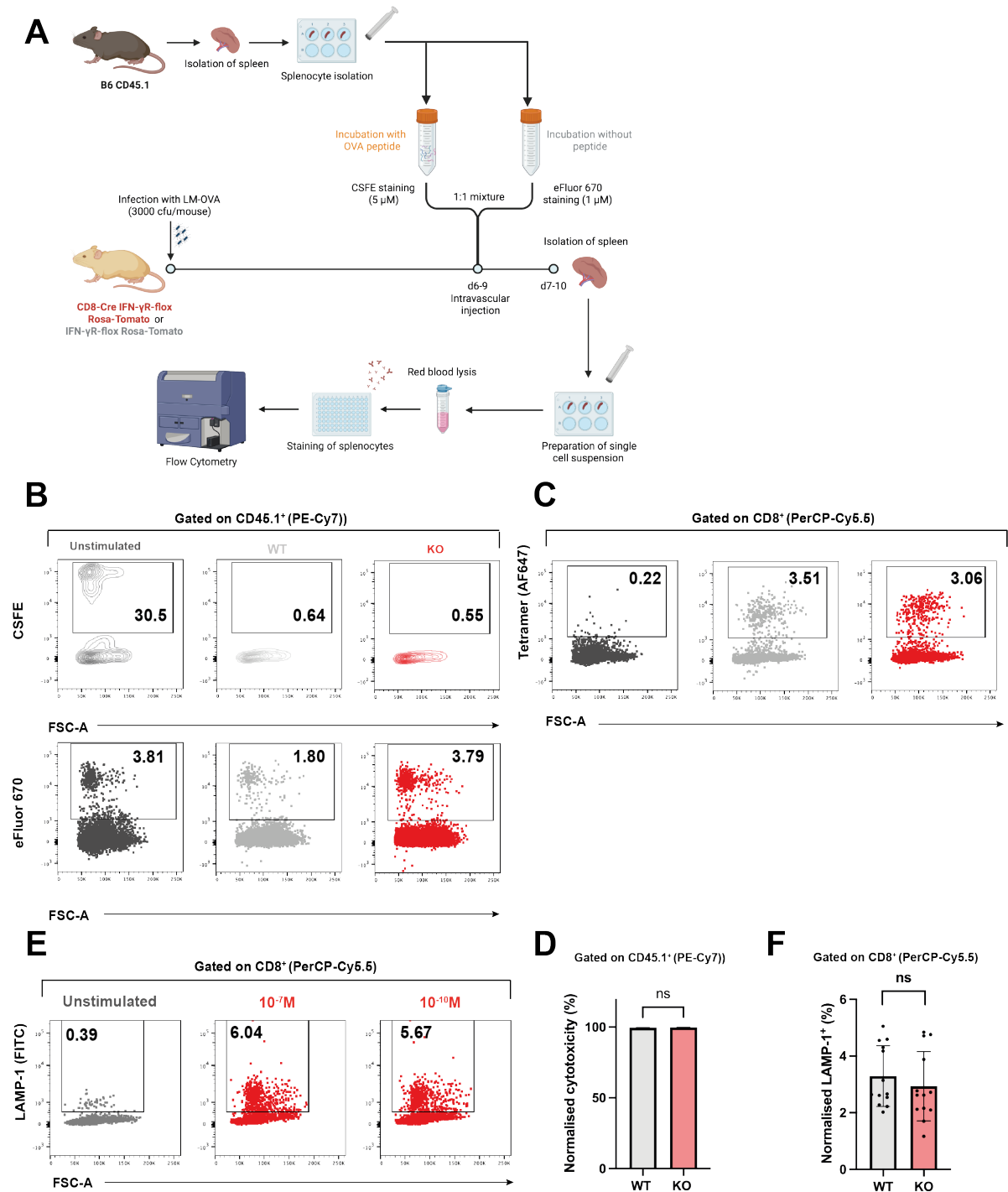


Figure 34. Absence of direct interferon-gamma (IFN- γ R) signalling does not alter the cytotoxic potential of effector CD8⁺ T cells. (A) *CD8 α -Cre IFN- γ R1^{flox/flox} Rosa26-tdTomato (KO)* or *CD8 α -Cre Rosa26-tdTomato (WT)* mice were infected with LM-OVA and injected with differentially labelled mixtures of peptide-loaded or unloaded target splenocytes at day 7 post-infection to assess *in vivo* cytotoxic functionality. (B, C, D) Representative dot plots of transferred target populations (B) and tetramer staining (C) and quantification (D) of normalised cytotoxicity. (E, F) Representative dot plots of LAMP-1 staining (E) and quantification (F) of LAMP-1 expression. Cytotoxicity and LAMP-1 expression was normalised to the relative abundance of tetramer⁺ CD8⁺ T cells within each independent experiment. Data are representative of 3 (B, C, D, $n = 3 \times 2$ technical replicates) or are pooled from 5 (E, F, $n = 3 \times 3$ technical replicates) independent experiments. Error bars indicate the mean $\pm$ s.d. Comparison between groups was calculated using the two-tailed unpaired Student's *t*-test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS, not significant.

The infectious dose of LM-OVA used in previous experiments guaranteed efficient activation of CD8⁺ T cells, without risking lethal infection. Weight loss was used as an approximate measure for how efficiently mice cleared an ongoing infection, however, no significant changes in weight was observed at this sublethal dose of LM-OVA.

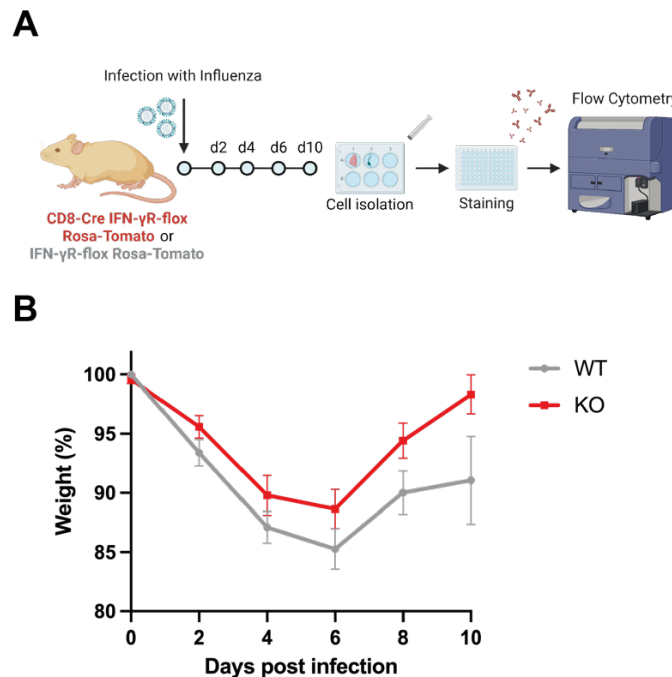


Figure 35. Absence of interferon-gamma (IFN-γR) alters the protective capacity of CD8⁺ T cells against influenza infection. (A) *CD8a*-Cre IFN-γR^{flox/flox} Rosa26-tdTomato (KO) or *CD8a*-Cre Rosa26-tdTomato (WT) mice were infected with X31-OVA and weight was monitored every two days. (B) Quantification of relative weight loss in KO and WT mice. Data are representative of 2 (n = 5 x 3 technical replicates) independent experiments. Error bars indicate the mean ± s.e.m.

We therefore decided to adopt an influenza virus infection model, which is known to be associated with significant changes in weight in the first days of infection. We infected WT and KO mice with the influenza strain X31 expressing OVA (X31-OVA) and measured the weight of infected mice every two days (Figure 35A). Notably, we measured reduced weight loss in KO mice compared to WT mice (Figure 35B). We concluded that the enhanced functional avidity observed in KO mice causes a more potent effector T cell response, helping mice to ward off infection more effectively. Follow-up studies to characterize the antigen-specific CD8⁺ T cell repertoire of virally infected KO and WT mice in more depth are currently ongoing.

3.3. Discussion

3.3.1. Efficient deletion of interferon-gamma receptor 1 in a CD8⁺-specific Cre mouse model

As shown in this chapter, the KO mouse model generated in our group leads to an efficient and specific genetic abrogation of IFN- γ R1 expression in CD8⁺ T cells (Figure 28). Cre recombination is one of the most widely used experimental tools to introduce targeted mutations in a cell-type specific and/or inducible manner. The usage of reporter alleles enables validation of cell and/or tissue specificity of successful Cre-mediated recombination at the single-cell level. However, one needs to be cautious using reporter expression as an approximation for productive target gene deletion and the fidelity of conditional Cre expression. The *loxP*-flanked STOP cassette of the reporter might differ in its sensitivity to Cre-mediated recombination to that of the *loxP*-flanked experimental target gene⁴⁸². To address this issue, we confirmed recombination of the chosen target gene in various immune cell subtypes, both on the protein level by flow cytometry analysis and on the transcript level by bulk RNA-sequencing (data not shown).

However, whilst offering many advantages, it is important to highlight that this system also comes with certain disadvantages, which could have potentially confounding effects on the observed phenotype. In our mouse model, Cre recombinase has been integrated into the endogenous gene locus, which comes with the risk of potentially impairing gene expression from at least one of the two alleles. The use of bacterial artificial chromosomes (BACs) containing a genetic locus engineered to express Cre allows to circumvent this potential problem. However, as previously reported, this approach does not necessarily guarantee the desired Cre expression pattern either⁴⁸². In addition, introducing Cre into a cell can give rise

to so-called Cre toxicities, caused by off-target genome cuts at endogenous pseudo-*loxP* sequences, which can eventually lead to cell death⁴⁸². The risk of introducing Cre toxicities can be minimised by either using self-deleting Cre expression vectors⁴⁸³ or employing systems of inducible Cre expression⁴⁸⁴. Both approaches come with their own disadvantages, such as decreased recombination efficiency⁴⁸². As discussed previously by others⁴⁸², the most crucial control to address the potential risk of introduced Cre toxicities may be the use of control mice, which carry the *cre* transgene but not its loxP-flanked target. Accordingly, we ensured to rely on *CD8α*-Cre mice as the most appropriate controls in our experiments.

3.3.2. Ablation of interferon-gamma receptor signalling in CD8⁺ T cells enhances the expansion of effector CD8⁺ T cells

In the aforementioned studies we used a twofold approach, investigating the function of IFN- γ at both the polyclonal, endogenous level and the monoclonal, transgenic level. Both approaches provided evidence that a loss of IFN- γ signalling results in augmented expansion of effector CD8⁺ T cells at the peak of the response against LM infection ([Figure 29](#)). Our results confirm previous studies that demonstrated a counter-regulatory effect of IFN- γ on CD8⁺ T cell expansion^{127,469}. However, while previous reports suggested an indirect mechanism through the action of IFN- γ on suppressive CD11b⁺ cells⁴⁶⁹, our study highlights that IFN- γ has a cell-intrinsic function on CD8⁺ T cells. The discrepancies between models, where IFN- γ limits effector expansion in some models^{133,378}, but not in all^{211,379}, may be explained by the specific balance between the pro-inflammatory and anti-inflammatory properties of IFN- γ in a given context. Our studies investigated the mechanism underlying this cell-intrinsic function of IFN- γ on CD8⁺ T cells will be described in more detail in the next chapter.

3.3.3. Ablation of interferon-gamma signalling in CD8⁺ T cells alters the functional avidity of the responding T cell repertoire

Comparison of the average tetramer binding profiles of antigen-specific WT and KO CD8⁺ T cells revealed a significant skewing towards higher tetramer MFIs in KO cells. The interpretation of these observed changes in tetramer binding is not straightforward and while having been used previously²⁶⁰ as a surrogate marker for the average avidity of the responding T cell population, it is per se not a readout for the avidity of T cells. Previous studies²⁶⁰ also often used the term “*affinity*” in association with tetramer binding studies. However, given that tetramer binding measures the binding of multivalent interactions, as opposed to monovalent interactions, it is important to emphasise that such binding more closely reflects the “*avidity*” of the T cells, rather than the intrinsic “*affinity*” of the TCR.

TCR avidity is mainly defined by functional readouts such as cytokine production measured after antigen-specific stimulation or lysis of target cells pulsed with limiting concentrations of peptide (“*functional avidity*”), and the results of those assays can be influenced by many factors, including TCR expression levels, adhesion molecules or coreceptors, and changes in components of the signalling cascade⁴⁸⁵. “*Structural avidity*” on the other hand is defined by the affinity of the TCR to pMHC molecules combined with co-receptor binding via CD8 or CD4 of surface-expressed TCRs and the most common technique to evaluate structural avidity is surface plasmon resonance (SPR), which comes with a number of challenges, most notably the need to provide pMHCs and TCRs in highly purified and correctly folded form. Alternative methods are based on pMHC multimer binding and dissociation^{249,486}. However, pMHC multimer staining intensity does not necessarily correlate with TCR binding avidity^{485,487}. Tetramer binding may result in receptor downregulation, a possibility we controlled for by

flow cytometry analysis of CD3 and/or TCR V β 5.1, 5.2 expression. The fact that we did not observe differences in CD3 and/or TCR expression, together with our results on the functional level assessing cytokine production after re-stimulation with cognate antigen ([Figure 30](#)), led us to conclude that IFN- γ modulates the functional avidity of the responding CD8⁺ T cell population. We have also sent tetramer⁺ CD8⁺ T cells isolated at the peak of the response for single-cell TCR-sequencing, which will allow us to definitively answer if the changes in functional avidity are associated with changes in the clonal composition and clonal breadth of the TCR repertoire. Of note, tetramer enrichment experiments performed on the naïve repertoire of WT and KO splenocytes did not show any differences in tetramer MFI and CD3 expression (data not shown), indicating that the functional avidity of the naïve T cell repertoire is not altered by the absence of IFN- γ R1 on mature peripheral CD8⁺ T cells.

In addition, we also measured the expansion and tetramer binding MFIs of responding CD8⁺ T cell populations specific for either the low-affinity OVA-peptide variant V4 or the LM-specific listeriolysin O antigen (LLO₉₂₋₉₉). However, in both cases only a very small tetramer⁺ population was observable, which made it very difficult to analyse and compare the two experimental conditions (data not shown). As our V4 and LLO₉₂₋₉₉ tetramer aliquots have been quite old by the time we performed these experiments, it is possible that the suboptimal binding may be caused by the fact that the tetramers were already partly denatured, and we are planning on repeating these experiments once we received new aliquots.

Of note, the group of Dirk Busch also developed an alternative tetramer binding assay based on reversible multimers, so-called Streptamers⁴⁸⁵, which enables measuring the dissociation kinetics of monomeric pMHCs from TCRs on living T cells, both by imaging and flow cytometry⁴⁸⁸. We collaborated with his laboratory to evaluate and compare the structural avidity of KO and WT effector CD8⁺ T cells using their “TCR-ligand *k_{off}-rate assay*”,

and despite preliminary promising results (Figure 36), we were unable to complete these studies in time to still be fully included in this thesis.

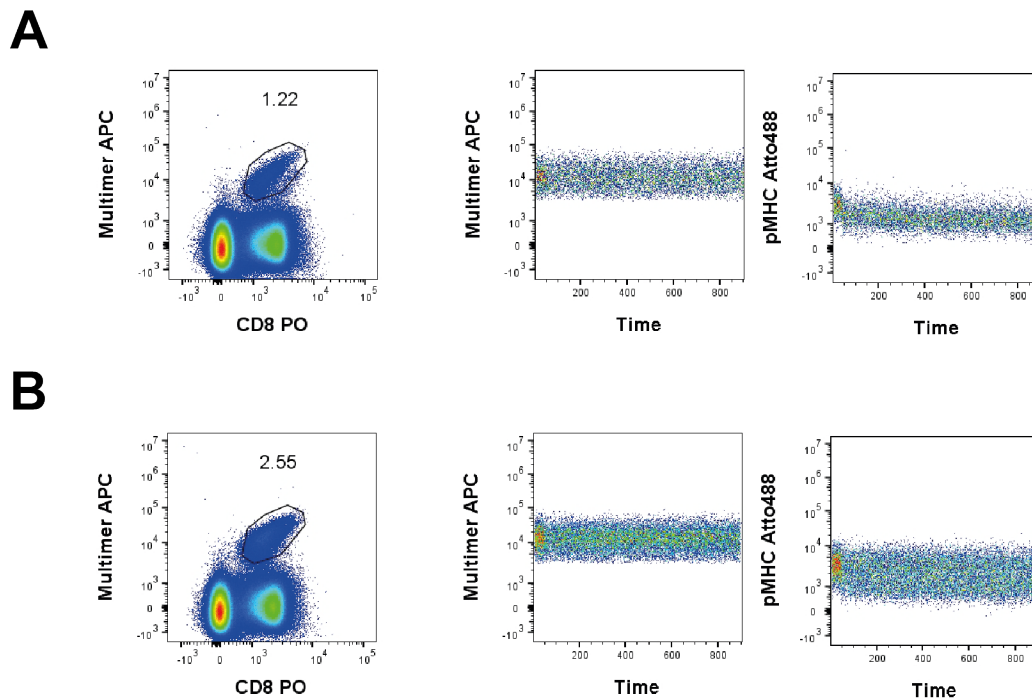


Figure 36. Flow Cytometry-based TCR-ligand k_{off} -rate assay. Splenocytes isolated at the peak of the response (d7-10) were stained with reversible multimers (comprising Steptactin-Atto488 and SIINFEKL-H2Kb-APC) for flow cytometry analysis. 2 mM D-biotin was added 30 seconds after start of acquisition and dissociation was observed for 10 minutes. Samples were gated on living $CD3^+ CD8^+$ lymphocytes and dissociation of Steptactin-Atto488 and MHC-APC from these cells were measured. Fluorescence values of Atto488 and APC staining of living cells are plotted over time of analysis. **(A, B)** Dissociation kinetics of *CD8α*-Cre Rosa26-tdTomato (WT) **(A)** and *CD8α*-Cre IFN- γ R1^{flox/flox} Rosa26-tdTomato (KO) **(B)** effector T cells. While WT mice displayed more rapid dissociation kinetics, KO mice exhibited an overall lower dissociation rate, indicative of longer TCR-pMHC binding half-lives and thus the presence of TCRs with higher affinity for antigen. Data were generated and provided by Elvira D'Ippolito based on protocols published by the group of Dirk Busch⁴⁸⁸.

3.3.4. Ablation of interferon-gamma receptor signalling in $CD8^+$ T cells alters the differentiation of the responding T cell population at the clonal level

We next studied the effect of direct IFN- γ signalling on the differentiation of $CD8^+$ T cells. It is important to highlight that T cell differentiation is a highly complex and dynamic process and dissecting the contribution of one specific cytokine to this process on the endogenous level is complicated by the fact that loss of one particular cytokine may potentially be compensated or partially masked by the contribution of other cytokines. At the population level we did not observe differences in the expansion of SLEC and MPEC subsets between KO

and WT. However, focusing on the tetramer binding of these two subpopulations, we noted a significant skewing in terminally differentiated effector cells of KO mice towards higher tetramer MFIs, suggesting that IFN- γ may regulate differentiation at the clonal level (Figure 31). Our group has furthermore explored the function of IFN- γ on a monoclonal population of CD8⁺ T cells, using adoptively transferred OT-I cells. These studies, which have already been published⁴⁸⁹, showed clearly that IFN- γ regulates the balance between terminally differentiated effector and memory precursor CD8⁺ T cells by limiting effector differentiation. It is known that the TCR of OT-I T cells exhibits notably high-affinity for its cognate antigen^{63,490}, whereas most antigen-specific T cells at the poly-clonal level will likely be of lower affinity^{286,491,492}. Taken together, it is thus conceivable that IFN- γ acts to preferentially limit the effector differentiation of very high-affinity clones.

The observed differences in tetramer binding of effector and memory CD8⁺ T cells between WT and KO prompted us to investigate if those changes translate into long-term differences of the resulting memory compartments. Investigating the memory CD8⁺ T cell repertoire comes with certain challenges but using two different experimental approaches we demonstrated that changes in tetramer binding that are measurable at the peak of the response are indeed translated into differences in the resulting memory pool. The functional relevance of these modifications in the context of secondary infection remains to be further investigated. Of note, so far, we did not investigate if IFN- γ also affects the differentiation of memory cells into different memory subsets.

3.3.5. The functional significance of interferon-gamma as a regulator of the cytolytic capacity of the responding CD8⁺ T cell population

Given the changes in functional avidity of the effector CD8⁺ T cell repertoire, we hypothesised that the loss of IFN- γ may also cause enhanced cytotoxicity of effector cells. While we did not detect significant differences in the cytotoxic potential of KO and WT effector cells in our optimised assay ([Figure 34](#)), it is conceivable that this was, at least partly, due to the sensitivity of our assay, given that complete killing was observed in both the WT and KO condition. We are currently aiming to optimise the assay further by injecting a higher number of peptide-loaded splenocytes or, alternatively, loading the target cells with a different peptide of lower affinity for the TCR of OVA-specific T cells, such as SIITFEKL (T4).

In addition, the comparison of cytolytic capacity *in vivo* is complicated by the fact that we cannot adjust the ratio of effector to target cells, as would be possible using an *in vitro* approach. We are also in the process of adapting an alternative *in vitro* assay²⁵⁷, which allows greater control over the effector to target ratio, which is currently still ongoing.

Lastly, it is also conceivable that enhanced functional avidity does not translate into increased cytotoxic capacity but instead modulates other aspects of response behaviour. It is commonly thought that T cells with high functional avidity are superior in clearing viral infections or inducing tumour regression^{485,493,494}. However, a recent *in vitro* study has discovered that T cell cytolytic capacity is independent of initial stimulation strength, which was revealed to mainly control how rapidly and simultaneously cells initiate activation²⁵⁷. Our single-cell sequencing data should again help us to answer this question definitively.

3.4. Conclusion

In summary, this chapter sought to examine the role of direct IFN- γ signalling on CD8⁺T cells and its effect on the responding CD8⁺ T cell response. The lack of direct IFN- γ signalling was revealed to promote the expansion of effector CD8⁺ T cells and to enhance the functional avidity of the antigen-specific repertoire. Having established critical functions of IFN- γ on CD8⁺ T cells, we sought to explore in the following chapter the cellular processes underlying this cytokine-mediated effect.

4. Activating CD8⁺ T cells communicate directly with each other through interferon-gamma

4.1. Introduction

4.1.1. Interferon-gamma-producing immune cell subsets

Numerous immune cell subsets, including T cells, NK cells, invariant NK T cells (iNKT), T_{reg} cells, $\gamma\delta$ T cells and B cells, have been shown to be capable of producing IFN- γ , which will be briefly reviewed in the following section.

4.1.1.1 T lymphocytes

IFN- γ is the signature cytokine of T_H1 cells, which also produce several other cytokines such as TNF, IL-2, lymphotoxin- α (LT α) and granulocyte-monocyte colony-stimulating factor (GM-CSF). Notably, whereas IFN- γ , IL-2, LT α and GM-CSF are released from T_H1 cells in a targeted fashion through synapses to APCs to promote survival³⁸⁵, TNF is released in a multidirectional manner to enhance dendritic cell maturation and macrophage activation at the synapse whilst simultaneously mediating local cytotoxicity⁴⁹⁵. Another CD4⁺ T cell subset reported to secrete IFN- γ amongst other effector cytokines are T_H17 cells³⁸⁵.

T_{reg} cells have also been shown to be capable of secreting IFN- γ . Surprisingly while FOXP3 is known to maintain T_{reg} cell suppressive identity through AKT inhibition via nuclear sequestration of FOXO1, which is thought to also repress *Ifng* transcription^{496,497}, FOXP3⁺IFN- γ ⁺ T_{reg} cells have been reported in various autoimmune diseases and in the context of cancer⁴⁹⁷. Those cells express a T_H1-like transcriptional programme, and while still showing

repressive activity against T_H1 cells in the context of infection^{444,498}, their suppressive function in cancer appears to be impaired, thus facilitating greater anti-tumour immunity⁴⁹⁹.

γδ T cells are T cells that express γ and δ chains and represent 1-4% of the T cell population. They secrete IFN-γ rapidly following activation mediated through T-bet and Eomes in a non-MHC restricted manner by lipids, glycoproteins, and phosphorous-containing compounds. IFN-γ⁺ γδ T cells have been shown to provide an early source of IFN-γ in tumour immunity. Interestingly, mice with IFN-γ-deficient were observed to display impaired IFN-γ production by αβ T cells⁵⁰⁰.

CD8⁺ cytotoxic T lymphocytes are well-known producers of IFN-γ during the effector phase of the response, which is a key component of their immune response to infection and cancer. IFN-γ is released from CTLs in a leaky synaptic manner, which ensures that the target cell receives a concentrated IFN-γ signal, while simultaneously also allowing some IFN-γ to reach neighbouring cells⁵⁰¹. Interestingly, this mode of secretion has been shown to differ from that for other cytotoxic molecules such as TNF and perforin, which are concentrated at the immunological synapse to mediate targeted cell killing³⁸⁵. In addition to its role as an effector cytokine expressed by CTLs, IFN-γ secretion by a subset of CD8⁺ T cells has furthermore been reported during the early phase of *L. monocytogenes* infection^{70,379,502}, which likely plays an important role in enhancing the cytotoxic function of both innate and adaptive cells to accelerate pathogen clearance. However, the precise function of this early wave of IFN-γ is much less well established and its potential role in regulating the CD8⁺ T cell response remains to be further investigated.

In addition, it has also been discovered that IFN-γ-secreting CD8⁺ T cells can form a synapse with neighbouring non-antigen-specific CD8⁺ T cells, which was shown to promote expansion and differentiation of bystander T cells, which in turn can become IFN-γ producers

themselves^{310,503,504}. Lastly, leaky synaptic release of IFN- γ in combination with comparably high stability and widespread distribution theoretically allows hours of IFN- γ exposure, which fulfils a crucial role in the context of cancer immunity by stimulating transcriptional programs that mediate effective killing of tumour cells³⁸⁵.

4.1.1.2 iNKT and MAIT cells

iNKT cells are innate-like T cells that express a TCR α chain which recognizes lipid antigens presented by the non-classical MHC molecule CD1d expressed on dendritic cells. iNKT cells can furthermore be subdivided into T_H1-, T_H2- and T_H17-like subsets. iNKT cells develop in the thymus and resemble memory-type T cells in the periphery, which may produce cytokines in the innate phase of immune responses⁵⁰⁵. It was suggested that the pattern of IFN- γ release by iNKT cells may be synaptic due to an iNKT cell-DC positive feedback loop in which IFN- γ secreted by iNKT cells promotes DC maturation through upregulation of co-stimulatory molecules^{385,506,507}.

Mucosal-associated invariant T (MAIT) cells recognize intermediates of riboflavin (vitamin B2) biosynthesis, which are presented on class I-related molecule 1 (MR1)^{508,509} by semi-variant TCRs comprising V α 7.2-Ja33, 12 or 20 with limited V β diversity^{510,511}. MAIT cells have been shown to produce IFN- γ both in a TCR-dependent⁵¹² and a TCR-independent manner⁵¹³.

4.1.1.3 NK cells and B cells

Apart from T cells, various other immune cells serve as important contributors to the available pool of IFN- γ . NK cells are innate cytotoxic cells recognizing non-self epitopes that provide the first line of defence against invading pathogen, and which mediate their cytotoxic function through IFN- γ expression. IFN- γ and TNF are stored in recycled endosomes, which

are delivered to localised compartments away from the target cell for multidirectional release⁵¹⁴, thus promoting the activation of inflammatory cells³⁸⁵.

B cells are best known for producing antibodies but are also capable of mediating antibody-independent functions via cytokine secretion⁵¹⁵. Innate CD11a^{hi}CD16/CD32^{hi} cells are a B cell subset shown to express IFN- γ during early stages of bacterial infection, similarly to NK cells⁵¹⁶. The mode of IFN- γ release in those cells remains to be further investigated.

4.1.2. Experimental models to investigate interferon-gamma expression by immune cells

Cytokines, including IFN- γ , are rapidly secreted and therefore cannot be reliably detected within the producing immune cell. However, recently activated T cells co-incubated with inhibitors for protein secretion, such as monensin or brefeldin A, accumulate proteins they would otherwise secrete, thereby allowing intracellular detection of cytokines at the single cell level^{517–519}, which can also be used to analyse IFN- γ expression by flow cytometry^{520,521}. The standard protocol used in our group involves intraperitoneal injection of 250 μ g BFA per animal 6 hours before organ isolation. However, due to repeated issues with this protocol, we decided to adapt a protocol from the group of David Withers, in which organs are harvested in complete media with added BFA, and single cell suspensions are cultured in the same mix for 3 hours before cells are subjected to intracellular cytokine staining⁵²². This protocol worked in general very well for our experimental purpose and ensured efficient staining of intracellular IFN- γ . Nevertheless, to not rely exclusively on protocols requiring prior blocking of cytokine secretion, we also utilised IFN- γ reporter mice known as GREAT mice. Integration of the coding sequencing for enhanced yellow fluorescent protein (eYFP)

downstream of the endogenous *Ifng* promoter⁵²³, enables *in vivo* quantification of IFN- γ expression through analysis of eYFP reporter expression.

4.1.3. Context and aim of this chapter

Given the wide range of candidate IFN- γ -secreting immune cells, it remains to be shown, if CD8⁺ T cells receive IFN- γ preferentially from one particular cell type, or alternatively, if multiple cell types can serve as potential sources of IFN- γ for CD8⁺ T cells. Furthermore, our results so far give no indication if the sensing of IFN- γ by CD8⁺ T cells is confined to a particular time window of the ensuing response. We thus aimed to dissect more thoroughly the temporal context of this signalling event. The aim of this chapter was therefore (1) to characterize IFN- γ expression in CD8⁺ T cells throughout the response using the aforementioned experimental protocols and models, (2) to pinpoint the temporal context of direct IFN- γ signalling in CD8⁺ T cells, (3) to investigate if CD8⁺ T cells preferentially receive IFN- γ from other CD8⁺ T cells and (4) to examine if IFN- γ is used as a mediator in direct CD8⁺ T cell-T cell communication.

4.2. Results

4.2.1. Global interferon-gamma expression in response to *L. monocytogenes* infection follows a biphasic pattern

To characterize the kinetics of IFN- γ expression in immune cells throughout the response to infection, we measured the expression of the IFN- γ reporter eYFP in GREAT mice at different time points following LM-OVA infection (**Figure 37A**). We observed an incremental increase in expression during the priming phase of the response, which was also reflected in an increased frequency of activated (CD69⁺) CD8⁺ T cells (**Figure 37B, C and D**). Similar kinetics were observed for global IFN- γ secretion (data not shown but available here⁴⁸⁹).

It is important to highlight, that eYFP expression in these reporter mice serves as a direct readout for IFN- γ gene transcription, whilst not necessarily reflecting the level of translated cytokine protein. However, the same general expression pattern during the early phase of the response was also observed by flow cytometry analysis of intracellular IFN- γ post BFA treatment (**Figure 37E and F**). Quantifying IFN- γ expression from day 3 to day 9 post-infection showed a significant increase in IFN- γ secretion from day 5 onwards before reaching peak expression at d7 and starting to decrease again by d9 post-infection (**Figure 37G and H**). Similar kinetics were also observed again for global IFN- γ secretion (data not shown but available here⁴⁸⁹). We concluded from these data that IFN- γ production in response to *L. monocytogenes* infection follows a biphasic pattern, characterized by an early wave of IFN- γ following activation, which is terminated within 2 days post-infection, and a second, more pronounced wave accompanying the peak of the effector response.

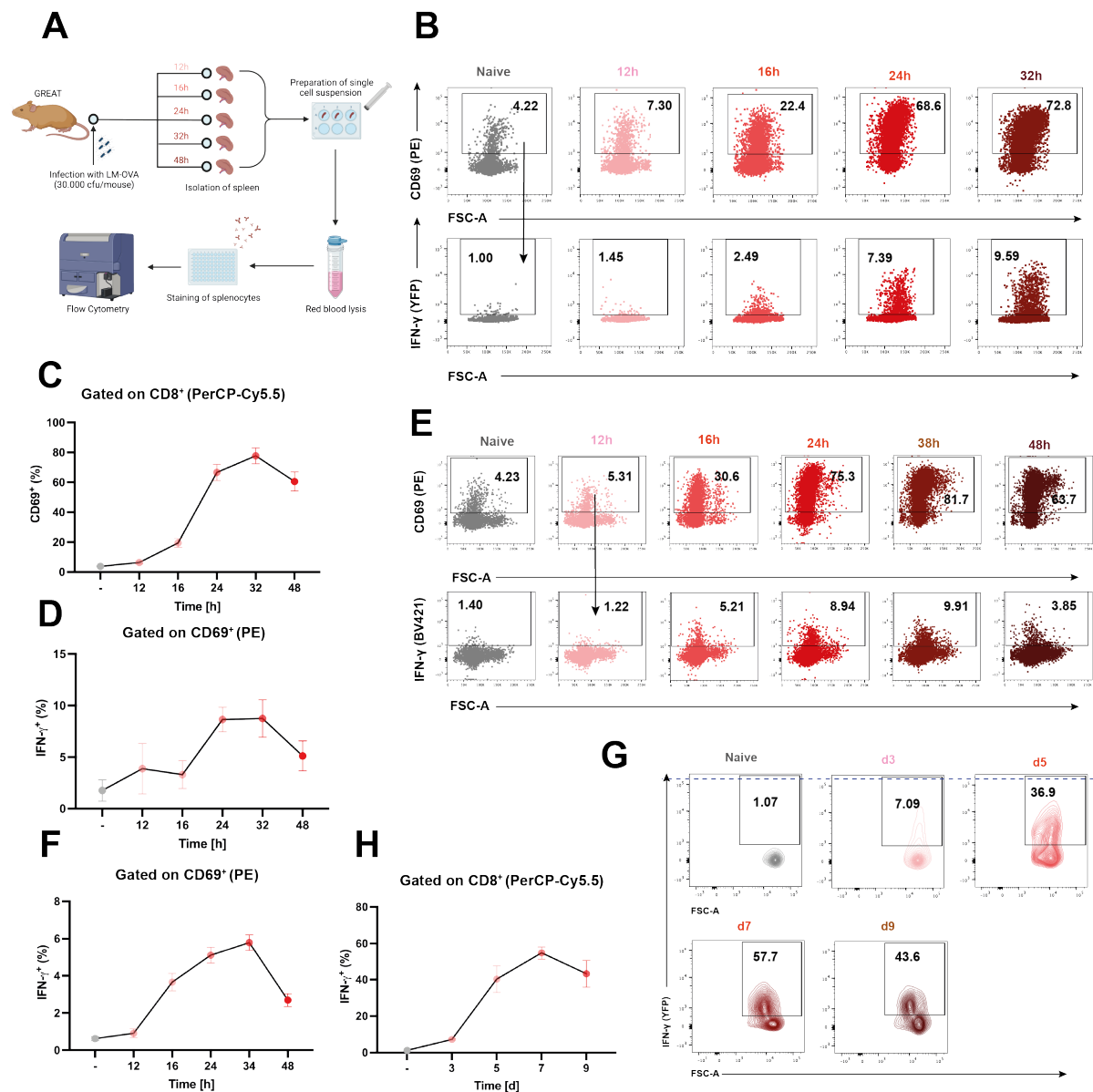


Figure 37. Kinetics of interferon-gamma (IFN- γ) expression in CD8 $^{+}$ T cells throughout the response. (A) GREAT mice were infected with LM-OVA and IFN- γ expression in splenocytes was assessed at the indicated time points. (B, C, D) Representative flow plots (B) and analysis of relative CD69 (C) and eYFP (D) expression in infected GREAT mice 12-48 hours post-infection. (E, F) Representative flow plots (E) and analysis of relative IFN- γ (F) expression in infected GREAT mice 12-48 hours post-infection following *in vitro* BFA treatment. (G, H) Representative flow plots (G) and analysis of relative eYFP (H) expression in infected GREAT mice 3-9 days post-infection. Data are representative of 2 (n = 3 x 3 technical replicates) independent experiments. Error bars indicate the mean $\pm$ s.d.

To furthermore investigate the dynamic changes in the sensitivity of CD8 $^{+}$ T cells to IFN- γ we also quantified the expression kinetics of both IFN- γ R chains in CD8 $^{+}$ T cells (Figure 38). Staining for the IFN γ -R1 requires treating the cells prior to staining with acid to remove any receptor-bound IFN- γ which may interfere with the binding of the staining antibody. To avoid increased cell death, cells can only be incubated for a short period of time in the acetic

stripping solution, before being washing extensively with 1x PBS prior to downstream processing. Altogether, we did not observe significant differences in IFN- γ R1 and -R2 expression during the priming phase of the response. We observed increased expression during the effector phase of the response, with peak expression at d5, before expression of both IFN- γ R1 and -R2 decreased again at d9.

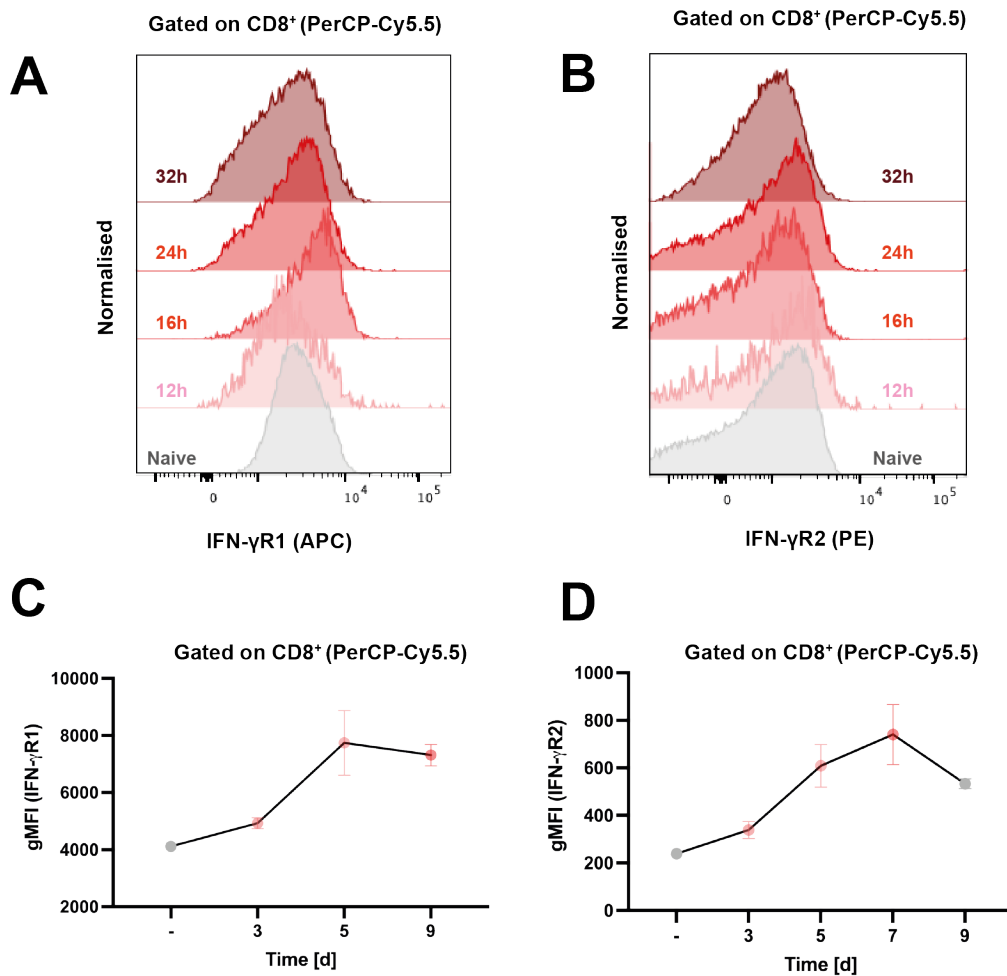


Figure 38. Kinetics of interferon-gamma (IFN- γ R) expression in CD8⁺ T cells throughout the response. (A, B) Representative histograms of IFN- γ R1 **(A)** and IFN- γ R2 **(B)** expression in C57BL/6J (B6) mice 12-32 hours post-infection. **(C, D)** Analysis of IFN- γ R1 **(C)** and IFN- γ R2 **(D)** expression in infected B6 mice 3-9 days post-infection. Data are representative of 2 ($n = 3 \times 3$ technical replicates) independent experiments. Error bars indicate the mean $\pm$ s.d.

4.2.1. CD8⁺ T cells need to sense interferon-gamma 16-24 hours post-infection to enhance effector CD8⁺ T cell expansion and the functional avidity of the endogenous T cell repertoire

Based on the biphasic pattern of global IFN- γ secretion, there are two candidate time windows during which IFN- γ could be sensed by CD8⁺ T cells. We therefore sought to determine the spatiotemporal context of direct IFN- γ signalling in CD8⁺ T cells that undergirds the modulatory functions of IFN- γ described in Chapter 3. To pinpoint if there is a specific temporal window at which IFN- γ sensing must occur, we transferred OT-I CD8⁺ T cells into C57BL/6 (B6) host mice and blocked IFN- γ 16-24- or 48-hours post-infection. Measuring OT-I cell numbers at the peak of the response showed a significant increase in antigen-specific cell numbers only when blocking IFN- γ 16-24 hours post-infection, whereas blockade of IFN- γ 48 hours post-infection did not have any effect on the total number of responding cells (**Figure 39A**). Similar results were obtained when analysis differentiation of antigen-specific cells at the peak of the response, with only 16-24 hours blockade of IFN- γ resulting in an increased SLEC/MPEC ratio (**Figure 39B**) and an increased total number of effector cells (**Figure 39C**).

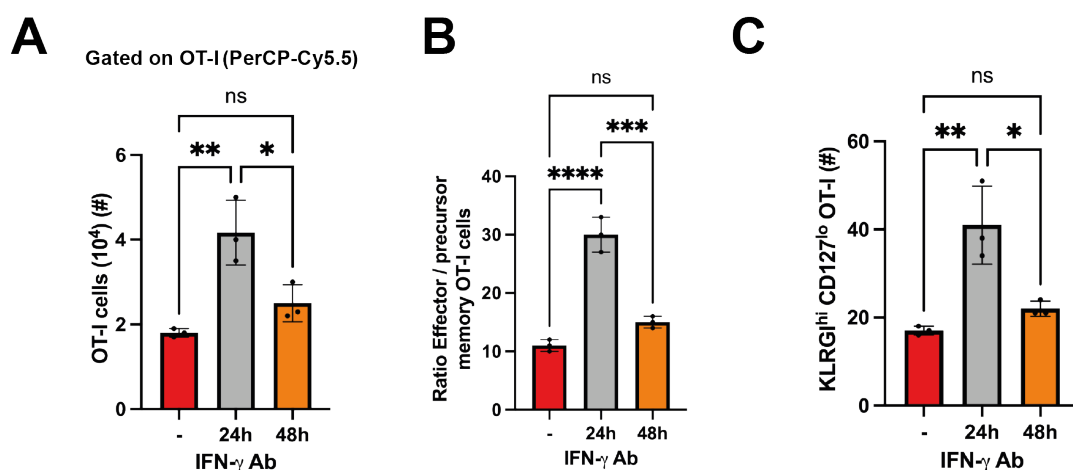


Figure 39. Interferon-gamma (IFN- γ) needs to be blocked 16-24 hours post-infection to modulate OT-I CD8⁺ T cells expansion and differentiation. (A) Blockade of IFN- γ 16-24 hours post-infection the enhanced expansion of adoptively transferred OT-I T cells. **(B)** Blockade of IFN- γ 16-24 hours post-infection increased the SLEC/MPEC ratio of adoptively transferred OT-I T cells at the peak of the response. **(C)** Blockade of IFN- γ 16-24 hours post-infection augmented the total

number of OT-I effector CD8⁺ T cells at the peak of the response. Data are representative of 3 (n = 3 x 3 technical replicates) independent experiments. Error bars indicate the mean ± s.d. Comparison between groups was calculated using the two-tailed unpaired Student's t-test. *P<0.05; **P<0.01; ***P<0.001; NS, not significant.

We next sought to reproduce these findings on the endogenous level, while simultaneously probing the effect of blocking IFN-γ secretion during the effector phase of the response. C57BL/6J (B6) mice were infected with LM-OVA and anti-IFN-γ blocking antibodies were administered either 16-24 hours post-infection or at day 5 and day 6 (Figure 40A). Increased relative abundance of antigen-specific CD8⁺ T cells was only observed when blocking IFN-γ 16-24 hours post-infection (Figure 40C and D), which was furthermore associated again with a skewing of the average tetramer binding of the responding T cell repertoire towards a higher tetramer MFI (Figure 40E), while no difference in CD3 expression in antigen-specific CD8⁺ T cells between the experimental conditions was observed (Figure 40G), indicating again that the changes in tetramer binding are not driven by differential TCR expression.

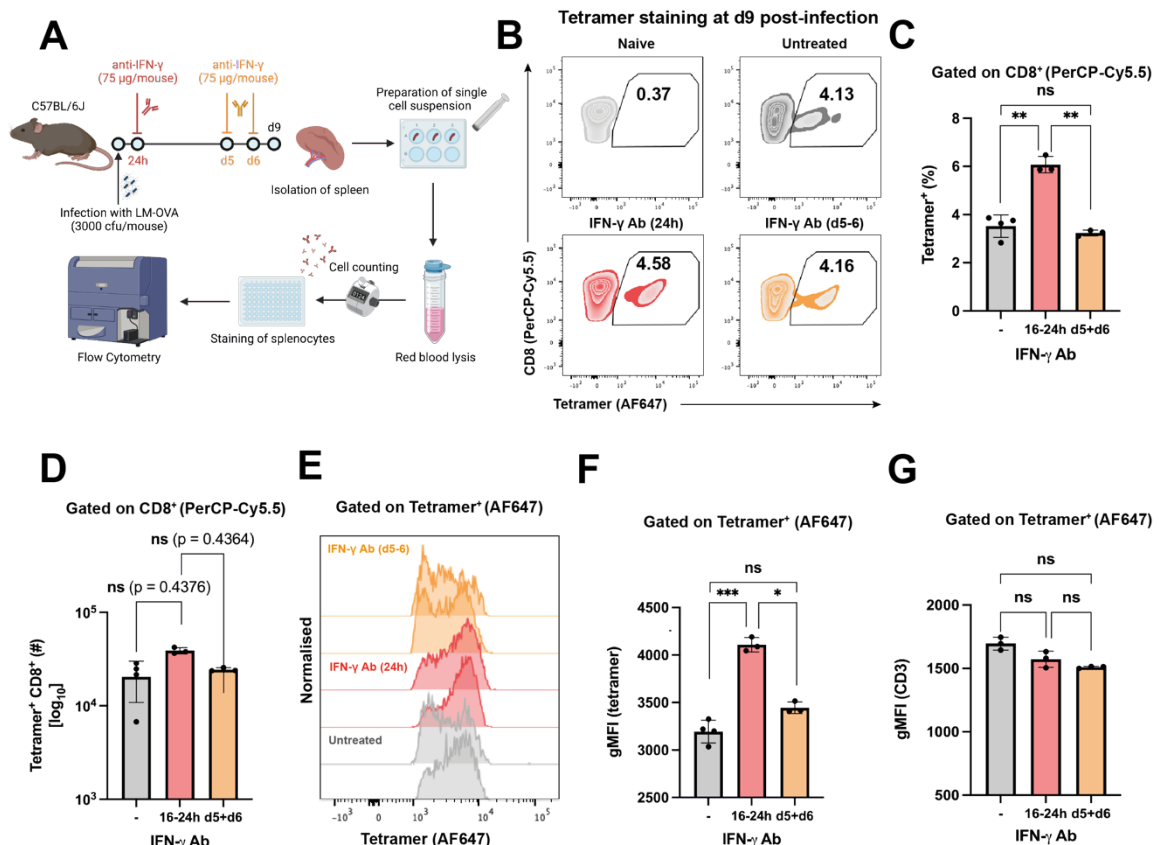


Figure 40. Interferon-gamma (IFN- γ) needs to be blocked 16-24 hours post-infection to modulate antigen-specific CD8⁺ T cells expansion at the endogenous level. (A) C57BL/6J (B6) mice were infected with LM-OVA and mice were either left untreated (grey) or IFN- γ was blocked 16-24 hours (red) or at day 5 and 6 (orange) post-infection. Splenocytes were isolated at the peak of the response (d7-10) for flow cytometry analysis. (B, C, D) Representative flow plots (B), relative abundance (C) and absolute numbers of antigen-specific CD8⁺ T cells at the peak of the response. (E, F) Representative histograms (E) and quantification (F) of tetramer-binding of antigen-specific CD8⁺ T cells at the peak of the response. (G) Quantification of CD3 expression in antigen-specific CD8⁺ T cells at the peak of the response. Data are representative of 3 (n = 3 x 3 technical replicates) independent experiments. Error bars indicate the mean $\pm$ s.d. Comparison between groups was calculated using the two-tailed unpaired Student's t-test. *P<0.05; **P<0.01; ***P<0.001; NS, not significant.

Blocking IFN- γ 16-24 hours post-infection also enhanced the relative abundance (Figure 41A) and absolute number (Figure 41B) of terminally differentiated effector CD8⁺ T cells at the peak of the response, whereas the relative frequency of memory precursor cells was significantly decreased (Figure 41C). Of note, we did not observe a change in total numbers of memory cells (Figure 41D), which may be explained, at least partly, by the overall increased CD8⁺ T cell number that we observed when blocking IFN- γ 16-24 hours post-infection. Thus, the difference may only become apparent in relative terms, while getting lost when quantifying absolute numbers. Lastly, blockade of IFN- γ 16-24 hours post-infection and measuring tetramer binding of SLECs and MPECs revealed again a significant skewing in the tetramer binding of the terminally differentiated effector population towards a higher tetramer MFI (Figure 41F). Taken together, we concluded from these data that IFN- γ needs to be sensed 16-24 hours post-infection to induce the observed changes in expansion, differentiation, and functional avidity.

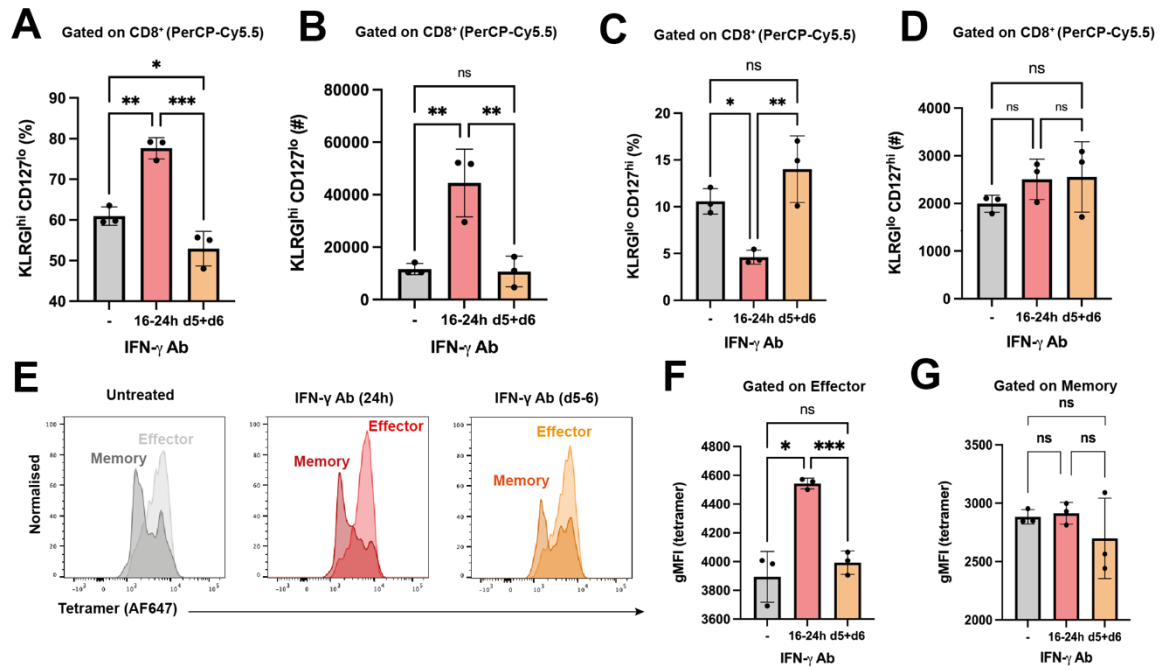


Figure 41. Blocking of interferon-gamma (IFN- γ) 16-24 hours post-infection modulates the differentiation of effector CD8⁺ T cells at the endogenous levels. C57BL/6J (B6) mice were infected with LM-OVA and mice were either left untreated (grey) or IFN- γ was blocked 16-24 hours (red) or at day 5 and 6 (orange) post-infection. Splenocytes were isolated at the peak of the response (d7-10) for flow cytometry analysis. **(A, B)** Blockade of IFN- γ 16-24 hours post-infection enhanced the relative frequency **(A)** and absolute number **(B)** of endogenous short-lived effector (KLRG1^{hi} CD127^{low}) CD8⁺ T cells. **(C, D)** Blockade of IFN- γ 16-24 hours post-infection reduced the relative frequency **(C)** of endogenous memory (KLRG1^{low} CD127^{hi}) CD8⁺ T cells, while no difference in absolute numbers **(D)** was observed. **(E, F, G)** Representative histograms **(E)** and quantification of tetramer binding MFIs of short-lived effector cells **(F)** and memory precursor cells **(G)**. Data are representative of 3 (n = 3 x 3 technical replicates) independent experiments. Error bars indicate the mean $\pm$ s.d. Comparison between groups was calculated using the two-tailed unpaired Student's t-test. *P<0.05; **P<0.01; ***P<0.001; NS, not significant.

4.2.2. Early interferon-gamma leads to accelerated CD25 downregulation in CD8⁺ T cells

The fact that early blockade of IFN- γ increased CD8⁺ T cell numbers at the peak of the effector response to infection suggested an indirect functional mechanism of IFN- γ on cell expansion or contraction. Earlier studies in our group (data available here⁴⁸⁹) suggested that IFN- γ regulated CD8⁺ T cell differentiation, per se, as opposed to cell expansion. However, surprisingly, IFN- γ did not modulate the ratio between the two transcriptional master regulators T-bet and Eomes (data not shown but available here⁴⁸⁹). This suggested that IFN- γ modulated CD8⁺ T cell differentiation through a noncanonical pathway. It has previously been shown that IFN- γ can synergise or antagonize the effect of growth factors and cytokines³⁸², and that inflammatory cytokines promote effector differentiation by prolonging the

expression of IL-2 receptor alpha chain CD25^{209,524}. We hypothesised that early IFN- γ in our context may function through a similar mechanism and thus investigated the function of early IFN- γ production on CD25 expression. We infected B6 with LM-OVA and blocked IFN- γ 16-24 hours post-infection (**Figure 42A**). CD25 expression in tetramer⁺ CD8⁺ T cells was assessed at day 5 post-infection and increased CD25 expression in antigen-specific cells following IFN- γ blockade was observed (**Figure 42G and H**). Prolonged CD25 expression at day 5 concurs with increased effector and decreased memory formation detected from day 7 onwards and is consistent with IFN- γ antagonizing, or targeting, the same pathway as inflammatory cytokines⁵²⁵. Notably, differences in tetramer binding profiles of antigen-specific CD8⁺ T cells became already apparent at day 5 (**Figure 42B, C, D, E and F**), indicating that the IFN- γ -mediated programming of the responding CD8⁺ T cell population occurs early on in the response.

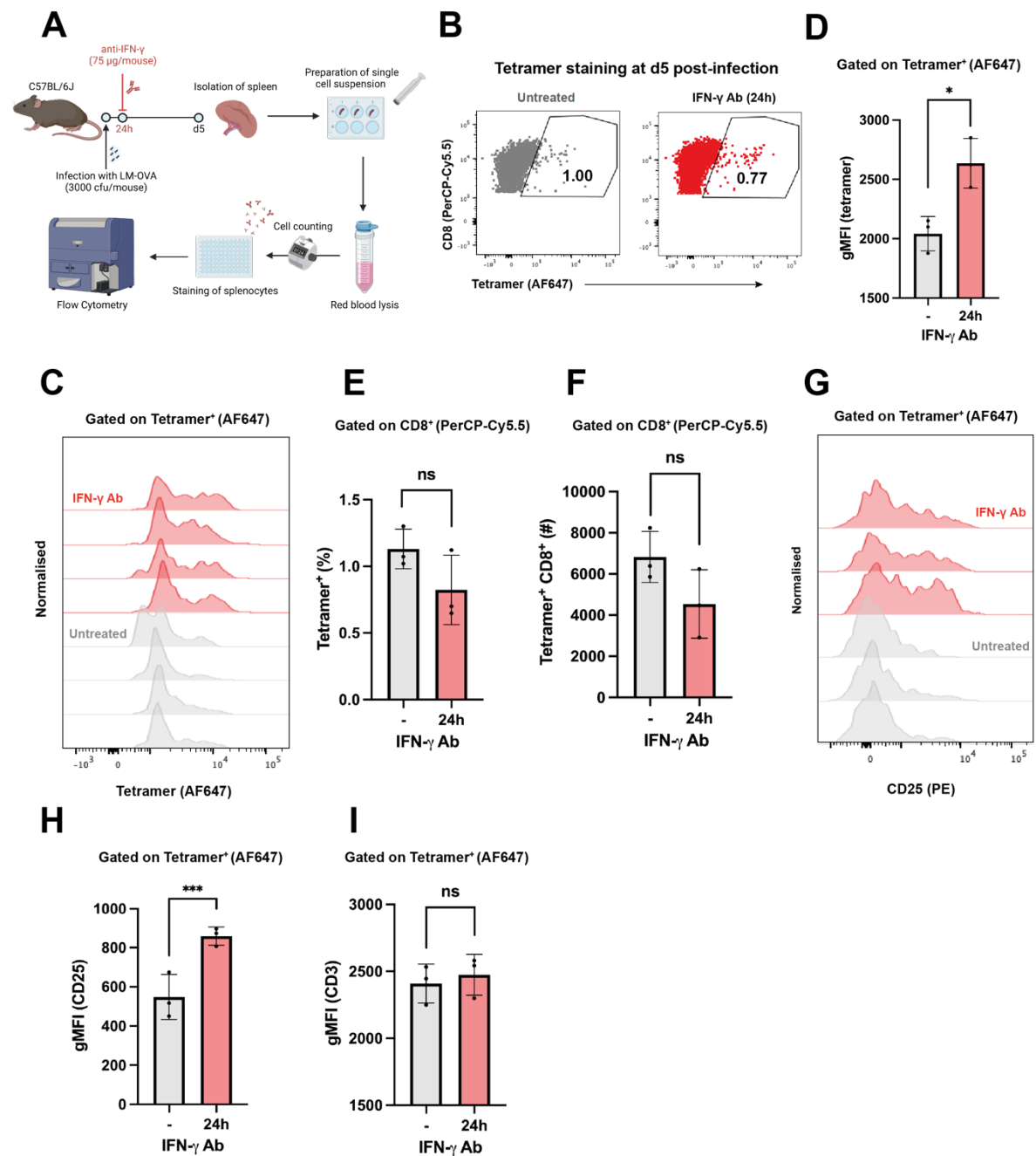


Figure 42. The effect of interferon-gamma (IFN- γ) on CD8⁺ T cells is mediated through a non-canonical signalling pathway modulating CD25 expression in antigen-specific CD8⁺ T cells. (A) C57BL/6J (B6) mice were infected with LM-OVA and mice were either left untreated (grey) or treated with anti-IFN- γ antibody 16-24 hours post-infection (red). Splenocytes were isolated at day 5 for flow cytometry analysis. (B, C, D) Representative flow plots (B), representative histograms (C) and quantification of tetramer binding mean fluorescence intensity (gMFI). (E, F) Analysis of relative abundance (E) and total number (F) of tetramer⁺ CD8⁺ T cells. (G, H) Representative histograms (G) and quantification (H) of CD25 expression in antigen-specific CD8⁺ T cells. (I) Quantification of CD3 expression in tetramer⁺ CD8⁺ T cells. Data are representative of 2 (n = 3 x 3 technical replicates) independent experiments. Error bars indicate the mean $\pm$ s.d. Comparison between groups was calculated using the two-tailed unpaired Student's t-test. *P<0.05; **P<0.01; ***P<0.001; NS, not significant.

Next, we also compared tetramer binding and CD25 expression at day 5 of antigen-specific CD8⁺ T cells in *CD8 α -Cre IFN γ R-flox^{-/-}* (KO) mice with *CD8 α -Cre* control (WT) mice (Figure 43A). A shift in tetramer binding was again observed in KO mice (Figure 43B, C and D), while CD25

expression displayed a trend towards higher expression in KO mice (**Figure 43F and G**). The variability between biological replicates of KO mice was much higher than previously measured for the temporal blockade of IFN- γ in B6 mice and may thus explain why we only observed a trend and not a clear difference in this context. These experiments were not carried out with *CD8 α -Cre IFN γ R-flox^{-/-} Rosa26-tdTomato* mice and it is thus possible that deletion was not fully penetrant in all of the CD8⁺ T cells of these mice, which may, at least partly, explain the higher variability. We are planning on repeating these experiments with *CD8 α -Cre IFN γ R-flox^{-/-} Rosa26-tdTomato* mice.

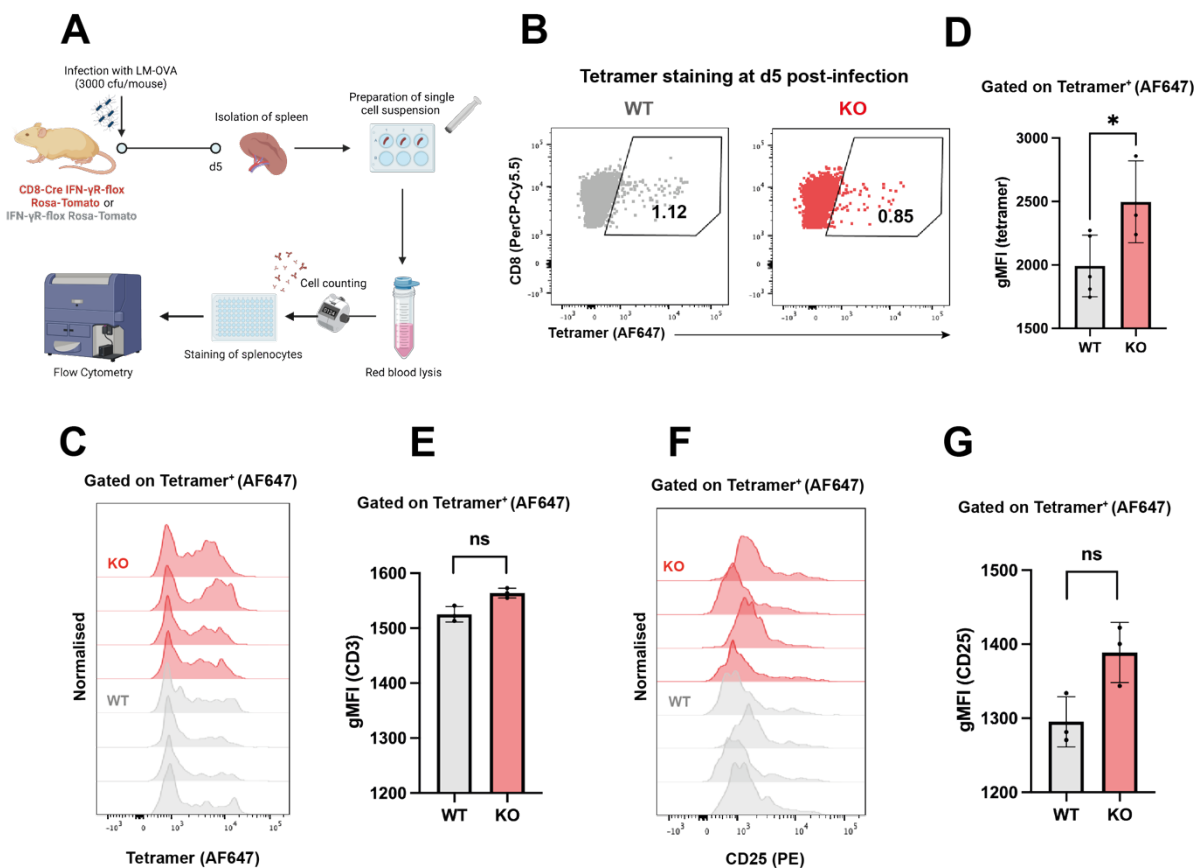


Figure 43. Interferon-gamma (IFN- γ) mediates its function on endogenous CD8⁺ T cells through a non-canonical signalling pathway modulating CD25 expression. (A) *CD8 α -Cre IFN γ R-flox^{-/-} Rosa26-tdTomato* (KO) mice (red) and *CD8 α -Cre IFN γ R-flox^{-/-} Rosa26-tdTomato* (WT) mice (grey) were infected with LM-OVA and splenocytes were isolated at day 5 for flow cytometry analysis. (B, C, D) Representative flow plots (B), representative histograms (C) and quantification (D) of tetramer binding of antigen-specific CD8⁺ T cells at the peak of the response. (E) Quantification of CD3 expression in antigen-specific CD8⁺ T cells at the peak of the response. (F, G) Representative histograms (F) and quantification (G) of CD25 expression in antigen-specific CD8⁺ T cells at the peak of the response. Data are representative of 1 (n = 3 x 3 technical replicates) independent experiment. Error bars indicate the mean $\pm$ s.d. Comparison between groups was calculated using the two-tailed unpaired Student's t-test. *P < 0.05; **P < 0.01; ***P < 0.001; NS, not significant.

4.2.1. NK and CD8⁺ T cells are the two main producers of interferon-gamma 24 hours post-infection

Following our observation that sensing of IFN- γ secretion by CD8⁺ T cells occurs in a specific time window 24 hours post-infection, we next sought to characterize the cell types producing IFN- γ in situ at this time. To analyse IFN- γ expression 24 hours post-infection, GREAT mice were infected with LM-OVA and eYFP expression was measured 24 hours post-infection by flow cytometry (**Figure 44A**) and analysed per cell type. NK(T) cells (analysed by NK1.1 staining and CD1d tetramer binding) comprised 4% of live cells in the spleen and CD8⁺ T cells up to 12 % (**Figure 44B and D**). These results also align with previously reported studies, such as the mouse phenome database (<https://phenome.jax.org>). Other immune cells in the spleen include CD4⁺ T cells, which comprise up to 18% of live splenocytes, and B cells, which comprise up to 58% of live splenocytes (data not shown).

Gating on IFN- γ -producing immune cells, NK(T) cells comprised 60% of IFN- γ -positive cells, followed by IFN- γ -producing CD8⁺ T cells, which comprised up to 20% of IFN- γ -producing cells (**Figure 44C and E**). IFN- γ expression by other immune cells, including CD4⁺ T cells and B cells, was responsible for the remaining 20% of IFN- γ secretion. We furthermore included MAIT cells in our flow cytometry analysis using MR1 tetramers loaded with the ligand 5-OP-RU⁵⁰⁹, which constituted less than 3% of live splenocytes and did not secrete notable amounts of IFN- γ at this early time point. These findings align with previously reported results⁵⁰².

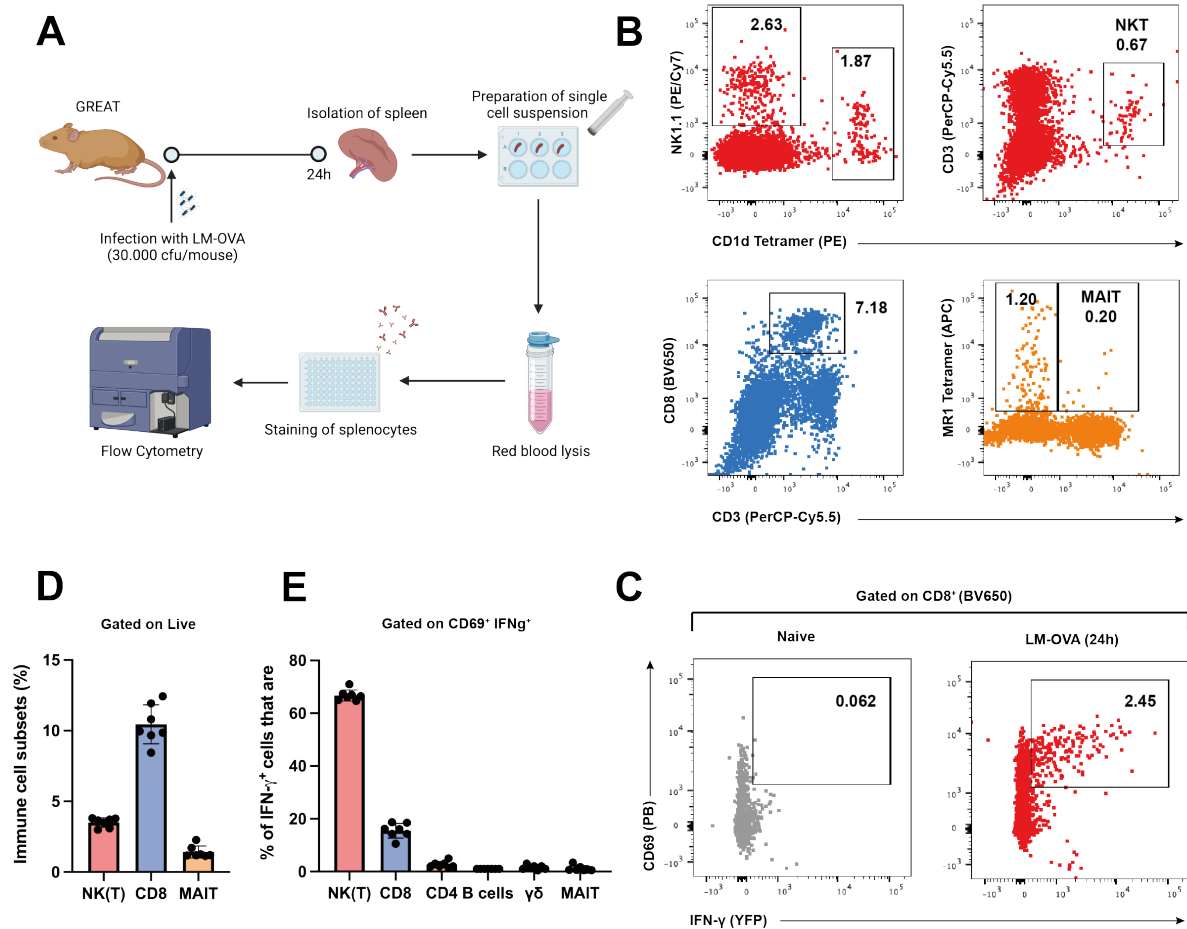


Figure 44. Characterization of interferon-gamma (IFN- γ)-producing immune cells 24 hours post-LM-OVA infection. (A) Splenocytes of LM-OVA infected GREAT mice were isolated 24 hours post-infection for flow cytometry analysis of IFN- γ expression. **(B, D)** Representative histograms **(B)** and quantification **(C)** of immune cell phenotyping 24 hours post-infection. **(C, E)** Representative histograms **(C)** and quantification of phenotyping of IFN- γ ⁺ immune cells 24 hours post-infection. Data are representative of 3 ($n = 3 \times 3$ technical replicates) independent experiments. Error bars indicate the mean $\pm$ s.d. Comparison between groups was calculated using the two-tailed unpaired Student's t-test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS, not significant.

4.2.1. CD8⁺ T cells sense early interferon-gamma in a cell-autonomous fashion

As CD4⁺ T cell-derived IFN- γ is sufficient to mediate T_H1 differentiation in the context of *Leishmania major* infection⁵²⁶, we hypothesised that CD8⁺ T cell-derived IFN- γ may likewise be the dominant source that regulates CD8⁺ T cell behaviour. We tested this hypothesis both at the monoclonal and endogenous level. First, we studied whether IFN- γ produced by wild-type (WT) OT-I cells could induce signalling in IFN- γ ^{-/-} OT-I cells, which would serve as a clear indication of paracrine signalling. We observed enhanced IFN- γ signalling in IFN- γ ^{-/-} OT-I cells

after *in vitro* co-culture (Figure 45B) and *in vivo* co-injection (Figure 45C and D) of IFN- γ ^{-/-} OT-I cells with WT OT-I cells, as assessed by STAT1 phosphorylation on Y701 [p(Y701)STAT1] 24 hours post activation, suggesting a paracrine mode of communication. We concluded that CD8⁺ T cells, at least on the monoclonal level, autonomously modulate certain aspects of their behaviour through early paracrine sensing of IFN- γ supplied by other CD8⁺ T cells.

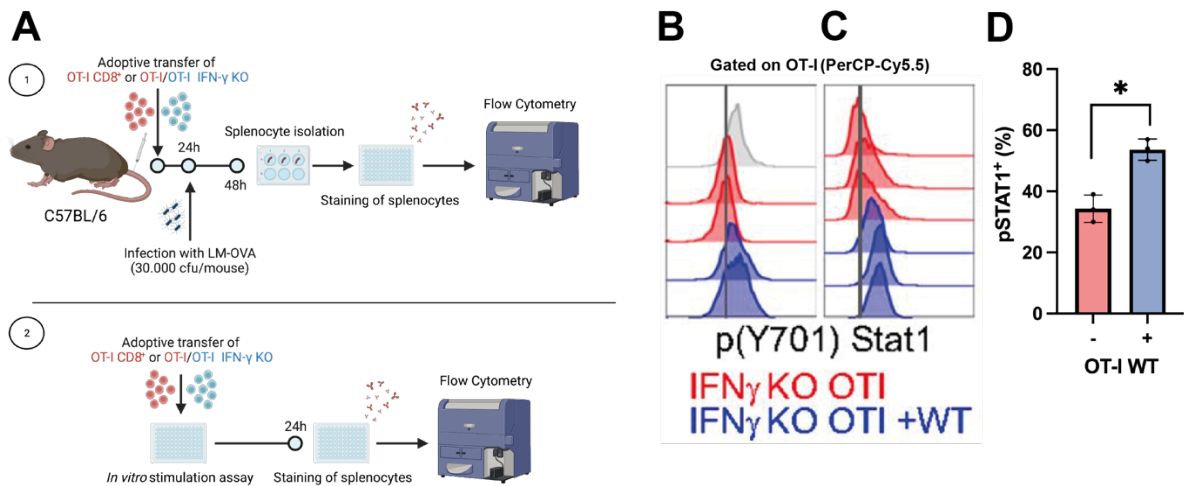


Figure 45. Monoclonal CD8⁺ T cells preferentially receive interferon-gamma (IFN- γ) directly from other CD8⁺ T cells. (A) Mice transferred with IFN- γ ^{-/-} OT-I cells, with or without WT OT-I cells (1:1 ratio), were infected with LM-OVA and analysed after 24 hours by flow cytometry (1). Alternatively, WT or IFN- γ ^{-/-} OT-I cells were stimulated with PMA/Ionomycin for 24 hours, either separated or in co-culture and analysed by flow cytometry (2). (B) Representative histograms show p(Y701)STAT1. WT OT-I cells are coloured grey, IFN- γ ^{-/-} OT-I cells are coloured red, and IFN- γ ^{-/-} OT-I cells are coloured blue when they are co-cultured with WT OT-I cells. (C) Representative histograms showing p(Y701)STAT1 staining in IFN- γ ^{-/-} OT-I cells. IFN- γ ^{-/-} OT-I cells only are coloured red, and IFN- γ ^{-/-} OT-I cells co-cultured with WT OT-I cells are coloured blue. (D) Graphs shows the percentage of p(Y701)STAT1-positive IFN- γ ^{-/-} OT-I cells. Data are representative of 2 (n = 6 x 3 technical replicates) independent experiments. Error bars indicate the mean $\pm$ s.d. Comparison between groups was calculated using the two-tailed unpaired Student's t-test. *P<0.05; **P<0.01; ***P<0.001; NS, not significant.

To test whether we could recapitulate these findings at the endogenous level, we created mixed bone marrow (BM) chimeras by reconstituting lethally irradiated C57BL/6 (B6) recipients with a 1:1 mixture of either *Ifng*^{-/-} (IFN- γ KO) and *Cd8a*^{-/-} (CD8 KO) donor BM or IFN- γ KO and B6 donor BM (Figure 46). Thus, we generated an experimental model, in which all immune cells, except CD8⁺ T cells, are capable of producing IFN- γ (referred to hereafter as KO mice) and a control model, in which all immune cells, including CD8⁺ T cells, are capable of producing IFN- γ (referred to hereafter as WT mice). We originally used exclusively B6 mice as hosts to limit variability between the two types of generated chimeras. However, we realised

that our first batch of chimeras has not been successfully irradiated, as a sizable fraction of host-derived BM cells could be detected in reconstituted mice (data not shown). After an equally unsuccessful second attempt, we decided to instead use IFN- γ KO mice as hosts for the KO condition and B6 mice only for the WT control. This strategy finally allowed us to successfully generate BM chimeras of the required phenotypes.

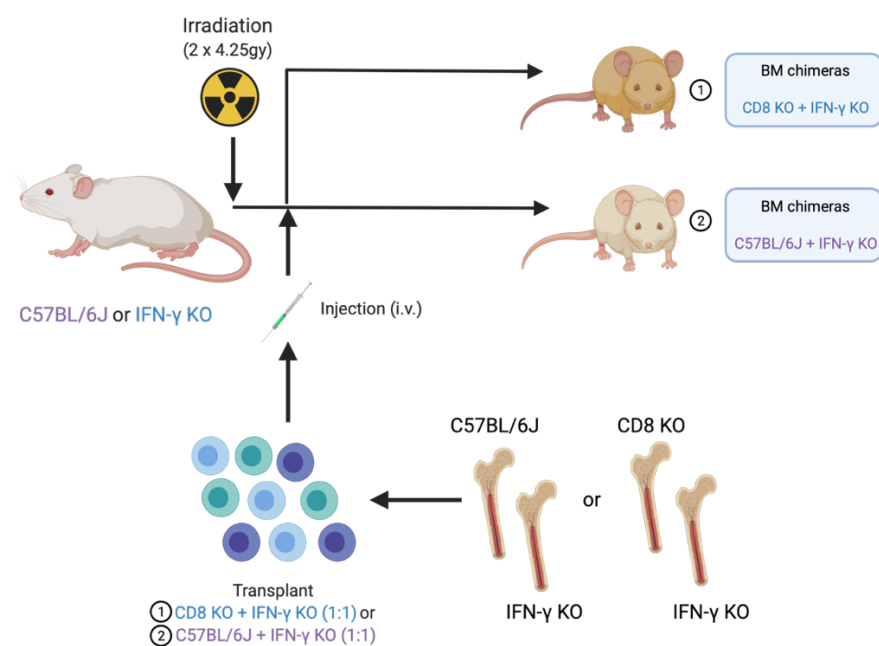


Figure 46. Generation of bone marrow (BM) chimera mice. Irradiated C57BL/6J (B6) or IFN- $\gamma^{-/-}$ (IFN- γ KO) host mice were reconstituted with a 1:1 mixture of either IFN- γ KO/B6 or IFN- γ KO/CD8 KO to generate BM chimeras in which all immune cells can produce IFN- γ (2) or all immune cells except CD8 T cells (1) are capable of secreting IFN- γ , respectively [Created with [BioRender.com](https://www.biorender.com/)].

After reconstitution of the hematopoietic compartment, mice were infected with LM-OVA and splenocytes were isolated 24 hours post-infection, which were either used to measure STAT1 phosphorylation or stimulated with PMA/Ionomycin (PI) to confirm successful reconstitution (**Figure 47A**). We quantified IFN- γ expression following *in vitro* stimulation in both NK and CD8 $^{+}$ T cells and measured no IFN- γ secretion by CD8 $^{+}$ T cells in our KO model, while IFN- γ expression by NK cells was observed in both WT and KO mice (**Figure 47B, C and D**).

STAT1 phosphorylation was used again as a surrogate marker for IFN- γ signalling in activated (CD69⁺) CD8⁺ T cells 24 hours post-infection. Infected IFN- γ ^{-/-} mice were used as control. We observed enhanced IFN- γ signalling in WT mice compared to KO mice (**Figure 47E and F**), suggesting that CD8⁺ T cells preferentially receive IFN- γ from other CD8⁺ T cells. Normalisation of pSTAT1 MFIs furthermore revealed significant inhibition of IFN- γ signalling in the absence of IFN- γ -producing CD8⁺ T cells (**Figure 47G**). While CD8⁺ T cells contribute to 15-20% of total IFN- γ secretion 24 hours post-infection (**Figure 44E**), ablation of CD8⁺-induced IFN- γ leads to 60% inhibition of IFN- γ signalling, as exemplified by STAT1 phosphorylation (**Figure 47G**). We therefore concluded that CD8⁺ T cells autonomously regulate their behaviour through paracrine IFN- γ secretion from other CD8⁺ T cells, not just at the monoclonal, but also at the endogenous level.

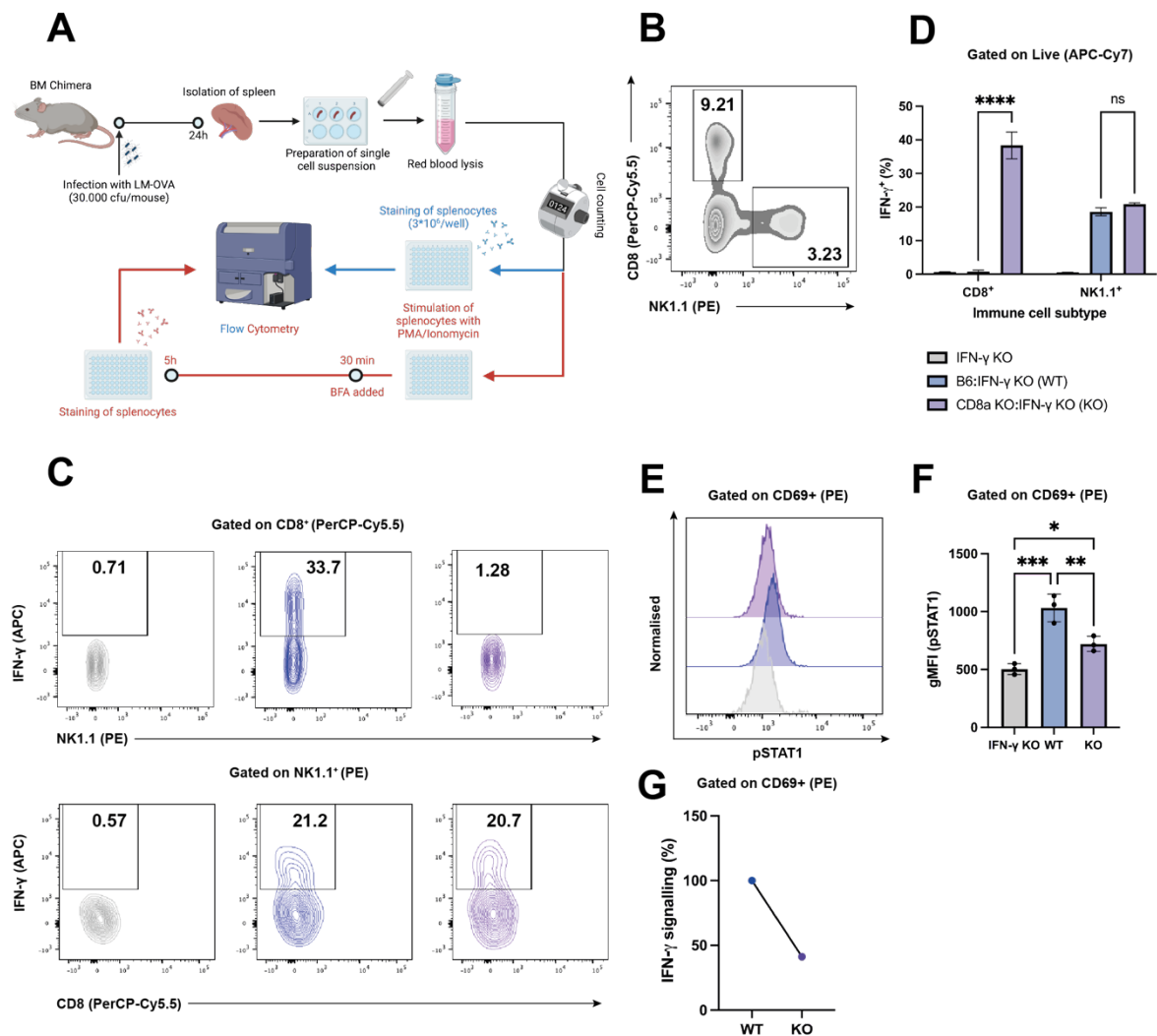


Figure 47. Endogenous CD8⁺ T cells preferentially receive interferon-gamma (IFN-γ) directly from other CD8⁺ T cells. (A) IFN-γ^{-/-} mice (control, grey), CD8a^{-/-}/IFN-γ^{-/-} (KO, purple) and B6/IFN-γ^{-/-} (WT, blue) BM chimeras were infected with LM-OVA and STAT1 phosphorylation in activated (CD69⁺) CD8⁺ T cells was quantified by flow cytometry 24 hours post-infection. (B, C, D) Representative dot plots of immune cell type gating (B) and IFN-γ staining (C) after re-stimulation and quantification (D) of IFN-γ secretion. (E, F) Representative histograms (E) and quantification (F) of STAT1 phosphorylation in activated CD8⁺ T cells 24 hours post-infection. (G) Analysis of IFN-γ signalling inhibition in KO mice. pSTAT1 MFIs of WT and KO mice were first normalised to control (IFN-γ^{-/-}) and obtained KO value was then normalised to WT (= 100% IFN-γ signalling). Data are representative of 3 (n = 1 × 3 technical replicates) independent experiments. Error bars indicate the mean ± s.d. Comparison between groups was calculated using the two-tailed unpaired Student's t-test. *P<0.05; **P<0.01; ***P<0.001; NS, not significant.

We next sought to study if we can extend these findings to other immune cell types. Staining for STAT1 phosphorylation on Y701 [p(Y701)STAT1] by flow cytometry requires permeabilization with methanol, which is known to destroy various fluorophores, such as APC, PE, PerCP and their respective conjugate forms, meaning that only a few fluorophores, such as Brilliant Violet or Alexa Fluor 488, can be used for flow cytometry analysis. Additionally, the changes in STAT1 phosphorylation measured by flow cytometry can be quite

subtle, making it sometimes difficult to draw definitive conclusions. We also considered quantifying STAT1 phosphorylation by western blotting, which is thought to be a more robust and sensitive method of quantifying pSTAT1. However, quantification by western blotting would require isolation of specific cell types, which may be associated with a significant loss of phosphorylation signal if not completed quickly enough. Thus, we tried to identify additional biomarkers, which may be used as surrogates for IFN- γ signalling. Based on the literature, we decided to focus on MHC I⁵²⁷, PD-L1 and IRF1 as potential candidates. Expression of these candidate markers was measured in T cells, macrophages, and dendritic cells 24 hours or 48 hours post-infection (**Figure 48A**). CD8⁺ T cells did not significantly upregulate MHC I in both WT and KO mice 24- or 48-hours post-infection (**Figure 48B, C and D**). Macrophages and dendritic cells on the other hand displayed significant upregulation of MHC Class I expression at both time points post-infection (**Figure 48C and D**). PD-L1 expression did not significantly differ in B6 mice compared with IFN- γ KO mice 24 hours post-infection (data not shown), suggesting that PD-L1 expression is not critically dependent on IFN- γ signalling at this early time point. We decided to not further pursue PD-L1 as a potential surrogate for IFN- γ signalling. The testing of IRF1 as a surrogate of IFN- γ signalling could not be completed in time but is currently ongoing. We concluded that MHC I expression is a useful surrogate to evaluate if macrophages and dendritic cells received IFN- γ but cannot be used to analyse T cells.

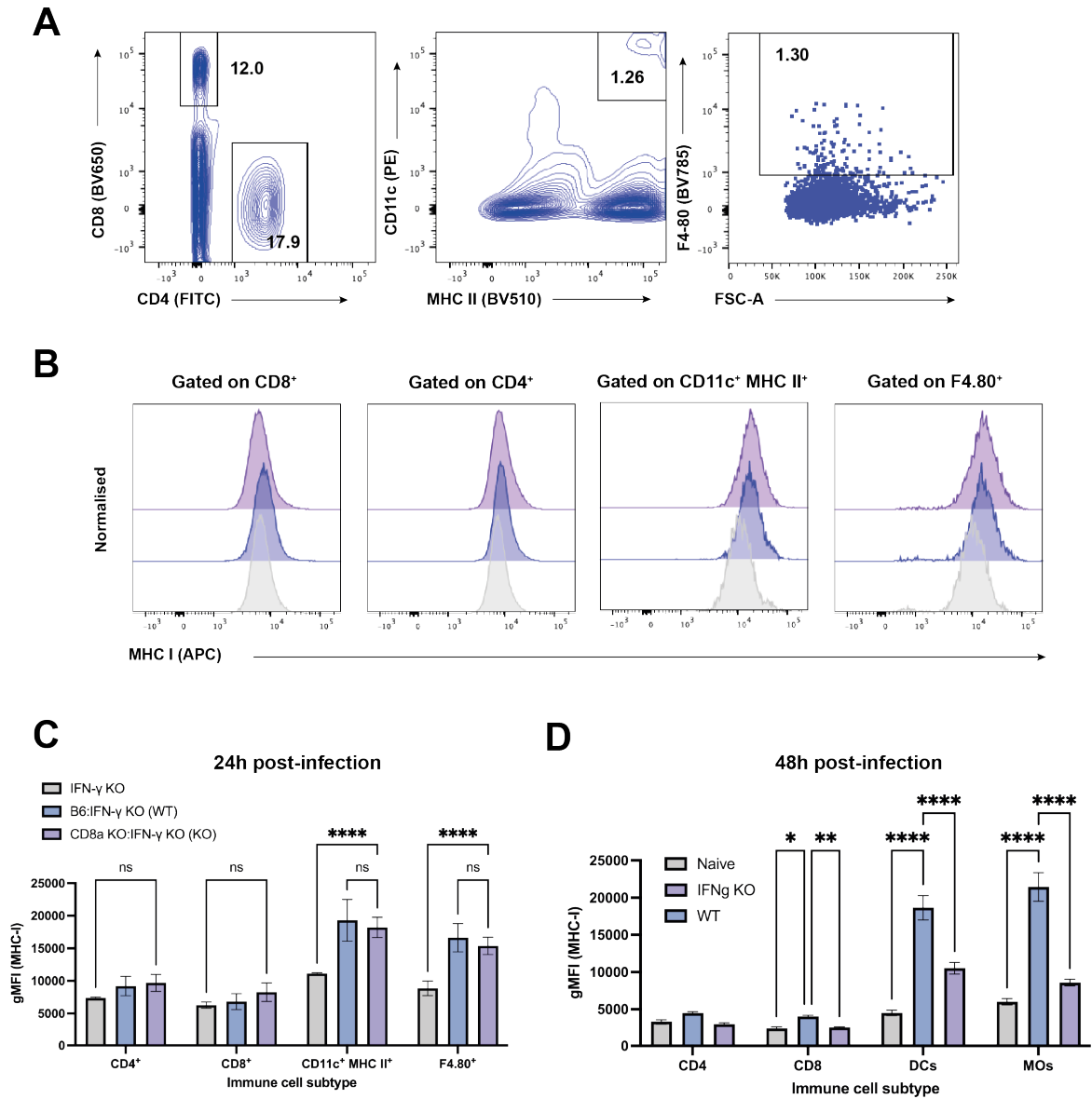


Figure 48. MHC I expression can be used as an alternative surrogate marker of interferon-gamma (IFN- γ) signalling. IFN- $\gamma^{-/-}$ mice (control, grey), CD8a $^{-/-}$ /IFN- $\gamma^{-/-}$ (KO, purple) and B6/IFN- $\gamma^{-/-}$ (WT, blue) BM chimeras were infected with LM-OVA and MHC class I expression in activated (CD69 $^{+}$) CD8 $^{+}$ T cells was quantified by flow cytometry 24- or 48-hours post-infection (**A**) Representative flow plots of gating strategy. (**B, C, D**) Representative histograms of MHC I expression (**B**) and quantification 24 hours (**C**) and 48 hours (**D**) post-infection. Data are representative of 2 ($n = 3 \times 3$ technical replicates) independent experiments. Error bars indicate the mean $\pm$ s.d. Comparison between groups was calculated using a Two-way ANOVA and Šidák's multiple comparison test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS, not significant.

4.3. Discussion

4.3.1. Functional specification of early interferon-gamma signalling in CD8⁺ T cells through a non-canonical signalling pathway

Based on previous findings of our group⁴⁸⁹, we hypothesised that IFN- γ exerts its modulatory function on CD8⁺ T cell expansion and differentiation through a noncanonical signalling pathway. Our studies at the endogenous level indeed suggest that IFN- γ acts through an unconventional signalling pathway. Ablation of IFN- γ R signalling in CD8⁺ T cells results in prolonged CD25 expression ([Figure 43](#)), and thus enhanced expansion of effector cells^{208,209} with a TCR of high functional avidity. The fact that we observe a lag of 4 days between IFN- γ secretion and CD25 downregulation indicates an indirect mode of action, but how IFN- γ precisely regulates CD25 expression remains to be further investigated.

Signalling by the JAK-STAT pathway, including activation of STAT1 by IFN- γ , is typically transient, with a peak signal occurring at 15-60 minutes and resolution back to baseline occurring at 2-4 hours after stimulation⁴⁰⁸. Accordingly, transcriptional responses of many direct STAT1 target genes are thought to peak within several hours of IFN- γ stimulation. However, in addition to its direct signalling effects, IFN- γ was shown to also induce a subset of ISGs with delayed kinetics in a manner dependent on new protein synthesis, thus implying indirect regulation, and to trigger a pattern of sustained gene expression that persists beyond the duration of immediate JAK-STAT signalling^{528,529}. A feedforward loop in which IFN- γ induces de novo expression of transcription factors, which cooperate to induce and sustain gene expression³⁸², may, at least partly, explain these delayed and sustained kinetics of gene induction.

Another possible mechanism for persistence of IFN- γ signalling may be capture of IFN- γ by cell surface phosphatidylserine on live cells, followed by its slow release to drive long-term transcription⁵³⁰, which was shown to elicit durable inflammatory responses in cancer cells. As TCR activation induces PS exposure at the surface of T cells⁵³¹, a similar mechanism may lead to long-term exposure of T cells to IFN- γ . Lastly, stability of gene expression that persists beyond the initiating signal could also be accomplished through epigenetic mechanisms and chromatin remodelling providing short-term memory^{532,533}. Several studies (reviewed here⁴⁰⁸) have started to elucidate such IFN- γ -mediated epigenetic mechanisms of cell reprogramming in macrophages. Whilst beyond the scope of this thesis, it is intriguing to speculate that IFN- γ may also induce remodelling of the chromatin landscape in CD8⁺ T cells to imprint them with a more stable and sustained transcriptional response.

It is intriguing to speculate that a convergence of additional signalling factors, such as TCR stimulation strength or additional cytokine signalling, programs a certain subset of responding CD8⁺ T cells for heightened IFN- γ sensitivity. Our finding that IFN- γ modulates differentiation at the clonal level indicates that additional signalling components play a crucial role in defining the precise signalling outcome of IFN- γ on T cells. Additional studies carried out in our group⁴⁸⁹ indeed suggest that T-T contacts dictate the responsiveness of activated CD8⁺ T cells to the IFN- γ they produce. As these studies have not been conducted by me and are thus not part of this thesis, they will only be briefly summarised for the purpose of discussion. In summary, our group has shown that blocking T-T contacts with LFA-1 blocking antibodies *in vitro* resulted in decreased p(Y701)STAT1 downstream of IFN- γ signalling, which was not due to an inability of cells to produce or detect IFN- γ , per se, as confirmed by the fact that phosphorylation of STAT1 at serine 727 was unaffected. Moreover, requirement of contact for optimum IFN- γ signalling was neither the consequence of a desensitization

mechanism due to chronic IFN- γ exposure, nor a result of IFN- γ down-regulation, as observed for CD4⁺ T cell differentiation into the T_H1 subset⁴³⁶. Artificial, integrin-independent clustering of cells treated with LFA-1 blocking antibody did not lead to increased IFN- γ sensitivity, and it was furthermore observed that integrins had to colocalise with IFN- γ R to facilitate signal integration. Collectively, our group has shown that integrin engagement potentiated IFN- γ signalling by fulfilling a critical co-stimulatory function that causes enhanced STAT1 phosphorylation and helps specify the signalling outcome controlling CD8⁺ T cell fate (Figure 49).

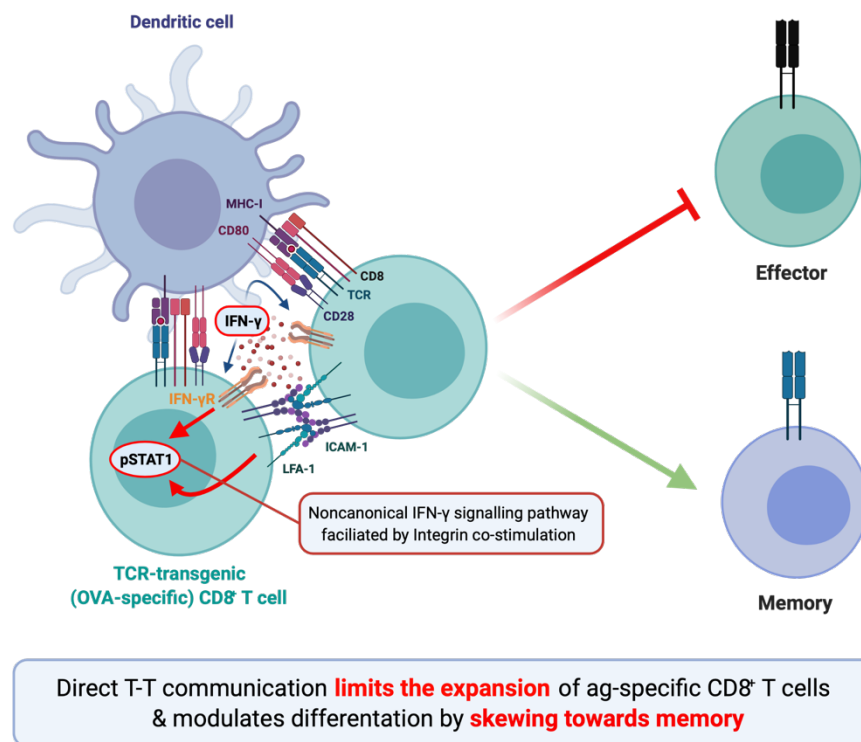


Figure 49. Paracrine co-stimulation of interferon-gamma (IFN- γ) signalling by integrins modulates CD8⁺ T cell differentiation. During bacterial infection, IFN- γ skews CD8⁺ T cell differentiation towards memory. Modulation of CD8⁺ T cell differentiation by IFN- γ relies on a nonconventional IFN- γ outcome that occurs specifically within 16-24 hours following infection. This is driven by IFN- γ co-stimulation by integrins at T-T synapses and leads to synergistic phosphorylation of the proximal STAT1 molecule and accelerated IL-2 receptor down-regulation⁴⁸⁹ [Created with [BioRender.com](https://www.biorender.com)].

These findings do not exclude the possibility that other additional factors may be required for signal integration, and that integrins may recruit IFN- γ R to specific lipid nanodomains at T cell-T cell synapses, thus enabling distinct downstream events. Additionally, given that these

studies were conducted on a monoclonal population of T cells using the high-affinity OT-I system, it is conceivable that TCR affinity may provide another crucial stimulus that helps to define and potentially fine-tune the ultimate signalling outcome.

Our findings help to explain the discrepancies reported between models of IFN- γ function on CD8⁺ T cells and suggest that the presence of cofactors represents one of a series of possible modifiers of IFN- γ signalling and function specification. This may also explain discrepancies reported between the localised and systemic effects of IFN- γ . Data from our group suggest that CD8⁺ T cells secrete early IFN- γ in a localised and polarized fashion, possibly to restrict harmful bystander responses while optimising efficient T cell-mediated pathogen clearance programs. It is plausible that multi-directional IFN- γ secretion by NK cells may be the source of systemic IFN- γ , possibly spreading IFN- γ throughout the spleen^{61,534}. Signal amplification and specification through integrin co-stimulation, which requires previous T cell activation³¹⁰, means that only cells in the correct state of activation and engaging integrins will adequately respond to this signalling cue.

4.3.2. Differential sensitivity of CD8⁺ T cells to interferon-gamma throughout the response

We initially hypothesised that differential sensitivity to IFN- γ may be regulated through cell-specific tuning of IFN- γ R expression. However, when quantifying the kinetics of IFN- γ R expression during the early phase of the response no notable changes in receptor expression were measured ([Figure 38](#)), suggesting that sensitivity towards early IFN- γ may be regulated mainly through other mechanisms. Our findings contrast earlier reports²⁰⁷, which have shown that the IFN- γ R2 chain is downmodulated beyond 12 hours post activation, rendering cells unresponsive to IFN- γ . mRNA expression levels for IFN- γ R1 and IFN- γ R2 were also shown in

another study to decline more than 8- to 10-fold within 24 hours of stimulation, at least *in vitro*^{379,535}. However, it is important to highlight that receptor downregulation in these earlier studies was only evaluated at the transcription level. It is therefore possible, that the receptor may be expressed for longer at the protein level. Our pSTAT1 data indeed supports the notion that the IFN- γ R may be expressed beyond 12 hours post activation.

Taken together, these findings suggest that IFN- γ signals that drive differentiation must be delivered early in the response. We have also shown that during that exact same time window a subset of CD8⁺ T cells secretes IFN- γ following activation (**Figure 37**). Our *in vivo* IFN- γ blocking experiments (**Figure 39**) furthermore demonstrated that the timing of the signalling event is critical in triggering the signalling effects described in **Chapter 3.2**. Only when receiving IFN- γ 16-24 hours post-infection do we observe the modulatory functions of IFN- γ in effector CD8⁺ T cells described earlier. Beyond this early time window following activation, CD8⁺ T cells were suggested to remain refractory to IFN- γ R signalling and to only regain partial responsiveness during the contraction phase of the response²⁰⁷. This does not exclude additional functions of IFN- γ secreted at later time points on CD8⁺ T cells, such as a previously described regulatory role in controlling the contraction phase of CTL responses via FAS-FASL-/Bim-mediated apoptosis, both of which are induced by STAT1 signalling⁴⁵⁹. CTL-derived IFN- γ was furthermore shown to enhance the motility and cytotoxicity of CTLs⁵³⁶, whilst high IFN- γ levels during expansion were implicating in limiting the size of the resulting memory population^{127,460}. Our findings do not contradict those reports but add an additional functional layer to the role of this pleiotropic cytokine in regulating CD8⁺ T cell responses.

It is important to note, that blocking IFN- γ does not phenocopy blocking T cell-T cell contacts as a whole, as reported previously³¹⁰. Whereas integration of IFN- γ and integrin signalling pathways restricts T cell effector differentiation, T cell-T cell contacts have been

shown to promote both effector and central memory differentiation³¹⁰. This discrepancy is likely due to the role T cell-T cell synapses play as a platform fostering a network of heterogeneous signals shared exclusively between responding T cells. Some of these may also be temporal and include contributions from other cell-cell contacts *in vivo*. Our study only provides one instance of such a platform function, facilitating in this case signal integration through co-stimulation of IFN- γ signalling by integrin co-engagement. Importantly, whereas integrin activation is dictated by the encounter with the antigen early during priming and is reflective of the strength of activation, IFN- γ secretion is determined by cell localisation and the specific environmental context.

4.4. Conclusion

In this chapter, we found evidence for a context-specific role of IFN- γ in the coordination of CD8⁺ T cell responses to infection. Both global and CD8⁺-mediated IFN- γ expression following infection with *L. monocytogenes* exhibits a biphasic pattern. While it has previously been shown that IFN- γ secretion at the peak of the response controls the contraction phase of activating CD8⁺ T cells, our data demonstrated a critical role of early IFN- γ in the regulation of the ensuing CD8⁺ T cell response. Our experimental approach combined observations on the endogenous and monoclonal level, which helped us to dissect the function of this early wave of IFN- γ in limiting effector expansion and tuning the functional avidity of the responding T cell repertoire. We have furthermore demonstrated that CD8⁺ T cells preferentially receive IFN- γ from other CD8⁺ T cells 16-24 hours post activation. Based on previous findings of our group on the monoclonal level⁴⁸⁹, we hypothesise that endogenous CD8⁺ T cells regulate each other's fate through targeted delivery of IFN- γ , which may be mediated and potentiated through transient, homotypic T-T synapses formed between

interacting T cells. We sought to explore in the next chapter if early IFN- γ production is restricted to a particular subset within the CD8⁺ T cell population and aimed to gain a better understanding of the inducible factors triggering IFN- γ secretion in those cells.

5. A specialised CD8⁺ T cell subset produces interferon-gamma following activation by TCR- and cytokine-stimulation

5.1. Introduction

5.1.1. General mechanisms of interferon-gamma stimulation expression in immune cells

IFN- γ expression is tightly regulated by epigenetic, transcriptional, post-transcriptional and post-translational modifications, which prevent IFN- γ expression by non-immune cells, naïve T cells and even some activated immune cells^{385,537}.

In naïve T cells *Ifng* has been shown to be actively silenced via methylation and hypoacetylation. Epigenetic regulation and cell-specific expression is facilitated through binding of specific transcription factors to conserved non-coding sequences within the proximal regulatory regions of *Ifng*⁵³⁸. Mechanistically, T-bet directly binds to the *Ifng* gene, and possibly cooperates with STAT4 for its gene regulatory function⁵³⁹. It was suggested that STAT4 promotes *Ifng* expression via histones H3 and H4 acetylation and binding to the *Ifng* promoter⁵⁴⁰. Of note, hyperacetylation of IFN- γ -encoding chromatin differs among T cells and can extend outside the *Ifng* locus, which likely enables cell-specific epigenetic regulation³⁸⁵.

Ifng transcription is promoted by various stimuli, including TCR engagement, and transcription factors, such as AP-1, T-bet, Eomes, NFAT and NF- κ B^{541–543}. Additional activated immune cells products, such as IL-2, hydrogen peroxide and leukotrienes, have furthermore been implicated in positive feedback loops that drive IFN- γ production via second messengers, such as protein kinase C and cyclic GMP^{544–546}. In addition, the cytokines IL-12 and IL-18 are known to be potent promoters of *Ifng* transcription⁵⁴⁷. IL-12 activates STAT4, whilst IL-18 signals via NF- κ B and activates the AP-1 subunit JUN, thus forming a STAT4-AP-1

complex, which enhances STAT4 binding to the *Ifng* promoter^{78,385,548}. IL-12 furthermore promotes transcription of IL-18 receptor, thus establishing a positive-feedback loop⁵⁴⁹ that promotes synergistic regulation of IFN- γ expression.

Fast translation and secretion upon appropriate stimulation is facilitated by the storage of preformed *Ifng* mRNA in both the nuclear and the cytoplasmic compartment. Induction of the mitogen-activated protein kinase p38 through IL-12 and IL-18 triggers its binding to an AU-rich element in the 3' untranslated region of *Ifng* mRNA, prompting, at least temporally, its stabilisation and protection from degradation^{550,551}. Negative regulation has also been reported and to be mediated indirectly by miR-29, miR-146a and miR-142-3P^{385,552–554}.

5.1.2. Immune cell type-specific regulatory mechanisms of interferon-gamma stimulation

Besides these mechanisms considered to drive IFN- γ secretion in a more generalised fashion, cell-specific differences in IFN- γ regulation between different immune cell subtypes have also been reported. IFN- γ secretion in NK cells is potently induced by IL-2 and IL-12 and potentiated by TNF^{555,556}. Another cytokine shown to promote IFN- γ expression in NK and $\gamma\delta$ T cells is the alarmin interleukin-33 (IL-33)⁵⁵⁷. iNKT cells have also been reported to release IFN- γ upon binding to IL-12⁵⁵⁸. Notably, iNKT cells have been observed to secrete IFN- γ in a TCR-independent fashion and to do so in response to DC-derived IL-12 in the absence of CD1d-presented antigen⁵⁵⁹. Induction of IFN- γ production in CD11a^{hi}CD16/CD32^{hi} B cells is dependent upon IFN- γ receptor, T-bet expression, and furthermore requires active Bruton's tyrosine kinase, which is activated via NF- κ B, IL-1 β and CD40-CD40L signalling⁵⁶⁰.

5.1.3. Interferon-gamma stimulation in CD8⁺ T cells

Interestingly, unlike for T_H1 cells, expression of IFN- γ by CTLs as well as their production of perforin, granzymes, TNF and IL-2, is independent of T-bet and requires the T-bet paralogue Eomes^{240,561}, which is induced by TCR stimulation³⁸⁵. However, as previously mentioned, CTLs are not the only CD8⁺ T cell subset reported to secrete IFN- γ . Rapid IFN- γ secretion by activating CD8⁺ T cells has been previously demonstrated to occur within only a few hours of lipopolysaccharide (LPS) injection⁵²⁶ or bacterial or viral infection^{379,502,562,563}, in an innate-like, non-antigenic specific fashion that is typical of other innate-like T cell subsets residing in peripheral tissues, such as NKT cells and mucosal-associated invariant T cells.

This mode of cytokine-mediated, so-called “*bystander activation*” of CD8⁺ T cells was first recognized in 1996 in a study demonstrating that poly(I:C) injection-induced type I IFN production, which promoted the proliferation and expansion of a diverse number of polyclonal CD8⁺ T cells independently of TCR and antigen specificity⁵⁶⁴. Follow-up studies have linked bacterial products such as LPS also to non-antigen-specific T cell stimulation⁵⁶⁵, and subsequent research revealed that type I IFNs indirectly regulate the antigen-independent proliferative capacity of memory CD8⁺ T cells via the production of IL-15⁵⁶⁶. Other groups, using murine models of viral infections with LCMV, and vaccinia virus confirmed the activation of antigen nonspecific T cells but concluded that bystander activation lacked biological significance^{567–569}, a notion that is currently heavily contested⁵⁷⁰.

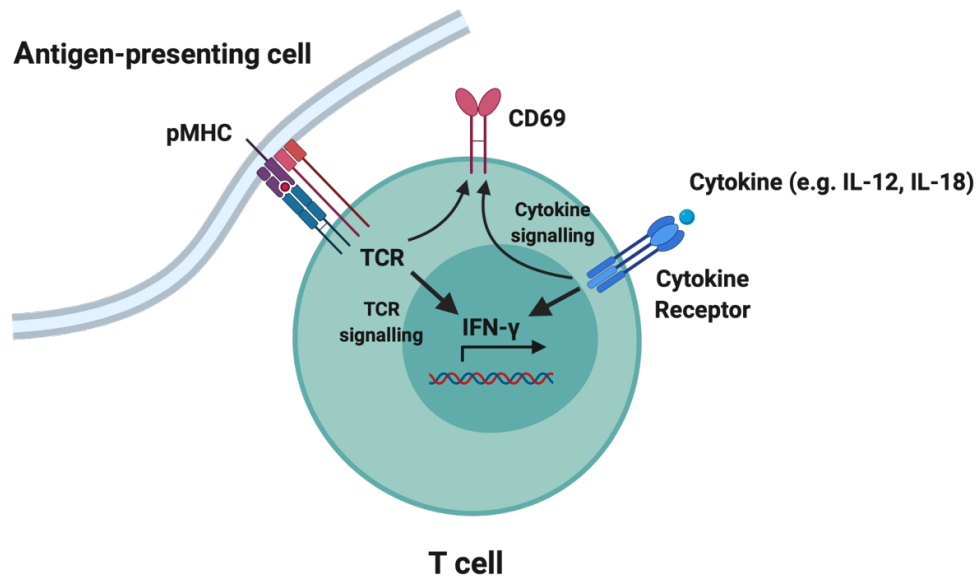


Figure 50. Interferon-gamma (IFN- γ) secretion by CD8⁺ T cells. Previous studies have shown that IFN- γ may be driven by either TCR or cytokine signalling or a combination of these triggers, both of which lead to upregulation of the activation marker CD69.

Rapid IFN- γ secretion by activating CD8⁺ T cells was observed to be independent of cognate antigen-TCR engagement and to be triggered in a cytokine-dependent, innate-like fashion early during infection. In the context of LM infection this mode of CD8⁺ bystander activation was suggested to be mainly driven by the pro-inflammatory cytokines IL-12, IL-18 and IL-15 from LM-infected monocytes and macrophages^{70,502,562,571–573}. Multiple studies have furthermore discovered these early IFN- γ -producing CD8⁺ cells to exhibit either an effector or memory phenotype, based on expression of the activation marker CD44, whilst a much lower percentage of antigenically naïve CD8⁺ T cells was reported to express IFN- γ upon cytokine activation^{70,574}. The heightened responsiveness by effector and memory CD8⁺ T cells, as opposed to naïve CD8⁺ T cells, may result from selective expression of the IL-18 receptor^{70,575}. Notably, memory CD8⁺ T cells were shown to display a gradual reduction in the expression of IL-12 and IL-18 receptors following initial antigen stimulation, which is consistent with an observed reduced ability to be activated by bystander signals, which was reversed upon repeated antigen stimulation⁵⁷⁶. Of note, a few studies also observed rapid cytokine-

independent IFN- γ expression in naïve CD8⁺ T cells upon TCR stimulation^{379,577}. However, it is important to highlight that these studies were conducted exclusively on the monoclonal, transgenic level using OT-I T cells.

It also important to highlight in this context, that in all of the aforementioned studies, rapid IFN- γ secretion was not a uniformly observed phenomenon. To the contrary, only a relatively small percentage of (mostly CD44^{hi}) CD8⁺ T cells have been shown to express IFN- γ following cytokine-mediated activation. What sets those cells apart from others has remained elusive, but it does indicate that a particular CD8⁺ T cell subset acts as primary producer of CD8⁺ T cell-derived IFN- γ early during the response. Indeed, more recent studies reported that IL-12 and IL-18 elicit rapid cytokine production and even bystander killing in an antigen-independent manner from a particular subset of CD8⁺ T cells known as virtual memory T cells^{578–580}, which will be described in more detail after a brief introduction of the cytokines IL-12, -18 and -15.

5.1.4. The cytokines interleukin-12, -18 and -15

5.1.4.1 Interleukin-12

The IL-12 family of cytokines are primarily secreted by cells of myeloid origin in response to inflammatory stimuli, such as microbial products or fungal infections, and execute a range of proinflammatory and immunosuppressive function⁵⁸¹. Whilst belonging to the type 1 hematopoietin family of cytokines, IL-12 family members are unique in that each member is comprised of heterodimers, in which either the α or β subunit is shared with other receptor complexes⁵⁴⁷. The functionality of each respective cytokine and cytokine receptor ranges from pro- to anti-inflammatory in a host of pathological and physiological conditions.

IL-12 consists of the subunits p35 and p40 and acts primarily on NK cells and T cells^{582,583}. Binding of the subunits IL-12R β 1 to IL-12R β 2 forms the IL-12 receptor complex^{583,584}. Upon

ligation, IL-12R β 1 binds to Tyrosine kinase 2 (Tyk2) whilst IL-12R β 2 binds to JAK2. Tyk2 and JAK2 then phosphorylate tyrosine residues primarily on STAT4. Ultimately, the STAT4 complex translocates to the nucleus and binds to the IFN- γ promoter. Additionally, Jun oncogene (c-Jun) is also recruited to the IFN- γ promoter via STAT4^{585–587}, potentiating IFN- γ transcription, and T_H1 differentiation in CD4⁺ T cells. IL-12 can furthermore promote expression of IRF1 and IRF4 in a STAT4-dependent manner^{588,589}.

Next to its role in T_H1 differentiation, IL-12/IL-12R was additionally shown to promote T-cell proliferation and adhesion during activation, at least partly through enhancing interleukin 2 receptor α (IL-2R α) expression by recruiting STAT4 and c-Jun to the promoter of IL-2R^{590,591}. IL-12-induced STAT4 activation also culminates in P-selectin ligand formation, which augments T cell adhesion during differentiation^{592–595}. Productive IL-12/IL-12R signalling furthermore triggers both positive and negative feedback mechanisms which can either strengthen or reduce IL-12 signalling, respectively^{596–598}.

5.1.4.2 Interleukin-18

IL-18 was initially discovered as an IFN- γ -inducing factor in T_H1⁷⁸ and is produced and accumulates in the cytoplasm as a biologically inactive precursor (pro-IL-18), which requires proteolytic processing by caspase-1 for its secretion in an active form⁵⁹⁹. IL-18 is mainly synthesised by activated macrophages and DCs, although production by other cell types has also been shown⁷⁹. In addition to stimulating T_H1 cells, IL-18 acts together with IL-12 to induce abundant IFN- γ production from memory CD8⁺ T cells^{70,526}. Synergistic activity with IL-12 furthermore promotes the proliferation of memory CD8⁺ T cells⁵⁷¹.

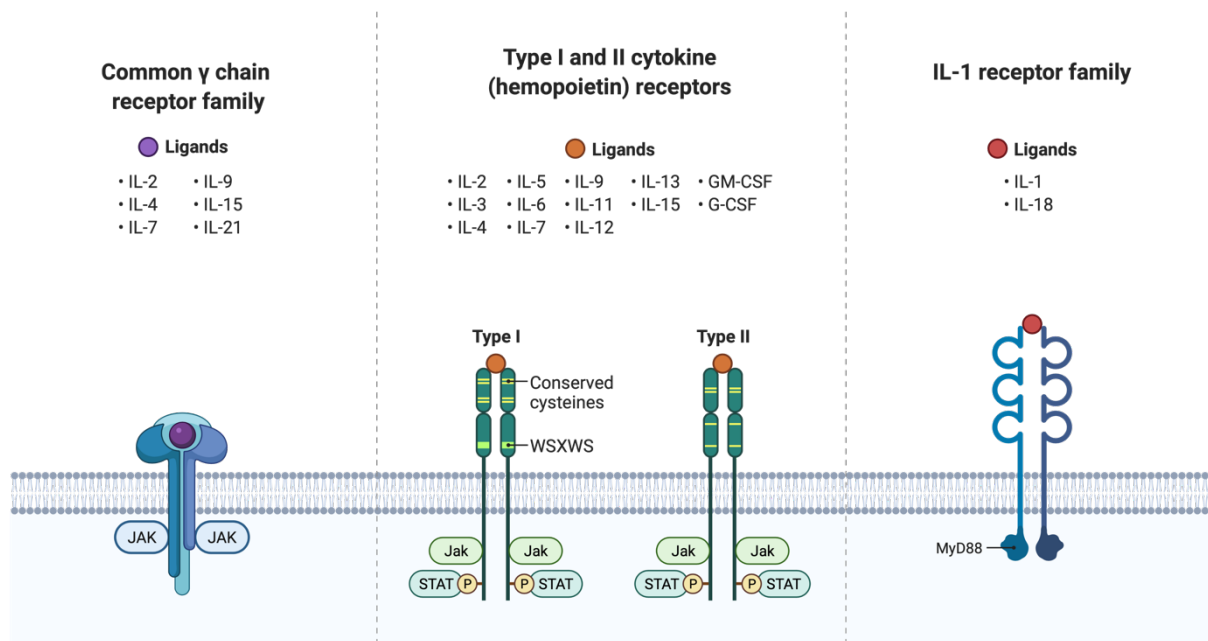


Figure 51. Interleukin-12, -18 and -15 belong to distinct cytokine families. IL-12 belongs to the hemopoietin family of cytokines, while IL-15 is a member of the common γ chain receptor family and IL-18 a member of the IL-1 receptor family [Created with [BioRender.com](https://www.biorender.com)].

5.1.4.3 Interleukin-15

IL-15 belongs to a group of cytokines that signal through a common gamma-chain (γ) cytokine receptor⁶⁰⁰ and is secreted by various cell types in response to microbial infection, as well as inflammatory and stress signals. It is *trans*-presented to NK or memory CD8⁺ T cells by the receptor IL-15R α ⁶⁰¹ and following IL-15 recognition by IL-15R β (CD122)- γ -chain (CD132) heterodimeric receptors on NK or memory CD8⁺ T cells, three parallel downstream signalling routes are activated, including JAK-STAT, Ras-Raf-MEK-mitogen activation protein kinase (MAPK) and PI3K-AKT-mTOR pathways. Activation of these pathways subsequently stimulates the proliferation, survival and activation of NK and memory CD8⁺ T cells^{602,603}. IL-15 furthermore sensitizes memory CD8⁺ T cells to enhance effector functions induced by TCR stimulation^{604,605}. In the absence of TCR stimulation, IL-15 can also activate memory CD8⁺ T cells to proliferate and exert effector functions⁶⁰⁶. Lastly, prolonged IL-15 exposure enables memory CD8⁺ T cells to acquire NK cell-like phenotypes and functions, including upregulated

expression of NK cell receptors and enhanced cytotoxicity, indicated by high expression of CD107a, perforin and granzymes. IL-15-stimulated memory CD8⁺ T cells have also been shown to kill target cells in a NK cell-like manner^{570,607–609}.

5.1.5. Memory phenotype CD8⁺ T cells

CD8⁺ T cells come in different flavours and are broadly differentiated into either antigen-inexperienced, naïve CD8⁺ T cells or antigen-experienced effector or memory CD8⁺ T cells. However, multiple studies have revealed a wide range of phenotypic heterogeneity within the CD8⁺ T cell population and led to the discovery of various T cell memory subsets with distinct and specialised modes of action ([Figure 52](#)). Antigen-experienced true memory CD8⁺ T cells arise in mice in response to infection. However, unimmunized mice also contain CD8⁺ T cells with a memory phenotype (T_{MP}), which are often termed virtual memory T cells (T_{VM}), which was inspired by its use in computing, where virtual memory describes a form of working memory, based on alternative utilization of existing space⁶¹⁰.

T_{MP} are classically described as displaying a CD44^{hi}CD122^{hi} phenotype^{611,612}, and while several studies included T_{VM} cells in this broader category, the majority of T_{MP} cells in naïve C57BL/6J (B6) mice has been reported to comprise of T_{VM} cells⁶¹³, which are commonly classified as exhibiting a CD44^{hi}CD122^{hi}CD49d^{low} phenotype, with the consistently observed low expression of CD49d first described by Haluszczak and colleagues to be a hallmark of T_{VM} cells⁶¹⁰. Another population of T_{MP} cells is known as innate-memory (T_{IM}) CD8⁺ T cells, which share many features with T_{VM} cells but are also distinct in others, most notably the dependence on IL-4 for their development^{611,614}. A few publications prefer using the more general term T_{MP}, whilst still mostly referring to T_{VM} cells. However, to avoid confusion, the term T_{MP} in the context of this thesis will be used to exclusively refer to the total population

of memory-phenotype CD8⁺ T cells, whereas we will use the term virtual memory CD8⁺ T cells to only refer to the specific memory phenotype subpopulation characterized by CD44^{hi}CD122^{hi}CD49d^{low} expression. Of note, an additional point of confusion in the field has been that T_{VM} cells are also CD62L^{hi}, which has often led to misclassification of T_{VM} cells as central memory CD8⁺ T cells, if CD49d is not included in the gating strategies^{613,615}.

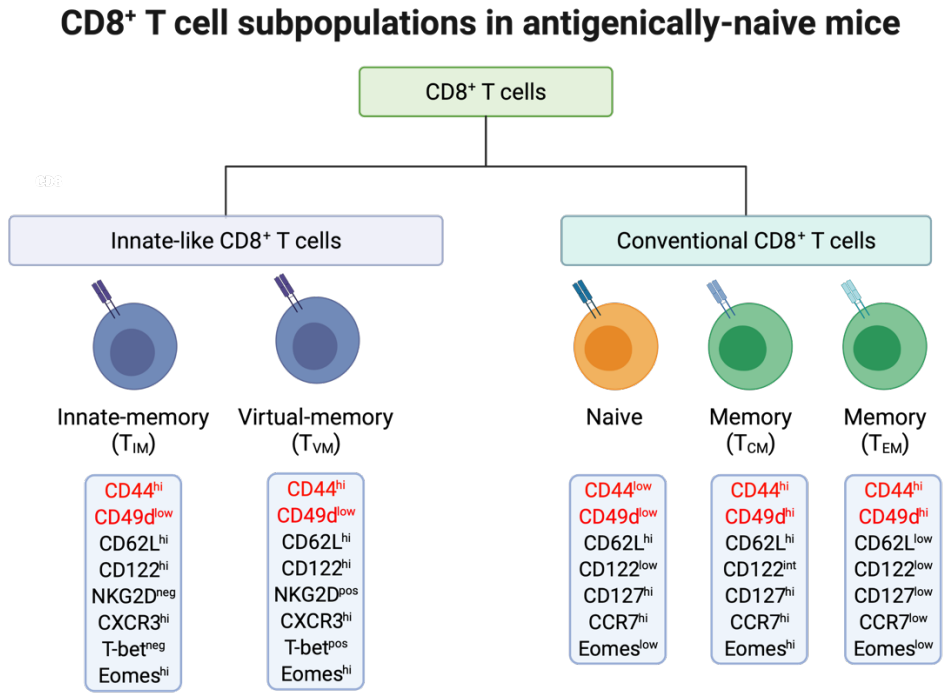


Figure 52. Antigenically-naïve CD8⁺ T cells can be subdivided into distinct subpopulations. Based on the expression level of certain surface markers and transcription factors, such as CD44 and CD49d, different subpopulations of CD8⁺ T cells in antigenically-naïve mice have been defined, which display distinct features and functionalities [Created with [BioRender.com](https://www.biorender.com)].

5.1.5.1 Virtual memory CD8⁺ T cells

T_{VM} cells comprise about 5-10% and 10-20% of the peripheral CD8⁺ T cell pool in the blood and spleen, respectively^{610,615}. Memory T cells are known to exhibit multiple qualitative advantages over their naïve counterparts with respect to their functional versatility, speed of response, and capacity to migrate to multiple sites besides lymphoid tissues⁴³. Consistent with their memory-like phenotype, T_{VM} cells exhibit memory-like functionality. Compared to naïve cells, activation of T_{VM} cells has been shown to occur more rapidly following stimulation

through their TCR, and their proliferation, cytokine secretion and cytotoxic qualities have been reported to follow similar kinetics and efficiencies as shown for conventional antigen-experienced memory CD8⁺ T cells^{579,616,617}, which reflects the fact that, similar to antigen-experienced memory CD8⁺ T cells, T_{VM} cells exhibit a poised state for entry into cell cycle⁵⁷⁹ and poised epigenetic profiles at the transcriptional start sites for many effector genes⁶¹⁸. However, notably, while effector T cells derived from naïve CD8⁺ T cells are known to be multifunctional⁶¹⁹, effector CD8⁺ T cells derived from T_{VM} cells have been observed to be predominantly monofunctional, and to secrete IFN-γ alone with limited TNF without expression of any IL-2⁶¹⁶. Another interesting feature of effector cells derived from T_{VM} cells is their propensity to differentiate into SLECs⁵⁷⁹, and to contract significantly upon resolution of the infection⁵⁷⁹, whereas contrastingly effector cells derived from naïve cells display a higher propensity of differentiation into MPECs⁵⁷⁹. This propensity towards a monofunctional, SLEC-like profile observed in effector T_{VM} cells was suggested to be another reflection of their semi-differentiated state prior to stimulation⁶¹⁵. In addition, T_{VM} cells also exhibit heightened responsiveness to cytokines, particularly to IL-12, IL-18 and γ cytokines^{610,615}, inducing rapid IFN-γ secretion. Altogether indicating that T_{VM} cells may represent a specialised CD8⁺ T cells capable of mediating innate-like responses in a cytokine-driven fashion.

The precise functional role of T_{VM} cells remains elusive, but it is thought that T_{VM} cells may offer a significant immunological advantage in newborns by providing a critical level of memory-like, protective capability without prior exposure to pathogens⁶¹⁵. Neonates must first survive a period of lymphopenia with few adaptive immune cells and then survive without the protection of established antigen-specific memory populations. By bridging the gap between innate and adaptive, T_{VM} cells are potentially capable of providing both innate-like and memory-like functionality during this period of immunological vulnerability⁶¹⁵. In the

context of several bacterial and viral infection models, T_{VM} cells have indeed been shown to be crucial for generating rapid protective immunity^{579,620–622}. Taken together, it has been proposed that T_{VM} cells may provide an additional layer of phenotypic and functional heterogeneity within naïve $CD8^+$ T cell populations, ensuring that the pool of antigen-inexperienced $CD8^+$ T cells is intrinsically diverse in terms of both antigen-specificity and pre-programmed response kinetics and functionality^{615,618}.

However, the ontogeny of T_{MP} cells, including T_{VM} cells, is still unclear, as are the exact mechanism that direct their role in the immune response to infection or cancer. $CD8^+$ T cell memory is typically generated by responding to foreign antigen, but studies investigating extreme lymphopenia have shown already more than two decades ago that functional memory T cells can also be produced from naïve cells through homeostatic mechanisms that bypass conventional T cell activation by foreign antigen^{623,624}. There is also evidence that homeostatic proliferation can occur in physiological circumstances of lymphopenia such as are found within unprimed neonatal mice^{625–627}.

T_{MP} cells derived from homeostatic proliferation in the lymphopenic environment are also known as T_{LIP} cells^{251,628}, and their generation was shown to be mainly driven by IL-7 signalling and low-affinity TCR ligands^{151,629} and to be furthermore modulated by cytokines, such as IL-2, IL-12 and IL-15^{630–632}. In contrast, the naturally occurring T_{VM} cell pool is believed to arise slowly over the weeks following birth in the periphery from conventional naïve cells⁵⁷⁸, in a process dependent upon IL-15 presentation by $CD8\alpha^+$ dendritic cells⁶³³. Given the pivotal role of IL-15 in T_{VM} cells generation, high expression of IL-15R β (also known as CD122 or IL-2R β) can therefore also be used as another phenotypic marker of T_{VM} cells^{615,633}. It was furthermore shown that IL-4 signalling can expand T_{VM} cell populations^{578,620,621,633}, and that T_{VM} cells express and maintain high levels of the IL-7 α chain (CD127), which is consistent with

a role in their survival^{615,616}. Lastly, it was also suggested that type I IFNs contributes to the homeostasis and innate functions of T_{MP} cells, both in the periphery and in the thymus, through inducing the expression of Eomes⁶³⁴.

Innate memory CD8⁺ T (T_{IM}) cells represent another population of T_{MP} cells, which has been investigated in several studies. An important distinction to T_{VM} cells is their dependence on IL-4, but not IL-15, for their development (reviewed here^{611,614}). Additionally, T_{IM} cells adopt a CD44^{hi} memory phenotype in the thymus rather than in the periphery. T_{IM} cells are observed in Balb/c mice and in some genetically deficient mouse strains with heightened IL-4 signalling. Apart from those reported differences, T_{IM} cells are understood to share many phenotypic and functional characteristics with T_{VM} cells, which highlights the importance of mouse strain selection and accurate use of phenotypic markers, when studying specific T_{MP}-cell subsets⁶¹⁵. Of note, recent findings⁶³⁵ suggest that T_{IM} and T_{VM} cells represent inherently distinct T_{MP} subpopulations with separate ontogeny and antigen specificities. Thus, it is important to interpret earlier studies equating these two subsets cautiously.

5.1.5.2 TCR signalling in T_{VM} cells

While the role of cytokines in the development and function of T_{VM} cells has been reasonably well elucidated, the nature of their TCR and the role of TCR signalling in those cells remains controversial. T_{VM} cells have been shown in a study by Haluszczak and colleagues to display a notable breadth of antigen specificities, and even though the frequency of T_{VM} cells varied greatly within any given specificity, T_{MP} cells were reliably found in unprimed animals for all of the foreign peptide-MHC ligands studied⁶¹⁰, suggesting that the antigen specificity of T_{VM} cells has similar diversity to the naïve pool. In addition, the precursor frequency of T_{VM} cells responding to nominal foreign antigens was discovered to be proportional to the

frequency of naïve phenotype precursors, suggesting the absence of a severe bias in the T_{VM} repertoire⁶¹⁰. Multiple studies have provided strong support for the notion that the generation of T_{VM} cells is not dependent upon exposure to environmental microbes or exogenous antigen^{578,580,610,616,636,637}

Current evidence furthermore suggests that naïve CD8⁺ T cells with higher basal TCR signalling and heightened self-reactivity may have a higher propensity to differentiate into T_{VM} cells^{580,615–617,637,638}. It was suggested that the threshold for this TCR signal may be higher than the tonic signal naïve T cells receive through their non-self-reactive TCRs that mediates their homeostatic survival, but below the threshold triggered by bona fide activation through cognate antigen binding⁶¹⁵. T_{VM} formation was furthermore suggested to be dependent on the induction of Eomes via low-affinity interaction with self-peptides^{252,638}, with high expression of Eomes and Bcl-2 also observed in mature T_{VM} cells^{580,616}. The recent discovery that high expression of Eomes endows low-affinity CD8⁺ T cell clones with a survival advantage during selection into effector and memory pools⁶⁴ supports the notion that high levels of Eomes may be crucial for the successful generation of T_{VM} cells.

Summarising the current evidence⁶¹⁵, Hussain and Quinn suggested a model of T_{VM} cell generation, in which IL-15 signalling in the neonatal periphery induces lymphopenia-induced proliferation and semi-differentiation in modestly self-reactive naïve CD8⁺ T cells to drive them towards a cytokine-driven, partial memory phenotype^{578,580,610,615}. Of note, despite exhibiting potentially heightened self-reactivity, T_{VM} cells have not been observed to compromise self-tolerance or enhance autoreactivity⁶³⁸.

Two studies recently questioned this model. The first one showed that the neonatal thymus, rather than the peripheral lymphopenic environments, appears to be mainly responsible for the programming of certain T cells towards becoming T_{VM} cells⁶¹⁸, whereas

the second one⁶³⁵ demonstrated that T_{VM} cell differentiation is triggered in a subset of T cells with distinct TCRs through recognition of self-ligands in the thymus, which is followed by a subsequent consolidation phase restricted to the periphery. Interestingly, such a two-step differentiation process has previously been shown to be required for the thymic differentiation of FoxP3⁺ regulatory T cells^{635,639}. Notably, IL-15 signalling, while most likely contributing to T_{VM} cell differentiation and survival^{580,633}, was shown to be insufficient in directing T_{VM} cell differentiation in unimmunized mice. Only select CD8⁺ T cell clones adopted the T_{VM} cell phenotype within the endogenous repertoire based on TCR specificity. However, these findings do not exclude the possibility that T_{VM} may still be reactive to any given foreign pMHC-I complex^{578,610} with innate-like effector functionality^{579,610}, as it is conceivable that T_{VM} cells reacting to foreign antigen in unprimed mice may represent cross-reactive T_{VM} cells, selected on self-pMHC-I ligands but responsive to foreign antigens due to the inherent plasticity of TCR-pMHC ligand recognition³.

Of note, most of the studies discussed so far have been predominantly carried out in mice. However, it is important to highlight that a putative T_{VM} cell has been identified in humans⁶⁴⁰, which is naïve-like, in that they express CD45RA, but also expresses Eomes and NK cell receptors, including NKG2A and/or KIR3DL1 and KIR2D, while also lacking expression of CCR7 and CD27^{580,640}. Human T_{VM} cells have furthermore been shown to share many features with murine T_{VM} cells^{640,616} and to exhibit innate-like immune functions during infection⁶⁴¹.

Complicating the study of T_{VM} cells in various contexts is that a core signature for both mouse and human T_{VM} cells across the lifespan remain to be identified. Reviewing the available data so far, Pribikova and colleagues suggested in 2018 that the only candidate marker, identified to be specifically upregulated in T_{IM}/T_{VM} cells in comparison to naïve, memory and effector T cells, is Drc1 (also known as Ccdc164)⁶⁴². However, evidence on the

protein levels is still missing. Nevertheless, the expression pattern of Dcr1, CD49d and NKG2D/Klrg1 was suggested to distinguish T_{IM}/T_{VM} cells clearly from true memory T cells, and to allow for their unambiguous identification in transcriptomic studies⁶⁴².

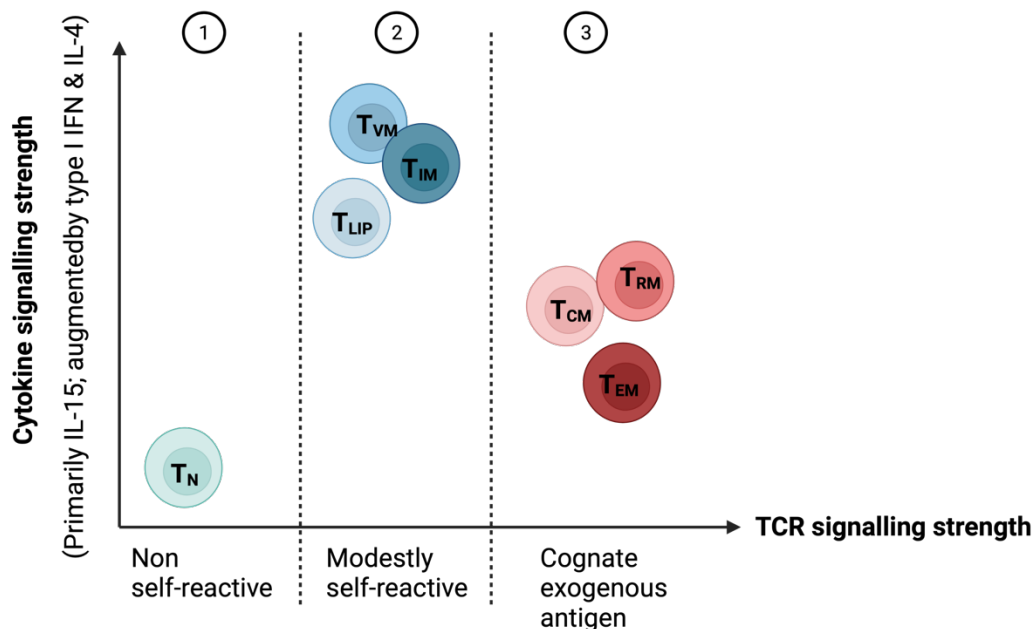


Figure 53. Current model of T_{VM} generation. Hussain and Quinn recently suggested a model of T_{VM} generation, in which the combined effects of cytokine and TCR signalling strength may lead to 3 main outcomes. **(1)** Naïve T cells are characterised by non-self-reactive TCRs and receive low cytokine signalling. **(2)** If cytokine signalling is high and $CD8^+$ T cells have modestly self-reactive TCRs, Eomes and IL-15R β expression are upregulated, thus making T cells receptive to IL-15 and permitting their cytokine-driven differentiation. While cytokines such as IL-15, type I IFN, and IL-4 all augment Eomes expression to potentiate IL-15 signalling and differentiation, each cytokine may engage additional pathways to result in subtly distinct phenotypic T_{MP} profiles of T_{VM} (IL-15, type I IFN), T_{IM} (IL-4) and T_{LIP} (IL-7) cells. **(3)** Cognate antigen encounter along with a degree of cytokine signalling will trigger differentiation into a variety of conventional memory cells. Of note, recent studies^{618,635} add important caveats to this model and suggest that developing T cells may already be programmed in the thymus to differentiate into T_{VM} cells in the periphery. T_{MP} = memory phenotype T cells, T_{VM} = virtual memory T cell, T_{IM} = innate memory T cells, T_{LIP} = Lymphopenia-induced proliferation-derived T cell [Created with BioRender.com, adapted from Hussain and Quinn 2018⁶¹⁵].

5.1.5.3 CD44 and CD49 as hallmark markers of T_{VM} cells

Murine virtual memory $CD8^+$ T cells are commonly differentiated from other $CD8^+$ T cell subtypes by high expression of CD44 and low expression of CD49d. These two markers reflect the semi-differentiated state of T_{VM} cells, as CD44 is rapidly upregulated on TCR- and cytokine-mediated stimulation, while CD49d upregulation requires more sustained, robust TCR stimulation from cognate antigen⁶¹⁵.

CD44 is a carbohydrate-binding type I transmembrane protein that is the product of a single gene and is represented by a family of six isoforms that arise by mRNA splicing of 10 variably expressed exons⁶⁴³, which can furthermore be glycosylated to introduce additional molecular heterogeneity on various cell types⁶⁴⁴. CD44 is constitutively expressed on the surface of many cell types, including resting T cells, which exclusively express the invariant or standard form. Most biological and signalling function of CD44 are associated with binding of the primary ligand, hyaluronic acid (HA), which is an integral part of the extracellular matrix⁶⁴⁵. Activated T cells upregulate surface CD44 expression in the earliest phases of clonal expansion and maintain high expression permanently thereafter. As such, CD44 is the most widely used indicator of prior antigen exposure. CD44 is best known as a critical regulator of biological processes involving migrating cells, including the recruitment of effector T cells and other leukocytes to sites of infection⁶⁴³. Besides its functional role in the migration of cells, multiple studies have also shown its critical involvement in the activation, differentiation and homeostasis of T cells recently reviewed here⁶⁴⁵).

CD49d (also known as the α_4 chain integrin) on the other hand constitutes one of the chains of $\alpha_4\beta_1$ (very late activating antigen-4 [VLA-4]) and $\alpha_4\beta_7$ integrins and mediates migration of T cells to extravascular spaces and into sites of inflammation⁶⁴⁶. Naïve cells express low levels of CD49d, which is upregulated upon antigen stimulation and typically remains high on the ensuing memory population^{647–649}.

5.1.6. Context and aim of this chapter

We have shown in the previous chapter that CD8⁺ T preferentially receive IFN- γ from other CD8⁺ T cells, and next sought to explore the signalling factors that induce IFN- γ expression in those cells. Additionally, given that only a small fraction of responding CD8⁺ T cells secreted

IFN- γ upon activation, we aimed to characterize this subset of early IFN- γ -producing CD8⁺ T cells more thoroughly. To this end, we (1) characterized IFN- γ -producing CD8⁺ T cells 24 hours post-infection by both flow-cytometric and scRNA-seq analysis, (2) investigated the triggers of IFN- γ -expression in CD8⁺ T cells, both *in vitro* and *in vivo* and (3) studied if additional licensing may be required to facilitate *in vivo* activation of this particular subset of IFN- γ -producing CD8⁺ T cells.

5.2. Results

5.2.1. Characterization of interferon-gamma-producing CD8⁺ T cell subtypes 24 hours post-infection

To investigate the relative contribution of the different CD8⁺ T cell subpopulations to the IFN- γ that is produced shortly after activation, we infected GREAT mice with either LM-OVA or LM-gp33 (**Figure 54A**) and characterized IFN- γ ⁺ CD69⁺ CD8⁺ T cells 24 hours post-infection (**Figure 54B**). Two different *L. monocytogenes* strains were used to rule out the possibility that the obtained results are somewhat skewed by antigenic bias. Notably, all IFN- γ ⁺ CD8⁺ T cells display very high CD69⁺ expression (**Figure 54C**), indicative of very potent activation. Comparing naïve with infected mice, we did not observe any differences in the relative frequency of the different subsets amongst the total CD8⁺ T cell population, based on CD44 and CD49d expression (**Figure 54E and F**). We furthermore observed a similar frequency of IFN- γ ⁺ CD8⁺ cells after infection with the two different antigens (**Figure 54D**). Gating on the different subpopulations, it became apparent that only T_{VM} and true memory (T_M) cells secrete IFN- γ at this early time point, with up to 30% of T_{VM} cells expressing IFN- γ 24 hours post-infection, and up to 20% of T_M cells secreting IFN- γ at this time IFN- γ (**Figure 54G**). Notably, gating on activating IFN- γ ⁺ CD8⁺ T cells we discovered that the vast majority (up to 70%) belong to the T_{VM} subpopulation (**Figure 54H**). We concluded that the majority of IFN- γ secretion by CD8⁺ T cells at this early time point stems from T_{VM} cells. Of note, we also investigated if CD8 IFN- γ R KO and CD8-Cre control mice differ in their composition of CD8⁺ T cell subpopulations but did not detect any differences in relative frequency (data not shown).

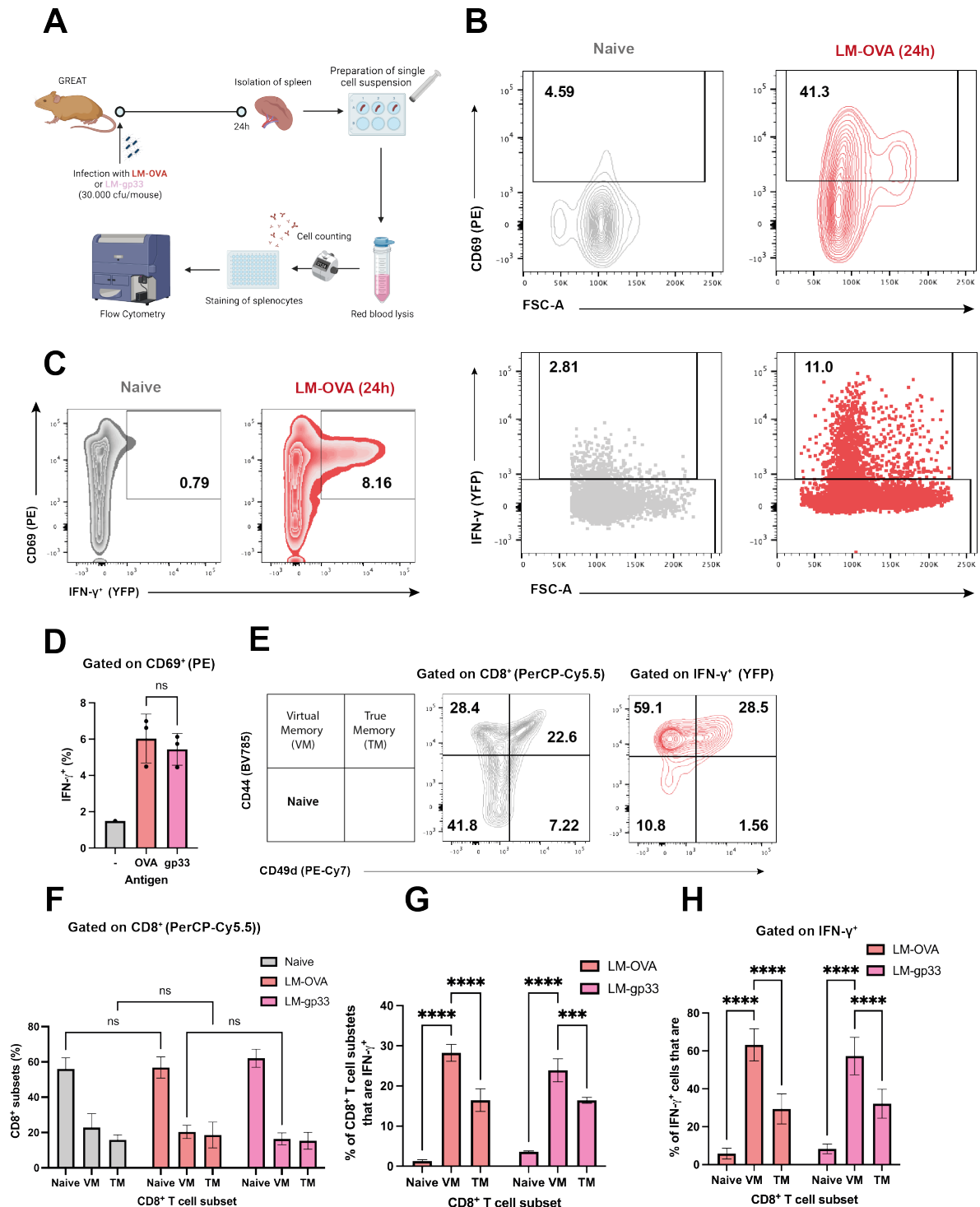


Figure 54. Virtual memory CD8⁺ T cells are the main producers of early interferon-gamma (IFN- γ). (A) GREAT mice were infected with either LM-OVA (red) or LM-gp33 (pink) and IFN- γ expression was evaluated 24 hours post-infection. (B, C) Representative flow plots of CD69 and IFN- γ expression. (D) Quantification of relative abundance of IFN- γ -secreting CD8⁺ T cells. (E, F) Representative flow plots (E) and quantification (F) of relative abundance of CD8⁺ T cell subpopulations. (G) Quantification of IFN- γ expression by CD8⁺ T cell subsets. (H) Quantification of phenotyping of IFN- γ ⁺ CD8⁺ T cell subsets. Data are representative of 3 (n = 3 x 3 technical replicates) independent experiments. Error bars indicate the mean $\pm$ s.d. Comparison between groups was calculated using the two-tailed unpaired Student's t-test. *P<0.05; **P<0.01; ***P<0.001; NS, not significant.

We next sought to study the kinetics of IFN- γ -secretion by CD8⁺ T cell subpopulations throughout the priming phase of the response. Phenotyping the activated IFN- γ ⁺ CD8⁺ population at different time points revealed that 12-16 hours post-infection both memory and T_{VM} cells contributed nearly equally to the pool of IFN- γ -secreting CD8⁺ T cells (**Figure 55A and B**). Of note, as shown also in the previous chapter (**p.133**), at those early time points the percentage of IFN- γ -producing CD8⁺ T cells is very small (**Figure 55C**). A sizeable percentage of activated CD8⁺ T cells producing IFN- γ emerges 24 hours post-infection, and from this time point onwards the vast majority of these cells also belong to the T_{VM} subpopulation, whose relative contribution even slightly increases up to 48 hours post-infection (**Figure 55B**).

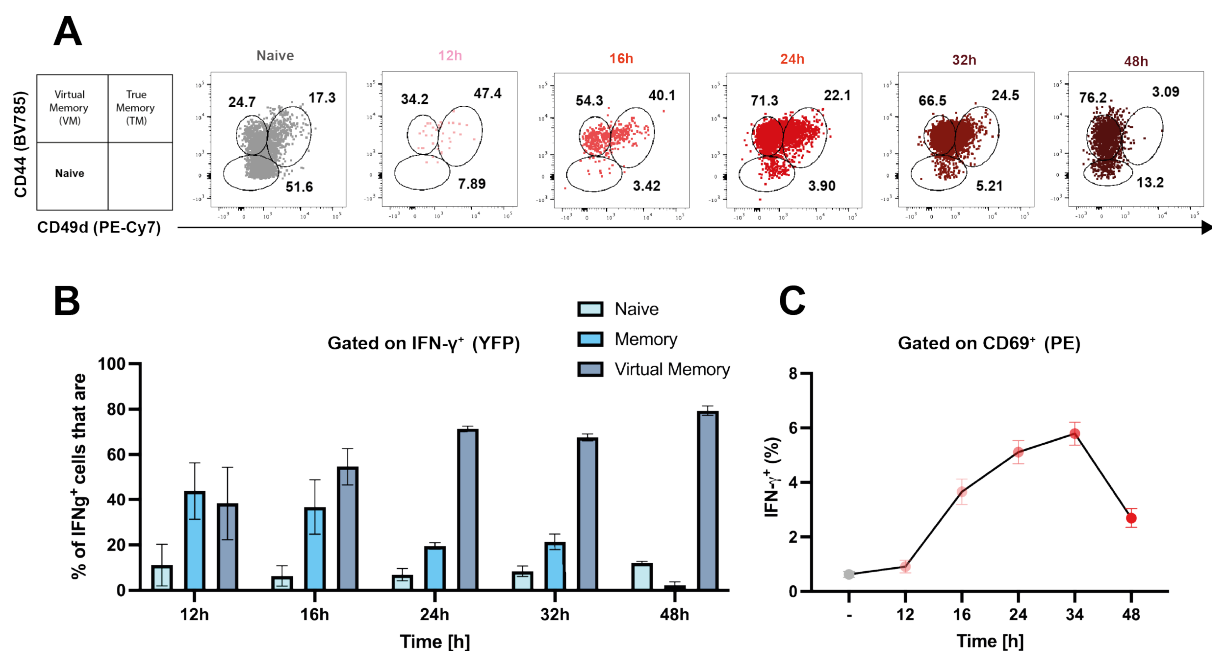


Figure 55. T_{VM} CD8⁺ T cells are the main producers of interferon-gamma (IFN- γ) throughout the priming phase. (A) Schematic of CD8⁺ T cell subpopulation gating strategy at different time points post LM-OVA infection. **(B)** Quantification of IFN- γ secretion by CD8⁺ T cell subpopulations at different time points. **(C)** Kinetics of IFN- γ secretion by CD69⁺ CD8⁺ T cells post LM-OVA infection. Data are representative of 3 (n = 3 x 3 technical replicates) independent experiments. Error bars indicate the mean $\pm$ s.d.

5.2.2. Characterization of interferon-gamma⁺ cells 24 hours post infection by scRNA-seq

Having shown that T_{VM} cells comprise the majority of IFN- γ -secreting CD8⁺ T cells during the early phase of the immune response to *L. monocytogenes* infection, we next sought to

investigate this subset in more depth. To this end, we isolated CD8⁺ T cells 24 hours post-infection and sent them for single-cell RNA-sequencing by 10x Genomics, together with CD8⁺ T cells isolated from naïve mice used as control cells. Unbiased hierarchical clustering confirmed that the vast majority of CD8⁺ T cells 24 hours post-infection displays a naïve phenotype, with T_{VM} and memory CD8⁺ T cells forming their own distinct clusters (**Figure 56A**). Analysing average gene expression revealed that T_{VM} cells share features with naïve T cells (T_N), such as upregulation of *Ccr7* and *Sell* (encoding for CD62L) and downregulation of *Cx3cr1*, and memory T cells (T_M), such as expression of *Cd44*, *Eomes* and *Cxcr3* (**Figure 56B**).

Analysing IFN- γ expression validated our previous results obtained by flow cytometry and confirmed that T_{VM} cells comprise the predominant subpopulation of IFN- γ ⁺ CD8⁺ T cells 24 hours post-infection (**Figure 56F**). Notably, the average IFN- γ expression of T_{VM} cells furthermore exhibits a much higher fold change compared to memory CD8⁺ T cells (**Figure 56E**), indicating much more potent induction of early IFN- γ production. Evaluating gene expression pathways more in depth did not show a strikingly distinct transcriptomic profile for T_{VM} cells.

Kyoto encyclopaedia of genes and genomes (KEGG) analysis revealed “viral protein interaction with cytokine and cytokine receptor”, “cytokine/cytokine receptor interaction” and “cell adhesion molecules” as the dominant cellular pathway in T_{VM} cells, which overlapped with T_N cells (**Figure 56H**). The data furthermore suggested that TCR signalling has been initiated in both T_N and T_{VM} cells, as opposed to memory cells. Notably, T_{VM} cells were unique in their expression of genes related to antigen processing and presentation (**Figure 56H**). How those genes are involved in T_{VM} functionality remains to be further investigated.

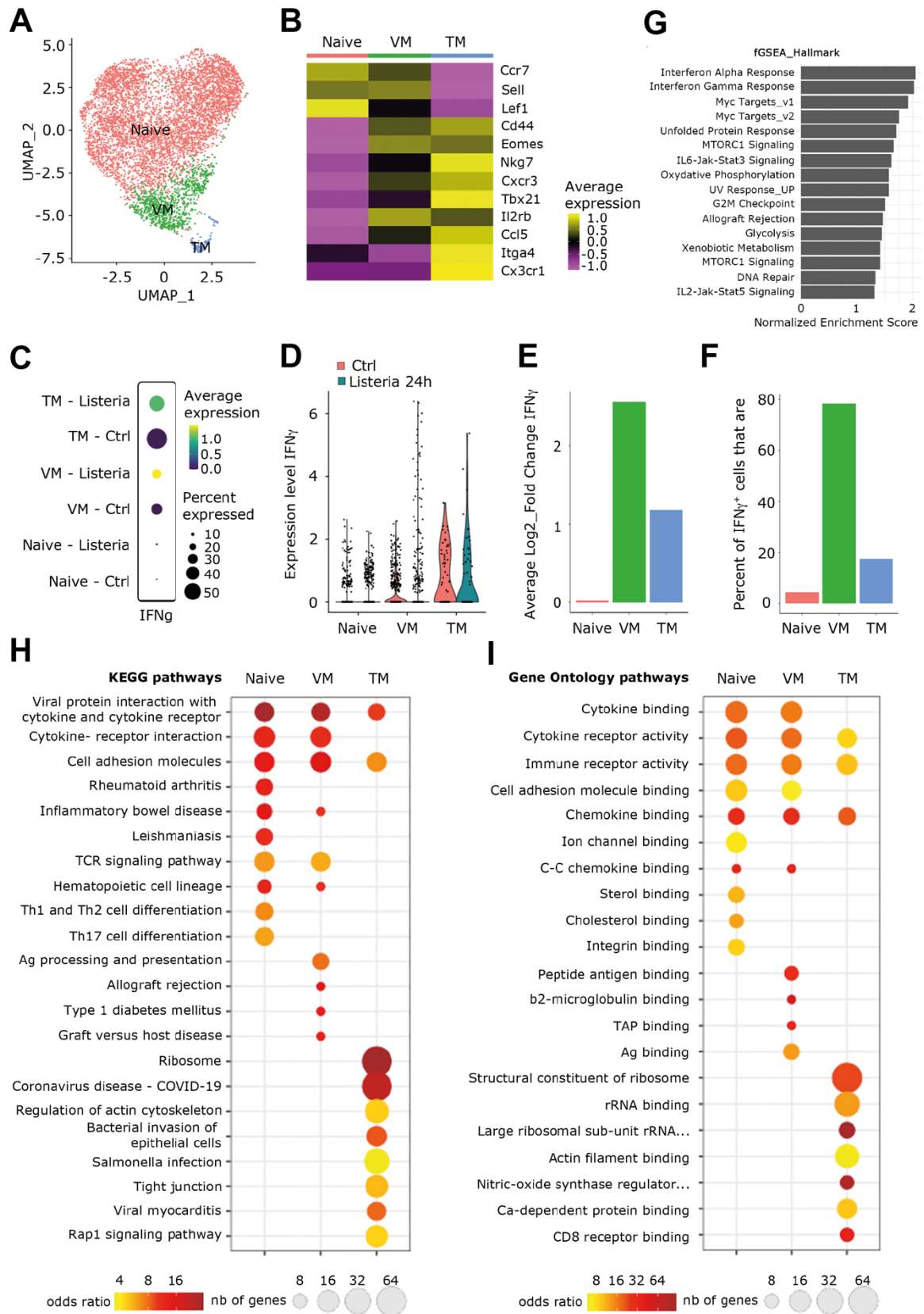


Figure 56. Single-cell RNA sequencing (scRNA-seq) of CD8⁺ T cells 24 hours post-infection. (A) Unbiased hierarchical clustering (UMAP) analysis of CD8⁺ T cells. Clusters high in *Ccr7*, *Sell* and *Lef1*, but devoid of *Cd44*, *Eomes* were labelled as naïve, whereas clusters with memory features (high *Cd44* and *Eomes* expression and low expression of *Ccr7*, *Sell* and *Lef1*) were labelled as memory. The third cluster which was high in *Cd44*, *Eomes* and also *Il2rb* expression and devoid of *Itga4*

(CD49d) expression was labelled as virtual memory. **(B, C)** Analysis of average gene expression. **(D)** IFN- γ expression levels. **(E)** Average fold change in expression of IFN- γ . **(F)** Phenotyping of IFN- γ -secreting CD8⁺ T cells. **(G)** Gene set enrichment analysis (GSEA). **(H)** Kyoto encyclopaedia of genes and genomes (KEGG) pathways analysis. **(I)** Gene ontology (GO) pathways analysis. Data are representative of 1 independent experiment.

Gene ontology (GO) analysis identified 6 cellular functions as significantly increased in both naïve T cells and T_{VM} cells, including “Cytokine binding”, “Cytokine receptor activity”, “Immune receptor activity”, “Cell adhesion molecule binding”, “Chemokine binding” and “C-C chemokine binding” (**Figure 56I**). Three of those (“Cytokine receptor activity”, “Immune receptor activity” and “Chemokine binding”) were also shared with memory cells. Lastly, gene set enrichment analysis (GSEA) showed that the most highly enriched gene sets in T_{VM} cells belong to the interferon alpha and gamma responses and targets of the cell cycle-regulating proto-oncoprotein Myc²⁷³ (**Figure 56G**).

Of note, highlighting the IFN- γ ⁺ fraction of CD8⁺ T cells within the subpopulation clusters revealed that IFN- γ ⁺ cells form their own sub-clusters within the T_{VM} and memory subpopulations, indicating that they may be distinct subsets (**Figure 57A**). Studying the integrin (**Figure 57B**) and chemokine (**Figure 57C**) profiles of the different populations showed that T_{VM} cells are more alike naïve than true memory cells. Shared downregulated integrin genes include *Itgal*, *Itga1*, *Itga2*, *Itga7* and *Itga9*, as well as *Itgb3*, *Itgb5* and *Itgb7*. Shared downregulated chemokine/chemokine receptor genes include *Xcr1*, *Cxcr1*, *Ccr2*, *Ccr6*, *Ccr10* and *Cxcr6*. Notably, our scRNA-seq data revealed T_{VM} cells to highly express *Cxcr5*, which is associated with the stemness in CD8⁺ T cells during chronic infections and cancer^{650–652}, and which has also been shown by a recent study⁶⁵³ to be a feature of T_{VM} cells. Altogether, our data suggest that naïve and virtual memory T cells may be located in the same microenvironment as naïve T cells, and we hypothesise that close proximity to naïve T cells may allow T_{VM} cells to play a critical role in regulating naïve T cell behaviour in response to infection.

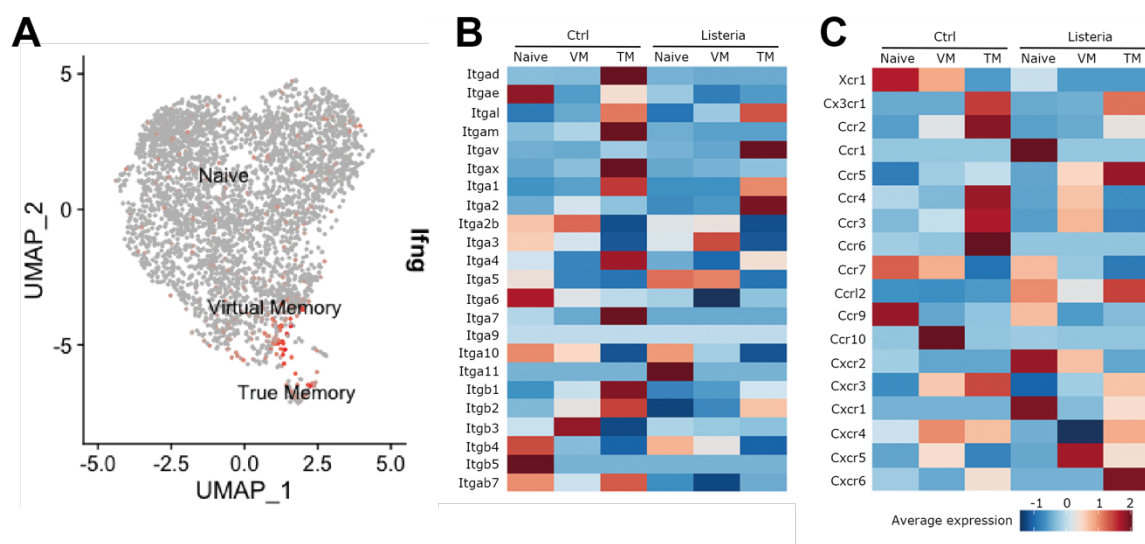


Figure 57. Single-cell RNA sequencing (scRNA-seq) of CD8⁺ T cells 24 hours post-infection. (A) Unbiased hierarchical clustering (UMAP) analysis. **(B, C)** Average gene expression analysis of integrin and chemokine/chemokine receptor expression in naïve, virtual memory (T_{VM}) and true memory (T_M) CD8⁺ T cells. Data are representative of 1 independent experiment.

5.2.3. NKG2D activity is dispensable for interferon-gamma production by T_{VM} cells

Having identified virtual memory T cells as the primary producers of early IFN- γ , we next sought to explore the inducing factors driving early IFN- γ expression in those cells. Based on previous studies demonstrating a critical role for natural killer (NK) group 2D (NKG2D) in the licencing of memory CD8⁺ T cells to express effector cytokines⁵⁶³, as well as previous reports demonstrating that T_{VM} cells express the innate receptor NKG2D^{580,615}, we hypothesised that NKG2D binding may induce IFN- γ expression by T_{VM} cells.

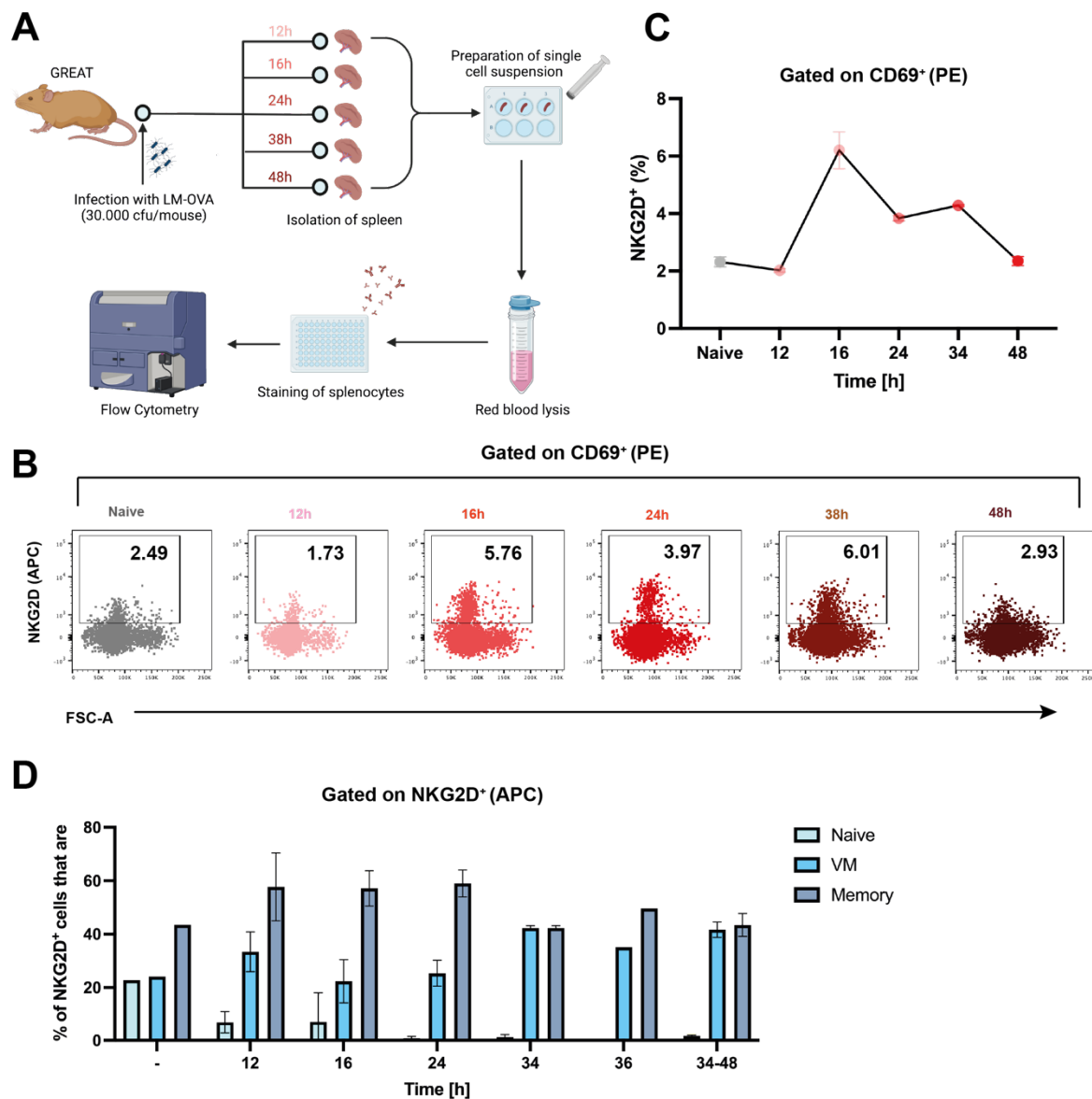


Figure 58. T_{VM} cells are not characterized by high NKG2D expression during priming. (A) GREAT mice were infected with LM-OVA and NKG2D expression was evaluated at different time points post-infection. (B, C) Representative flow plots (B) and analysis (C) of NKG2D expression. (D) Quantification of NKG2D⁺ CD8⁺ T cell phenotyping. Data are representative of 3 (n = 3 x 3 technical replicates) independent experiments. Error bars indicate the mean $\pm$ s.d.

NKG2D, encoded by KLRK1, is one of several innate-like receptors, which have been identified on T cells²⁸⁶ (reviewed here⁶⁵⁴). NKG2D is a type II transmembrane protein, which serves as an activating receptor capable of binding several diverse non-classical MHC I-like ligands including human MHC class I polypeptide-related sequence A (MICA), MICB, UL16 binding proteins (ULBPs), murine ribonucleic acid export 1 (RAE1), histocompatibility 60 (H60), and murine UL16-binding protein-like transcript (MULT) proteins⁶⁵⁵. These ligands

possess elements of heat shock promoters and are generally absent in uninfected hosts but can become upregulated as a consequence of stress conditions, such as bacterial or viral infections⁶⁵⁶. STAT-dependent cytokines such as IL-2⁶⁵⁷, IL-7⁶⁵⁸, IL-12³⁸³ and IL-15^{572,659} have been shown to upregulate NKG2D protein expression in both naïve and memory CD8⁺ T cells in the absence of TCR signalling. It has furthermore been suggested that NKG2D mediates a costimulatory function in CD8⁺ T cells in the presence of TCR engagement^{658,660,661}, and signalling downstream of NKG2D-NKG2DL interactions have been hypothesised to regulate the activation of antigen-stimulated effector/memory T cells in the local tissue environment^{662,663}, and to be linked to increased memory CD8⁺ T cell function independently of CD4⁺ T cell help⁶⁶⁴. In addition, engagement of TCR and NKG2D has been revealed to result in enhanced cytokine production and proliferation in effector⁶⁶² and memory CD8⁺ T cells^{660,661}. Lastly, NKG2D signalling can also elicit cytolytic function in the absence of TCR engagement⁶⁶⁵ and has been shown to be involved in antigen-independent killing of infected target cells by antigen-experienced memory T cells⁵⁶³.

We first decided to measure the expression kinetics of NKG2D in activating CD8⁺ T cells throughout the priming phases of the response (**Figure 58A, B and C**). Expression of NKG2D spiked around 16 hours post-infection before decreasing again and returning to baseline levels 48 hours post-infection. Analysing expression by subpopulation revealed that it is predominantly memory CD8⁺ T cells which upregulate NKG2D, while T_{VM} cells do not display a significant upregulation in expression of this surface protein (**Figure 58D**).

We concluded that licencing of T_{VM} cells is most likely not regulated by NKG2D binding. Our data align with a recent report, which showed that true memory cells can be distinguished in mice from memory phenotype T cells by surface expression of NKG2D, with NKG2D surface expression being restricted to true memory CD8⁺ T cells. The authors noted

that while T_{VM} cells express higher levels of mRNA encoding NKG2D compared with naïve cells, they are mostly NKG2D^{neg} at the protein level⁶⁶⁶.

5.2.4. Interferon-gamma expression requires both TCR and cytokine signalling *in vitro*

After excluding NKG2D as a trigger of IFN- γ production by T_{VM} cells, we decided to elucidate the factors that induce IFN- γ expression in T_{VM} cells more thoroughly *in vitro*. Earlier studies have suggested that IFN- γ can be produced by CD8⁺ T cells in an innate-like fashion early during LM infection upon cytokine-mediated activation through IL-12 and IL-18^{502,562}. To investigate the priming factors inducing IFN- γ production by antigenically naïve, endogenous CD8⁺ T cells, IFN- γ secretion in CD8⁺ T cell upon stimulation with several different stimuli was examined *in vitro*. CD8⁺ T cells were isolated from the lymph nodes and spleens of naïve, unprimed GREAT mice and incubated overnight with the TCR and co-stimulatory agonists, anti-CD3 ϵ /CD28 (referred to hereafter simply as “TCR stimulation”), and/or inflammatory cytokines (Figure 59A).

Expression of IFN- γ was evaluated the next day by flow cytometry analysis of eYFP expression (Figure 59B). Our first experiment aimed to assess the IFN- γ inducing effects of the previously reported cytokines IL-12 and IL-18, and to test if IFN- γ induction requires additional TCR triggering. While we did not observe any IFN- γ expression upon TCR activation alone, potent expression was measured when adding the two cytokines to the stimulation mix (Figure 59C). IL-12 and IL-18 alone only triggered comparatively minor IFN- γ secretion, and the same result was obtained when adding them in combination (Figure 59D).

Higher IFN- γ levels were detected upon combined TCR + IL-12 + IL-18 stimulation than for TCR + IL-12 or TCR + IL-18 stimulation summed together, indicating that potent IFN- γ induction may not simply result from the additive effects of cytokine receptor signalling, but

may likely involve the induction of additional feedback mechanisms ([Figure 59C](#)). Of note, the levels of IFN- γ secretion also corresponded with the expression levels of the T cell activation marker CD69 of IFN- γ^+ CD8 $^+$ T cells (data not shown), suggesting that IFN- γ production requires CD8 $^+$ T cell activation, either directly through TCR engagement or indirectly via cytokine stimulation. Additionally, we also studied if there is a specific sequence to the signalling events by adding each additional stimulus 6 hours apart from each other, but no differences in IFN- γ production were observed upon testing different stimulation sequences and sequence combinations (data not shown).

Next, we sought to study the IFN- γ -inducing effect of the integrin ICAM-1. LFA-1 has been reported to enhance CD8 $^+$ T cell activation and IFN- γ production through sustaining TCR-activated ERK signalling and by additionally directly activating ERK⁶⁶⁷. LFA-1-mediated IFN- γ induction was also observed in other innate T cell subsets, with high ICAM-1 density shown to be sufficient for driving IFN- γ production in the absence of TCR stimulation⁶⁶⁸. When examining the contribution of ICAM-1 alone, we measured only very low levels of IFN- γ expression in stimulated cells ([Figure 59D](#)). However, despite leading to much less potent IFN- γ induction that was observed for TCR + IL-12 + IL-18 stimulation, ICAM-1 appeared to synergize with TCR stimulation to drive IFN- γ secretion ([Figure 59D](#)). Notably, this synergistic effect was lost when adding IL-12 and IL-18 to the stimulation mix. We concluded from these data that ICAM-1, albeit displaying synergy with TCR signalling, is unlikely to be a major contributing factor to early IFN- γ production.

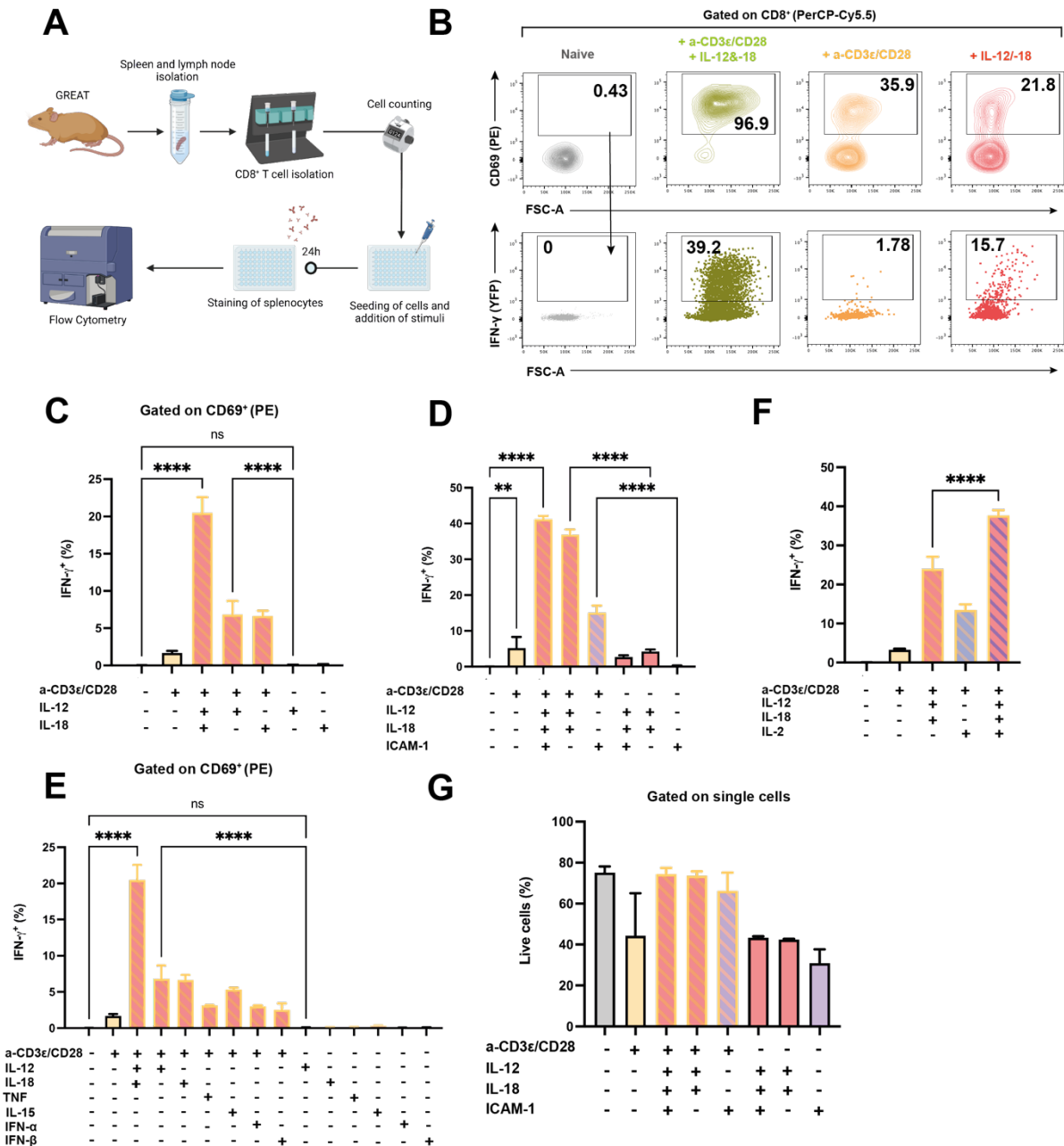


Figure 59. Interferon-gamma (IFN- γ) expression in CD8⁺ T cells requires both T cell receptor (TCR) and cytokine signalling. (A) CD8⁺ T were isolated from GREAT mice by negative selection and stimulated *in vitro* with either TCR or cytokine stimuli or a combination of both. **(B)** Representative flow plots of CD69 and IFN- γ expression. **(C, D, F, E)** Quantification of IFN- γ expression by CD8⁺ T cells following TCR and/or IL-12 and/or IL-18 stimulation **(C)**, TCR and/or IL-12 and/or IL-18 and/or ICAM-1 stimulation **(D)**, TCR and/or IL-12 and/or IL-18 and/or TNF and/or IL-15 and/or Type I IFN stimulation **(E)**, TCR and/or IL-12 and/or IL-18 and/or IL-2 stimulation **(F)**. **(G)** Quantification of live CD8⁺ T cells. Data are representative of 5 (n = 3 x 2 technical replicates) independent experiments. Error bars indicate the mean $\pm$ s.d. Comparison between groups was calculated using a Two-way ANOVA and Šidák's multiple comparison test. *P<0.05; **P<0.01; ***P<0.001; NS, not significant.

5.2.5. IL-2, IL-7 and IL-15 enhance interferon-gamma secretion by CD8⁺ T cells *in vitro*

A functional synergistic role for early IFN- γ secretion in CD8⁺ T cells has also previously been revealed for IL-2⁵⁰², and we therefore decided to test its potential contribution to early IFN- γ production by CD8⁺ T cells. Notably, reduced IFN- γ secretion was observed when adding IL-2 on its own, regardless of whether additional TCR stimulation was provided simultaneously (**Figure 59F**). However, when being paired with TCR + IL-12 + IL-18 stimulation, potent IFN- γ induction was observed (**Figure 59F**). The enhanced IFN- γ secretion was unlikely to result from heightened CD8⁺ T cell activation, as the levels of CD69 were only marginally different across these conditions, indicating similar degrees of cell activation (**Figure 60D**). Notably, observing this effect required a relatively high dose of IL-2 (100 U/mL). In contrast, a lower dose (10 U/mL) did not induce any synergistic effect on IFN- γ induction (data not shown). This may be, at least partly, due to the relatively low responsiveness of naïve CD8⁺ T cells to IL-2 in overnight incubation, as CD25, the high-affinity subunit of the IL-2 receptor complex, remains minimally expressed during the early stage of T cell priming, and displays marked upregulated following 2 days of TCR stimulation²⁰⁹.

We then decided to further investigate the potential role of a range of additional cytokines. Cytokines were selected based on earlier studies that characterised the cytokine milieu of LM infection 24 hours post-infection, as well as preliminary single-cell transcriptomic data showing elevated expression of specific cytokine receptors on T_{VM} cells (data not shown). Based on these criteria, TNF, IL-1 β , type I IFNs, IL-7, IL-15 and IL-33 were tested for their effects on IFN- γ production, both individually and in various combinations.

Using the combination with the most IFN- γ -inducing effect (TCR stimulation paired with IL-12 + IL-18 stimulation) as a positive control, the effects of other inflammatory cytokines were

subsequently assessed. When added to CD8⁺ T cells individually in the absence of TCR stimulation, none of TNF, IFN- α , IFN- β or IL-15 was able to induce IFN- γ secretion beyond that of the unstimulated naïve control (**Figure 59E**). Testing TNF, IFN- α and IFN- β in combination with each other, as well as paired with TCR + IL-12 + IL-18 stimulation did not result in enhanced IFN- γ production in any of the conditions tested (data not shown). Similar results were obtained for IL-33, which was suggested to enhance IFN- γ secretion⁶⁶⁹ but did not have a discernible effect when being tested in our *in vitro* system (data not shown). The role of the pro-inflammatory cytokine IL-1 β was also tested, whose receptor belongs to the same receptor family as IL-18 (see also **p.162**). However, no effect on IFN- γ production was observed, neither when added individually or in combination with other cytokines or TCR + IL-12 + IL-18 stimulation (data not shown).

Of all these cytokines tested, only IL-15 appeared to promote IFN- γ inducing effects of TCR + IL-12 + IL-18 stimulation, albeit to a lesser extent than our positive control (**Figure 59E**). The receptors for IL-2 and IL-15 are both members of the common γ chain family, to which also IL-7 belongs. Having already shown that both IL-2 and IL-15 display a synergistic effect on IFN- γ induction when added to TCR + IL-12 + IL-18 stimulation, we next studied the contribution of these three cytokines in more detail.

Notably, all three cytokines significantly enhanced IFN- γ induction by TCR + IL-12 + IL-18 stimulation when added as an additional stimulus (**Figure 60A, B and C**). This synergistic effect was again unlikely to result from increased CD8⁺ T cell activation, as CD69 levels differed only marginally between conditions (**Figure 60D**). All three cytokines also enhanced the IFN- γ -inducing effect of combined IL-12 + IL-18 in the absence of TCR signalling, reaching a similar level as our positive control (**Figure 60A, B and C**). However, given the markedly increased CD69 level relative to IL-12 + IL-18 stimulation, elevated IFN- γ production in these conditions

may simply reflect a higher proportion of activated CD8⁺ T cells rather than increased IFN- γ expression on a single-cell level.

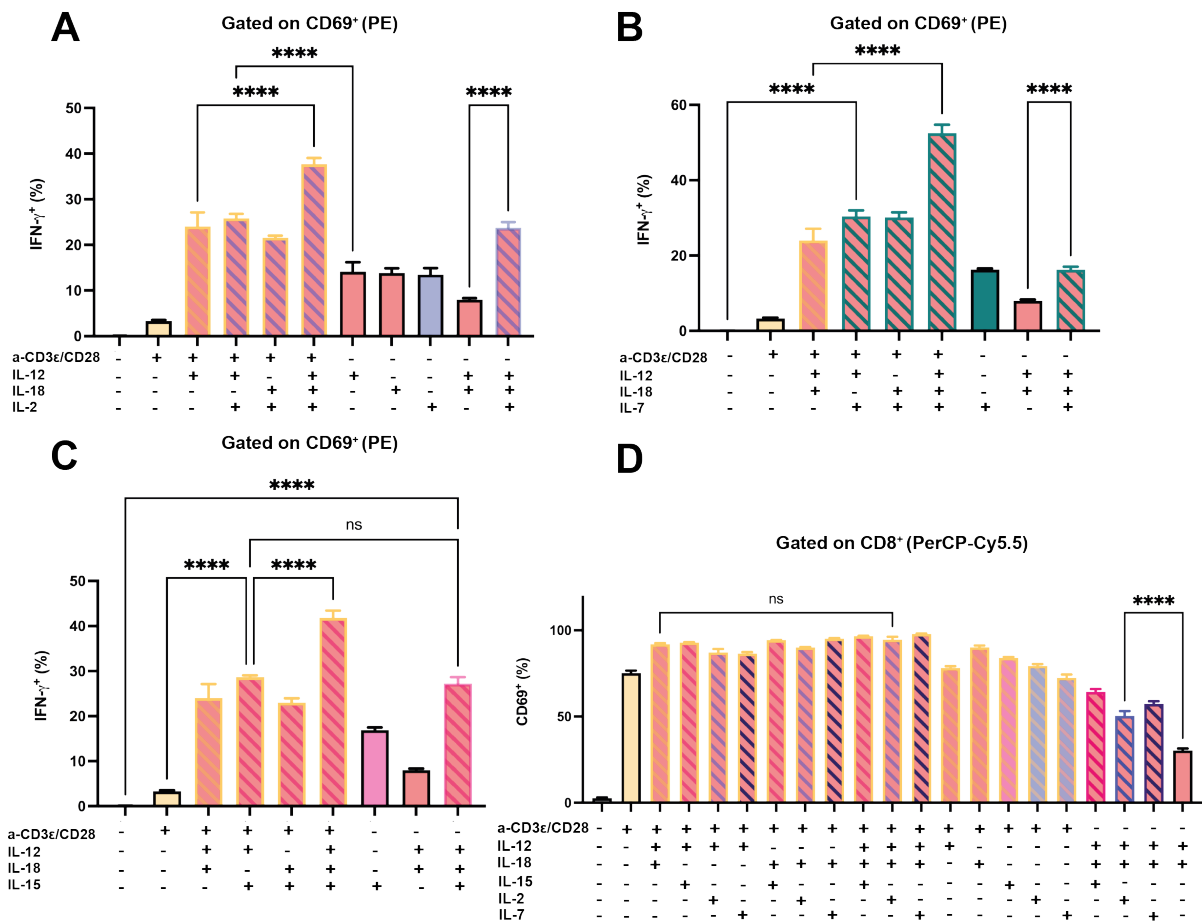


Figure 60. Interleukin-2 (IL-2), IL-7 and IL-15 enhance interferon-gamma (IFN- γ) production by CD8⁺ T cells. (A) Quantification of IFN- γ expression by CD8⁺ T cells following TCR and/or IL-12 and/or IL-18 and/or IL-2 stimulation. (B) Quantification of IFN- γ expression by CD8⁺ T cells following TCR and/or IL-12 and/or IL-18 and/or IL-7 stimulation. (C) Quantification of IFN- γ expression by CD8⁺ T cells following TCR and/or IL-12 and/or IL-18 and/or IL-15 stimulation. Data are representative of 5 (n = 3 x 2 technical replicates) independent experiments. Error bars indicate the mean $\pm$ s.d. Comparison between groups was calculated using a Two-way ANOVA and Šidák's multiple comparison test. *P<0.05; **P<0.01; ***P<0.001; NS, not significant.

5.2.6. CD44 and CD49d expression dynamics *in vitro* and *in vivo*

Given that we identified T_{VM} cells as the primary source of early CD8⁺ T cell-derived IFN- γ , it would have been intriguing to dissect the mode of activation and IFN- γ induction of T_{VM} cells more in depth *in vitro*. However, we noted that the levels of CD44 varied drastically across experimental conditions following both overnight (24 hours) incubation and a reduced

incubation period of 16 hours (data not shown), which suggests that under certain conditions *in vitro* the expression of CD44 is rapidly upregulated following *in vitro* stimulation. The observed variation rendered its use as a phenotypic marker in this context unreliable. CD44 induction within 24 hours after *in vitro* TCR-initiated signalling has been reported previously⁶⁷⁰ and rapid upregulation of CD44 expression on CD8⁺ T cells *in vitro* was observed to occur as early as 16 hours after activation³³⁷.

It is reasonable to assume that expression kinetics after *in vitro* stimulation exhibit an advanced time course of activation compared with *in vivo* infection, and indeed CD44 upregulation and downregulation of CD62L in CD4⁺ cells was reported to occur *in vivo* around day 4 post activation, once cells started to divide and at a time when early activation markers such as CD69, CD25 and Fas had disappeared^{100,671,672}. CD49d upregulation on the other hand was also shown to require at least 2-6 cell divisions, depending on the pathogen^{100,646,672–674}. Expansion of activating CD4⁺ and CD8⁺ T cells *in vivo* was reported to start around day 3-4 post-infection⁶⁷⁵. Thus, a change in CD44 and CD49d upregulation should not be observed prior to that time.

We sought to investigate the *in vivo* kinetics of CD44 and CD49d expression in our LM-OVA infection model to confirm that those markers can be relied upon to distinguish between the different CD8⁺ T cell subpopulations. Comparing IFN- γ^{neg} to IFN- γ^{pos} CD8⁺ T cells in infected mice confirmed that nearly all IFN- γ^{pos} cells are CD44⁺ (**Figure 61B**). Measuring CD44 expression levels in naïve vs LM-OVA infected mice showed no significant differences, both in terms of the relative abundance of CD44^{pos} and CD44^{neg} CD8⁺ T cells (**Figure 61B**). Using mice of the same age and gender that have been co-housed in the same cage, we furthermore confirmed that there was no significant change in the distribution of the different subsets

between naïve and infected mice 48 hours post-infection (**Figure 61E**), indicating that CD44 and CD49d are only upregulated after at least day 2 post-infection.

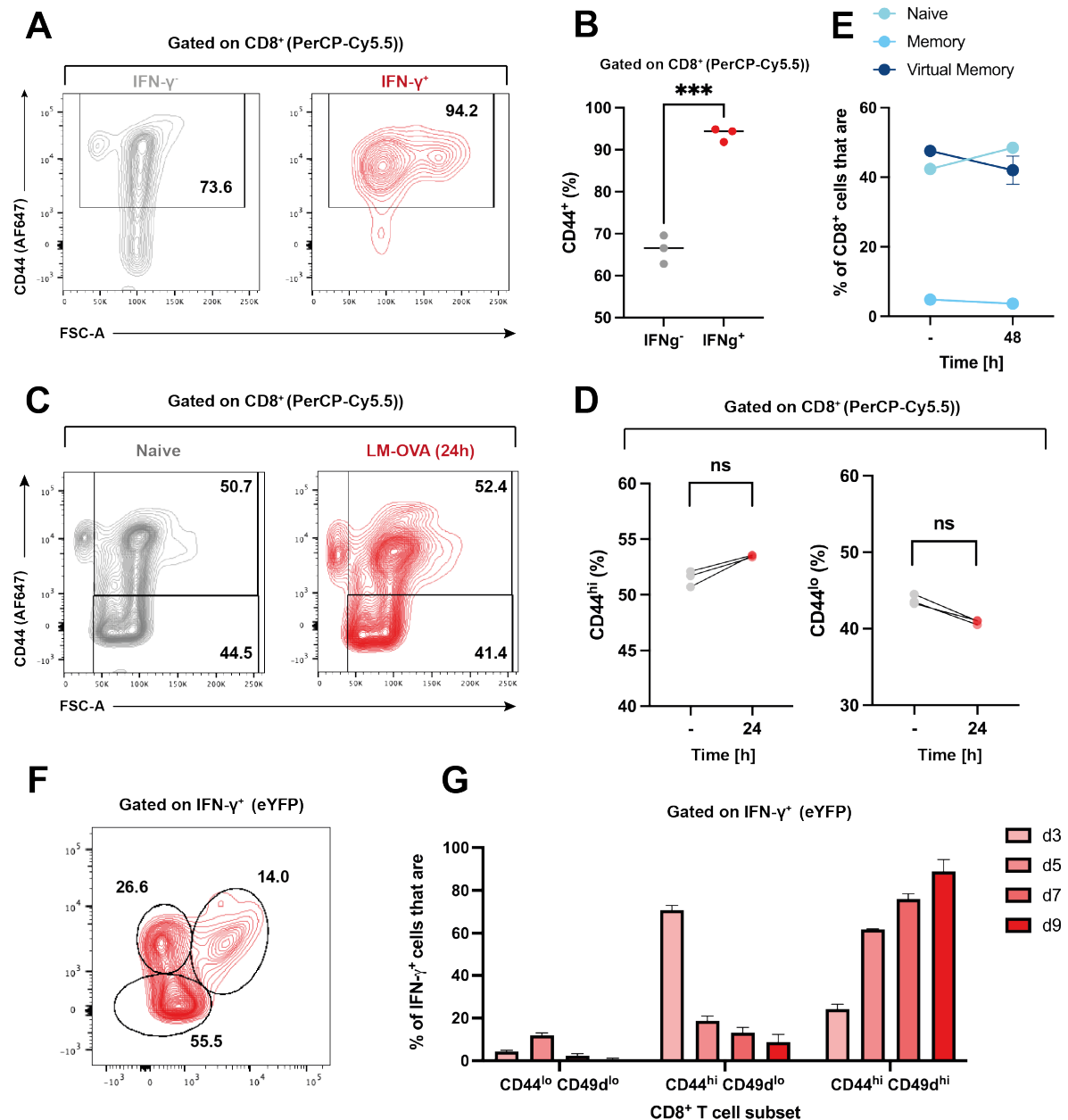


Figure 61. CD44 and CD49 are suitable markers for CD8⁺ T cell phenotyping *in vivo*. (**A, B**) Representative flow plots (**A**) and quantification (**B**) of CD44^{pos} cells amongst IFN- γ ^{pos} or IFN- γ ^{neg} CD8⁺ T cells 24 hours post-infection. (**C, D**) Representative flow plots (**C**) and quantification (**D**) of CD44^{pos} and CD44^{neg} CD8⁺ T cells. (**F, G**) Representative flow plot (**F**) and quantification of CD44 and CD49d expression kinetics during the effector phase of the response. Data are representative of 3 (n = 3 x 2 technical replicates) independent experiments. Error bars indicate the mean $\pm$ s.d. Comparison between groups was calculated using the two-tailed unpaired Student's t-test. *P<0.05; **P<0.01; ***P<0.001; NS, not significant

Finally, we also measured CD44 and CD49d expression during the effector phase of the response. Phenotyping of IFN- γ ⁺ CD8⁺ cells from day 3 post-infection onwards showed a clear

shift in surface expression of CD44 and CD49d (**Figure 61G**). Whilst the majority of IFN- γ ⁺ cells at day 3 post-infection exhibited predominantly a CD44^{hi}CD49d^{lo} phenotype, a marked shift was observable from day 5 onwards towards a CD44^{hi}CD49d^{hi} phenotype, which gradually increased until day 9 (**Figure 61G**). We concluded that while not being a reliable marker *in vitro* due to rapid upregulation, CD44 and CD49d are suitable and reliable markers to distinguish between the different subsets in the early phases of the response.

5.2.7. Interferon-gamma expression requires both TCR and cytokine signalling *in vivo*

Our *in vitro* results have shown that early IFN- γ expression in CD8⁺ T cells relies upon both TCR- and cytokine-stimulation. We next sought to validate those findings *in vivo*. To evaluate, whether IFN- γ production is dependent on TCR stimulation *in vivo*, adoptive transfer experiments were conducted to assess the level of IFN- γ secretion by antigen-specific CD8⁺ T cells upon cognate or non-cognate antigen stimulation. CD8⁺ T cells from antigenically naïve GREAT OT-I mice were transferred into C57BL/6J (B6) mice carrying the CD45.1 congenic marker (**Figure 62**). One day post adoptive transfer host mice were infected with either LM-OVA or LM-gp33. Their spleens were harvested 24 hours post infection and processed and stained for flow cytometric analysis of eYFP expression, which was again used as a surrogate marker for IFN- γ production. The activation marker CD69 was potently upregulated in both experimental conditions (**Figure 62D**), which could reflect either productive TCR engagement or cytokine stimulation or both. Of note, LM-gp33 infection even resulted in a higher percentage of CD69⁺ CD8⁺ T cells 24 hours post-infection, which may be due to differences in infectivity of the two strains (**Figure 62D**). However, IFN- γ expression in OT-I cells was only observed after infection with LM-OVA, while LM-gp33 did not elicit any detectable levels of eYFP expression (**Figure 62E**), confirming the requirement of cognate TCR-epitope interaction

for early IFN- γ induction *in vivo*. These findings were also reproduced using CD8 $^{+}$ T cells from a different TCR transgenic mouse strain, P14, in which CD8 $^{+}$ T cells express a transgenic TCR specific for the LCMV-derived peptide gp33. As observed previously, only under conditions of cognate antigen stimulation was IFN- γ secretion measurably induced in adoptively transferred TCR transgenic CD8 $^{+}$ T cells (**Figure 62E**), whilst transferred CD8 $^{+}$ T cells displayed potent CD69 upregulation in all tested conditions (**Figure 62D**).

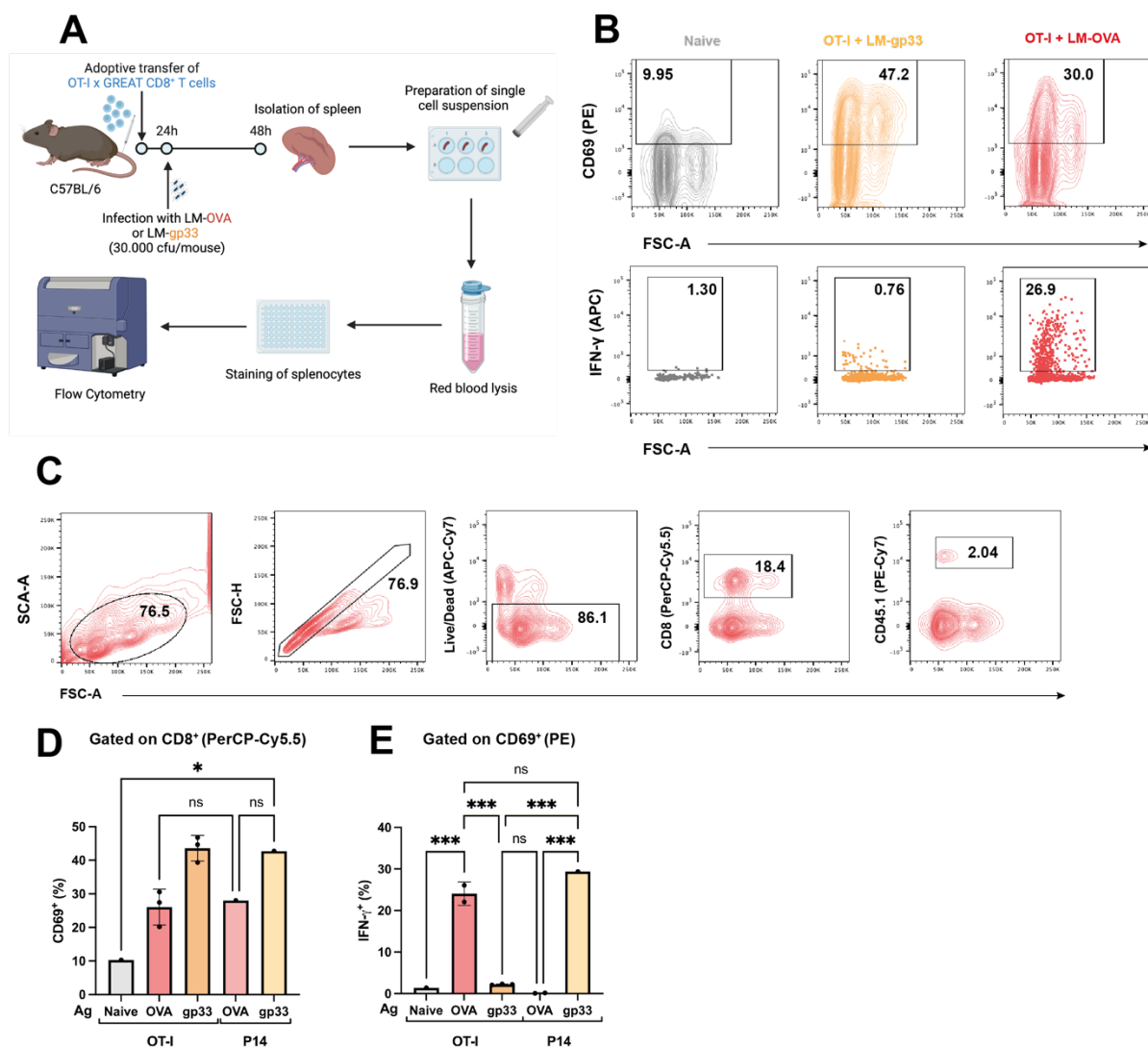


Figure 62. *In vivo* interferon-gamma (IFN- γ) induction in CD8 $^{+}$ T cells requires T cell receptor (TCR) and cytokine signalling. (A) C57BL/6J (B6) mice were infected with either LM-OVA (red) or LM-gp33 (orange) and CD69 and IFN- γ expression were evaluated 24 hours post-infection. (B) Representative flow plots of CD69 and IFN- γ expression. (C) Gating strategy for adoptively transferred cells. (D, E) Quantification of CD69 (D) and IFN- γ (E) expression in adoptively transferred cells 24 hours post-infection. Data are representative of 3 ($n = 3 \times 2$ technical replicates) independent experiments. Error bars indicate the mean $\pm$ s.d. Comparison between groups was calculated using the two-tailed unpaired Student's t-test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS, not significant.

To confirm these findings at the endogenous level, we used the Nur77-GFP reporter mouse model. Expression of the nuclear orphan receptor Nur77 in these reporter mice drives expression of the fluorescent reporter GFP⁶⁷⁶. First, we evaluated whether Nur77-GFP expression is TCR-specific or additionally upregulated by cytokine signalling. To test this, we compared primed CD8⁺ T cells from LM-OVA infected mice to CD8⁺ T cells from mice that were injected subcutaneously with DCs loaded with the peptide N4. As expected, we detected much fewer CD69⁺ CD8⁺ T cells in the pathogen-free setting, whilst observing potent upregulation in the infection setting (**Figure 63B**). However, those fewer activated CD8⁺ T cells in the pathogen-free condition were all GFP⁺, while only approximately a third of activated CD8⁺ T cells were GFP⁺ after LM-OVA infection (**Figure 63B**).

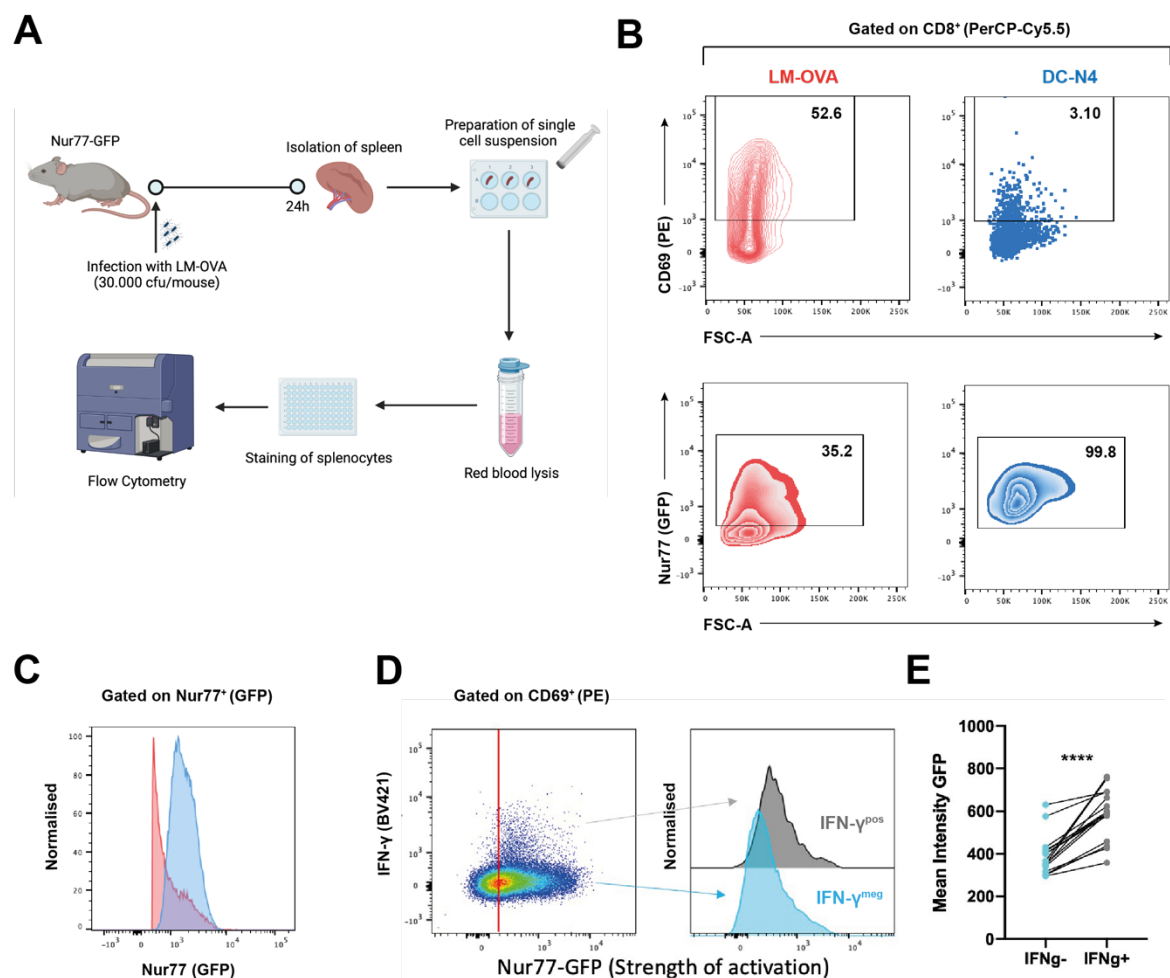


Figure 63. Interferon-gamma (IFN- γ)^{pos} CD8⁺ T cells upregulate Nur77. (A) Nur77-GFP mice were infected with LM-OVA and Nur77-GFP and IFN- γ expression was evaluated 24 hours post-infection. (B) Representative flow plots of CD69 and Nur77-GFP expression in mice either infected with LM-OVA (red) or injected with peptide-loaded dendritic cells (blue) 24 hours post-infection. (C) Representative histograms of Nur77-GFP expression. (D, E) Representative flow plot (D) and quantification of Nur77-GFP expression in IFN- γ ^{neg} and IFN- γ ^{pos} CD8⁺ T cells. Data are representative of 3 (n = 3 x 3 technical replicates) independent experiments. Error bars indicate the mean $\pm$ s.d. Comparison between groups was calculated using the two-tailed unpaired Student's t-test. *P<0.05; **P<0.01; ***P<0.001; NS, not significant.

Having confirmed that Nur77 expression is indeed TCR-dependent, we then infected Nur77-GFP mice with LM-OVA and quantified IFN- γ expression in CD69⁺ CD8⁺ T cells (Figure 63C, D and E). We observed a significant shift in GFP expression in IFN- γ ⁺ as compared to IFN- γ ⁻ CD8⁺ T cells (Figure 63E), indicating that early IFN- γ expression in CD8⁺ T cells indeed requires productive TCR stimulation.

5.2.8. Interferon-gamma expression is independent of TCR stimulation strength

To examine whether the strength of TCR-antigen interaction affects IFN- γ induction, mice with transferred GREAT OT-I CD8⁺ T cells were infected with LM expressing altered OVA peptides of different TCR binding affinities (Figure 64A). Altered peptide ligands N4, T4 and V4 represent the high-, intermediate- and low-affinity OVA peptides, respectively. While N4 constitutes the original OVA₂₅₇₋₂₆₄ peptide binding with high avidity to the transgenic TCR, T4 (SIITFEKL) is of considerably lower binding avidity, and V4 (SIIVFEKL) is estimated to be about 700-fold less potent than N4⁶³, so that in terms of TCR signal intensity V4 < T4 < N4. The differential TCR stimulation strength of these peptides is also reflected in their potency to drive CD69 upregulation (Figure 64B). Notably, we didn't observe statistically significant differences in IFN- γ expression by OT-I cells in response to APLs, indicating that the strength of TCR engagement does not critically modulate early IFN- γ induction during priming (Figure 64C).

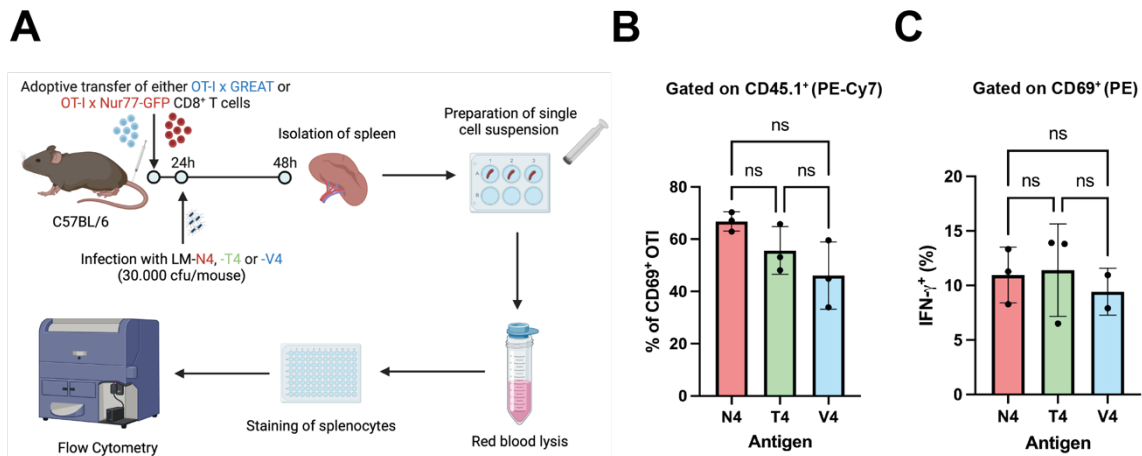


Figure 64. Induction of interferon-gamma (IFN-γ) expression by CD8⁺ T cells is independent of T cell receptor (TCR) stimulation strength. (A) GREAT OT-I CD8⁺ T cells were adoptively transferred into C57BL/6J host mice and infected with either LM-N4, -T4 or -V4. IFN-γ expression in adoptively transferred CD8⁺ T cells was assessed 24 hours later by flow cytometry. (B) Quantification of CD69 expression in adoptively transferred cells 24 hours post-infection. (C) Quantification of IFN-γ expression in adoptively transferred cells 24 hours post-infection. Data are representative of 3 (n = 3 x 2 technical replicates) independent experiments. Error bars indicate the mean ± s.d. Comparison between groups was calculated using the two-tailed unpaired Student's t-test. *P<0.05; **P<0.01; ***P<0.001; NS, not significant.

Notably, it was discovered that Nur77 expression is TCR-dependent and proportional to TCR signal strength (Figure 65)⁶⁷⁶, thus enabling the use of GFP expression levels as a surrogate marker for the strength of TCR stimulation.

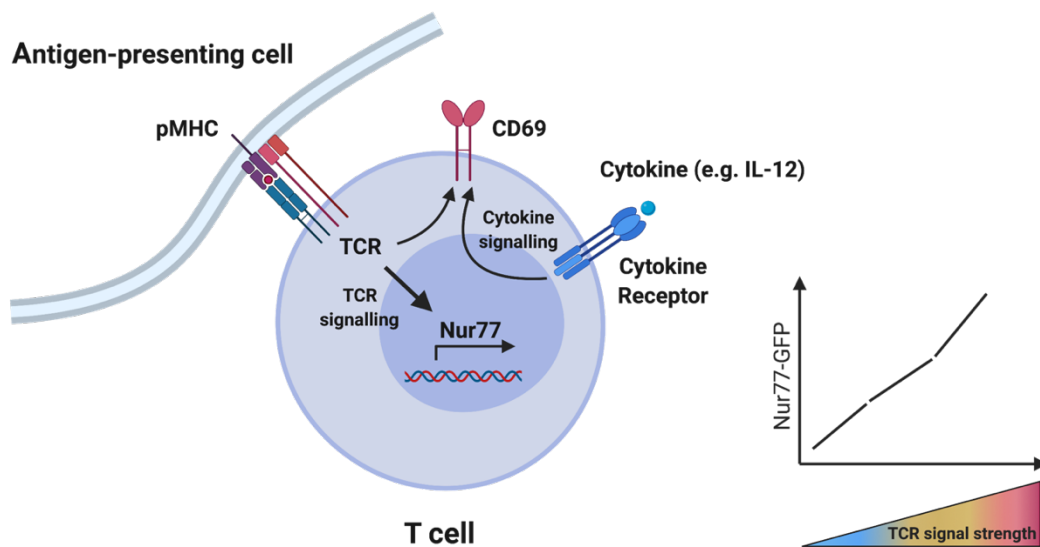


Figure 65. Nur77-GFP reporter mice. Nur77 is a nuclear orphan receptor that is upregulated upon TCR priming in a TCR strength-dependent manner. Nur77 expression in these reporter mice is coupled to GFP expression [Created with BioRender.com].

We crossed Nur77-GFP animals with OT-I mice to generate OVA-specific CD8⁺ T cells carrying the Nur77 reporter transgene. CD8⁺ T cells isolated from these mice were then

adoptively transferred into B6 hosts and subsequently infected with LM expressing OVA-derived APLs differing in their potency for transgenic TCR stimulation. Based on CD69 expression level we inferred that all transferred cells got successfully activated following infection with the different LM strains.

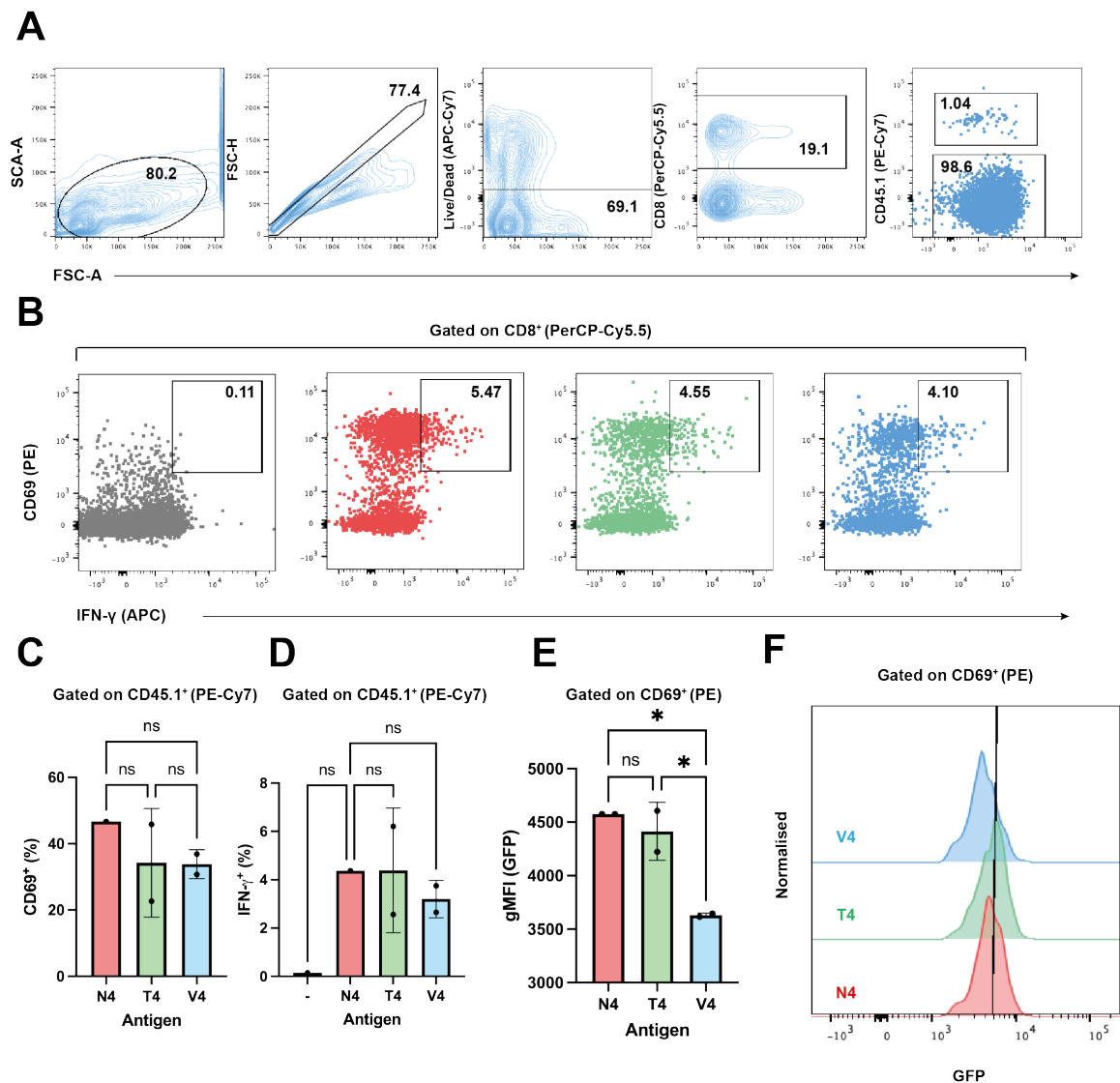


Figure 66. Induction of interferon-gamma (IFN-γ) expression by CD8⁺ T cells is independent of T cell receptor (TCR) stimulation strength. (A) Representative gating strategy to gate on adoptively transferred cells. (B, C, D) Representative flow plots (B) and quantification of CD69 (C) and IFN-γ (D) expression in adoptively transferred cells 24 hours post-infection. (E, F) Representative histograms (F) and quantification (E) of Nur77-GFP expression in adoptively transferred cells 24 hours post-infection. Data are representative of 2 (n = 3 x 2 technical replicates) independent experiments. Error bars indicate the mean ± s.d. Comparison between groups was calculated using the two-tailed unpaired Student's t-test. *P<0.05; **P<0.01; ***P<0.001; NS, not significant.

IFN-γ secretion was detectable in all experimental conditions. However, we again did not detect any significant differences between the differentially activated TCR transgenic CD8⁺ T

cells (**Figure 66C**). Altogether we concluded that IFN- γ induction is not dependent upon a specific threshold level of TCR stimulation strength.

5.2.9. *In vivo* blockade of TCR and cytokine signalling reduces interferon-gamma expression by CD8⁺ T cells

Lastly, we aimed to validate our *in vitro* findings by blocking the identified stimuli *in vivo*. MHC Class I and/or IL-12 + IL-18 were blocked in animals prior to LM-OVA infection by intravenous injection of blocking antibodies and IFN- γ production was evaluated 24 hours post LM-OVA infection (**Figure 67A**). Blocking only IL-12 + IL-18 did not result in a significant reduction of CD69⁺ CD8⁺ T cells, while blocking MHC Class I did cause a significant reduction in activation. A similar reduction in activation was measured when blocking both MHC Class I and cytokines (**Figure 67B and C**). Gating on activated cells revealed a significant reduction in IFN- γ expression following either MHC Class I or MHC-I + IL-12 + IL-18 blockade (**Figure 67C**). The fact that we did not observe much difference between these two conditions, and furthermore did not see a significant reduction in activation upon blockade of IL-12 + IL-18 alone (**Figure 67B and D**), may be due to suboptimal cytokine blocking *in vivo*. Alternatively, additional cytokines may compensate for the loss of IL-12/IL-18 signalling. There was not sufficient time left to optimise this experiment before submission of this thesis, but we are planning on repeating this experiment as soon as possible to be able to conclude definitively if both TCR- and cytokine-signalling are indeed absolute requirements for early IFN- γ induction in CD8⁺ T cells *in vivo*.

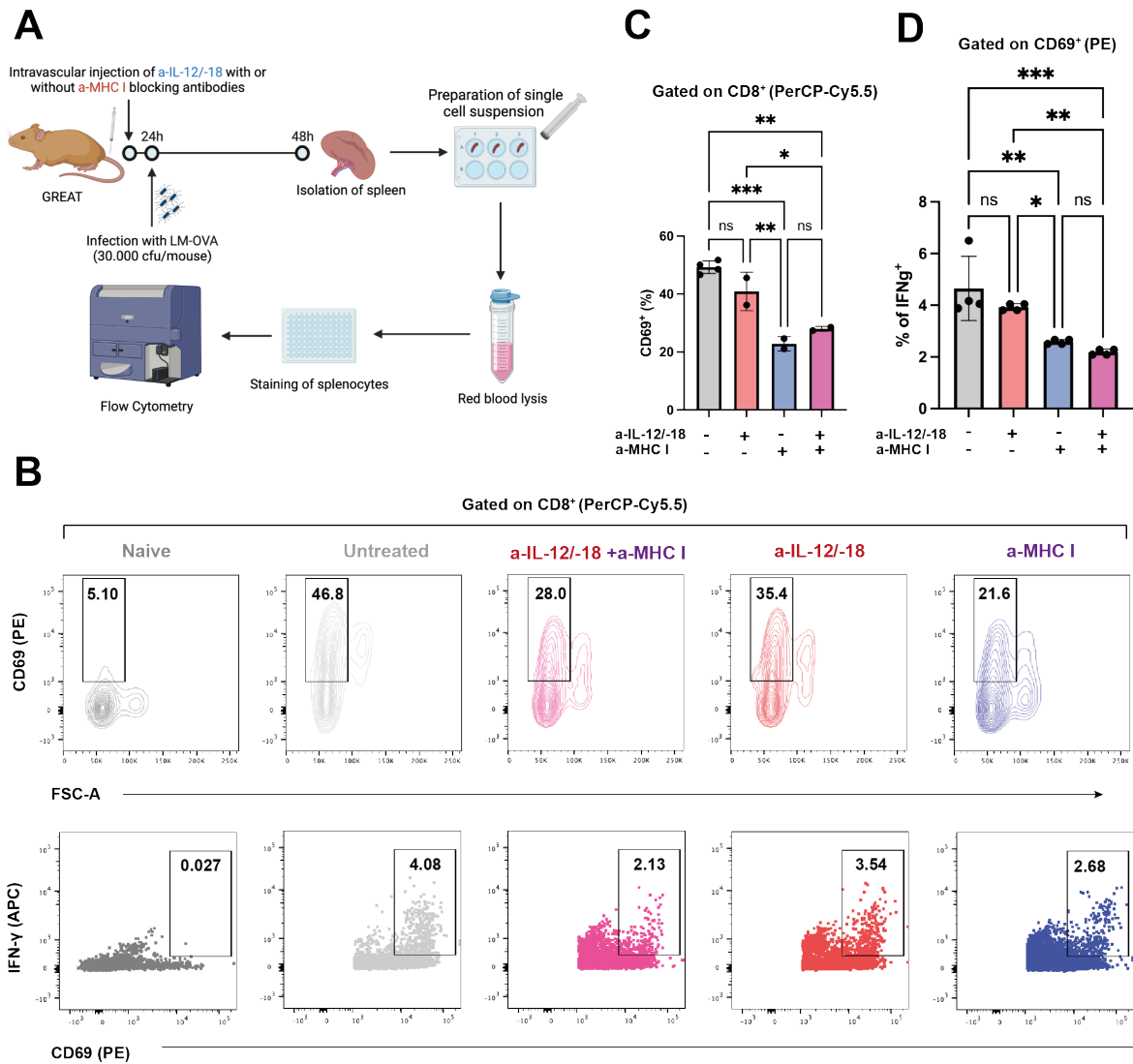


Figure 67. Blockade of T cell receptor (TCR) and cytokine signalling reduces interferon-gamma (IFN- γ) secretion by CD8⁺ T cells. (A) GREAT mice were injected with blocking antibodies and infected with LM-OVA the day after. IFN- γ secretion was evaluated in CD8⁺ T cells 24 hours post-infection. (B, C, D) Representative flow plots (B) and quantification of CD69 (C) and IFN- γ (D) expression in CD8⁺ T cells 24 hours post-infection. Data are representative of 1 (n = 3 x 3 technical replicates) independent experiments. Error bars indicate the mean $\pm$ s.d. Comparison between groups was calculated using the two-tailed unpaired Student's t-test. *P<0.05; **P<0.01; ***P<0.001; NS, not significant.

5.3. Discussion

5.3.1. Virtual Memory CD8⁺ T cells as the main subset producing early interferon-gamma

In this chapter we have shown that the primary CD8⁺ T cell subpopulation producing IFN- γ upon activation are virtual memory T cells, which belong to the broader family of memory phenotype CD8⁺ T cells. Diverse and dichotomous functions have been attributed to T_{MP} cells, including innate-like effector functions in the early stages of pathogen clearance^{579,610}, as well as a role in the maintenance of immune homeostasis at steady state⁶⁷⁷. However, their precise function for the CD8⁺ T cell immune response remains elusive. It furthermore remains controversial, what factors induce early IFN- γ secretion by CD8⁺ T cells.

Previous studies attributed IFN- γ production by CD8⁺ T cells 24 hours post-infection mostly to effector or memory CD8⁺ T cells and to be driven by so-called “*bystander activation*” via IL-12 and/or IL-18 cytokine signalling⁷⁰. Of note, IFN- γ secretion by a small percentage of phenotypically naïve CD8⁺ T cells was also observed in earlier studies⁵⁰². However, the percentage of IFN- γ production reported in nonprimed, adoptively transferred OT-I T cells was significantly lower compared with the memory CD44^{hi} OT-I population. Additionally, these nonprimed, IFN- γ -secreting OT-I cells were shown to alter their surface phenotype with respect to CD44, CD94 and other memory markers in response to stimulation, whereas non-transferred OT-I did not secrete IFN- γ upon stimulation⁵⁰², suggesting that the *in vivo* environment may imprint or enforce a memory-phenotype in these transferred cells. Early IFN- γ secretion by antigenically naïve, CD44^{low} OT-I has also been reported by another study³⁷⁹, and it is possible that this function may be an artifact of the OVA-specific OT-I (V β 5/V α 2) system and their comparatively high-affinity TCR. In this context it is also worth highlighting that OT-I cells have been shown to undergo strong homeostatic proliferation, and

thus typically display a higher proportion of CD44^{hi} cells than CD8⁺ T cells derived from other TCR-transgenic mice⁶⁷⁸. However, altogether, we hypothesise that the memory-phenotype of the vast majority of IFN- γ -producing OT-I T cells described in previous studies, as well as their innate-like manner of IFN- γ secretion, may likely be indicative of their identity as T_{VM} cells.

Characterizing the population of IFN- γ -producing CD8⁺ T cells 24 hours post-infection, both by flow cytometry (**Figure 54**) and scRNA-sequencing (**Figure 56**), indeed revealed that T_{VM} cells comprise the predominant fraction of IFN- γ -secreting CD8⁺ T cells at this early time point during the response. A notable fraction of T_{VM} cells is also detectable in naïve mice, confirming that this phenotype is not an artifact of infection. However, the obtained results could potentially be an artifact of the OT-I system, or specific to *L. monocytogenes* infections. Hence, we tested if we could detect a similar fraction of IFN- γ -secreting CD8⁺ T cells with a T_{VM} phenotype in response to another antigen, LCMV-derived gp33, which was indeed the case. Whilst we did not test different pathogens, other groups have confirmed the presence of T_{VM} CD8⁺ T cells in diverse infection models such as vaccinia virus and other pathogens⁵⁷⁹. An alternative infection model, influenza virus, has been established in our group and could be used to confirm our findings in a different infection context.

Our characterization by scRNA-seq analysis confirmed that T_{VM} cells are transcriptionally distinct from naïve and true memory cells, while exhibiting significant overlap with both subsets, as reported previously^{616,638}. A few studies have furthermore assessed the transcriptional profile and chromatin accessibility of T_{VM} cells at different life stages^{580,616,638,679}. Key signatures of T_{VM} cells that have been identified in these studies included high expression of transcripts related to NK cells, granzymes and cell killing, inflammatory cytokines and cytokine sensing, as well as differential expression of chemokine and cell adhesion markers⁶¹⁵. In addition, several transcription factors have also been

reported to be differentially, if not exclusively, expressed in T_{VM} cells, such as high expression of *Eomes*, *Runx2* and *Bhlhe40* and decreased expression of *Sox4*^{580,616,618,680}, which altogether comprise a key transcription factor hub for memory differentiation⁶⁸¹. Lastly, T_{VM} cells also exhibited a clear transcriptional signal for IL-15 signalling mediators and regulators, such as increased expression of *Eomes*, *IL15*, *IL12rb*, *IL15ra* and *Bcl2*^{580,616,618}.

Of note, CD44^{hi} CD8⁺ T cells can also be detected in TCR transgenic mice on a RAG-expressing background and their presence has mostly been attributed to the expression of endogenous TCR α chains^{617,642}. One critical control experiment we are still missing is to validate our findings by adoptively transferring antigenically naïve CD8⁺ T cells from Rag^{-/-} mice into host mice and quantifying IFN- γ expression 24 hours post-infection in transferred cells, which would allow us to conclude definitively if naïve cells contribute to the pool of available IFN- γ during priming. Of note, CD44^{hi} T cells have previously been reported to be present in unprimed TCR transgenic Rag^{-/-} mice, and suggested to arise from homeostatic proliferation⁶¹⁰, which highlights the importance of sorting CD44^{hi} cells prior to adoptive transfer or using isolation kits specifically designed for isolation of antigenically naïve CD8⁺ T cells.

It is important to highlight that our studies so far did not investigate the effector role and protective quality of T_{VM} cells in response to infection. It has previously been shown that the T_{VM} pool can provide potent and antigen-specific protective immunity against pathogen infection^{579,580,613,635,653,682}, and that T_{VM} cells furthermore differ in their expansion and differentiation patterns compared with naïve-derived effector populations^{579,579,635,653,682}. However, investigating the effector functions of T_{VM} cells lay beyond the scope of this thesis.

5.3.2. Induction of interferon-gamma secretion by CD8⁺ T cells requires both TCR and cytokine signalling

Investigating the priming factors inducing IFN- γ production by antigenically naïve, endogenous CD8⁺ T cells, both *in vitro* (Figure 59) and *in vivo* (Figure 62), revealed that IFN- γ secretion 24 hours post-activation required both TCR stimulation and cytokine signalling, with IL-12 and IL-18 having been identified as the most potent triggers of early IFN- γ secretion. Additionally, IL-15, IL-2, and IL-7, all of which belong to the γ family of cytokines, displayed synergistic effects on IFN- γ induction. Based on these and previously described findings, we propose a mechanistic model, in which T_{VM} cells contribute to IFN- γ production early during immune responses, through integration of TCR and inflammatory signalling during priming.

IFN- γ secretion by CD8⁺ T cells has previously been described to be induced upon cytokine-mediated signalling within the local microenvironment, suggested to be driven predominantly by IL-12/IL-18 production by LM-infected macrophages⁵⁰², which is consistent with our *in vitro* results showing that the combination of IL-12 and IL-18 had the most potent IFN- γ -inducing effect on antigenically naïve CD8⁺ T cells. Notably, we did not observe an inducing or enhancing effect on IFN- γ by other inflammatory cytokines, such as TNF, IL-33, and type I IFNs. Type I IFN signalling was shown by earlier studies to contribute to various innate functions, including IFN- γ production by T_{VM} cells⁶³⁴. While our data cannot rule out that type I IFN may serve an important function in contributing to the long-term homeostasis or fitness of T_{VM} cells in the periphery and the differentiation of innate memory cells in the thymus, our data suggest that it is not critically involved in the induction of early IFN- γ secretion by T_{VM} cells.

However, as shown by other studies examining the landscape of potential T cell:cytokine interactions during infection⁶⁸³, the response of T cells to individual signals is typically highly

context dependent. It is thus possible that certain cytokines only exert a synergistic or inhibitory effect when paired with a specific set of cytokine partners, and whilst studying a wide range of distinct combinations, we did not examine more complex cytokine interactions.

It is also important to highlight that our *in vitro* setup, albeit having the advantage of controlling stimulant concentrations and testing specific cytokine combinations, is an overly simplified recapitulation of the situation encountered *in vivo* and does not fully replicate all the unique microenvironmental cues CD8⁺ T cells are exposed to *in vivo*. Certain inflammatory cytokines, such as TNF and type I IFNs, may furthermore require concomitant activity of additional signalling factors missing in our well-controlled *in vitro* assay, which may offer another explanation why they failed to induce potent IFN- γ production *in vitro*, but may do so under certain conditions *in vivo*. *In vivo* infection is also associated with the dissemination of PAMPs into the local microenvironment, leading to inflammatory signalling via TLRs, which have been reported to provide important signals for bystander activation^{41,684}, and to function as co-stimulatory receptors on T cells, which lower the threshold for TCR activation^{663,684,685}. Our results do not exclude that TLR signalling is involved in driving early IFN- γ secretion in CD8⁺ T cells *in vivo*⁶⁸⁶.

However, in contrast to previous findings, our data clearly indicate IFN- γ secretion to be dependent upon concomitant TCR stimulation, whilst showing only low inducibility of IFN- γ expression upon cytokine signalling alone. This observed discrepancy may be due to differences in experimental conditions. Whereas Berg and colleagues used non-purified splenocytes for stimulation, we directly stimulated isolated CD8⁺ T cells. It is worth noting that TCR stimulation, despite exhibiting profound synergies with cytokine signalling *in vitro*, did not result in much detectable IFN- γ secretion by itself. TCR-dependence for IFN- γ secretion was also confirmed *in vivo*. We furthermore validated the IFN- γ -inducing

capabilities of certain priming factors identified by the *in vitro* stimulation assay *in vivo*, by antibody blocking of specific cytokines and MHC I in a spatially specific manner. It is interesting to speculate what kind of TCR stimulus T_{VM} cells require, and while our *in vivo* studies offer certain hints, this remains to be further elucidated. The increased responsiveness of T_{VM} cells to IL-12 and IL-18 may be explained by their increased self-reactivity, as suggested previously⁶³⁵, even though our data suggest that cognate antigen may be required to trigger early IFN- γ secretion.

Of note, cytokine-independent induction of IFN- γ expression upon *in vitro* TCR stimulation in naïve and memory-like OT-I T cells has also previously been reported^{379,577}. A study byurtsinger and co-workers showed rapid induction of IFN- γ within 4 hours post-activation, with a decline in expression levels observed beyond 8 hours after stimulation, a time window which we did not evaluate in our studies. Purification of naïve $CD44^{low}$ OT-I cells by negative selection and subsequent TCR stimulation showed that a small fraction of $CD44^{low/int}$ cells produces IFN- γ , albeit distinctly less than $CD44^{hi}$ cells³⁷⁹. As mentioned previously, this effect may be an artifact of the OT-I system, which we are currently investigating.

5.3.3. Proposed mechanism of early interferon-gamma induction in $CD8^+$ T cells

How the observed synergy between TCR and cytokine signalling exactly operates is an open question, but our *in vitro* data suggest that the effect of both signalling cascades is not simply additive. TCR stimulation has previously been shown to induce upregulation of functional IL-12R complex expression in naïve $CD8^+$ T cells, allowing them to sense IL-12⁵⁴⁸. Earlier studies using polarized T_H1 $CD4^+$ T cell lines have furthermore demonstrated that IL-12 upregulates expression of the IL-18 receptor, while IL-18 concomitantly augments the expression of the IL-12R β 2 subunit^{687–689}, and a similar type of synergism was suggested to occur in $CD8^+$ T

cells⁵⁰², which seems to be indeed the case, with studies showing that IL-12R signalling drives the expression of IL-18R complex through the activation of STAT4, thereby enhancing responsiveness to IL-18.

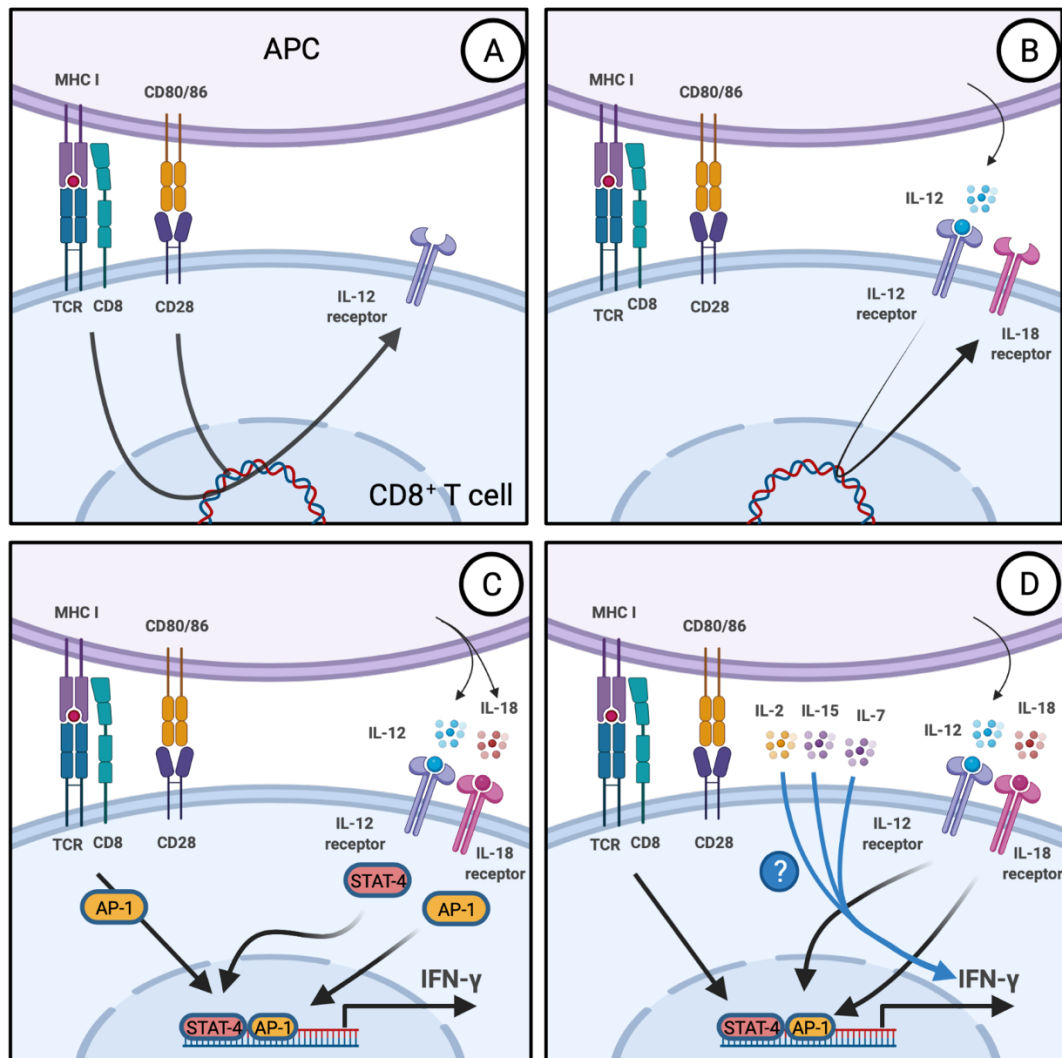


Figure 68. Proposed mechanism of early interferon-gamma (IFN- γ) production by CD8⁺ T cells. (A) Cognate T cell receptor (TCR)-peptide MHC I interaction, in combination with concurrent CD28-CD80/CD86 co-stimulation, drives the upregulation of Interleukin-12 (IL-12) receptor (IL-12R). (B) IL-2 receptor signalling in turn upregulates the IL-18R in a STAT4-dependent manner, allowing the CD8⁺ T cell to respond to both IL-12 and IL-18. (C) STAT4, activated downstream of IL-12 receptor, forms a complex with AP-1 activated downstream of both TCR and IL-18 receptor signalling. This STAT4-AP-1 complex can bind to an AP-1 responsive region within the *Ifng* promoter with high-affinity, inducing IFN- γ expression. (D) IFN- γ induction may be further enhanced by IL-2, IL-15, and IL-7, through uncharacterised mechanisms. Their enhancing effects on IFN- γ induction are likely influenced by other extrinsic factors such as local cell density and presence of other immune cell types and cytokines [Created with BioRender.com; Figure generated with the help of Han Cai]

This functional “licencing” role of TCR triggering on the cytokine-responsiveness of CD8⁺ T cells is also consistent with our observation that TCR stimulation alone did not induce significant IFN- γ production, similar to conditions including only IL-12 and IL-18. The capacity

of CD8⁺ T cells to rapidly secrete IFN-γ may depend, at least partly, on the intrinsic ability of T cells to upregulate receptors for IL-12 and IL-18 in the absence of infection⁵⁰², which is supported by findings showing that CD8⁺ T cells with lower expression levels of IL-12R and IL-18R exhibit impaired IFN-γ-secreting capability⁶⁹⁰. IL-12 and IL-18 can synergistically induce IFN-γ production through the formation of the STAT4-AP-1 complex, which binds to the AP-1-responsive region within the *Ifng* promoter⁵⁴⁸, indicating that IL-12-activated STAT4, which does not directly bind to the *Ifng* promoter, may exert its IFN-γ-inducing effect via enhancing the binding affinity of IL-18-induced AP-1 to the AP-1-responsive region⁵⁴⁸. A similar mode of action was reported for the synergistic effect of IL-12-dependent STAT4 activation with AP-1 activated downstream of TCR stimulation⁴⁵⁸. However, the synergy between IL-12 and TCR signalling alone is insufficient at driving potent IFN-γ expression, indicating that IL-18, either through driving AP-1 phosphorylation or additional signalling pathways, is still necessary for eliciting potent IFN-γ secretion.

Taken together, the following model of mechanistic collaboration to induce early IFN-γ production by CD8⁺ T cells emerges (**Figure 68**): Upregulation of cytokine receptor expression in naïve CD8⁺ T cells via TCR stimulation heightens responsiveness to IL-12 and IL-18, which promote IFN-γ secretion in largely cytokine-driven fashion through the synergistic activities of STAT4 and AP-1. Due to the rapid upregulation of CD44 following *in vitro* stimulation, we could not further examine if our results are a specific function of T_{VM} cells. To specifically examine IFN-γ production by T_{VM} cells may require prior sorting of CD44^{hi}CD49d^{lo} CD8⁺ T cells. However, taken together with our *in vivo* investigation, we hypothesise that T_{VM} cells are indeed the primary source of early IFN-γ.

5.3.4. Synergistic effects of IL-2, IL-7, and IL-15 on interferon-gamma production

Our *in vitro* data also indicated a significant synergistic function of IL-15, IL-2, and IL-7 at enhancing early IFN- γ production by activating CD8⁺ T cells ([Figure 60](#)). Augmentation of IL-12- and IL-18-induced IFN- γ secretion by either IL-2 or IL-15 has been previously described⁵⁰². Mechanistically, IL-15 is found to synergize with IL-12 and IL-18 in human T and NK cells to promote IFN- γ secretion, both directly through transcriptional upregulation of IFN- γ and indirectly via the activation of STAT5, which in turn upregulates IL-12R and IL-18R, promoting the aforementioned IL-12 and IL-18 synergy^{691,692}. In human samples, IL-15 was furthermore revealed to directly induce IFN- γ expression through activation of STAT1, STAT3, STAT4 and STAT5, which bind to activation sites within both the *Ifng* promoter and the first intron⁶⁹². Additionally, IL-15 also enhances STAT4 phosphorylation, which indicates a possible synergistic effect with IL-18 for IFN- γ induction. IL-15 is also a well-known promoter of proliferation for memory-phenotype CD8⁺ T cells⁵⁶⁶, and thus might function through increasing the number of IFN- γ -secreting CD8⁺ T cells. However, proliferation is only observed between day 2-3 post-activation, while IFN- γ secretion is observed as early as 16 hours post-activation *in vitro*. Lastly, it is also intriguing to speculate that the transcription factor Runx2 may serve as the critical point of signal integration for TCR, IL-12, IL-18 and IL-15 signalling, as previously proposed for the signalling mechanisms driving the generation of T_{VM} cells⁶¹⁵. Runx2 is highly upregulated in T_{VM} cells⁶¹⁸, and expressed downstream of type I IFN, IL-12 and IL-15 signalling, and its expression furthermore promotes Eomes expression in CD8⁺ T cells⁶⁹³. Importantly, Runx2 is augmented when cytokine signals are combined with TCR signals, but this requires modest as opposed to strong TCR signalling⁶⁹³. Self-reactive TCRs could therefore deliver modest TCR signals alongside cytokine stimulation to promote Runx2-mediated

Eomes expression, which may lead to selective IL-15R β expression in T_{VM} cells rendering them exquisitely sensitive to IL-15 signalling.

Synergistic effects on IFN- γ induction have also been described between IL-2 and IL-12 in human NK cells, with IL-2 being implied in the release of nuclear *Ifng* mRNA that is otherwise retained by IL-12 signalling, facilitating its translation and subsequent secretion⁵⁵¹. In addition, IL-2 has been shown to induce IFN- γ secretion *in vivo* in NK cells⁶⁹⁴ and it was suggested that IL-2 contributes to IFN- γ secretion through upregulation of STAT4 expression⁷⁰, as STAT4 leads to transcriptional activation of the *Ifng* promoter⁶⁹⁵. Finally, IL-12 induced expression of the IL-2R α chain on polarized CD4⁺ T cells⁶⁹⁶ and IFN- γ -secreting CD8⁺ T cells⁷⁰ has also been reported.

Altogether, it becomes clear that the transcriptional regulation of IFN- γ production involves a highly dynamic and complex network of molecular interactions, with diverse transcription factors and epigenetic changes fine-tuning its expression. It is highly plausible, that different cytokine combinations can activate distinct sets of transcription factors which differ in their modes of interactions and IFN- γ -inducing activities, thus leading to variable expression of this cytokine⁶⁹⁷. Given that IL-2 displayed a dose-dependent effect on IFN- γ induction *in vitro*, which was not observed for IL-12 and IL-18, the capacity of CD8⁺ T cells to produce IFN- γ may not only reflect its intrinsic responsiveness to cytokines, but also extrinsic factors such as local cytokine availabilities⁶⁹⁰. Complementing our previously described model of IFN- γ induction in CD8⁺ T cells ([Figure 68](#)), we hypothesise that additional cytokines, such as IL-2, IL-7, and IL-15, may conditionally enhance the expression of IFN- γ driven by the aforementioned TCR and cytokine synergy. The enhancing effects of these three cytokines may be influenced by their spatial distribution and temporal availability determined by the balance between their local production and consumption within a particular

microenvironment, thus imparting an additional layer of tuneable control over IFN- γ production⁶⁹⁸.

5.3.5. Early interferon-gamma induction by virtual memory T cells does not appear to be bystander-driven

Our data support the notion of a TCR-dependent activation mechanism for the induction of early IFN- γ , which contrasts earlier findings, such as a recent study by White and co-workers⁵⁸⁰, and thus warrants further discussion. It is important to highlight that the earlier study by White and colleagues has not directly measured IFN- γ expression in transferred T_{VM} cells but relied on splenic bacterial counts measured 4 days post-infection for their conclusions. It is thus conceivable that the observed protective effect was due to other effector functions, such as IL-12/IL-18/IL-15-induced expression of granzyme B and NKG2D⁵⁸⁰ or IFN- γ secretion at later time points. Of note, maintaining expression of the effector molecules necessary for the protective function was shown by White et al. to be dependent upon the continued presence of IL-15. IL-15 has previously been shown to confer cytolytic ability on memory CD8⁺ T cells by promoting expression of NK-activating receptors, such as NKG2D, and cytolytic molecules such as granzyme B and perforin^{572,608}, thus expanding memory CD8⁺ T cells towards innate-like or NK-like cells. Intriguingly, concurrent TCR activation was shown to abrogate IL-15-induced upregulation of NKG2D on the surface of memory CD8⁺ T cells⁶⁹⁹, suggesting that NKG2D-mediated functions are preferentially activated in the absence of TCR stimulation. Another important signal that downregulates NKG2D expression, at least during viral infection, is type I IFN⁷⁰⁰, which, altogether, suggests that NKG2D expression in CD8⁺ T cells may be modulated through the balanced action of various proinflammatory cytokines (e.g., IL-15 and/or type I IFNs) and TCR signalling, leaving

open the possibility that T_{VM} cells are capable of mediating bystander-like killing under specific circumstances.

The findings that T_{VM} cells are self-reactive may furthermore offer another explanation why some studies concluded that their activation occurs independent of TCR signalling. Self-reactive T cells could be cross-reactive or recognize pathogen epitopes due so-called molecular mimicry, mediated through the presence of a common epitope structure or sequence between host and microbe^{41,701}. However, it is important to emphasise that our data do not rule out that under certain conditions T_{VM} cells are capable of bypassing TCR signalling to produce effector cytokines.

5.3.6. The spatiotemporal context of T_{VM} activity

T_{VM} cells exhibit a memory-like phenotype, which suggests that they may be endowed with similar migratory patterns as observed for true memory cells, thus permitting them to co-localise with infected cells^{70,502,574}. However, our scRNA-seq results showed that T_{VM} cells express a unique signature of chemokine receptors and adhesion molecules collectively defining their tissue access (**Figure 57**). T_{VM} cells expressed some markers associated with antigen naivety, including CD62L, which would permit circulation of T_{VM} cells to the lymph node, but they downregulated transcription of *Ccr7*⁵⁸⁰, which may limit residency and retention in the lymph node. Whilst it has been shown that a subset of conventional true memory T cells expresses C-X-3C chemokine receptor (CX3CR)1, expression of this receptor was not observed in T_{VM} cells⁶³⁸, which may limit their ability to survey peripheral tissues. However, both T_{VM} and true memory T cells were reported to express a high level of C-X-X chemokine receptor (CXCR)3 in steady state⁵⁸⁰, which may enable circulating T_{VM} cells to migrate toward type I IFN-induced C-X-C chemokine ligand (CXCL)9/10, enabling rapid access

to sites of inflammation. Given the overlap in integrin and chemokine/chemokine receptor expression with naïve T cells, we hypothesise that T_{VM} cells may be preferentially located in close proximity to T_N cells, which we are currently further investigating by confocal imaging.

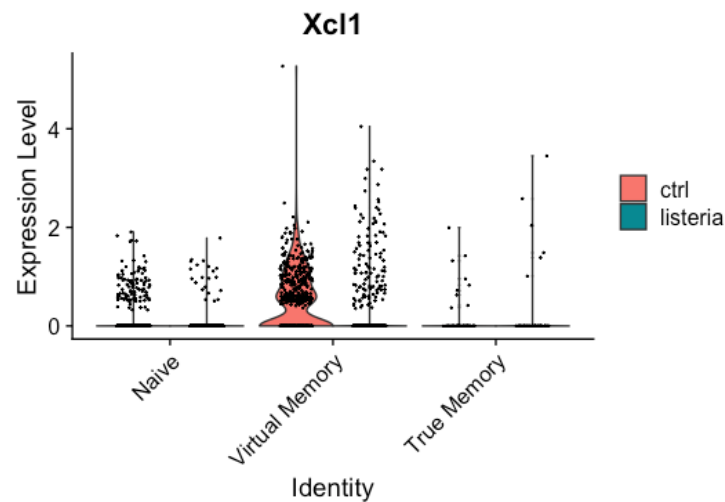


Figure 69. Single-cell sequencing data show that virtual memory T cells express high levels of *Xcl1*.

Notably, analysis of our scRNA-seq data revealed T_{VM} cells to highly express *Xcl1* (XC-chemokine ligand 1) (Figure 69), which binds to XC-chemokine receptor 1 (XCR1) expressed by conventional DCs (cDCs), which have been shown to display optimal antigen cross-presentation capacity⁷⁰². Earlier studies have furthermore revealed that CD8⁺ T cells in a virus infection model, that have completed the first priming step, which is mediated by an infected or migratory cDC1, produce XCL1 to recruit uninfected, lymph-node resident XCR1⁺ cDC1c^{703,704}, presumably to facilitate the transfer of antigens from the initial (dying) migratory cDC1 to the lymph-node resident cDC1, thus mediating antigen cross-presentation. It is intriguing to speculate that T_{VM} cells may play a similar role in the orchestration of CD8⁺ T cell immune responses to infection.

5.4. Conclusion

In summary, this chapter sought to characterize the precise CD8⁺ T cell subset that is capable of rapidly secreting IFN- γ upon activation, and further investigate the inducing factors driving IFN- γ expression in those cells. Combining *in vitro* and *in vivo* experiments, as well as flow cytometry and single-cell RNA-sequencing analysis, we demonstrated that T_{VM} cells are the main source of early IFN- γ in the context of *L. monocytogenes* infection. In addition, our data revealed that rapid IFN- γ expression by CD8⁺ T cells requires both TCR and cytokine stimulation.

6. General Discussion and Conclusion

6.1. Interferon-gamma as a critical, cell-autonomous mediator in the regulation of CD8⁺ T cell responses to infection

In this thesis, we describe a critical role of IFN- γ as a cell-autonomous modulator of CD8⁺ T cell responses against infection that regulates the expansion, differentiation, and functional avidity of the ensuing CD8⁺ T cell response. Individual CD8⁺ T cell clones exhibit a remarkable degree of functional and phenotypic plasticity, which is an indispensable prerequisite for the orchestration of effective adaptive immune responses^{50,99,705}. However, such malleability at the single-cell level necessitates regulatory mechanisms ensuring robustness at the population-wide level and we hypothesise that IFN- γ may mediate such mechanisms in a cell-autonomous fashion. It is also important to highlight that the direct function of IFN- γ on CD8⁺ T cells has to date mostly been studied using a monoclonal TCR-transgenic population, whereas our study, at least to our knowledge, presents the first investigation of the specific effect of IFN- γ R signalling on endogenous, polyclonal CD8⁺ T cells. Our findings furthermore align with the notion that this signalling event is operative during the priming phase of the response, when CD8⁺ T cells have been shown to be responsive to IFN- γ R signalling²⁰⁷.

6.1.1. The signal 3 function of interferon-gamma during CD8⁺ T cell activation

Inflammation is a critical modulator of CD8⁺ T cell activation, function, and memory formation^{28,133,378,379} and several pro-inflammatory cytokines, such as IL-12 and type I IFN, have been reported to directly promote CD8⁺ T cell responses through enhancing proximal TCR signalling²⁸ and thus heightening the antigen sensitivity of responding CD8⁺ T cells⁷⁰⁶.

IFN- γ is a major effector cytokine produced in large quantities by fully differentiated CD8⁺ T cells upon antigen encounter⁷⁰⁷. While the role of IFN- γ as an effector cytokine is well-established, its relative importance for modulating CD8⁺ T cell expansion and differentiation is controversial. While some studies demonstrated a direct, growth-promoting effect of IFN- γ for specific CD8⁺ T cell clones²¹¹, others have shown CD8⁺ T cell expansion to be independent of IFN- γ ^{378,461}, altogether suggesting that IFN- γ signalling specification in CD8⁺ T cells may critically depend upon the specific priming context, which is determined by the nature of the invading pathogen and the triggered immune response.

Following up on these previous studies, we have shown in this thesis that early IFN- γ limits the expansion of endogenous antigen-specific CD8⁺ T cells in a clone-specific manner. Using an adoptive transfer model of OT-I T cells, our group has furthermore demonstrated that IFN- γ promotes memory differentiation of high-affinity clones, whilst limiting the expansion of SLECs⁴⁸⁹. In addition, published findings by our group revealed that IFN- γ modulates OT-I cell differentiation and expansion through a non-conventional pathway facilitated by integrin co-stimulation⁴⁸⁹.

Loss of IFN- γ R signalling on endogenous CD8⁺ T cells did not affect the relative abundance of SLECs and MPECs at the peak of the response. However, analysing the tetramer binding profiles of SLEC and MPEC subsets revealed that IFN- γ appears to modulate differentiation of CD8⁺ T cells at the clonal level, by limiting selection of high-affinity clones into the SLEC compartment, while the MPEC compartment is dominated by low-affinity clones. We furthermore validated that these observed changes also led to long-term modifications in the functional avidity of the memory pool.

The functional relevance of this mechanism for re-infection or heterologous immunity remains to be further investigated. Infection with influenza virus is frequently used as a model

to study heterologous immunity. The influenza strain PR8 generates a dominant response against the viral epitope ASNENMETM (METM), but a minor fraction of cells is also able to bind the altered peptide ligand ASNENMDAM (MDAM). These cells are derived from different clones and have a lower affinity for D^bMETM than cells without cross-reactive capacity^{64,260}. Secondary infection with influenza HKx31, which expresses the METM epitope, or with HK2/68, which expressed MDAM, makes it possible to study the response of antigen-specific memory cells against a heterologous antigen. Future studies in our group will investigate if the differential clonality observed upon ablated IFN- γ R expression confers enhanced protection against heterologous infection. Alternatively, our *in vivo* cytotoxic assay could be used to assess cytolytic capacity of “*mutated*” low-affinity targets by comparison of injected CFSE-labelled splenocytes pulsed with either N4 or T4 peptides, as performed previously⁶⁴.

6.1.2. Skewing of the functional avidity of CD8⁺ T cell responses to infection through interferon-gamma

In addition to its regulatory role in limiting the expansion and differentiation of antigen-specific CD8⁺ T cells, our data furthermore revealed that IFN- γ modulates the functional avidity of the ensuing response by skewing the antigen-specific TCR repertoire towards higher average tetramer binding. Earlier studies have already shown that IFN- γ controls the immunodominance of the response against infection, with CD8⁺ memory cells specific for subdominant epitopes accumulating to unusually high numbers in IFN- γ R^{-/-} mice¹³³. However, the precise mechanism of this regulatory function of IFN- γ on CD8⁺ T cells remains unclear.

Our findings that loss of IFN- γ signalling skews towards antigen-specific cells with an average higher tetramer binding profile suggests that IFN- γ may be involved in modulating the affinity-based selection of antigen-specific T cell clones to ensure appropriate effector

and memory responses. This notion is also supported by an earlier study³⁷⁷, which suggested that IFN- γ exerts a TCR affinity-dependent modulatory effect on the differentiation of long-lived memory OT-I T cells, by blocking their generation in response to weak TCR agonists. Notably, high-affinity clones which are activated through strong TCR signals remained unaffected. The authors speculated that sufficient mTOR activation under conditions of high TCR signalling intensity may offset the regulatory function of IFN- γ ^{282,377}. IFN- γ was furthermore suggested to modulate expansion, rather than differentiation capability per se, and to help determine the poly-clonality of the memory pool by limiting differentiation of low-affinity clones into long-lived memory cells³⁷⁷. This finding is in alignment with our data showing that a loss of IFN- γ signalling during priming results in a memory pool that displays lower functional avidity. However, our findings furthermore reveal a negative effect of IFN- γ on high-affinity T cells at the endogenous level.

Of note, we have not investigated mTOR signalling activity and other metabolic pathways in our CD8⁺ IFN- γ R system, but it is intriguing to speculate that IFN- γ may exert some of its functions, at least partly, through cellular metabolic reprogramming, which is known to affect the effector and memory potential of T cells^{209,708}. Several studies identified mTOR as an important signalling module in T cells that senses environmental cues, integrates signals from multiple receptor systems^{709,710}, determines Eomes and T-bet levels, and modulates CD8⁺ T cell differentiation^{238,708}. IFN- γ R signalling has previously been shown to promote mTOR function in a PI3K- and protein kinase B/AKT-dependent fashion, at least in mouse embryonic fibroblast and human cell lines^{711–713}. It remains to be further investigated if differential regulation of mTOR activity through converging TCR and IFN- γ R signalling pathways serves as a critical mediator in leading to clone-specific signalling outcomes.

Studying adoptively transferred OT-I T cells after blocking of IFN- γ , our group has shown that IFN- γ does not modulate the ratio of T-bet to Eomes expression at the monoclonal level⁴⁸⁹. However, ablation of IFN- γ R signalling may affect a heterogenous polyclonal population of T cells differently and it is possible that TCR agonists may serve a similar role to integrins in specifying the precise outcome of IFN- γ R signalling in clones of distinct antigen affinity. Given recent findings that Eomes is critical in granting an essential survival advantage to low-affinity clones in becoming memory⁶⁴, it is intriguing to speculate that IFN- γ may modulate Eomes levels in a TCR affinity-dependent manner. This notion is supported by previous studies showing that IFN- γ promotes the rapid upregulation of T-bet in CD8⁺ T cells¹²⁰ and furthermore controls both Eomes and T-bet levels in IFN- γ ^{-/-} OT-I cells³⁷⁷.

Taken together, we hypothesise that IFN- γ may serve an important autoregulatory role, by both broadening the effector T cell pool, whilst also acting as a safeguarding mechanism against the risk of developing autoimmune reactions through the activity of low-affinity, possibly self-reactive T cell clones. We sent *CD8 α -Cre* IFN- γ R^{-/-} (KO) and *CD8 α -Cre* (WT) tetramer⁺ effector CD8⁺ T cells for single-cell TCR-sequencing, which should allow us to conclude definitively if the shift in functional avidity reflects changes in the poly-clonality of the CD8⁺ T cell response and additionally provide us with clues if these cells have been differentially programmed in the absence of early IFN- γ signalling. Preliminary analysis of our single-cell TCR-sequencing data indeed appears to confirm our hypothesis that specific effector clones are selectively expanded in KO mice compared with WT mice.

It is also important to emphasise that the precise composition of the cytokine milieu differs strongly between different types of pathogens^{120,129,160,281,706}. The relative importance of the crosstalk between TCR and IFN- γ R signalling for CD8⁺ T cell expansion and differentiation may therefore be heavily dependent on the specific experimental conditions, which may critically

contribute to defining the exact signalling outcome of IFN- γ on CD8⁺ T cells through various cell-intrinsic and cell-extrinsic factors (e.g. by integrins acting as co-stimulants⁴⁸⁹ or additional cytokines which directly modulate proximal TCR signalling⁷⁰⁶).

6.1.3. The functional relevance of early interferon-gamma signalling on CD8⁺ T cell responses

The functional relevance of this early wave of IFN- γ for the effector phase of CD8⁺ T cell responses was assessed both in the context of infection with *L. monocytogenes* and influenza virus. Using an optimised *in vivo* cytotoxicity assay to evaluate killing capacity, we did not measure a significant difference between endogenous IFN- γ R KO and WT CD8⁺ T cells. It is possible that the IFN- γ -mediated effect on the cytolytic capacity of responding CD8⁺ T cells is more subtle than our cytotoxicity assay allows us to detect. Alternatively, other inflammatory cytokines, such as IL-12, may play a more dominant role in establishing the effector functionality of CTLs and thus compensate for the lack of IFN- γ R signalling during the early priming phase³⁷⁹. It is also possible that IFN- γ R KO and WT effector cells do not differ in their cytolytic capacity, but only in their rate of activation, as was shown previously²⁵⁷. To ascertain and complement our results obtained *in vivo*, future experiments may be performed *in vitro* by co-culturing sorted tetramer⁺ CD8⁺ T cells and peptide-loaded target splenocytes at specified ratios⁷¹⁴.

6.2. Virtual memory CD8⁺ T cells as a novel regulatory CD8⁺ T cell subset

Having demonstrated that CD8⁺ T cells preferentially receive IFN- γ from other CD8⁺ T cells 16-24 hours post-infection, we next characterized the specific CD8⁺ T cell subset that is capable of secreting IFN- γ during the priming phase of the CD8⁺ T cell response. Using a combination of scRNA-seq and flow cytometry analysis, we identified virtual memory CD8⁺ T cells as the main drivers of this early wave of IFN- γ . Classically, it was thought that immunological memory is mediated through antigen-specific interactions, but it is becoming more and more apparent that mechanisms of immunological memory can be co-opted by innate or antigen-inexperienced immune cells to generate additional layers of heterogeneity in immune responses⁶¹⁵. Antigen-naïve, semi-differentiated T_{VM} cells represent a unique subset of CD8⁺ T cells with memory-like characteristics. Previous studies have reported overlapping features with conventional memory T cells, such as a poised cell cycle and differentiation state and a readiness towards effector functions, which is reflected in their transcriptional profile and epigenetic landscape^{580,615,616}. In addition, they exhibit many hallmarks of memory, such as modest expansion, marked differentiation, a steady-state survival advantage, accelerated TCR-driven responses, decreased signalling thresholds and tissue-specific localisation⁶¹⁵.

We next characterized the IFN- γ -inducing factors in more detail. As discussed in detail in the previous chapter, we discovered IFN- γ expression to be dependent upon concomitant TCR- and cytokine-stimulation. These findings contrast previous reports suggesting that T_{VM} cells act in a bystander-like fashion in response to pro-inflammatory cytokines such as IL-12 and IL-18^{580,615}. Of note, CD8⁺ T cells can exhibit different functional consequences according to the specific inflammatory context, and it was suggested that cytokine secretion and

cytolytic activity may be independently regulated processes⁷¹⁵. This functional differentiation may also be found in bystander-activated T cells, plausibly controlled by diverse factors such as pathogen load, chronicity of inflammation, location of CD8⁺ T cells, and surrounding cytokine milieu⁶⁶³. Altogether, we hypothesise that the functional properties of T_{VM} cells are heavily context-dependent, and thus it is conceivable that T_{VM} cells may exhibit bystander functionality under certain conditions. Notably, terminally differentiated, or replicative senescent memory CD8⁺ T cells were shown to display IL-15 hyper-responsiveness as compared with early differentiated memory CD8⁺ T cells^{716,717}. Considering the accumulation of memory T cells with age, particularly terminally differentiated or replicative senescent memory T cells, it is conceivable that bystander activation may be more exaggerated in older individuals as compared with younger individuals⁵⁷⁰. In this context, adult mice maintained under specific pathogen-free conditions should be considered a counterpart to human neonates rather than human adults^{615,718}.

Taken together, we hypothesise that requiring both TCR and cytokine signals may serve as an additional layer of regulation to prevent spurious IFN- γ secretion and to help the immune response balancing two competing demands: effective clearance of invading pathogens, whilst simultaneously preventing an over-reactive response causing immunopathology.

In addition to identifying T_{VM} cells as the primary source of early IFN- γ , we furthermore observed that only a small fraction of T_{VM} cells was triggered to secrete IFN- γ upon activation. Our characterization by single-cell sequencing suggests that it is indeed a particular subset of T_{VM} cells, which is activated to produce IFN- γ during priming. Of note, previous studies have shown that only a small fraction of memory phenotype CD8⁺ T cells are able to respond to IL-12/IL-18-induced IFN- γ expression⁷⁰, which was suggested to be regulated by the level of IL-12 and/or IL-18 receptors on subpopulations of CD8⁺ memory T cells. The hypothesis that T_{VM}

cells themselves represent a heterogeneous population with diverse functional properties is also supported by a recent study showing that T_{VM} cells can be sub-divided into two subsets according to CCR2 expression⁶⁵³. These subsets were furthermore shown to be heterogeneous in terms of their effector and memory potentials⁶⁵³. What drives a particular subset of T_{VM} cells to produce IFN- γ during priming remains to be further investigated.

Of note, we did not determine the precise type of stimulus required to trigger TCR stimulation in $CD8^+$ T cells. However, our data demonstrated that antigen affinity does not appear to be a determining factor. Moreover, IFN- γ secretion in our experimental model required cognate antigen stimulation, which suggests that IFN- γ production by T_{VM} cells may not be triggered by self-antigen. This finding contrasts a recent study showing that T_{VM} cells harbour self-reactive TCRs⁶³⁵. The observation that T_{VM} cells can orchestrate an extra-lymphoid primary response to execute early viral control⁶⁵³ lends additional support to the notion that the immune system harnesses unavoidable self-affinities to better fight microbial invasions^{719,720}. It is conceivable that the abundance of self-antigen in a given inflammatory context, and thus the frequency and intensity by which a T cell sees self-antigen could be a critical determining factor in driving early IFN- γ secretion by T_{VM} cells. Further studies of the precise nature of the TCR trigger for early IFN- γ secretion by T_{VM} cells are thus warranted.

6.3. CD8⁺ T cell-T cell collectivity as a critical mechanism in the autoregulation of global T cell behaviour

In the first part of this thesis, we have shown that IFN- γ plays an instrumental role in modulating the expansion and fate determination of responding CD8⁺ T cells, while furthermore shaping the functional avidity of the response. We identified an early time window as the temporal context of this signalling event and went on to identify T_{VM} cells as the primary source of this early wave of IFN- γ . Our observation that IFN- γ controls the functional avidity of the endogenous response suggests that IFN- γ may exert a TCR signal strength-dependent effect on endogenous poly-clonal CD8⁺ T cells.

Due to the readiness of T_{VM} cells to secrete IFN- γ and other effector cytokines upon activation, it was suggested that those cells may participate in both innate and adaptive immune responses during a primary immune response⁶¹⁰. We believe that our data may indicate an additional regulatory role of T_{VM} cells in tuning CD8⁺ T cell responses according to the given inflammatory context, by modulating the expansion, differentiation, and functional avidity of the responding T cell repertoire through early IFN- γ secretion. Our transcriptomic analysis also suggests that T_{VM} may be located in the same microenvironment as naïve CD8⁺ T cells, thus potentially providing them with an opportunity to readily shape the response and behaviour of naïve activating CD8⁺ T cells through rapid IFN- γ secretion.

As revealed by earlier studies^{310,311}, clustering of CD8⁺ T cells during the priming phase of the response results in heightened sensitivity towards local cytokines, such as IL-2 and IFN- γ . A similar contact-dependent mechanism was identified by our group to be instrumental in specifying the functional outcome of early IFN- γ on CD8⁺ T cells, at least when studying a monoclonal population of T cells⁴⁸⁹. If a similar mechanism acts on the endogenous level and

if T_{VM} and activating CD8⁺ T cells are directly interacting with each other will be the focus of future studies. The precise local cytokine availabilities during the priming stage may likely be critical determinants of early IFN- γ production by T_{VM} cells, in addition to the TCR-dependent responsiveness to the pro-inflammatory cytokines IL-12 and IL-18. IL-2 signalling, for instance, is locally intensified within homotypic CD8⁺ T cell clusters and provides the molecular basis behind the quorum sensing-like regulation of CD8⁺ T cell expansion³²⁸, and may also be involved in tuning local IFN- γ production, as suggested by the results of our *in vitro* assay.

A regulatory role of CD8⁺ T cells has been previously suggested^{677,721–725}. The discovery by Miller and co-workers⁶³⁵ that the differentiation programme of T_{VM} cells resembles the one regulatory T cells undergo supports the idea that T_{VM} cells may fulfil an important autoregulatory role in the immune system, in addition to their previously reported effector functions. Immune autoregulatory functions have also previously been reported for IFN- γ ⁷²⁶. The IFN- γ -secreting subset of T_{VM} cells we identified may furthermore contribute to the initial shaping of the local inflammatory cytokine milieu, thereby potentially enhancing the cytotoxic functions of both innate and adaptive cells to expedite pathogen clearance. However, this hypothesis awaits further experimental proof. Lastly, T_{VM} cells have also been reported to display increased expression of *Ifngr1*⁵⁸⁰, which suggests that they may be highly sensitive for their own IFN- γ . It is intriguing to speculate that in addition to modulating activating CD8⁺ T cell behaviour, T_{VM} cells may also regulate their own fate through IFN- γ .

6.4. Thesis Summary and Outlook

CD8⁺ T cell immune responses exhibit a remarkable degree of plasticity at the single-cell level, whilst their overall population response is consistent and robust. How heterogeneous individual T cell responses are integrated at the population-level remains incompletely understood, but previous research has highlighted that direct T cell-T cell communication during the priming phase plays a critical role in the regulation of the ensuing T cell response^{310,328}. One of the cytokines identified to be expressed by a subset of activated CD8⁺ T cells during priming has been IFN- γ ³²⁸. However, whereas its role during the effector phase of the response has been well-studied, the function of this early wave of IFN- γ on the CD8⁺ T cell response remained incompletely understood.

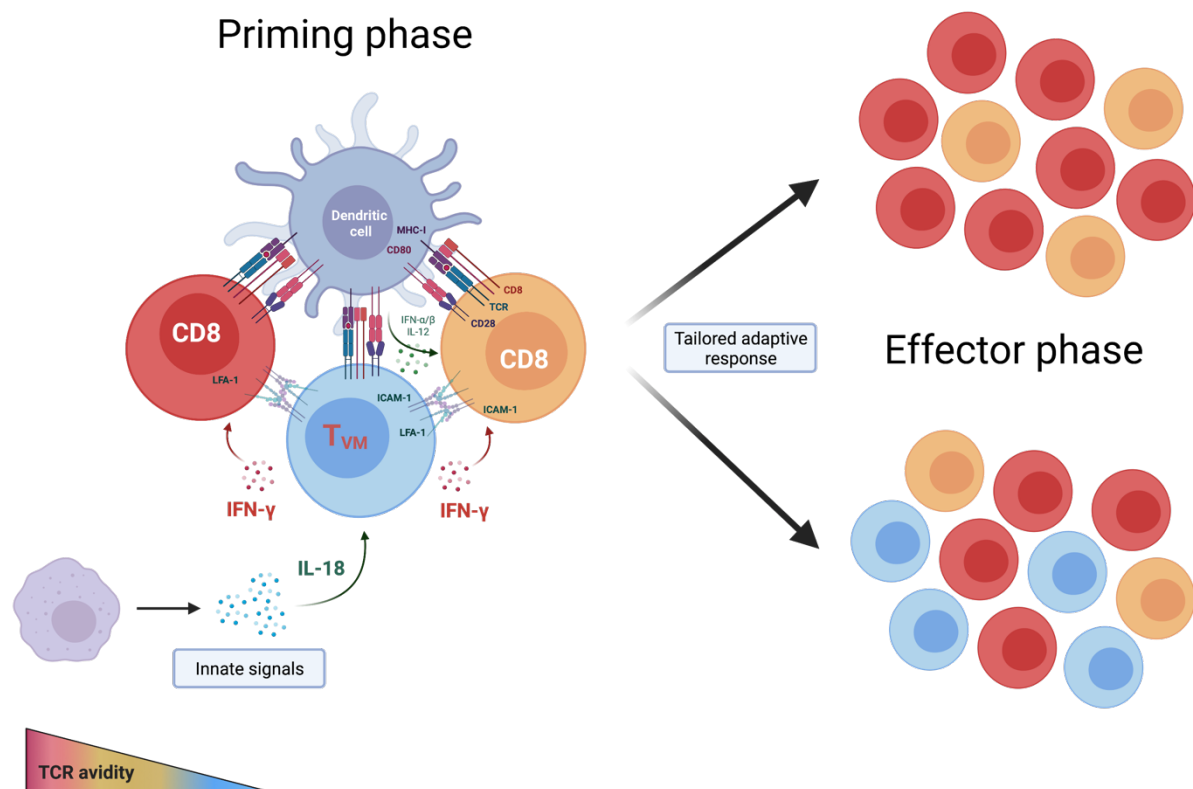


Figure 70. Graphical thesis summary. Virtual memory CD8⁺ T cells (T_{VM}) produce interferon-gamma (IFN- γ) during the priming phase of the response in a T cell receptor (TCR)- and cytokine-dependent manner. Early IFN- γ functions to modulate the expansion and differentiation of antigen-specific CD8⁺ T cells and helps to tailor the response to the specific inflammatory context [Created with [BioRender.com](https://www.biorender.com)].

In this thesis evidence is presented which reveals that IFN- γ modulates distinct aspects of the ensuing CD8⁺ T cell response. Our data revealed a cell-autonomous function of IFN- γ on the endogenous T cell response by modulating the expansion, differentiation, and functional avidity of the effector T cell population in a clone-specific manner. We furthermore demonstrated that direct IFN- γ signalling in CD8⁺ T cells is restricted to a specific temporal context and identified virtual memory T cells as the main source of this early wave of IFN- γ , which require both TCR and cytokine signalling to induce IFN- γ production. We hypothesise that T_{VM} cells fulfil a critical immunoregulatory role in modulating collective T cell behaviour in response to the specific inflammatory context. Taken together, this study has revealed a novel cell-autonomous function of IFN- γ on CD8⁺ T cells during the priming phase of the response and a novel regulatory role of virtual memory T cells in modulating CD8⁺ T cell responses to infection. Future studies remain to be carried out to further elucidate the precise mechanism of this regulatory role and its relevance for shaping the response to infection.

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8. References

1. Nikolich-Zugich, J., Slifka, M. K. & Messaoudi, I. The many important facets of T-cell repertoire diversity. *Nat. Rev. Immunol.* **4**, 123–132 (2004).
2. Cantrell, D. Signaling in lymphocyte activation. *Cold Spring Harb. Perspect. Biol.* **7**, 1–14 (2015).
3. Garcia, K. C. & Adams, E. J. How the T cell receptor sees antigen - A structural view. *Cell* **122**, 333–336 (2005).
4. Germain, R. N. T-cell development and the CD4–CD8 lineage decision. *Nat. Rev. Immunol.* **2**, 309–322 (2002).
5. Hwang, J.-R., Byeon, Y., Kim, D. & Park, S.-G. Recent insights of T cell receptor-mediated signaling pathways for T cell activation and development. *Exp. & Mol. Med.* **52**, 750–761 (2020).
6. Starr, T. K., Jameson, S. C. & Hogquist, K. A. Positive and Negative Selection of T Cells. *Annu. Rev. Immunol.* **21**, 139–176 (2003).
7. O'Shea, J. J. & Paul, W. E. Mechanisms Underlying Lineage Commitment and Plasticity of Helper CD4+ T Cells. *Science (80-.)* **327**, 1098–1102 (2010).
8. Ghaedi, M. *et al.* Common-Lymphoid-Progenitor-Independent Pathways of Innate and T Lymphocyte Development. *Cell Rep.* **15**, 471–480 (2016).
9. Kondo, M., Weissman, I. L. & Akashi, K. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell* **91**, 661–672 (1997).
10. Smith-Garvin, J. E., Koretzky, G. A. & Jordan, M. S. T cell activation. *Annual Review of Immunology* **27**, 591–619 (2009).
11. Dolton, G. *et al.* More tricks with tetramers: a practical guide to staining T cells with peptide–MHC multimers. *Immunology* **146**, 11–22 (2015).
12. Krogsaard, M. & Davis, M. M. How T cells 'see' antigen. *Nat. Immunol.* **6**, 239–245 (2005).
13. Krangel, M. S. Mechanics of T cell receptor gene rearrangement. *Current Opinion in Immunology* **21**, 133–139 (2009).
14. Rock, B. E. P., Sibbald, P., Davis, M. M. & Chien, Y. CDR3 Length in Antigen-specific Immune Receptors By Edwin P. Rock,* Peter g. Sibbald,~ Mark M. Davis,* and Yueh-hsiu Chien. *Methods* **179**, (1994).
15. Love, P. E. & Hayes, S. M. ITAM-mediated Signaling by the T-Cell Antigen Receptor. *Cold Spring Harb. Perspect. Biol.* **2**, a002485 (2010).
16. Letourneur, F. & Klausner, R. D. Activation of T Cells by a Tyrosine Kinase Activation Domain in the Cytoplasmic Tail of CD3 ϵ . *Science (80-.)* **255**, 79–82 (1992).
17. Bu, J. Y., Shaw, A. S. & Chan, A. C. Analysis of the interaction of ZAP-70 and syk protein-tyrosine kinases with the T-cell antigen receptor by plasmon resonance. *Proc. Natl. Acad. Sci. U. S. A.* **92**, 5106–5110 (1995).
18. Courtney, A. H., Lo, W.-L. & Weiss, A. TCR Signaling: Mechanisms of Initiation and Propagation. *Trends Biochem. Sci.* **43**, 108–123 (2017).
19. Greenwald, R. J., Freeman, G. J. & Sharpe, A. H. THE B7 FAMILY REVISITED. *Annu. Rev. Immunol.* **23**, 515–548 (2005).
20. Watts, T. H. TNF/TNFR FAMILY MEMBERS IN COSTIMULATION OF T CELL RESPONSES. *Annu. Rev. Immunol.* **23**, 23–68 (2005).
21. Brownlie, R. J. & Zamoyska, R. T cell receptor signalling networks: Branched, diversified and bounded. *Nat. Rev. Immunol.* **13**, 257–269 (2013).
22. Siller-Farfán, J. A. & Dushek, O. Molecular mechanisms of T cell sensitivity to antigen. *Immunol. Rev.* **285**, 194–205 (2018).
23. Richard, A. C., Frazer, G. L., Ma, C. Y. & Griffiths, G. M. Staggered starts in the race to T cell activation. *Trends Immunol.* **42**, 994–1008 (2021).
24. Mescher, M. F. *et al.* Signals required for programming effector and memory development by CD8+ T cells. *Immunol. Rev.* **211**, 81–92 (2006).
25. Aichele, P. *et al.* Cutting Edge: CD8 T Cells Specific for Lymphocytic Choriomeningitis Virus Require Type I IFN Receptor for Clonal Expansion. *J. Immunol.* **176**, 4525–4529 (2006).
26. Kolumam, G. A., Thomas, S., Thompson, L. J., Sprent, J. & Murali-Krishna, K. Type I interferons act directly on CD8 T cells to allow clonal expansion and memory formation in response to viral infection. *J. Exp. Med.* **202**, 637–650 (2005).

27. Xiao, Z., Casey, K. A., Jameson, S. C., Curtsinger, J. M. & Mescher, M. F. Programming for CD8 T Cell Memory Development Requires IL-12 or Type I IFN. *J. Immunol.* **182**, 2786–2794 (2009).
28. Curtsinger, J. M. & Mescher, M. F. Inflammatory cytokines as a third signal for T cell activation. *Curr. Opin. Immunol.* **22**, 333–340 (2010).
29. Hamilton, S. E. & Jameson, S. C. The nature of the lymphopenic environment dictates protective function of homeostatic-memory CD8+ T cells. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 18484–18489 (2008).
30. Keppler, S. J., Theil, K., Vucikuj, S. & Aichele, P. Effector T-cell differentiation during viral and bacterial infections: Role of direct IL-12 signals for cell fate decision of CD8 + T cells. *Eur. J. Immunol.* **39**, 1774–1783 (2009).
31. Cho, Y. Bin, Lee, I. G., Joo, Y. H., Hong, S. H. & Seo, Y. J. Tcr transgenic mice: a valuable tool for studying viral immunopathogenesis mechanisms. *Int. J. Mol. Sci.* **21**, 1–12 (2020).
32. Holst, J. *et al.* Generation of T-cell receptor retrogenic mice. *Nat. Protoc.* **1**, 406–417 (2006).
33. Schuldt, N. J. & Binstadt, B. A. Dual TCR T Cells: Identity Crisis or Multitaskers? *J. Immunol.* **202**, 637–644 (2019).
34. Padovan, E. *et al.* Expression of two T cell receptor α chains: Dual receptor T cells. *Science (80-.)*. **262**, 422–424 (1993).
35. Padovan, E., Casorati, G., Dellabona, P., Giachino, C. & Lanzavecchia, A. Dual receptor T-cells. Implications for alloreactivity and autoimmunity. *Ann. N. Y. Acad. Sci.* **756**, 66–70 (1995).
36. Heath, W. R. *et al.* Expression of two T cell receptor α chains on the surface of normal murine T cells. *Eur. J. Immunol.* **25**, 1617–1623 (1995).
37. Ni, P. P., Solomon, B., Hsieh, C.-S., Allen, P. M. & Morris, G. P. The Ability To Rearrange Dual TCRs Enhances Positive Selection, Leading to Increased Allo- and Autoreactive T Cell Repertoires. *J. Immunol.* **193**, 1778–1786 (2014).
38. Sewell, A. K. Why must T cells be cross-reactive? *Nat. Rev. Immunol.* **12**, 669–677 (2012).
39. Hinz, T., Weidmann, E. & Kabelitz, D. Dual TCR-Expressing T Lymphocytes in Health and Disease. *Int. Arch. Allergy Immunol.* **125**, 16–20 (2001).
40. Cusick, M. F., Libbey, J. E. & Fujinami, R. S. Molecular mimicry as a mechanism of autoimmune disease. *Clin. Rev. Allergy Immunol.* **42**, 102–111 (2012).
41. Whiteside, S. K., Snook, J. P., Williams, M. A. & Weis, J. J. Bystander T Cells: A Balancing Act of Friends and Foes. *Trends Immunol.* **39**, 1021–1035 (2018).
42. Kearney, E. R., Pape, K. A., Loh, D. Y. & Jenkins, M. K. Visualization of peptide-specific T cell immunity and peripheral tolerance induction in vivo. *Immunity* **1**, 327–339 (1994).
43. Harty, J. T. & Badovinac, V. P. Shaping and reshaping CD8+ T-cell memory. *Nat. Rev. Immunol.* **8**, 107–119 (2008).
44. Badovinac, V. P., Haring, J. S. & Harty, J. T. Initial T Cell Receptor Transgenic Cell Precursor Frequency Dictates Critical Aspects of the CD8+ T Cell Response to Infection. *Immunity* **26**, 827–841 (2007).
45. Hataye, J., Moon, J. J., Khoruts, A., Reilly, C. & Jenkins, M. K. Naïve and Memory CD4 + T Cell Survival Controlled by Clonal Abundance. *Science (80-.)*. **312**, 114–116 (2006).
46. Kemp, R. A., Powell, T. J., Dwyer, D. W. & Dutton, R. W. Cutting Edge: Regulation of CD8 + T Cell Effector Population Size. *J. Immunol.* **173**, 2923–2927 (2004).
47. Marzo, A. L. *et al.* Initial T cell frequency dictates memory CD8+ T cell lineage commitment. *Nat. Immunol.* **6**, 793–799 (2005).
48. Probst, H. C., Dumrese, T. & van den Broek, M. F. Cutting Edge: Competition for APC by CTLs of Different Specificities Is Not Functionally Important During Induction of Antiviral Responses. *J. Immunol.* **168**, 5387–5391 (2002).
49. Buchholz, V. R. & Flossdorf, M. Single-Cell Resolution of T Cell Immune Responses. *Adv. Immunol.* **137**, 1–41 (2018).
50. Buchholz, V. R., Schumacher, T. N. M. & Busch, D. H. T Cell Fate at the Single-Cell Level. *Annu. Rev. Immunol.* **34**, 1–28 (2016).
51. Hopkins, F. G. On the separation of a pure albumin from egg-white. *J. Physiol.* **25**, 306–330 (1900).
52. Tiselius, A. & Kabat, E. A. An electrophoretic study of immune sera and purified antibody preparations. *J. Exp. Med.* **69**, 119–132 (1939).
53. HEIDELBERGER, M., PEDERSEN, K. A. I. O. & TISELIUS, A. Ultracentrifugal and Electrophoretic Studies on Antibodies. *Nature* **138**, 165 (1936).
54. Jones, S. B. Equilibria in precipitin reactions. The coexistence of a single free antigen and its antibody in the same serum. *J. Exp. Med.* **25**, 837–853 (1917).
55. Hooker, S. B. & Boyd, W. C. The Existence of Antigenic Determinants of Diverse Specificity in a Single

- Protein : III . Further Notes on Crystalline. *J. Immunol.* **26 no**, 469–479 (1933).
56. Boyd, W. C. & Bernard, H. Quantitative Changes in Antibodies and Globulin Fractions in Sera of Rabbits Injected with Several Antigens. *J. Immunol.* **33**, 111–122 (1937).
 57. Hogquist, K. A. *et al.* T cell receptor antagonist peptides induce positive selection. *Cell* **76**, 17–27 (1994).
 58. Boehmer, H. Von. Deciphering Thymic Development. *Front. Immunol.* **5**, 424 (2014).
 59. Miyagawa, F., Gutermuth, J., Zhang, H. & Katz, S. I. The use of mouse models to better understand mechanisms of autoimmunity and tolerance. *J. Autoimmun.* **35**, 192–198 (2010).
 60. Wilson, E. H. *et al.* Behavior of Parasite-Specific Effector CD8+ T Cells in the Brain and Visualization of a Kinesis-Associated System of Reticular Fibers. *Immunity* **30**, 300–311 (2009).
 61. Pope, C. *et al.* Organ-Specific Regulation of the CD8 T Cell Response to *Listeria monocytogenes* Infection. *J. Immunol.* **166**, 3402–3409 (2001).
 62. Yachi, P. P., Ampudia, J., Zal, T. & Gascoigne, N. R. J. Altered Peptide Ligands Induce Delayed CD8-T Cell Receptor Interaction—a Role for CD8 in Distinguishing Antigen Quality. *Immunity* **25**, 203–211 (2006).
 63. Zehn, D., Lee, S. Y. & Bevan, M. J. Complete but curtailed T-cell response to very low-affinity antigen. *Nature* **458**, 211–214 (2009).
 64. Kavazović, I. *et al.* Eomes broadens the scope of CD8 T-cell memory by inhibiting apoptosis in cells of low affinity. *PLOS Biol.* **18**, e3000648 (2020).
 65. Murray, E. G. D., Webb, R. A. & Swann, M. B. R. A disease of rabbits characterised by a large mononuclear leucocytosis, caused by a hitherto undescribed bacillus *Bacterium monocytogenes* (n.sp.). *J. Pathol. Bacteriol.* **29**, 407–439 (1926).
 66. Hamon, M., Bierne, H. & Cossart, P. *Listeria monocytogenes*: a multifaceted model. *Nat. Rev. Microbiol.* **4**, 423–434 (2006).
 67. Condotta, S. A., Richer, M. J., Badovinac, V. P. & Harty, J. T. Chapter 5 Probing CD8 T Cell Responses with *Listeria monocytogenes* Infection. *Adv. Immunol.* **113**, 51–80 (2012).
 68. Portnoy, D. A. Innate immunity to a facultative intracellular bacterial pathogen. *Curr. Opin. Immunol.* **4**, 20–24 (1992).
 69. Mombaerts, P., Arnoldi, J., Russ, F., Tonegawa, S. & Kaufmann, S. H. E. Different roles of $\alpha\beta$ and $\gamma\delta$ T cells in immunity against an intracellular bacterial pathogen. *Nature* **365**, 53–56 (1993).
 70. Berg, R. E., Crossley, E., Murray, S. & Forman, J. Memory CD8+ T Cells Provide Innate Immune Protection against *Listeria monocytogenes* in the Absence of Cognate Antigen. *J. Exp. Med.* **198**, 1583–1593 (2003).
 71. Rogers, H. W., Tripp, C. S., Schreiber, R. D. & Unanue, E. R. Endogenous IL-1 is required for neutrophil recruitment and macrophage activation during murine listeriosis. *J. Immunol.* **153**, 2093–101 (1994).
 72. Havell, E. A. & Sehgal, P. B. Tumor necrosis factor-independent IL-6 production during murine listeriosis. *J. Immunol.* **146**, 756–61 (1991).
 73. Dalrymple, S. A. *et al.* Interleukin-6-deficient mice are highly susceptible to *Listeria monocytogenes* infection: Correlation with inefficient neutrophilia. *Infect. Immun.* **63**, 2262–2268 (1995).
 74. Ehlers, S. *et al.* The Lymphotoxin β Receptor Is Critically Involved in Controlling Infections with the Intracellular Pathogens *Mycobacterium tuberculosis* and *Listeria monocytogenes*. *J. Immunol.* **170**, 5210–5218 (2003).
 75. Rothe, J. *et al.* Mice lacking the tumour necrosis factor receptor 1 are resistant to IMF-mediated toxicity but highly susceptible to infection by *Listeria monocytogenes*. *Nature* **364**, 798–802 (1993).
 76. Tripp, C. S., Gately, M. K., Hakimi, J., Ling, P. & Unanue, E. R. Neutralization of IL-12 decreases resistance to *Listeria* in SCID and C.B-17 mice. Reversal by IFN- γ . *J. Immunol.* **152**, 1883–7 (1994).
 77. Tripp, C. S., Wolf, S. F. & Unanue, E. R. Interleukin 12 and tumor necrosis factor α are costimulators of interferon γ production by natural killer cells in severe combined immunodeficiency mice with listeriosis, and interleukin 10 is a physiologic antagonist. *Proc. Natl. Acad. Sci. U. S. A.* **90**, 3725–3729 (1993).
 78. Okamura, H. *et al.* Cloning of a new cytokine that induces IFN- γ production by T cells. *Nature* **378**, 88–91 (1995).
 79. Nakanishi, K., Yoshimoto, T., Tsutsui, H. & Okamura, H. Interleukin-18 is a unique cytokine that stimulates both Th1 and Th2 responses depending on its cytokine milieu. *Cytokine Growth Factor Rev.* **12**, 53–72 (2001).
 80. Huang, S. *et al.* Immune response in mice that lack the interferon-gamma receptor. *Science* (80-.). **259**, 1742–1745 (1993).
 81. Bancroft, G. J., Schreiber, R. D., Bosma, G. C., Bosma, M. J. & Unanue, E. R. A T cell-independent mechanism of macrophage activation by interferon-gamma. *J. Immunol.* **139**, 1104–1107 (1987).
 82. Harty, J. T. & Bevan, M. J. Specific immunity to *Listeria monocytogenes* in the absence of IFN γ . *Immunity* **3**, 109–117 (1995).

83. Dai, W. J. *et al.* Impaired macrophage listericidal and cytokine activities are responsible for the rapid death of *Listeria monocytogenes*-infected IFN-gamma receptor-deficient mice. *J. Immunol.* **158**, 5297–304 (1997).
84. Mackaness, G. B. THE IMMUNOLOGICAL BASIS OF ACQUIRED CELLULAR RESISTANCE. *J. Exp. Med.* **120**, 105–120 (1964).
85. Mackaness, G. B. CELLULAR RESISTANCE TO INFECTION. *J. Exp. Med.* **116**, 381–406 (1962).
86. Kaech, S. M. & Ahmed, R. Memory CD8+ T cell differentiation: Initial antigen encounter triggers a developmental program in naïve cells. *Nat. Immunol.* **2**, 415–422 (2001).
87. Pircher, H. *et al.* Molecular analysis of the antigen receptor of virus-specific cytotoxic T cells and identification of a new V α family. *Eur. J. Immunol.* **17**, 1843–1846 (1987).
88. Altman, J. D. *et al.* Phenotypic Analysis of Antigen-Specific T Lymphocytes. *Science (80-.)*. **274**, 94–96 (1996).
89. Newell, E. W. *et al.* Combinatorial tetramer staining and mass cytometry analysis facilitate T-cell epitope mapping and characterization. *Nat. Biotechnol.* **31**, 623–629 (2013).
90. Laugel, B. *et al.* Different T Cell Receptor Affinity Thresholds and CD8 Coreceptor Dependence Govern Cytotoxic T Lymphocyte Activation and Tetramer Binding Properties*. *J. Biol. Chem.* **282**, 23799–23810 (2007).
91. Sabatino, J. J., Huang, J., Zhu, C. & Evavold, B. D. High prevalence of low affinity peptide–MHC II tetramer–negative effectors during polyclonal CD4+ T cell responses. *J. Exp. Med.* **208**, 81–90 (2011).
92. Dolton, G. *et al.* Comparison of peptide–major histocompatibility complex tetramers and dextramers for the identification of antigen-specific T cells. *Clin. Exp. Immunol.* **177**, 47–63 (2014).
93. Steinman, R. M., Hawiger, D. & Nussenzweig, M. C. Tolerogenic Dendritic Cells. *Annu. Rev. Immunol.* **21**, 685–711 (2003).
94. Heath, W. R. & Carbone, F. R. Cross-presentation, dendritic cells, tolerance and immunity. *Annual Review of Immunology* **19**, 47–64 (2001).
95. Haring, J. S., Badovinac, V. P. & Harty, J. T. Inflaming the CD8+ T Cell Response. *Immunity* **25**, 19–29 (2006).
96. Parish, I. A. & Kaech, S. M. Diversity in CD8+ T cell differentiation. *Current Opinion in Immunology* **21**, 291–297 (2009).
97. Annunziato, F., Romagnani, C. & Romagnani, S. The 3 major types of innate and adaptive cell-mediated effector immunity. *J. Allergy Clin. Immunol.* **135**, 626–635 (2015).
98. Williams, M. A. & Bevan, M. J. Effector and Memory CTL Differentiation. *Annu. Rev. Immunol.* **25**, 171–192 (2007).
99. Kaech, S. M. & Cui, W. Transcriptional control of effector and memory CD8+ T cell differentiation. *Nat. Rev. Immunol.* **12**, 749–761 (2012).
100. Román, E. *et al.* CD4 Effector T Cell Subsets in the Response to Influenza. *J. Exp. Med.* **196**, 957–968 (2002).
101. Marshall, H. D. *et al.* Differential Expression of Ly6C and T-bet Distinguish Effector and Memory Th1 CD4+ Cell Properties during Viral Infection. *Immunity* **35**, 633–646 (2011).
102. Pepper, M., Pagán, A. J., Igyártó, B. Z., Taylor, J. J. & Jenkins, M. K. Opposing Signals from the Bcl6 Transcription Factor and the Interleukin-2 Receptor Generate T Helper 1 Central and Effector Memory Cells. *Immunity* **35**, 583–595 (2011).
103. Mayya, V. & Dustin, M. L. What Scales the T Cell Response? *Trends Immunol.* **37**, 513–522 (2016).
104. Uhl, L. F. K. & Gérard, A. Modes of Communication between T Cells and Relevance for Immune Responses. *Int. J. Mol. Sci.* **21**, 2674 (2020).
105. Jenkins, M. K. & Moon, J. J. The Role of Naive T Cell Precursor Frequency and Recruitment in Dictating Immune Response Magnitude. *J. Immunol.* **188**, 4135–4140 (2012).
106. Heijst, J. W. J. van *et al.* Recruitment of Antigen-Specific CD8+ T Cells in Response to Infection Is Markedly Efficient. *Science (80-.)*. **325**, 1265–1269 (2009).
107. Jenkins, M. K., Chu, H. H., McLachlan, J. B. & Moon, J. J. On the Composition of the Preimmune Repertoire of T Cells Specific for Peptide–Major Histocompatibility Complex Ligands. *Annu. Rev. Immunol.* **28**, 275–294 (2010).
108. Badovinac, V. P. & Harty, J. T. CD8+ T-cell homeostasis after infection: Setting the ‘curve’. *Microbes Infect.* **4**, 441–447 (2002).
109. Badovinac, V. P., Porter, B. B. & Harty, J. T. Programmed contraction of CD8+ T cells after infection. *Nat. Immunol.* **3**, 619–626 (2002).
110. Butz, E. A. & Bevan, M. J. Massive expansion of antigen-specific CD8+ T cells during an acute virus infection. *Immunity* **8**, 167–175 (1998).

111. Burnet, F. M. A modification of jerne's theory of antibody production using the concept of clonal selection. *CA. Cancer J. Clin.* **26**, 119–121 (1957).
112. Khan, S. H. & Badovinac, V. P. *Listeria monocytogenes*: a model pathogen to study antigen-specific memory CD8 T cell responses. *Semin. Immunopathol.* **37**, 301–310 (2015).
113. Mercado, R. *et al.* Early Programming of T Cell Populations Responding to Bacterial Infection. *J. Immunol.* **165**, 6833–6839 (2000).
114. Van Stipdonk, M. J. B., Lemmens, E. E. & Schoenberger, S. P. Naïve CTLs require a single brief period of antigenic stimulation for clonal expansion and differentiation. *Nat. Immunol.* **2**, 423–429 (2001).
115. Wong, P. & Pamer, E. G. Cutting Edge: Antigen-Independent CD8 T Cell Proliferation. *J. Immunol.* **166**, 5864–5868 (2001).
116. Wherry, E. J., Puorro, K. A., Porgador, A. & Eisenlohr, L. C. The induction of virus-specific CTL as a function of increasing epitope expression: responses rise steadily until excessively high levels of epitope are attained. *J. Immunol.* **163**, 3735–45 (1999).
117. Bevan, M. J. Helping the CD8+ T-cell response. *Nat. Rev. Immunol.* **4**, 595–602 (2004).
118. Sun, J. C., Williams, M. A. & Bevan, M. J. CD4+ T cells are required for the maintenance, not programming, of memory CD8+ T cells after acute infection. *Nat. Immunol.* **5**, 927–933 (2004).
119. Curtsinger, J. M., Johnson, C. M. & Mescher, M. F. CD8 T Cell Clonal Expansion and Development of Effector Function Require Prolonged Exposure to Antigen, Costimulation, and Signal 3 Cytokine. *J. Immunol.* **171**, 5165–5171 (2003).
120. Joshi, N. S. *et al.* Inflammation Directs Memory Precursor and Short-Lived Effector CD8+ T Cell Fates via the Graded Expression of T-bet Transcription Factor. *Immunity* **27**, 281–295 (2007).
121. Sarkar, S. *et al.* Functional and genomic profiling of effector CD8 T cell subsets with distinct memory fates. *J. Exp. Med.* **205**, 625–640 (2008).
122. Badovinac, V. P., Messingham, K. A. N., Jabbari, A., Haring, J. S. & Harty, J. T. Accelerated CD8+ T-cell memory and prime-boost response after dendritic-cell vaccination. *Nat. Med.* **11**, 748–756 (2005).
123. D'Souza, W. N. & Hedrick, S. M. Cutting Edge: Latecomer CD8 T Cells Are Imprinted with a Unique Differentiation Program. *J. Immunol.* **177**, 777–781 (2006).
124. Cui, W., Joshi, N. S., Jiang, A. & Kaech, S. M. Effects of Signal 3 during CD8 T cell priming: Bystander production of IL-12 enhances effector T cell expansion but promotes terminal differentiation. *Vaccine* **27**, 2177–2187 (2009).
125. Badovinac, V. P. & Harty, J. T. Manipulating the Rate of Memory CD8 + T Cell Generation after Acute Infection. *J. Immunol.* **179**, 53–63 (2007).
126. Prlic, M., Hernandez-Hoyos, G. & Bevan, M. J. Duration of the initial TCR stimulus controls the magnitude but not functionality of the CD8+ T cell response. *J. Exp. Med.* **203**, 2135–2143 (2006).
127. Badovinac, V. P., Porter, B. B. & Harty, J. T. CD8+ T cell contraction is controlled by early inflammation. *Nat. Immunol.* **5**, 809–817 (2004).
128. Pearce, E. L. & Shen, H. Generation of CD8 T Cell Memory Is Regulated by IL-12. *J. Immunol.* **179**, 2074–2081 (2007).
129. Thompson, L. J., Kolumam, G. A., Thomas, S. & Murali-Krishna, K. Innate Inflammatory Signals Induced by Various Pathogens Differentially Dictate the IFN- γ Dependence of CD8 T Cells for Clonal Expansion and Memory Formation. *J. Immunol.* **177**, 1746–1754 (2006).
130. Curtsinger, J. M. *et al.* Inflammatory cytokines provide a third signal for activation of naive CD4+ and CD8+ T cells. *J. Immunol.* **162**, 3256–62 (1999).
131. Marrack, P., Kappler, J. & Mitchell, T. Type I interferons keep activated T cells alive. *J. Exp. Med.* **189**, 521–529 (1999).
132. Buchmeier, N. A. & Schreiber, R. D. Requirement of endogenous interferon- γ production for resolution of *Listeria monocytogenes* infection. *Proc. Natl. Acad. Sci. U. S. A.* **82**, 7404–7408 (1985).
133. Badovinac, V. P., Tvinnereim, A. R. & Harty, J. T. Regulation of Antigen-Specific CD8+ T Cell Homeostasis by Perforin and Interferon- γ . *Science (80-.)*. **290**, 1354–1357 (2000).
134. Huang, S. *et al.* Immune Response in Mice that Lack the Interferon- γ Receptor. *Science (80-.)*. **259**, 1742–1745 (1993).
135. Bancroft, G. J., Sheehan, K. C., Schreiber, R. D. & Unanue, E. R. Tumor necrosis factor is involved in the T cell-independent pathway of macrophage activation in scid mice. *J. Immunol.* **143**, 127–30 (1989).
136. Fuller, M. J., Khanolkar, A., Tebo, A. E. & Zajac, A. J. Maintenance, Loss, and Resurgence of T Cell Responses During Acute, Protracted, and Chronic Viral Infections. *J. Immunol.* **172**, 4204–4214 (2004).
137. Wherry, E. J., Barber, D. L., Kaech, S. M., Blattman, J. N. & Ahmed, R. Antigen-independent memory CD8 T cells do not develop during chronic viral infection. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 16004–16009

- (2004).
138. Porter, B. B. & Harty, J. T. *Listeria monocytogenes* Infection and the CD8⁺ T-Cell Hierarchy. in *Immunodominance* 147–162 (Wiley-VCH Verlag GmbH & Co. KGaA, 2006). doi:10.1002/3527608028.ch7
 139. Prlic, M. & Bevan, M. J. Exploring regulatory mechanisms of CD8⁺ T cell contraction. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 16689–16694 (2008).
 140. Pellegrini, M., Belz, G., Bouillet, P. & Strasser, A. Shutdown of an acute T cell immune response to viral infection is mediated by the proapoptotic Bcl-2 homology 3-only protein Bim. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 14175–14180 (2003).
 141. Grayson, J. M., Weant, A. E., Holbrook, B. C. & Hildeman, D. Role of Bim in Regulating CD8⁺ T-Cell Responses during Chronic Viral Infection. *J. Virol.* **80**, 8627–8638 (2006).
 142. Wojciechowski, S. *et al.* Bim mediates apoptosis of CD127^{lo} effector T cells and limits T cell memory. *Eur. J. Immunol.* **36**, 1694–1706 (2006).
 143. Wojciechowski, S. *et al.* Bim/Bcl-2 balance is critical for maintaining naive and memory T cell homeostasis. *J. Exp. Med.* **204**, 1665–1675 (2007).
 144. Zhan, Y., Carrington, E. M., Zhang, Y., Heinzel, S. & Lew, A. M. Life and Death of Activated T Cells: How Are They Different from Naïve T Cells? *Front. Immunol.* **8**, 1809 (2017).
 145. Liu, N. *et al.* Serine protease inhibitor 2A is a protective factor for memory T cell development. *Nat. Immunol.* **5**, 919–926 (2004).
 146. Zhang, M. *et al.* Serine Protease Inhibitor 6 Protects Cytotoxic T Cells from Self-Inflicted Injury by Ensuring the Integrity of Cytotoxic Granules. *Immunity* **24**, 451–461 (2006).
 147. Ahmed, R. & Gray, D. Immunological Memory and Protective Immunity: Understanding Their Relation. *Science (80-.)*. **272**, 54–60 (1996).
 148. Wherry, E. J. *et al.* Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat. Immunol.* **4**, 225–234 (2003).
 149. Kaech, S. M. *et al.* Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. *Nat. Immunol.* **4**, 1191–1198 (2003).
 150. Joshi, N. S. & Kaech, S. M. Effector CD8 T Cell Development: A Balancing Act between Memory Cell Potential and Terminal Differentiation. *J. Immunol.* **180**, 1309–1315 (2008).
 151. Schluns, K. S., Kieper, W. C., Jameson, S. C. & Lefrançois, L. Interleukin-7 mediates the homeostasis of naïve and memory CD8 T cells in vivo. *Nat. Immunol.* **1**, 426–432 (2000).
 152. Huster, K. M. *et al.* Selective expression of IL-7 receptor on memory T cells identifies early CD40L-dependent generation of distinct CD8⁺ memory T cell subsets. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 5610–5615 (2004).
 153. Hikono, H. *et al.* Activation phenotype, rather than central– or effector–memory phenotype, predicts the recall efficacy of memory CD8⁺ T cells. *J. Exp. Med.* **204**, 1625–1636 (2007).
 154. Croom, H. A. *et al.* Memory precursor phenotype of CD8⁺ T cells reflects early antigenic experience rather than memory numbers in a model of localized acute influenza infection. *Eur. J. Immunol.* **41**, 682–693 (2011).
 155. Obar, J. J. *et al.* Pathogen-Induced Inflammatory Environment Controls Effector and Memory CD8 + T Cell Differentiation . *J. Immunol.* **187**, 4967–4978 (2011).
 156. Haring, J. S. *et al.* Constitutive Expression of IL-7 Receptor α Does Not Support Increased Expansion or Prevent Contraction of Antigen-Specific CD4 or CD8 T Cells following *Listeria monocytogenes* Infection . *J. Immunol.* **180**, 2855–2862 (2008).
 157. Hand, T. W., Morre, M. & Kaech, S. M. Expression of IL-7 receptor α is necessary but not sufficient for the formation of memory CD8 T cells during viral infection. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 11730–11735 (2007).
 158. Sun, J. C., Lehar, S. M. & Bevan, M. J. Augmented IL-7 Signaling during Viral Infection Drives Greater Expansion of Effector T Cells but Does Not Enhance Memory. *J. Immunol.* **177**, 4458–4463 (2006).
 159. Lacombe, M.-H., Hardy, M.-P., Rooney, J. & Labrecque, N. IL-7 Receptor Expression Levels Do Not Identify CD8 + Memory T Lymphocyte Precursors following Peptide Immunization . *J. Immunol.* **175**, 4400–4407 (2005).
 160. Pham, N.-L. L., Badovinac, V. P. & Harty, J. T. A Default Pathway of Memory CD8 T Cell Differentiation after Dendritic Cell Immunization Is Deflected by Encounter with Inflammatory Cytokines during Antigen-Driven Proliferation. *J. Immunol.* **183**, 2337–2348 (2009).
 161. Pham, N.-L. L. *et al.* Exploiting cross-priming to generate protective CD8 T-cell immunity rapidly. *Proc. Natl. Acad. Sci.* **107**, 12198–12203 (2010).
 162. Herndler-Brandstetter, D. *et al.* KLRG1⁺ Effector CD8⁺ T Cells Lose KLRG1, Differentiate into All Memory

- T Cell Lineages, and Convey Enhanced Protective Immunity. *Immunity* **48**, 716–729.e8 (2018).
163. Surh, C. D. & Sprent, J. Homeostasis of Naive and Memory T Cells. *Immunity* **29**, 848–862 (2008).
 164. Becker, T. C. *et al.* Interleukin 15 is required for proliferative renewal of virus-specific memory CD8 T cells. *J. Exp. Med.* **195**, 1541–1548 (2002).
 165. Goldrath, A. W. *et al.* Cytokine Requirements for Acute and Basal Homeostatic Proliferation of Naive and Memory CD8+ T Cells. *J. Exp. Med.* **195**, 1515–1522 (2002).
 166. Tan, J. T. *et al.* Interleukin (IL)-15 and IL-7 jointly regulate homeostatic proliferation of memory phenotype CD8+ cells but are not required for memory phenotype CD4+ cells. *J. Exp. Med.* **195**, 1523–1532 (2002).
 167. Klenerman, P. & Hill, A. T cells and viral persistence: lessons from diverse infections. *Nat. Immunol.* **6**, 873–879 (2005).
 168. Wherry, E. J., Blattman, J. N., Murali-Krishna, K., van der Most, R. & Ahmed, R. Viral Persistence Alters CD8 T-Cell Immunodominance and Tissue Distribution and Results in Distinct Stages of Functional Impairment. *J. Virol.* **77**, 4911–4927 (2003).
 169. Wherry, E. J. & Ahmed, R. Memory CD8 T-Cell Differentiation during Viral Infection. *J. Virol.* **78**, 5535–5545 (2004).
 170. Alfei, F. & Zehn, D. T Cell Exhaustion: An Epigenetically Imprinted Phenotypic and Functional Makeover. *Trends Mol. Med.* **23**, 769–771 (2017).
 171. Jameson, S. C. & Masopust, D. Diversity in T Cell Memory: An Embarrassment of Riches. *Immunity* **31**, 859–871 (2009).
 172. Sallusto, F., Lenig, D., Förster, R., Lipp, M. & Lanzavecchia, A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* **401**, 708–712 (1999).
 173. Masopust, D., Vezys, V., Marzo, A. L. & Lefrançois, L. Preferential localization of effector memory cells in nonlymphoid tissue. *Science* **291**, 2413–7 (2001).
 174. Gebhardt, T. *et al.* Memory T cells in nonlymphoid tissue that provide enhanced local immunity during infection with herpes simplex virus. *Nat. Immunol.* **10**, 524–530 (2009).
 175. Gebhardt, T. *et al.* Different patterns of peripheral migration by memory CD4+ and CD8+ T cells. *Nature* **477**, 216–219 (2011).
 176. Steinert, E. M. *et al.* Quantifying Memory CD8 T Cells Reveals Regionalization of Immunosurveillance. *Cell* **161**, 737–749 (2015).
 177. Andrian, U. H. von & Mempel, T. R. Homing and cellular traffic in lymph nodes. *Nat. Rev. Immunol.* **3**, 867–878 (2003).
 178. Mueller, S. N., Gebhardt, T., Carbone, F. R. & Heath, W. R. Memory T Cell Subsets, Migration Patterns, and Tissue Residence. *Annu. Rev. Immunol.* **31**, 137–161 (2013).
 179. MacKay, L. K. *et al.* The developmental pathway for CD103+ CD8+ tissue-resident memory T cells of skin. *Nat. Immunol.* **14**, 1294–1301 (2013).
 180. Masopust, D. *et al.* Dynamic T cell migration program provides resident memory within intestinal epithelium. *J. Exp. Med.* **207**, 553–564 (2010).
 181. Strydom, G. *et al.* A mucosal vaccine against Chlamydia trachomatis generates two waves of protective memory T cells. *Science (80-.)*. **348**, (2015).
 182. Jiang, X. *et al.* Skin infection generates non-migratory memory CD8+ TRM cells providing global skin immunity. *Nature* **483**, 227–231 (2012).
 183. Zhang, Y., Joe, G., Hexner, E., Zhu, J. & Emerson, S. G. Host-reactive CD8+ memory stem cells in graft-versus-host disease. *Nat. Med.* **11**, 1299–1305 (2005).
 184. Gattinoni, L. *et al.* Wnt signaling arrests effector T cell differentiation and generates CD8+ memory stem cells. *Nat. Med.* **15**, 808–813 (2009).
 185. Graef, P. *et al.* Serial Transfer of Single-Cell-Derived Immunocompetence Reveals Stemness of CD8+ Central Memory T Cells. *Immunity* **41**, 116–126 (2014).
 186. Gattinoni, L. *et al.* A human memory T cell subset with stem cell-like properties. *Nat. Med.* **17**, 1290–1297 (2011).
 187. Jandus, C., Usatorre, A. M., Viganò, S., Zhang, L. & Romero, P. The vast universe of T cell diversity: Subsets of memory cells and their differentiation. *Methods Mol. Biol.* **1514**, 1–17 (2017).
 188. Newell, E. W. & Davis, M. M. Beyond model antigens: high-dimensional methods for the analysis of antigen-specific T cells. *Nat. Biotechnol.* **32**, 149–157 (2014).
 189. Hickman, H. D., Bennink, J. R. & Yewdell, J. W. Caught in the Act: Intravital Multiphoton Microscopy of Host-Pathogen Interactions. *Cell Host Microbe* **5**, 13–21 (2009).
 190. Mueller, S. N. & Hickman, H. D. In vivo imaging of the T cell response to infection. *Current Opinion in Immunology* **22**, 293–298 (2010).

191. Germain, R. N., Miller, M. J., Dustin, M. L. & Nussenzweig, M. C. Dynamic imaging of the immune system: progress, pitfalls and promise. *Nat. Rev. Immunol.* **6**, 497–507 (2006).
192. Denk, W., Strickler, J. H. & Webb, W. W. Two-photon laser scanning fluorescence microscopy. *Science (80-.)*. **248**, 73–76 (1990).
193. Gerlach, C. *et al.* One naive T cell, multiple fates in CD8+ T cell differentiation. *J. Exp. Med.* **207**, 1235–1246 (2010).
194. Schumacher, T. N. M., Gerlach, C. & Van Heijst, J. W. J. Mapping the life histories of T cells. *Nat. Rev. Immunol.* **10**, 621–631 (2010).
195. Stemberger, C. *et al.* A Single Naive CD8+ T Cell Precursor Can Develop into Diverse Effector and Memory Subsets. *Immunity* **27**, 985–997 (2007).
196. Buchholz, V. R. *et al.* Disparate individual fates compose robust CD8+ T cell immunity. *Science (80-.)*. **340**, 630–635 (2013).
197. Tubo, N. J. *et al.* Single Naive CD4+ T Cells from a Diverse Repertoire Produce Different Effector Cell Types during Infection. *Cell* **153**, 785–796 (2013).
198. Plumlee, C. R., Sheridan, B. S., Cicek, B. B. & Lefrançois, L. Environmental cues dictate the fate of individual CD8+ T cells responding to infection. *Immunity* **39**, 347–356 (2013).
199. Gerlach, C. *et al.* Heterogeneous differentiation patterns of individual CD8+ T cells. *Science (80-.)*. **340**, 635–639 (2013).
200. Mousavi, S. F. *et al.* OX40 Costimulatory Signals Potentiate the Memory Commitment of Effector CD8 + T Cells . *J. Immunol.* **181**, 5990–6001 (2008).
201. Hendriks, J. *et al.* CD27 is required for generation and long-term maintenance of T cell immunity. *Nat. Immunol.* **1**, 433–440 (2000).
202. Pulle, G., Vidric, M. & Watts, T. H. IL-15-Dependent Induction of 4-1BB Promotes Antigen-Independent CD8 Memory T Cell Survival. *J. Immunol.* **176**, 2739–2748 (2006).
203. Salek-Ardakani, S. *et al.* The TNFR family members OX40 and CD27 link viral virulence to protective T cell vaccines in mice. *J. Clin. Invest.* **121**, 296–307 (2011).
204. Chen, L. & Flies, D. B. Molecular mechanisms of T cell co-stimulation and co-inhibition. *Nat. Rev. Immunol.* **13**, 227–242 (2013).
205. Hendriks, J., Xiao, Y. & Borst, J. CD27 Promotes Survival of Activated T Cells and Complements CD28 in Generation and Establishment of the Effector T Cell Pool. *J. Exp. Med.* **198**, 1369–1380 (2003).
206. Hendriks, J. *et al.* During Viral Infection of the Respiratory Tract, CD27, 4-1BB, and OX40 Collectively Determine Formation of CD8 + Memory T Cells and Their Capacity for Secondary Expansion . *J. Immunol.* **175**, 1665–1676 (2005).
207. Haring, J. S., Corbin, G. A. & Harty, J. T. Dynamic Regulation of IFN- γ Signaling in Antigen-Specific CD8+ T Cells Responding to Infection. *J. Immunol.* **174**, 6791–6802 (2005).
208. Kalia, V. *et al.* Prolonged Interleukin-2R α Expression on Virus-Specific CD8+ T Cells Favors Terminal-Effector Differentiation In Vivo. *Immunity* **32**, 91–103 (2010).
209. Pipkin, M. E. *et al.* Interleukin-2 and Inflammation Induce Distinct Transcriptional Programs that Promote the Differentiation of Effector Cytolytic T Cells. *Immunity* **32**, 79–90 (2010).
210. Whitmire, J. K., Eam, B., Benning, N. & Whitton, J. L. Direct Interferon- γ Signaling Dramatically Enhances CD4+ and CD8+ T Cell Memory. *J. Immunol.* **179**, 1190–1197 (2007).
211. Whitmire, J. K., Tan, J. T. & Whitton, J. L. Interferon- γ acts directly on CD8+ T cells to increase their abundance during virus infection. *J. Exp. Med.* **201**, 1053–1059 (2005).
212. Bonilla, W. V *et al.* The Alarmin Interleukin-33 Drives Protective Antiviral CD8+ T Cell Responses. *Science (80-.)*. **335**, 984–989 (2012).
213. Corse, E., Gottschalk, R. A. & Allison, J. P. Strength of TCR–Peptide/MHC Interactions and In Vivo T Cell Responses. *J. Immunol.* **186**, 5039–5045 (2011).
214. Valitutti, S., Miller, S., Cella, M., Padovan, E. & Lanzavecchia, A. Serial triggering of many T-cell receptors by a few peptide-MHC complexes. *Nature* **375**, 148–151 (1995).
215. Viola, A., Schroeder, S., Sakakibara, Y. & Lanzavecchia, A. T lymphocyte costimulation mediated by reorganization of membrane microdomains. *Science (80-.)*. **283**, 680–682 (1999).
216. Corbin, G. A. & Harty, J. T. Duration of Infection and Antigen Display Have Minimal Influence on the Kinetics of the CD4+ T Cell Response to *Listeria monocytogenes* Infection. *J. Immunol.* **173**, 5679–5687 (2004).
217. Starbeck-Miller, G. R., Xue, H.-H. & Harty, J. T. IL-12 and type I interferon prolong the division of activated CD8 T cells by maintaining high-affinity IL-2 signaling in vivo. *J. Exp. Med.* **211**, 105–120 (2014).
218. Restifo, N. P. & Gattinoni, L. Lineage relationship of effector and memory T cells. *Curr. Opin. Immunol.* **25**,

- 556–563 (2013).
219. Kaech, S. M., Hemby, S., Kersh, E. & Ahmed, R. Molecular and functional profiling of memory CD8 T cell differentiation. *Cell* **111**, 837–851 (2002).
 220. Nayar, R. *et al.* Graded Levels of IRF4 Regulate CD8 + T Cell Differentiation and Expansion, but Not Attrition, in Response to Acute Virus Infection. *J. Immunol.* **192**, 5881–5893 (2014).
 221. Au-Yeung, B. B. *et al.* IL-2 Modulates the TCR Signaling Threshold for CD8 but Not CD4 T Cell Proliferation on a Single-Cell Level. *J. Immunol.* **198**, 2445–2456 (2017).
 222. Preston, G. C. *et al.* Single cell tuning of Myc expression by antigen receptor signal strength and interleukin-2 in T lymphocytes. *EMBO J.* **34**, 2008–2024 (2015).
 223. Altan-Bonnet, G. & Germain, R. N. Modeling T cell antigen discrimination based on feedback control of digital ERK responses. *PLoS Biol.* **3**, 1925–1938 (2005).
 224. Balyan, R. *et al.* Modulation of Naive CD8 T Cell Response Features by Ligand Density, Affinity, and Continued Signaling via Internalized TCRs. *J. Immunol.* **198**, 1823–1837 (2017).
 225. Navarro, M. N., Feijoo-Carnero, C., Arandilla, A. G., Trost, M. & Cantrell, D. A. Protein kinase D2 is a digital amplifier of T cell receptor-stimulated diacylglycerol signaling in naïve CD8+ T cells. *Sci. Signal.* **7**, ra99 (2014).
 226. Lanzavecchia, A. & Sallusto, F. Progressive differentiation and selection of the fittest in the immune response. *Nat. Rev. Immunol.* **2**, 982–987 (2002).
 227. Sallusto, F., Geginat, J. & Lanzavecchia, A. Central Memory and Effector Memory T Cell Subsets: Function, Generation, and Maintenance. *Annu. Rev. Immunol.* **22**, 745–763 (2004).
 228. Gett, A. V., Sallusto, F., Lanzavecchia, A. & Geginat, J. T cell fitness determined by signal strength. *Nat. Immunol.* **4**, 355–360 (2003).
 229. Chang, J. T. *et al.* Asymmetric T lymphocyte division in the initiation of adaptive immune responses. *Science (80-.)*. **315**, 1687–1691 (2007).
 230. Chang, J. T. *et al.* Asymmetric Proteasome Segregation as a Mechanism for Unequal Partitioning of the Transcription Factor T-bet during T Lymphocyte Division. *Immunity* **34**, 492–504 (2011).
 231. Ciocca, M. L., Barnett, B. E., Burkhardt, J. K., Chang, J. T. & Reiner, S. L. Cutting Edge: Asymmetric Memory T Cell Division in Response to Rechallenge. *J. Immunol.* **188**, 4145–4148 (2012).
 232. King, C. G. *et al.* T Cell Affinity Regulates Asymmetric Division, Effector Cell Differentiation, and Tissue Pathology. *Immunity* **37**, 709–720 (2012).
 233. Flossdorf, M., Rössler, J., Buchholz, V. R., Busch, D. H. & Höfer, T. CD8+ T cell diversification by asymmetric cell division. *Nat. Immunol.* **16**, 891–893 (2015).
 234. Intlekofer, A. M. *et al.* Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin. *Nat. Immunol.* **6**, 1236–1244 (2005).
 235. Banerjee, A. *et al.* Cutting Edge: The Transcription Factor Eomesodermin Enables CD8+ T Cells To Compete for the Memory Cell Niche. *J. Immunol.* **185**, 4988–4992 (2010).
 236. Intlekofer, A. M. *et al.* Requirement for T-bet in the aberrant differentiation of unhelped memory CD8+ T cells. *J. Exp. Med.* **204**, 2015–2021 (2007).
 237. Intlekofer, A. M. *et al.* Anomalous type 17 response to viral infection by CD8+ T cells lacking T-bet and eomesodermin. *Science (80-.)*. **321**, 408–411 (2008).
 238. Rao, R. R., Li, Q., Odunsi, K. & Shrikant, P. A. The mTOR Kinase Determines Effector versus Memory CD8+ T Cell Fate by Regulating the Expression of Transcription Factors T-bet and Eomesodermin. *Immunity* **32**, 67–78 (2010).
 239. Takemoto, N., Intlekofer, A. M., Northrup, J. T., Wherry, E. J. & Reiner, S. L. Cutting Edge: IL-12 Inversely Regulates T-bet and Eomesodermin Expression during Pathogen-Induced CD8 + T Cell Differentiation. *J. Immunol.* **177**, 7515–7519 (2006).
 240. Pearce, E. L. *et al.* Control of Effector CD8+ T Cell Function by the Transcription Factor Eomesodermin. *Science (80-.)*. **302**, 1041–1043 (2003).
 241. Cruz-Guilloty, F. *et al.* Runx3 and T-box proteins cooperate to establish the transcriptional program of effector CTLs. *J. Exp. Med.* **206**, 51–59 (2009).
 242. Nayar, R. *et al.* TCR signaling via Tec kinase ITK and interferon regulatory factor 4 (IRF4) regulates CD8+ T-cell differentiation. *Proc. Natl. Acad. Sci. U. S. A.* **109**, (2012).
 243. Man, K. *et al.* The transcription factor IRF4 is essential for TCR affinity-mediated metabolic programming and clonal expansion of T cells. *Nat. Immunol.* **14**, 1155–1165 (2013).
 244. Knudson, K. M., Goplen, N. P., Cunningham, C. A., Daniels, M. A. & Teixeira, E. Low-Affinity T cells are programmed to maintain normal primary responses but are impaired in their recall to low-affinity ligands. *Cell Rep.* **4**, 554–565 (2013).

245. Rao, R. R., Li, Q., Bupp, M. R. G. & Shrikant, P. A. Transcription Factor Foxo1 Represses T-bet-Mediated Effector Functions and Promotes Memory CD8⁺ T Cell Differentiation. *Immunity* **36**, 374–387 (2012).
246. Joshi, N. S. *et al.* Increased Numbers of Preexisting Memory CD8 T Cells and Decreased T-bet Expression Can Restrain Terminal Differentiation of Secondary Effector and Memory CD8 T Cells. *J. Immunol.* **187**, 4068–4076 (2011).
247. Zhou, X. *et al.* Differentiation and Persistence of Memory CD8⁺ T Cells Depend on T Cell Factor 1. *Immunity* **33**, 229–240 (2010).
248. Wiesel, M. & Oxenius, A. From crucial to negligible: Functional CD8⁺ T-cell responses and their dependence on CD4⁺ T-cell help. *Eur. J. Immunol.* **42**, 1080–1088 (2012).
249. Busch, D. H. & Pamer, E. G. T cell affinity maturation by selective expansion during infection. *J. Exp. Med.* **189**, 701–709 (1999).
250. Masopust, D., Kaech, S. M., Wherry, E. J. & Ahmed, R. The role of programming in memory T-cell development. *Curr. Opin. Immunol.* **16**, 217–225 (2004).
251. Hamilton, S. E., Walkers, M. C., Schoenberger, S. P. & Jameson, S. C. The generation of protective memory-like CD8⁺ T cells during homeostatic proliferation requires CD4⁺ T cells. *Nat. Immunol.* **7**, 475–481 (2006).
252. Goldrath, A. W. & Bevan, M. J. Low-affinity ligands for the TCR drive proliferation of mature CD8⁺ T cells in lymphopenic hosts. *Immunity* **11**, 183–190 (1999).
253. Hommel, M. & Hodgkin, P. D. TCR Affinity Promotes CD8⁺ T Cell Expansion by Regulating Survival. *J. Immunol.* **179**, 2250–2260 (2007).
254. Wensveen, F. M., van Gisbergen, K. P. J. M. & Eldering, E. The fourth dimension in immunological space: How the struggle for nutrients selects high-affinity lymphocytes. *Immunol. Rev.* **249**, 84–103 (2012).
255. Wensveen, F. M. *et al.* Apoptosis Threshold Set by Noxa and Mcl-1 after T Cell Activation Regulates Competitive Selection of High-Affinity Clones. *Immunity* **32**, 754–765 (2010).
256. Feinerman, O., Veiga, J., Dorfman, J. R., Germain, R. N. & Altan-Bonnet, G. Variability and Robustness in T Cell Activation from Regulated Heterogeneity in Protein Levels. *Science (80-.)*. **321**, 1081–1084 (2008).
257. Richard, A. C. *et al.* T cell cytolytic capacity is independent of initial stimulation strength. *Nat. Immunol.* **19**, 849–858 (2018).
258. Marchingo, J. M. *et al.* Antigen affinity, costimulation, and cytokine inputs sum linearly to amplify T cell expansion. *Science (80-.)*. **346**, 1123–1127 (2014).
259. Marchingo, J. M. *et al.* T-cell stimuli independently sum to regulate an inherited clonal division fate. *Nat. Commun.* **7**, 13540 (2016).
260. van Gisbergen, K. P. J. M. *et al.* The costimulatory molecule CD27 maintains clonally diverse CD8⁺ T cell responses of low antigen affinity to protect against viral variants. *Immunity* **35**, 97–108 (2011).
261. Abu-Shah, E. *et al.* Human CD8⁺ T Cells Exhibit a Shared Antigen Threshold for Different Effector Responses. *J. Immunol. Author Choice* **205**, 1503–1512 (2020).
262. Gmünder, H. & Lesslauer, W. A 45-kDa human T-cell membrane glycoprotein functions in the regulation of cell proliferative responses. *Eur. J. Biochem.* **142**, 153–60 (1984).
263. Acuto, O. & Michel, F. CD28-mediated co-stimulation: a quantitative support for TCR signalling. *Nat. Rev. Immunol.* **3**, 939–951 (2003).
264. Kündig, T. M. *et al.* Duration of TCR stimulation determines costimulatory requirement of T cells. *Immunity* **5**, 41–52 (1996).
265. Peperzak, V., Xiao, Y., Veraar, E. A. M. & Borst, J. CD27 sustains survival of CTLs in virus-infected nonlymphoid tissue in mice by inducing autocrine IL-2 production. *J. Clin. Invest.* **120**, 168–178 (2010).
266. Voisinne, G. *et al.* T Cells Integrate Local and Global Cues to Discriminate between Structurally Similar Antigens. *Cell Rep.* **11**, 1208–1219 (2015).
267. Tkach, K. E. *et al.* T cells translate individual, quantal activation into collective, analog cytokine responses via time-integrated feedbacks. *Elife* **3**, e01944 (2014).
268. Ross, S. H. & Cantrell, D. A. Signaling and Function of Interleukin-2 in T Lymphocytes. *Annu. Rev. Immunol.* **36**, 411–433 (2018).
269. Wensveen, F. M. *et al.* CD70-Driven Costimulation Induces Survival or Fas-Mediated Apoptosis of T Cells Depending on Antigenic Load. *J. Immunol.* **188**, 4256–4267 (2012).
270. Penalzoza-MacMaster, P. *et al.* Opposing Effects of CD70 Costimulation during Acute and Chronic Lymphocytic Choriomeningitis Virus Infection of Mice. *J. Virol.* **85**, 6168–6174 (2011).
271. Matter, M., Odermatt, B., Yagita, H., Nuoffer, J. M. & Ochsenbein, A. F. Elimination of chronic viral infection by blocking CD27 signaling. *J. Exp. Med.* **203**, 2145–2155 (2006).
272. Tan, T. C. J. *et al.* Suboptimal T-cell receptor signaling compromises protein translation, ribosome

- biogenesis, and proliferation of mouse CD8 T cells. *Proc. Natl. Acad. Sci.* **114**, E6117–E6126 (2017).
273. Heinzel, S. *et al.* A Myc-dependent division timer complements a cell-death timer to regulate T cell and B cell responses. *Nat. Immunol.* **18**, 96–103 (2017).
 274. Casrouge, A. *et al.* Size Estimate of the $\alpha\beta$ TCR Repertoire of Naive Mouse Splenocytes. *J. Immunol.* **164**, 5782–5787 (2000).
 275. Bousso, P. *et al.* Individual variations in the murine T cell response to a specific peptide reflect variability in naive repertoires. *Immunity* **9**, 169–178 (1998).
 276. Blattman, J. N. *et al.* Estimating the precursor frequency of naive antigen-specific CD8 T cells. *J. Exp. Med.* **195**, 657–664 (2002).
 277. Arstila, T. P. *et al.* A direct estimate of the human $\alpha\beta$ T cell receptor diversity. *Science (80-.)*. **286**, 958–961 (1999).
 278. Wensveen, F. M. *et al.* Pro-Apoptotic Protein Noxa Regulates Memory T Cell Population Size and Protects against Lethal Immunopathology. *J. Immunol.* **190**, 1180–1191 (2013).
 279. Shih, T. A. Y., Meffre, E., Roederer, M. & Nussenzweig, M. C. Role of BCR affinity in T cell-dependent antibody responses in vivo. *Nat. Immunol.* **3**, 399–406 (2002).
 280. Shih, T. A. Y., Roederer, M. & Nussenzweig, M. C. Role of antigen receptor affinity in T cell-independent antibody responses in vivo. *Nat. Immunol.* **3**, 399–406 (2002).
 281. Zehn, D., Roepke, S., Weakly, K., Bevan, M. J. & Prlic, M. Inflammation and TCR Signal Strength Determine the Breadth of the T Cell Response in a Bim-Dependent Manner. *J. Immunol.* **192**, 200–205 (2014).
 282. Yao, S. *et al.* Interferon regulatory factor 4 sustains CD8+ T cell expansion and effector differentiation. *Immunity* **39**, 833–845 (2013).
 283. Brehm, M. A. *et al.* T cell immunodominance and maintenance of memory regulated by unexpectedly cross-reactive pathogens. *Nat. Immunol.* **3**, 627–634 (2002).
 284. Koup, R. A. Virus escape from CTL recognition. *J. Exp. Med.* **180**, 779–782 (1994).
 285. Pantaleo, G. *et al.* The qualitative nature of the primary immune response to HIV infection is a prognosticator of disease progression independent of the initial level of plasma viremia. *Proc. Natl. Acad. Sci. U. S. A.* **94**, 254–258 (1997).
 286. Kavazović, I., Polić, B. & Wensveen, F. M. Cheating the Hunger Games; Mechanisms Controlling Clonal Diversity of CD8 Effector and Memory Populations. *Front. Immunol.* **9**, 2831 (2018).
 287. Carrio, R., Rolle, C. E. & Malek, T. R. Non-redundant role for IL-7R signaling for the survival of CD8+ memory T cells. *Eur. J. Immunol.* **37**, 3078–3088 (2007).
 288. Day, E. K. *et al.* Rapid CD8 + T Cell Repertoire Focusing and Selection of High-Affinity Clones into Memory Following Primary Infection with a Persistent Human Virus: Human Cytomegalovirus . *J. Immunol.* **179**, 3203–3213 (2007).
 289. Pizzolla, A. *et al.* Influenza-specific lung-resident memory t cells are proliferative and polyfunctional and maintain diverse TCR profiles. *J. Clin. Invest.* **128**, 721–733 (2018).
 290. Klarenbeek, P. L. *et al.* Deep Sequencing of Antiviral T-Cell Responses to HCMV and EBV in Humans Reveals a Stable Repertoire That Is Maintained for Many Years. *PLoS Pathog.* **8**, (2012).
 291. Kedzierska, K., Turner, S. J. & Doherty, P. C. Conserved T cell receptor usage in primary and recall responses to an immunodominant influenza virus nucleoprotein epitope. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 4942–4947 (2004).
 292. Iancu, E. M. *et al.* Clonotype Selection and Composition of Human CD8 T Cells Specific for Persistent Herpes Viruses Varies with Differentiation but Is Stable Over Time. *J. Immunol.* **183**, 319–331 (2009).
 293. Beuneu, H. *et al.* Visualizing the Functional Diversification of CD8+ T Cell Responses in Lymph Nodes. *Immunity* **33**, 412–423 (2010).
 294. Ozga, A. J. *et al.* pMHC affinity controls duration of CD8+ T cell–DC interactions and imprints timing of effector differentiation versus expansion. *J. Exp. Med.* jem.20160206 (2016). doi:10.1084/jem.20160206
 295. Ochel, A. *et al.* Effective intrahepatic CD8+ T-cell immune responses are induced by low but not high numbers of antigen-expressing hepatocytes. *Cell. Mol. Immunol.* **13**, 805–815 (2016).
 296. Utzschneider, D. T. *et al.* High antigen levels induce an exhausted phenotype in a chronic infection without impairing T cell expansion and survival. *J. Exp. Med.* **213**, 1819–1834 (2016).
 297. Krummey, S. M. *et al.* Low-Affinity Memory CD8+ T Cells Mediate Robust Heterologous Immunity. *J. Immunol.* **196**, 2838–2846 (2016).
 298. Kedzierska, K., La Gruta, N. L., Davenport, M. P., Turner, S. J. & Doherty, P. C. Contribution of T cell receptor affinity to overall avidity for virus-specific CD8+ T cell responses. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 11432–11437 (2005).
 299. Gras, S. *et al.* Cross-reactive CD8+ T-cell immunity between the pandemic H1N1-2009 and H1N1-1918

- influenza A viruses. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 12599–12604 (2010).
300. Cukalac, T. *et al.* Reproducible selection of high avidity CD8+ T-cell clones following secondary acute virus infection. *Proc. Natl. Acad. Sci. U. S. A.* **111**, 1485–1490 (2014).
 301. Doğaner, B. A., Yan, L. K. Q. & Youk, H. Autocrine Signaling and Quorum Sensing: Extreme Ends of a Common Spectrum. *Trends Cell Biol.* **26**, 262–271 (2016).
 302. Ågren, J. A., Davies, N. G. & Foster, K. R. Enforcement is central to the evolution of cooperation. *Nat. Ecol. Evol.* **3**, 1018–1029 (2019).
 303. Amado, I. F. *et al.* IL-2 coordinates IL-2–producing and regulatory T cell interplay. *J. Exp. Med.* **210**, 2707–2720 (2013).
 304. Foster, K. R. The sociobiology of molecular systems. *Nat. Rev. Genet.* **12**, 193–203 (2011).
 305. Hu, J. K., Kagari, T., Clingan, J. M. & Matloubian, M. Expression of chemokine receptor CXCR3 on T cells affects the balance between effector and memory CD8 T-cell generation. *Proc. Natl. Acad. Sci.* **108**, E118–E127 (2011).
 306. Kurachi, M. *et al.* Chemokine receptor CXCR3 facilitates CD8+ T cell differentiation into short-lived effector cells leading to memory degeneration. *J. Exp. Med.* **208**, 1605–1620 (2011).
 307. Kohlmeier, J. E. *et al.* Inflammatory chemokine receptors regulate CD8+ T cell contraction and memory generation following infection. *J. Exp. Med.* **208**, 1621–1634 (2011).
 308. Gérard, A., Beemiller, P., Friedman, R. S., Jacobelli, J. & Krummel, M. F. Evolving immune circuits are generated by flexible, motile, and sequential immunological synapses. *Immunol. Rev.* **251**, 80–96 (2013).
 309. Borst, J., Ahrends, T., Bąbała, N., Melief, C. J. M. & Kastenmüller, W. CD4+ T cell help in cancer immunology and immunotherapy. *Nat. Rev. Immunol.* **18**, 635–647 (2018).
 310. Gérard, A. *et al.* Secondary T cell–T cell synaptic interactions drive the differentiation of protective CD8+ T cells. *Nat. Immunol.* **14**, 356–363 (2013).
 311. Sabatos, C. A. *et al.* A Synaptic Basis for Paracrine Interleukin-2 Signaling during Homotypic T Cell Interaction. *Immunity* **29**, 238–248 (2008).
 312. Zumwalde, N. A., Domae, E., Mescher, M. F. & Shimizu, Y. ICAM-1–Dependent Homotypic Aggregates Regulate CD8 T Cell Effector Function and Differentiation during T Cell Activation. *J. Immunol.* **191**, 3681–3693 (2013).
 313. Wakil, A. E., Wang, Z.-E., Ryan, J. C., Fowell, D. J. & Locksley, R. M. Interferon γ Derived from CD4+ T Cells Is Sufficient to Mediate T Helper Cell Type 1 Development. *J. Exp. Med.* **188**, 1651–1656 (1998).
 314. Antonielli, L., Blandizzi, C., Pacher, P., Williams, M. & Haskó, G. Quorum sensing in the immune system. *Nat. Rev. Immunol.* **18**, 537–538 (2018).
 315. Rutherford, S. T. & Bassler, B. L. Bacterial Quorum Sensing: Its Role in Virulence and Possibilities for Its Control. *Cold Spring Harb. Perspect. Med.* **2**, a012427 (2012).
 316. Postat, J. & Bousso, P. Quorum Sensing by Monocyte-Derived Populations. *Front. Immunol.* **10**, 2140 (2019).
 317. Antonielli, L., Blandizzi, C., Pacher, P., Williams, M. & Haskó, G. Rethinking Communication in the Immune System: The Quorum Sensing Concept. *Trends Immunol.* **40**, 88–97 (2019).
 318. Almeida, A. R. M. *et al.* Quorum-Sensing in CD4+ T Cell Homeostasis: A Hypothesis and a Model. *Front. Immunol.* **3**, 125 (2012).
 319. Morgan, D. A., Ruscetti, F. W. & Gallo, R. Selective in Vitro Growth of T Lymphocytes from Normal Human Bone Marrows. *Science (80-.)*. **193**, 1007–1008 (1976).
 320. Smith, K. A. Interleukin-2: Inception, Impact, and Implications. *Science (80-.)*. **240**, 1169–1176 (1988).
 321. Malek, T. R. The Biology of Interleukin-2. *Annu. Rev. Immunol.* **26**, 453–479 (2008).
 322. Malek, T. R. & Castro, I. Interleukin-2 Receptor Signaling: At the Interface between Tolerance and Immunity. *Immunity* **33**, 153–165 (2010).
 323. Schimpl, A. *et al.* IL-2 and autoimmune disease. *Cytokine Growth Factor Rev.* **13**, 369–378 (2002).
 324. Dooms, H., Wolslegel, K., Lin, P. & Abbas, A. K. Interleukin-2 enhances CD4+ T cell memory by promoting the generation of IL-7R α –expressing cells. *J. Exp. Med.* **204**, 547–557 (2007).
 325. Boyman, O. & Sprent, J. The role of interleukin-2 during homeostasis and activation of the immune system. *Nat. Rev. Immunol.* **12**, 180–190 (2012).
 326. Feinerman, O. *et al.* Single-cell quantification of IL-2 response by effector and regulatory T cells reveals critical plasticity in immune response. *Mol. Syst. Biol.* **6**, 437 (2010).
 327. Savir, Y., Waysbort, N., Antebi, Y. E., Tlusty, T. & Friedman, N. Balancing speed and accuracy of polyclonal T cell activation: a role for extracellular feedback. *BMC Syst. Biol.* **6**, 111 (2012).
 328. Zenke, S. *et al.* Quorum Regulation via Nested Antagonistic Feedback Circuits Mediated by the Receptors CD28 and CTLA-4 Confers Robustness to T Cell Population Dynamics. *Immunity* **52**, 313–327.e7 (2020).

329. Feau, S., Arens, R., Togher, S. & Schoenberger, S. P. Autocrine IL-2 is required for secondary population expansion of CD8⁺ memory T cells. *Nat. Immunol.* **12**, 908–913 (2011).
330. Hart, Y. *et al.* Paradoxical Signaling by a Secreted Molecule Leads to Homeostasis of Cell Levels. *Cell* **158**, 1022–1032 (2014).
331. Waysbort, N., Russ, D., Chain, B. M. & Friedman, N. Coupled IL-2–Dependent Extracellular Feedbacks Govern Two Distinct Consecutive Phases of CD4 T Cell Activation. *J. Immunol.* **191**, 5822–5830 (2013).
332. Polonsky, M. *et al.* Induction of CD4 T cell memory by local cellular collectivity. *Science (80-.).* **360**, eaaj1853 (2018).
333. Verma, N. K. *et al.* LFA-1/ICAM-1 Ligation in Human T Cells Promotes Th1 Polarization through a GSK3 β Signaling–Dependent Notch Pathway. *J. Immunol.* **197**, 108–118 (2016).
334. Ramming, A., Thümmel, K., Schulze-Koops, H. & Skapenko, A. Homotypic T-cell/T-cell interaction induces T-cell activation, proliferation, and differentiation. *Hum. Immunol.* **70**, 873–881 (2009).
335. Thümmel, K. *et al.* Immune regulation by peripheral suppressor T cells induced upon homotypic T cell/T cell interactions. *J. Leukoc. Biol.* **88**, 1041–1050 (2010).
336. Thaventhiran, J. E. D. *et al.* Activation of the Hippo pathway by CTLA-4 regulates the expression of Blimp-1 in the CD8⁺ T cell. *Proc. Natl. Acad. Sci.* **109**, E2223–E2229 (2012).
337. Zhou, J. *et al.* A kinetic investigation of interacting, stimulated T cells identifies conditions for rapid functional enhancement, minimal phenotype differentiation, and improved adoptive cell transfer tumor eradication. *PLoS One* **13**, e0191634 (2018).
338. Klebanoff, C. A. *et al.* Memory T cell–driven differentiation of naive cells impairs adoptive immunotherapy. *J. Clin. Invest.* **126**, 318–334 (2016).
339. Fooksman, D. R. *et al.* Functional Anatomy of T Cell Activation and Synapse Formation. *Annu Rev Immunol* **28**, 79–105 (2010).
340. Campi, G., Varma, R. & Dustin, M. L. Actin and agonist MHC–peptide complex–dependent T cell receptor microclusters as scaffolds for signaling. *J. Exp. Med.* **202**, 1031–1036 (2005).
341. Varma, R., Campi, G., Yokosuka, T., Saito, T. & Dustin, M. L. T Cell Receptor–Proximal Signals Are Sustained in Peripheral Microclusters and Terminated in the Central Supramolecular Activation Cluster. *Immunity* **25**, 117–127 (2006).
342. Yokosuka, T. *et al.* Newly generated T cell receptor microclusters initiate and sustain T cell activation by recruitment of Zap70 and SLP-76. *Nat. Immunol.* **6**, 1253–1262 (2005).
343. Moseman, E. A. & McGavern, D. B. The great balancing act: regulation and fate of antiviral T-cell interactions. *Immunol. Rev.* **255**, 110–124 (2013).
344. Alarcón, B., Mestre, D. & Martínez-Martín, N. The immunological synapse: a cause or consequence of T-cell receptor triggering? *Immunology* **133**, 420–425 (2011).
345. Hommel, M. & Kyewski, B. Dynamic Changes During the Immune Response in T Cell–Antigen-presenting Cell Clusters Isolated from Lymph Nodes. *J. Exp. Med.* **197**, 269–280 (2003).
346. Bousso, P. & Robey, E. Dynamics of CD8⁺ T cell priming by dendritic cells in intact lymph nodes. *Nat. Immunol.* **4**, 579–585 (2003).
347. Ingulli, E., Mondino, A., Khoruts, A. & Jenkins, M. K. In vivo detection of dendritic cell antigen presentation to CD4⁺ T cells. *J. Exp. Med.* **185**, 2133–2141 (1997).
348. Doh, J. & Krummel, M. F. Immunological Synapses Within Context: Patterns of Cell–Cell Communication and Their Application in T–T Interactions. in 25–50 (Springer, Berlin, Heidelberg, 2010). doi:10.1007/978-3-642-03858-7_2
349. Rothlein, R., Dustin, M. L., Marlin, S. D. & Springer, T. A. A human intercellular adhesion molecule (ICAM-1) distinct from LFA-1. *J. Immunol.* **137**, 1270–4 (1986).
350. Van Kooyk, Y., Weder, P., Heije, K., De Waal Malefijt, R. & Figdor, C. G. Role of intracellular ca²⁺ levels in the regulation of CD11a/CD18 mediated cell adhesion. *Cell Commun. Adhes.* **1**, 21–32 (1993).
351. Walling, B. L. & Kim, M. LFA-1 in T cell migration and differentiation. *Front. Immunol.* **9**, (2018).
352. Makgoba, M. W. *et al.* ICAM-1 a ligand for LFA-1-dependent adhesion of B, T and myeloid cells. *Nature* **331**, 86–88 (1988).
353. Brod, S. A., Purvee, M., Benjamin, D. & Hafler, D. A. T–T cell interactions are mediated by adhesion molecules. *Eur. J. Immunol.* **20**, 2259–2268 (1990).
354. Gérard, A., Cope, A. P., Kemper, C., Alon, R. & Köchl, R. LFA-1 in T cell priming, differentiation, and effector functions. *Trends Immunol.* (2021). doi:10.1016/j.it.2021.06.004
355. Chirathaworn, C. *et al.* Stimulation Through Intercellular Adhesion Molecule-1 Provides a Second Signal for T Cell Activation. *J. Immunol.* **168**, 5530–5537 (2002).
356. Dragovich, M. A. *et al.* SLAMF6 clustering is required to augment T cell activation. *PLoS One* **14**, e0218109

- (2019).
357. McCoy-Simandle, K., Hanna, S. J. & Cox, D. Exosomes and nanotubes: Control of immune cell communication. *Int. J. Biochem. & Cell Biol.* **71**, 44–54 (2016).
 358. Sowinski, S. *et al.* Membrane nanotubes physically connect T cells over long distances presenting a novel route for HIV-1 transmission. *Nat. Cell Biol.* **10**, 211–219 (2008).
 359. Rustom, A., Saffrich, R., Markovic, I., Walther, P. & Gerdes, H.-H. Nanotubular Highways for Intercellular Organelle Transport. *Science (80-.)*. **303**, 1007–1010 (2004).
 360. Oyler-Yaniv, A. *et al.* A Tunable Diffusion-Consumption Mechanism of Cytokine Propagation Enables Plasticity in Cell-to-Cell Communication in the Immune System. *Immunity* **46**, 609–620 (2017).
 361. Noben-Trauth, N., Hu-Li, J. & Paul, W. E. Conventional, Naive CD4+ T Cells Provide an Initial Source of IL-4 During Th2 Differentiation. *J. Immunol.* **165**, 3620–3625 (2000).
 362. Le Gros, G., Ben-Sasson, S. Z., Seder, R., Finkelman, F. D. & Paul, W. E. Generation of interleukin 4 (IL-4)-producing cells in vivo and in vitro: IL-2 and IL-4 are required for in vitro generation of IL-4-producing cells. *J. Exp. Med.* **172**, 921–929 (1990).
 363. Sofi, M. H., Li, W., Kaplan, M. H. & Chang, C.-H. Elevated IL-6 expression in CD4 T cells via PKC θ and NF- κ B induces Th2 cytokine production. *Mol. Immunol.* **46**, 1443–1450 (2009).
 364. Murakami, M., Kamimura, D. & Hirano, T. Pleiotropy and Specificity: Insights from the Interleukin 6 Family of Cytokines. *Immunity* **50**, 812–831 (2019).
 365. Tanaka, T., Narazaki, M. & Kishimoto, T. IL-6 in Inflammation, Immunity, and Disease. *Cold Spring Harb. Perspect. Biol.* **6**, a016295 (2014).
 366. Donkor, M. K., Sarkar, A. & Li, M. O. Tgf- β 1 produced by activated CD4+ T Cells Antagonizes T Cell Surveillance of Tumor Development. *Oncimmunology* **1**, 162–171 (2012).
 367. Li, M. O., Wan, Y. Y. & Flavell, R. A. T Cell-Produced Transforming Growth Factor- β 1 Controls T Cell Tolerance and Regulates Th1- and Th17-Cell Differentiation. *Immunity* **26**, 579–591 (2007).
 368. Saraiva, M. & O’Garra, A. The regulation of IL-10 production by immune cells. *Nat. Rev. Immunol.* **10**, 170–181 (2010).
 369. Griffith, J. W., Sokol, C. L. & Luster, A. D. Chemokines and chemokine receptors: positioning cells for host defense and immunity. *Annu. Rev. Immunol.* **32**, 659–702 (2014).
 370. Castellino, F. *et al.* Chemokines enhance immunity by guiding naive CD8+ T cells to sites of CD4+ T cell–dendritic cell interaction. *Nature* **440**, 890–895 (2006).
 371. Montecalvo, A. *et al.* Exosomes As a Short-Range Mechanism to Spread Alloantigen between Dendritic Cells during T Cell Allorecognition. *J. Immunol.* **180**, 3081–3090 (2008).
 372. Mittelbrunn, M. *et al.* Unidirectional transfer of microRNA-loaded exosomes from T cells to antigen-presenting cells. *Nat. Commun.* **2**, 282 (2011).
 373. Montecalvo, A. *et al.* Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes. *Blood* **119**, 756–766 (2012).
 374. Blanchard, N. *et al.* TCR Activation of Human T Cells Induces the Production of Exosomes Bearing the TCR/CD3/ ζ Complex. *J. Immunol.* **168**, 3235–3241 (2002).
 375. Admyre, C., Johansson, S. M., Paulie, S. & Gabrielsson, S. Direct exosome stimulation of peripheral human T cells detected by ELISPOT. *Eur. J. Immunol.* **36**, 1772–1781 (2006).
 376. Zhang, H. *et al.* CD4+ T cell-released exosomes inhibit CD8+ cytotoxic T-lymphocyte responses and antitumor immunity. *Cell. & Mol. Immunol.* **8**, 23–30 (2011).
 377. Stoycheva, D. *et al.* IFN- γ Regulates CD8+ Memory T Cell Differentiation and Survival in Response to Weak, but Not Strong, TCR Signals. *J. Immunol.* **194**, 553–559 (2015).
 378. Sercan, Ö., Stoycheva, D., Hämmerling, G. J., Arnold, B. & Schüler, T. IFN- γ Receptor Signaling Regulates Memory CD8+ T Cell Differentiation. *J. Immunol.* **184**, 2855–2862 (2010).
 379. Curtsinger, J. M., Agarwal, P., Lins, D. C. & Mescher, M. F. Autocrine IFN- γ Promotes Naive CD8 T Cell Differentiation and Synergizes with IFN- α To Stimulate Strong Function. *J. Immunol.* **189**, 659–668 (2012).
 380. Wheelock, E. F. Interferon-Like Virus-Inhibitor Induced in Human Leukocytes by Phytohemagglutinin. *Science (80-.)*. **149**, 310–311 (1965).
 381. Billiau, A. & Matthys, P. Interferon- γ : A historical perspective. *Cytokine & Growth Factor Rev.* **20**, 97–113 (2009).
 382. Hu, X. & Ivashkiv, L. B. Cross-regulation of Signaling Pathways by Interferon- γ : Implications for Immune Responses and Autoimmune Diseases. *Immunity* **31**, 539–550 (2009).
 383. Zhang, C. *et al.* Interleukin-12 improves cytotoxicity of natural killer cells via upregulated expression of NKG2D. *Hum. Immunol.* **69**, 490–500 (2008).

384. Ealick, S. E. *et al.* Three-dimensional structure of recombinant human interferon- γ . *Science* (80-.). **252**, 698–702 (1991).
385. Gocher, A. M., Workman, C. J. & Vignali, D. A. A. Interferon- γ : teammate or opponent in the tumour microenvironment? *Nat. Rev. Immunol.* 1–15 (2021). doi:10.1038/s41577-021-00566-3
386. Miyakawa, N. *et al.* Prolonged circulation half-life of interferon γ activity by gene delivery of interferon γ -serum albumin fusion protein in mice. *J. Pharm. Sci.* **100**, 2350–2357 (2011).
387. Charych, D. *et al.* Modeling the receptor pharmacology, pharmacokinetics, and pharmacodynamics of NKTR-214, a kinetically-controlled interleukin-2 (IL2) receptor agonist for cancer immunotherapy. *PLoS One* **12**, e0179431 (2017).
388. Molinas, F. C., Wietzerbin, J. & Falcoff, E. Human platelets possess receptors for a lymphokine: demonstration of high specific receptors for HuIFN-gamma. *J. Immunol.* **138**, 802–6 (1987).
389. Farrar, M. A. & Schreiber, R. D. The molecular cell biology of interferon- γ and its receptor. *Annu. Rev. Immunol.* **11**, 571–611 (1993).
390. Gough, D. J., Levy, D. E., Johnstone, R. W. & Clarke, C. J. IFN γ signaling-Does it mean JAK-STAT? *Cytokine Growth Factor Rev.* **19**, 383–394 (2008).
391. Mendoza, J. L. *et al.* Structure of the IFN γ receptor complex guides design of biased agonists. *Nature* **567**, 56–60 (2019).
392. Plataniias, L. C. Mechanisms of type-I- and type-II-interferon-mediated signalling. *Nat. Rev. Immunol.* **5**, 375–386 (2005).
393. Valente, G. *et al.* Distribution of interferon- γ receptor in human tissues. *Eur. J. Immunol.* **22**, 2403–2412 (1992).
394. Schmiedel, B. J. *et al.* Impact of Genetic Polymorphisms on Human Immune Cell Gene Expression. *Cell* **175**, 1701–1715.e16 (2018).
395. Monaco, G. *et al.* RNA-Seq Signatures Normalized by mRNA Abundance Allow Absolute Deconvolution of Human Immune Cell Types. *Cell Rep.* **26**, 1627–1640.e7 (2019).
396. Ebensperger, C. *et al.* GENOMIC ORGANIZATION AND PROMOTER ANALYSIS OF THE GENE ifngr2 ENCODING THE SECOND CHAIN OF THE MOUSE INTERFERON- γ RECEPTOR. *Scand. J. Immunol.* **44**, 599–606 (1996).
397. Green, D. S., Young, H. A. & Valencia, J. C. Current prospects of type II interferon γ signaling and autoimmunity. *J. Biol. Chem.* **292**, 13925–13933 (2017).
398. Larkin, J., Johnson, H. M. & Subramaniam, P. S. Differential nuclear localization of the IFNGR-1 and IFNGR-2 subunits of the IFN- γ receptor complex following activation by IFN- γ . *J. Interf. Cytokine Res.* **20**, 565–576 (2000).
399. Bernabei, P. *et al.* Interferon-gamma receptor 2 expression as the deciding factor in human T, B, and myeloid cell proliferation or death. *J. Leukoc. Biol.* **70**, 950–60 (2001).
400. Castro, F., Cardoso, A. P., Gonçalves, R. M., Serre, K. & Oliveira, M. J. Interferon-Gamma at the Crossroads of Tumor Immune Surveillance or Evasion. *Front. Immunol.* **9**, 847 (2018).
401. Greenlund, A. C., Farrar, M. A., Viviano, B. L. & Schreiber, R. D. Ligand-induced IFN(γ) receptor tyrosine phosphorylation couples the receptor to its signal transduction system (p91). *EMBO J.* **13**, 1591–1600 (1994).
402. Decker, T., Lew, D. J., Mirkovitch, J. & Darnell, J. E. Cytoplasmic activation of GAF, an IFN- γ -regulated DNA-binding factor. *EMBO J.* **10**, 927–932 (1991).
403. Ramana, C. V. *et al.* Stat1-independent regulation of gene expression in response to IFN- γ . *Proc. Natl. Acad. Sci. U. S. A.* **98**, 6674–6679 (2001).
404. Stephens, J. M., Lumpkin, S. J. & Fishman, J. B. Activation of signal transducers and activators of transcription 1 and 3 by leukemia inhibitory factor, oncostatin-M, and interferon- γ in adipocytes. *J. Biol. Chem.* **273**, 31408–31416 (1998).
405. Greenlund, A. C. *et al.* Stat recruitment by tyrosine-phosphorylated cytokine receptors: An ordered reversible affinity-driven process. *Immunity* **2**, 677–687 (1995).
406. Qi, Y. feng *et al.* Elucidating the crosstalk mechanism between IFN-gamma and IL-6 via mathematical modelling. *BMC Bioinformatics* **14**, 41 (2013).
407. Chatterjee-Kishore, M., Wright, K. L., Ting, J. P. Y. & Stark, G. R. How Stat1 mediates constitutive gene expression: A complex of unphosphorylated Stat1 and IRF1 supports transcription of the LMP2 gene. *EMBO J.* **19**, 4111–4122 (2000).
408. Ivashkiv, L. B. IFN γ : signalling, epigenetics and roles in immunity, metabolism, disease and cancer immunotherapy. *Nat. Rev. Immunol.* **18**, 545–558 (2018).
409. O'Shea, J. J. & Plenge, R. JAK and STAT Signaling Molecules in Immunoregulation and Immune-Mediated

- Disease. *Immunity* **36**, 542–550 (2012).
410. Wegrzyn, J. *et al.* Function of mitochondrial Stat3 in cellular respiration. *Science* (80-.). **323**, 793–797 (2009).
 411. Gough, D. J. *et al.* Mitochondrial STAT3 Supports Ras-Dependent Oncogenic Transformation. *Science* (80-.). **324**, 1713–1716 (2009).
 412. Endo, T. A. *et al.* A new protein containing an SH2 domain that inhibits JAK kinases. *Nature* **387**, 921–924 (1997).
 413. Starr, R. *et al.* A family of cytokine-inducible inhibitors of signalling. *Nature* **387**, 917–921 (1997).
 414. Palmer, D. C. & Restifo, N. P. Suppressors of cytokine signaling (SOCS) in T cell differentiation, maturation, and function. *Trends Immunol.* **30**, 592–602 (2009).
 415. Kamizono, S. *et al.* The SOCS Box of SOCS-1 Accelerates Ubiquitin-dependent Proteolysis of TEL-JAK2*. *J. Biol. Chem.* **276**, 12530–12538 (2001).
 416. Zhang, J.-G. *et al.* The conserved SOCS box motif in suppressors of cytokine signaling binds to elongins B and C and may couple bound proteins to proteasomal degradation. *Proc. Natl. Acad. Sci.* **96**, 2071–2076 (1999).
 417. Chang, J. H. *et al.* Ubc13 maintains the suppressive function of regulatory T cells and prevents their conversion into effector-like T cells. *Nat. Immunol.* **13**, 481–490 (2012).
 418. Choudhury, G. G. A linear signal transduction pathway involving phosphatidylinositol 3-kinase, protein kinase C ϵ , and MAPK in mesangial cells regulates interferon- γ -induced STAT1 α transcriptional activation. *J. Biol. Chem.* **279**, 27399–27409 (2004).
 419. Qing, Y. & Stark, G. R. Alternative activation of STAT1 and STAT3 in response to interferon- γ . *J. Biol. Chem.* **279**, 41679–41685 (2004).
 420. Meinke, A., Barahmand-Pour, F., Wöhr, S., Stoiber, D. & Decker, T. Activation of different Stat5 isoforms contributes to cell-type-restricted signaling in response to interferons. *Mol. Cell. Biol.* **16**, 6937–6944 (1996).
 421. Deb, A., Haque, S. J., Mogensen, T., Silverman, R. H. & Williams, B. R. G. RNA-Dependent Protein Kinase PKR Is Required for Activation of NF- κ B by IFN- γ in a STAT1-Independent Pathway. *J. Immunol.* **166**, 6170–6180 (2001).
 422. Lewis, M., Amento, E. P. & Unemori, E. N. Transcriptional inhibition of stromelysin by interferon- γ in normal human fibroblasts is mediated by the AP-1 domain. *J. Cell. Biochem.* **72**, 373–386 (1999).
 423. Barber, D. L., Wherry, E. J. & Ahmed, R. Cutting Edge: Rapid In Vivo Killing by Memory CD8 T Cells. *J. Immunol.* **171**, 27–31 (2003).
 424. Fallarino, F. *et al.* T cell apoptosis by tryptophan catabolism. *Cell Death Differ.* **9**, 1069–1077 (2002).
 425. Bhat, M. Y. *et al.* Comprehensive network map of interferon gamma signaling. *J. Cell Commun. Signal.* **12**, 745–751 (2018).
 426. Samarajiwa, S. A., Forster, S., Auchettl, K. & Hertzog, P. J. INTERFEROME: the database of interferon regulated genes. *Nucleic Acids Res.* **37**, D852–D857 (2009).
 427. Der, S. D., Zhou, A., Williams, B. R. G. & Silverman, R. H. Identification of genes differentially regulated by interferon α , β , or γ using oligonucleotide arrays. *Proc. Natl. Acad. Sci.* **95**, 15623–15628 (1998).
 428. Schneider, W. M., Chevillotte, M. D. & Rice, C. M. Interferon-Stimulated Genes: A Complex Web of Host Defenses. *Annu. Rev. Immunol.* **32**, 513–545 (2014).
 429. Szabo, S. J. *et al.* A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* **100**, 655–669 (2000).
 430. Bradley, L. M., Dalton, D. K. & Croft, M. A direct role for IFN- γ in regulation of Th1 cell development. *J. Immunol.* **157**, 1350–8 (1996).
 431. Girdlestone, J. & Wing, M. Autocrine activation by interferon- γ of STAT factors following T cell activation. *Eur. J. Immunol.* **26**, 704–709 (1996).
 432. Lighvani, A. A. *et al.* T-bet is rapidly induced by interferon- γ in lymphoid and myeloid cells. *Proc. Natl. Acad. Sci. U. S. A.* **98**, 15137–15142 (2001).
 433. Afkarian, M. *et al.* T-bet is a STAT1-induced regulator for IL-12R expression in naïve CD4⁺ T cells. *Nat. Immunol.* **3**, 549–557 (2002).
 434. Schulz, E. G., Mariani, L., Radbruch, A. & Höfer, T. Sequential Polarization and Imprinting of Type 1 T Helper Lymphocytes by Interferon- γ and Interleukin-12. *Immunity* **30**, 673–683 (2009).
 435. Pernis, A. *et al.* Lack of interferon γ receptor β chain and the prevention of interferon γ signaling in TH1 Cells. *Science* (80-.). **269**, 245–247 (1995).
 436. Bach, E. A. *et al.* Ligand-induced autoregulation of IFN- γ receptor β chain expression in T helper cell subsets. *Science* (80-.). **270**, 1215–1218 (1995).

437. Maggi, E. *et al.* Reciprocal regulatory effects of IFN- γ and IL-4 on the in vitro development of human Th1 and Th2 clones. *J. Immunol.* **148**, 2142–2147 (1992).
438. Hwang, E. S., Szabo, S. J., Schwartzberg, P. L. & Glimcher, L. H. T Helper Cell Fate Specified by Kinase-Mediated Interaction of T-bet with GATA-3. *Science* (80-.). **307**, 430–433 (2005).
439. Naka, T. *et al.* SOCS-1/SSI-1-deficient NKT cells participate in severe hepatitis through dysregulated cross-talk inhibition of IFN- γ and IL-4 signaling in vivo. *Immunity* **14**, 535–545 (2001).
440. Lazarevic, V. *et al.* T-bet represses TH17 differentiation by preventing Runx1-mediated activation of the gene encoding ROR γ t. *Nat. Immunol.* **12**, 96–104 (2011).
441. Xu, X., Fu, X. Y., Plate, J. & Chong, A. S. F. IFN- γ induces cell growth inhibition by fas-mediated apoptosis: Requirement of STAT1 protein for up-regulation of Fas and FasL expression. *Cancer Res.* **58**, 2832–2837 (1998).
442. Rakshit, S. *et al.* Interferon-gamma induced cell death: Regulation and contributions of nitric oxide, cJun N-terminal kinase, reactive oxygen species and peroxynitrite. *Biochim. Biophys. Acta - Mol. Cell Res.* **1843**, 2645–2661 (2014).
443. Zhou, Y., Weyman, C. M., Liu, H., Almassan, A. & Zhou, A. IFN- γ induces apoptosis in HL-60 cells through decreased Bcl-2 and increased Bak expression. *J. Interf. Cytokine Res.* **28**, 65–72 (2008).
444. Koch, M. A. *et al.* The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. *Nat. Immunol.* **10**, 595–602 (2009).
445. Putz, E. M. *et al.* Novel non-canonical role of STAT1 in Natural Killer cell cytotoxicity. *Oncoimmunology* **5**, e1186314 (2016).
446. Fröh, K. & Yang, Y. Antigen presentation by MHC class I and its regulation by interferon γ . *Curr. Opin. Immunol.* **11**, 76–81 (1999).
447. Steimle, V., Siegrist, C.-A., Mottet, A., Lisowska-Grospierre, B. & Mach, B. Regulation of MHC Class II Expression by Interferon- γ Mediated by the Transactivator Gene CIITA. *Science* (80-.). **265**, 106–109 (1994).
448. Meissner, T. B. *et al.* NLR family member NLRC5 is a transcriptional regulator of MHC class I genes. *Proc. Natl. Acad. Sci.* **107**, 13794–13799 (2010).
449. Collins, A. V. *et al.* The interaction properties of costimulatory molecules revisited. *Immunity* **17**, 201–210 (2002).
450. Pan, J. *et al.* Interferon- γ is an autocrine mediator for dendritic cell maturation. *Immunol. Lett.* **94**, 141–151 (2004).
451. Liu, M. *et al.* Targeting the IDO1 pathway in cancer: from bench to bedside. *J. Hematol. & Oncol.* **11**, 100 (2018).
452. Yan, Y. *et al.* IDO Upregulates Regulatory T Cells via Tryptophan Catabolite and Suppresses Encephalitogenic T Cell Responses in Experimental Autoimmune Encephalomyelitis. *J. Immunol.* **185**, 5953–5961 (2010).
453. Jackson, S. W. *et al.* B cell IFN- γ receptor signaling promotes autoimmune germinal centers via cell-intrinsic induction of BCL-6. *J. Exp. Med.* **213**, 733–750 (2016).
454. Metzger, D. W. *et al.* Interleukin-12 acts as an adjuvant for humoral immunity through interferon- γ -dependent and -independent mechanisms. *Eur. J. Immunol.* **27**, 1958–1965 (1997).
455. Martinez, F. O., Helming, L. & Gordon, S. Alternative Activation of Macrophages: An Immunologic Functional Perspective. *Annu. Rev. Immunol.* **27**, 451–483 (2009).
456. Wu, C. *et al.* IFN- γ Primes Macrophage Activation by Increasing Phosphatase and Tensin Homolog via Downregulation of miR-3473b. *J. Immunol.* **193**, 3036–3044 (2014).
457. Zhang, N. & Bevan, M. J. CD8 $^{+}$ T Cells: Foot Soldiers of the Immune System. *Immunity* **35**, 161–168 (2011).
458. Park, S. S. *et al.* IFN- γ enhances TRAIL-induced apoptosis through IRF-1. *Eur. J. Biochem.* **271**, 4222–4228 (2004).
459. Refaelli, Y., Parijs, L. Van, Alexander, S. I. & Abbas, A. K. Interferon γ Is Required for Activation-induced Death of T Lymphocytes. *J. Exp. Med.* **196**, 999–1005 (2002).
460. Prabhu, N. *et al.* Gamma Interferon Regulates Contraction of the Influenza Virus-Specific CD8 T Cell Response and Limits the Size of the Memory Population. *J. Virol.* **87**, 12510–12522 (2013).
461. Tewari, K., Nakayama, Y. & Suresh, M. Role of Direct Effects of IFN- γ on T Cells in the Regulation of CD8 T Cell Homeostasis. *J. Immunol.* **179**, 2115–2125 (2007).
462. Ouyang, W., Beckett, O., Flavell, R. A. & Li, M. O. An Essential Role of the Forkhead-Box Transcription Factor Foxo1 in Control of T Cell Homeostasis and Tolerance. *Immunity* **30**, 358–371 (2009).
463. Qiang, L., Banks, A. S. & Accili, D. Uncoupling of Acetylation from Phosphorylation Regulates FoxO1 Function Independent of Its Subcellular Localization*. *J. Biol. Chem.* **285**, 27396–27401 (2010).

464. Tau, G. Z., Cowan, S. N., Weisburg, J., Braunstein, N. S. & Rothman, P. B. Regulation of IFN- γ Signaling Is Essential for the Cytotoxic Activity of CD8+ T Cells. *J. Immunol.* **167**, 5574–5582 (2001).
465. Rodriguez, F., Harkins, S., Slifka, M. K. & Whitton, J. L. Immunodominance in Virus-Induced CD8 + T-Cell Responses Is Dramatically Modified by DNA Immunization and Is Regulated by Gamma Interferon . *J. Virol.* **76**, 4251–4259 (2002).
466. Liu, F., Whitton, J. L. & Slifka, M. K. The Rapidity with Which Virus-Specific CD8 + T Cells Initiate IFN- γ Synthesis Increases Markedly over the Course of Infection and Correlates with Immunodominance . *J. Immunol.* **173**, 456–462 (2004).
467. Bonaventura, P. *et al.* Cold Tumors: A Therapeutic Challenge for Immunotherapy. *Front. Immunol.* **10**, 168 (2019).
468. Colvin, R. A., Campanella, G. S. V, Sun, J. & Luster, A. D. Intracellular Domains of CXCR3 That Mediate CXCL9, CXCL10, and CXCL11 Function*. *J. Biol. Chem.* **279**, 30219–30227 (2004).
469. Sercan, Ö., Hämmerling, G. J., Arnold, B. & Schüler, T. Cutting Edge: Innate Immune Cells Contribute to the IFN- γ -Dependent Regulation of Antigen-Specific CD8 + T Cell Homeostasis . *J. Immunol.* **176**, 735–739 (2006).
470. Lohman, B. L. & Welsh, R. M. Apoptotic Regulation of T Cells and Absence of Immune Deficiency in Virus-Infected Gamma Interferon Receptor Knockout Mice. *J. Virol.* **72**, 7815–7821 (1998).
471. Maekawa, Y. *et al.* Notch2 integrates signaling by the transcription factors RBP-J and CREB1 to promote T cell cytotoxicity. *Nat. Immunol.* **9**, 1140–1147 (2008).
472. Madisen, L. *et al.* A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. *Nat. Neurosci.* **13**, 133–140 (2010).
473. Kannan, K. *et al.* Lysosome-Associated Membrane Proteins h-LAMP1 (CD107a) and h-LAMP2 (CD107b) Are Activation-Dependent Cell Surface Glycoproteins in Human Peripheral Blood Mononuclear Cells Which Mediate Cell Adhesion to Vascular Endothelium. *Cell. Immunol.* **171**, 10–19 (1996).
474. Fukuda, M. Lysosomal membrane glycoproteins: Structure, biosynthesis, and intracellular trafficking. *J. Biol. Chem.* **266**, 21327–21330 (1991).
475. Peters, P. J. *et al.* Cytotoxic T lymphocyte granules are secretory lysosomes, containing both perforin and granzymes. *J. Exp. Med.* **173**, 1099–1109 (1991).
476. Winchester, B. G. Lysosomal membrane proteins. *Eur. J. Paediatr. Neurol.* **5**, 11–19 (2001).
477. Betts, M. R. *et al.* Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. *J. Immunol. Methods* **281**, 65–78 (2003).
478. Krzewski, K., Gil-Krzewska, A., Nguyen, V., Peruzzi, G. & Coligan, J. E. LAMP1/CD107a is required for efficient perforin delivery to lytic granules and NK-cell cytotoxicity. *Blood* **121**, 4672–4683 (2013).
479. Cohnen, A. *et al.* Surface CD107a/LAMP-1 protects natural killer cells from degranulation-associated damage. *Blood* **122**, 1411–1418 (2013).
480. Aktas, E., Kucuksezer, U. C., Bilgic, S., Erten, G. & Deniz, G. Relationship between CD107a expression and cytotoxic activity. *Cell. Immunol.* **254**, 149–154 (2009).
481. Alter, G., Malenfant, J. M. & Altfeld, M. CD107a as a functional marker for the identification of natural killer cell activity. *J. Immunol. Methods* **294**, 15–22 (2004).
482. Schmidt-Suppran, M. & Rajewsky, K. Vagaries of conditional gene targeting. *Nat. Immunol.* **8**, 665–668 (2007).
483. Pfeifer, A., Brandon, E. P., Kootstra, N., Gage, F. H. & Verma, I. M. Delivery of the Cre recombinase by a self-deleting lentiviral vector: Efficient gene targeting in vivo. *Proc. Natl. Acad. Sci. U. S. A.* **98**, 11450–11455 (2001).
484. Branda, C. S. & Dymecki, S. M. Talking about a Revolution. *Dev. Cell* **6**, 7–28 (2004).
485. Nauerth, M. *et al.* TCR-Ligand koff Rate Correlates with the Protective Capacity of Antigen-Specific CD8+ T Cells for Adoptive Transfer. *Sci. Transl. Med.* **5**, 192ra87--192ra87 (2013).
486. Lyons, D. S. *et al.* A TCR binds to antagonist ligands with lower affinities and faster dissociation rates than to agonists. *Immunity* **5**, 53–61 (1996).
487. Wang, X. L. & Altman, J. D. Caveats in the design of MHC class I tetramer/antigen-specific T lymphocytes dissociation assays. *J. Immunol. Methods* **280**, 25–35 (2003).
488. Nauerth, M. *et al.* Flow cytometry-based TCR-ligand Koff-rate assay for fast avidity screening of even very small antigen-specific T cell populations ex vivo. *Cytom. Part A* **89**, 816–825 (2016).
489. Krummel, M. F. *et al.* Paracrine costimulation of IFN- γ signaling by integrins modulates CD8 T cell differentiation. *Proc. Natl. Acad. Sci.* **115**, 201804556 (2018).
490. Segal, G., Prato, S., Zehn, D., Mintern, J. D. & Villadangos, J. A. Target Density, Not Affinity or Avidity of Antigen Recognition, Determines Adoptive T Cell Therapy Outcomes in a Mouse Lymphoma Model. *J.*

- Immunol.* **196**, 3935–3942 (2016).
491. Rius, C. *et al.* Peptide-MHC Class I Tetramers Can Fail To Detect Relevant Functional T Cell Clonotypes and Underestimate Antigen-Reactive T Cell Populations. *J. Immunol.* **200**, 2263–2279 (2018).
 492. Martinez, R. J., Andargachew, R., Martinez, H. A. & Evavold, B. D. Low-affinity CD4⁺ T cells are major responders in the primary immune response. *Nat. Commun.* **7**, 13848 (2016).
 493. Derby, M. A., Alexander-Miller, M. A., Tse, R. & Berzofsky, J. A. High-Avidity CTL Exploit Two Complementary Mechanisms to Provide Better Protection Against Viral Infection Than Low-Avidity CTL. *J. Immunol.* **166**, 1690–1697 (2001).
 494. Zeh, H. J., Perry-Lalley, D., Dudley, M. E., Rosenberg, S. A. & Yang, J. C. High avidity CTLs for two self-antigens demonstrate superior in vitro and in vivo antitumor efficacy. *J. Immunol.* **162**, 989–94 (1999).
 495. Huse, M., Lillemeier, B. F., Kuhns, M. S., Chen, D. S. & Davis, M. M. T cells use two directionally distinct pathways for cytokine secretion. *Nat. Immunol.* **7**, 247–255 (2006).
 496. Ouyang, W. *et al.* Novel Foxo1-dependent transcriptional programs control T reg cell function. *Nature* **491**, 554–559 (2012).
 497. Lucca, L. E. *et al.* TIGIT signaling restores suppressor function of Th1 Tregs. *JCI Insight* **4**, (2019).
 498. Levine, A. G. *et al.* Stability and function of regulatory T cells expressing the transcription factor T-bet. *Nature* **546**, 421–425 (2017).
 499. Overacre-Delgoffe, A. E. *et al.* Interferon- γ Drives Treg Fragility to Promote Anti-tumor Immunity. *Cell* **169**, 1130–1141.e11 (2017).
 500. Gao, Y. *et al.* $\gamma\delta$ T cells provide an early source of interferon γ in tumor immunity. *J. Exp. Med.* **198**, 433–442 (2003).
 501. Sanderson, N. S. R. *et al.* Cytotoxic immunological synapses do not restrict the action of interferon- γ to antigenic target cells. *Proc. Natl. Acad. Sci.* **109**, 7835–7840 (2012).
 502. Berg, R. E., Cordes, C. J. & Forman, J. Contribution of CD8⁺ T cells to innate immunity: IFN- γ secretion induced by IL-12 and IL-18. *Eur. J. Immunol.* **32**, 2807–2816 (2002).
 503. Thibaut, R. *et al.* Bystander IFN- γ activity promotes widespread and sustained cytokine signaling altering the tumor microenvironment. *Nat. Cancer* **1**, 302–314 (2020).
 504. Hoekstra, M. E. *et al.* Long-distance modulation of bystander tumor cells by CD8⁺ T-cell-secreted IFN- γ . *Nat. Cancer* **1**, 291–301 (2020).
 505. Dong, C. Cytokine Regulation and Function in T Cells. *Annu. Rev. Immunol.* **39**, 1–26 (2021).
 506. Das, R. *et al.* The adaptor molecule SAP plays essential roles during invariant NKT cell cytotoxicity and lytic synapse formation. *Blood* **121**, 3386–3395 (2013).
 507. Wolf, B. J., Choi, J. E. & Exley, M. A. Novel Approaches to Exploiting Invariant NKT Cells in Cancer Immunotherapy. *Front. Immunol.* **9**, 384 (2018).
 508. Kjer-Nielsen, L. *et al.* MR1 presents microbial vitamin B metabolites to MAIT cells. *Nature* **491**, 717–723 (2012).
 509. Corbett, A. J. *et al.* T-cell activation by transitory neo-antigens derived from distinct microbial pathways. *Nature* **509**, 361–365 (2014).
 510. Godfrey, D. I., Uldrich, A. P., McCluskey, J., Rossjohn, J. & Moody, D. B. The burgeoning family of unconventional T cells. *Nat. Immunol.* **16**, 1114–1123 (2015).
 511. Legoux, F., Salou, M. & Lantz, O. Unconventional or preset $\alpha\beta$ T cells: Evolutionarily conserved tissue-resident T cells recognizing nonpeptidic ligands. *Annu. Rev. Cell Dev. Biol.* **33**, 511–535 (2017).
 512. Godfrey, D. I., Koay, H.-F., McCluskey, J. & Gherardin, N. A. The biology and functional importance of MAIT cells. *Nat. Immunol.* **20**, 1110–1128 (2019).
 513. Ussher, J. E. *et al.* CD161⁺⁺CD8⁺ T cells, including the MAIT cell subset, are specifically activated by IL-12+IL-18 in a TCR-independent manner. *Eur. J. Immunol.* **44**, 195–203 (2014).
 514. Reefman, E. *et al.* Cytokine Secretion Is Distinct from Secretion of Cytotoxic Granules in NK Cells. *J. Immunol.* **184**, 4852–4862 (2010).
 515. Fillatreau, S. B cells and their cytokine activities implications in human diseases. *Clin. Immunol.* **186**, 26–31 (2018).
 516. Ballesteros-Tato, A., Stone, S. L. & Lund, F. E. Innate IFN γ -producing B cells. *Cell Res.* **24**, 135–136 (2014).
 517. Jung, T., Schauer, U., Heusser, C., Neumann, C. & Rieger, C. Detection of intracellular cytokines by flow cytometry. *J. Immunol. Methods* **159**, 197–207 (1993).
 518. Schmitz, J., Assenmacher, M. & Radbruch, A. Regulation of T helper cell cytokine expression: functional dichotomy of antigen-presenting cells. *Eur. J. Immunol.* **23**, 191–199 (1993).
 519. Openshaw, P. *et al.* Heterogeneity of intracellular cytokine synthesis at the single-cell level in polarized T helper 1 and T helper 2 populations. *J. Exp. Med.* **182**, 1357–1367 (1995).

520. Foster, B., Prussin, C., Liu, F., Whitmire, J. K. & Whitton, J. L. Detection of Intracellular Cytokines by Flow Cytometry. *Curr. Protoc. Immunol.* **78**, 6.24.1--6.24.21 (2007).
521. Liu, F. & Whitton, J. L. Cutting Edge: Re-evaluating the In Vivo Cytokine Responses of CD8 + T Cells during Primary and Secondary Viral Infections. *J. Immunol.* **174**, 5936--5940 (2005).
522. Gajdasik, D. W. *et al.* Th1 responses in vivo require cell-specific provision of OX40L dictated by environmental cues. *Nat. Commun.* **11**, 3421 (2020).
523. Reinhardt, R. L., Liang, H.-E. & Locksley, R. M. Cytokine-secreting follicular T cells shape the antibody repertoire. *Nat. Immunol.* **10**, 385--393 (2009).
524. Kalia, V. *et al.* Prolonged Interleukin-2R α Expression on Virus-Specific CD8+ T Cells Favors Terminal-Effector Differentiation In Vivo. *Immunity* **32**, 91--103 (2010).
525. Belz, G. T. & Masson, F. Interleukin-2 Tickles T Cell Memory. *Immunity* **32**, 7--9 (2010).
526. Kambayashi, T., Assarsson, E., Lukacher, A. E., Ljunggren, H.-G. & Jensen, P. E. Memory CD8 + T Cells Provide an Early Source of IFN- γ . *J. Immunol.* **170**, 2399--2408 (2003).
527. Zhou, F. Molecular mechanisms of IFN- γ to up-regulate MHC class I antigen processing and presentation. *Int. Rev. Immunol.* **28**, 239--260 (2009).
528. Levy, D. E., Kessler, D. S., Pine, R., Reich, N. & Darnell, J. E. Interferon-induced nuclear factors that bind a shared promoter element correlate with positive and negative transcriptional control. *Genes Dev.* **2**, 383--393 (1988).
529. Levy, D. E., Lew, D. J., Decker, T., Kessler, D. S. & Darnell, J. E. Synergistic interaction between interferon-alpha and interferon-gamma through induced synthesis of one subunit of the transcription factor ISGF3. *EMBO J.* **9**, 1105--1111 (1990).
530. Oyler-Yaniv, J. *et al.* Catch and Release of Cytokines Mediated by Tumor Phosphatidylserine Converts Transient Exposure into Long-Lived Inflammation. *Mol. Cell* **66**, 635--647.e7 (2017).
531. Fischer, K. *et al.* Antigen recognition induces phosphatidylserine exposure on the cell surface of human CD8+ T cells. *Blood* **108**, 4094--4101 (2006).
532. Monticelli, S. & Natoli, G. Short-term memory of danger signals and environmental stimuli in immune cells. *Nat. Immunol.* **14**, 777--784 (2013).
533. Foster, S. L., Hargreaves, D. C. & Medzhitov, R. Gene-specific control of inflammation by TLR-induced chromatin modifications. *Nature* **447**, 972--978 (2007).
534. Kim, D., Reilly, A. & Lawrence, D. A. Relationships between IFN γ , IL-6, corticosterone, and *Listeria monocytogenes* pathogenesis in BALB/c mice. *Cell. Immunol.* **207**, 13--18 (2001).
535. Agarwal, P. *et al.* Gene Regulation and Chromatin Remodeling by IL-12 and Type I IFN in Programming for CD8 T Cell Effector Function and Memory. *J. Immunol.* **183**, 1695--1704 (2009).
536. Bhat, P., Leggatt, G., Waterhouse, N. & Frazer, I. H. Interferon- γ derived from cytotoxic lymphocytes directly enhances their motility and cytotoxicity. *Cell Death & Dis.* **8**, e2836 (2017).
537. Young, H. A., Dray, J. F. & Farrar, W. L. Expression of transfected human interferon-gamma DNA: evidence for cell-specific regulation. *J. Immunol.* **136**, 4700--3 (1986).
538. Soutto, M., Zhou, W. & Aune, T. M. Cutting Edge: Distal Regulatory Elements Are Required to Achieve Selective Expression of IFN- γ in Th1/Tc1 Effector Cells. *J. Immunol.* **169**, 6664--6667 (2002).
539. Nakayamada, S. *et al.* Early Th1 Cell Differentiation Is Marked by a Tfh Cell-like Transition. *Immunity* **35**, 919--931 (2011).
540. Fields, P. E., Kim, S. T. & Flavell, R. A. Cutting Edge: Changes in Histone Acetylation at the IL-4 and IFN- γ Loci Accompany Th1/Th2 Differentiation. *J. Immunol.* **169**, 647--650 (2002).
541. Kiani, A. *et al.* Regulation of interferon- γ gene expression by nuclear factor of activated T cells. *Blood* **98**, 1480--1488 (2001).
542. Dong, C., Davis, R. J. & Flavell, R. A. MAP kinases in the immune response. *Annu. Rev. Immunol.* **20**, 55--72 (2002).
543. Beals, C. R., Sheridan, C. M., Turck, C. W., Gardner, P. & Crabtree, G. R. Nuclear Export of NF-ATc Enhanced by Glycogen Synthase Kinase-3. *Science (80-.)*. **275**, 1930--1933 (1997).
544. Johnson, H. M., Torres, B. A., Smith, E. M., Dion, L. D. & Blalock, J. E. Regulation of lymphokine (gamma-interferon) production by corticotropin. *J. Immunol.* **132**, 246--50 (1984).
545. Johnson, W. J. *et al.* Cytolytic interactions between murine macrophages, tumor cells, and monoclonal antibodies: Characterization of lytic conditions and requirements for effector activation. *J. Immunol.* **136**, 4704--4713 (1986).
546. Kasahara, T., Hooks, J. J., Dougherty, S. F. & Oppenheim, J. J. Interleukin 2-mediated immune interferon (IFN-gamma) production by human T cells and T cell subsets. *J. Immunol.* **130**, 1784--9 (1983).
547. Vignali, D. A. A. & Kuchroo, V. K. IL-12 family cytokines: immunological playmakers. *Nat. Immunol.* **13**,

- 722–728 (2012).
548. Nakahira, M. *et al.* Synergy of IL-12 and IL-18 for IFN- γ Gene Expression: IL-12-Induced STAT4 Contributes to IFN- γ Promoter Activation by Up-Regulating the Binding Activity of IL-18-Induced Activator Protein 1. *J. Immunol.* **168**, 1146–1153 (2002).
 549. Rex, D. A. B. *et al.* A comprehensive pathway map of IL-18-mediated signalling. *J. Cell Commun. Signal.* **14**, 257–266 (2020).
 550. Mavropoulos, A., Sully, G., Cope, A. P. & Clark, A. R. Stabilization of IFN- γ mRNA by MAPK p38 in IL-12– and IL-18–stimulated human NK cells. *Blood* **105**, 282–288 (2005).
 551. Hodge, D. L., Martinez, A., Julias, J. G., Taylor, L. S. & Young, H. A. Regulation of Nuclear Gamma Interferon Gene Expression by Interleukin 12 (IL-12) and IL-2 Represents a Novel Form of Posttranscriptional Control. *Mol. Cell. Biol.* **22**, 1742–1753 (2002).
 552. Steiner, D. F. *et al.* MicroRNA-29 Regulates T-Box Transcription Factors and Interferon- γ Production in Helper T Cells. *Immunity* **35**, 169–181 (2011).
 553. Wang, H. *et al.* Regulation of Human Natural Killer Cell IFN- γ Production by MicroRNA-146a via Targeting the NF- κ B Signaling Pathway. *Front. Immunol.* **9**, 293 (2018).
 554. Ma, N. *et al.* MicroRNA-142-3p inhibits IFN- γ production via targeting of RICTOR in *Aspergillus fumigatus* activated CD4+ T cells. *Ann. Transl. Med.* **7**, 649–649 (2019).
 555. Kubota, A., Kubota, S., Lohwasser, S., Mager, D. L. & Takei, F. Diversity of NK cell receptor repertoire in adult and neonatal mice. *J. Immunol.* **163**, 212–6 (1999).
 556. Almishri, W. *et al.* TNF α Augments Cytokine-Induced NK Cell IFN γ Production through TNFR2. *J. Innate Immun.* **8**, 617–629 (2016).
 557. Liang, Y. *et al.* IL-33 promotes innate IFN- γ production and modulates dendritic cell response in LCMV-induced hepatitis in mice. *Eur. J. Immunol.* **45**, 3052–3063 (2015).
 558. Moreno, M. *et al.* IFN- γ -Producing Human Invariant NKT Cells Promote Tumor-Associated Antigen-Specific Cytotoxic T Cell Responses. *J. Immunol.* **181**, 2446–2454 (2008).
 559. Brigl, M., Bry, L., Kent, S. C., Gumperz, J. E. & Brenner, M. B. Mechanism of CD1d-restricted natural killer T cell activation during microbial infection. *Nat. Immunol.* **4**, 1230–1237 (2003).
 560. Harris, D. P., Goodrich, S., Mohrs, K., Mohrs, M. & Lund, F. E. Cutting Edge: The Development of IL-4-Producing B Cells (B Effector 2 Cells) Is Controlled by IL-4, IL-4 Receptor α , and Th2 Cells. *J. Immunol.* **175**, 7103–7107 (2005).
 561. Szabo, S. J. *et al.* Distinct Effects of T-bet in TH1 Lineage Commitment and IFN- γ Production in CD4 and CD8 T Cells. *Science (80-.).* **295**, 338–342 (2002).
 562. Lertmemongkolkhai, G., Cai, G., Hunter, C. A. & Bancroft, G. J. Bystander Activation of CD8+ T Cells Contributes to the Rapid Production of IFN- γ in Response to Bacterial Pathogens. *J. Immunol.* **166**, 1097–1105 (2001).
 563. Chu, T. *et al.* Bystander-Activated Memory CD8 T Cells Control Early Pathogen Load in an Innate-like, NKG2D-Dependent Manner. *Cell Rep.* **3**, 701–708 (2013).
 564. Tough, D. F., Borrow, P. & Sprent, J. Induction of Bystander T Cell Proliferation by Viruses and Type I Interferon in Vivo. *Science (80-.).* **272**, 1947–1950 (1996).
 565. Tough, D. F., Sun, S. & Sprent, J. T cell stimulation in vivo by lipopolysaccharide (LPS). *J. Exp. Med.* **185**, 2089–2094 (1997).
 566. Zhang, X., Sun, S., Hwang, I., Tough, D. F. & Sprent, J. Potent and selective stimulation of memory-phenotype CD8+ T cells in vivo by IL-15. *Immunity* **8**, 591–599 (1998).
 567. Zarozinski, C. C. & Welsh, R. M. Minimal bystander activation of CD8T cells during the virus-induced polyclonal T cell response. *J. Exp. Med.* **185**, 1629–1639 (1997).
 568. Ehl, S., Hombach, J., Aichele, P., Hengartner, H. & Zinkernagel, R. M. Bystander activation of cytotoxic T cells: Studies on the mechanism and evaluation of in vivo significance in a transgenic mouse model. *J. Exp. Med.* **185**, 1241–1251 (1997).
 569. Murali-Krishna, K. *et al.* Counting antigen-specific CD8 T cells: A reevaluation of bystander activation during viral infection. *Immunity* **8**, 177–187 (1998).
 570. Lee, H., Jeong, S. & Shin, E.-C. Significance of bystander T cell activation in microbial infection. *Nat. Immunol.* 1–10 (2021). doi:10.1038/s41590-021-00985-3
 571. Raué, H. P., Beadling, C., Haun, J. & Slifka, M. K. Cytokine-Mediated Programmed Proliferation of Virus-Specific CD8+ Memory T Cells. *Immunity* **38**, 131–139 (2013).
 572. Soudja, S. M., Ruiz, A. L., Marie, J. C. & Lauvau, G. Inflammatory Monocytes Activate Memory CD8+ T and Innate NK Lymphocytes Independent of Cognate Antigen during Microbial Pathogen Invasion. *Immunity* **37**, 549–562 (2012).

573. Kupz, A. *et al.* NLRC4 inflammasomes in dendritic cells regulate noncognate effector function by memory CD8⁺ T cells. *Nat. Immunol.* **13**, 162–169 (2012).
574. Berg, R. E., Crossley, E., Murray, S. & Forman, J. Relative Contributions of NK and CD8 T Cells to IFN- γ Mediated Innate Immune Protection against *Listeria monocytogenes*. *J. Immunol.* **175**, 1751–1757 (2005).
575. Raué, H.-P., Brien, J. D., Hammarlund, E. & Slifka, M. K. Activation of Virus-Specific CD8 + T Cells by Lipopolysaccharide-Induced IL-12 and IL-18. *J. Immunol.* **173**, 6873–6881 (2004).
576. Martin, M. D., Shan, Q., Xue, H.-H. & Badovinac, V. P. Time and Antigen-Stimulation History Influence Memory CD8 T Cell Bystander Responses. *Front. Immunol.* **8**, 634 (2017).
577. Araújo-Souza, P. S. *et al.* Differential interferon- γ production by naive and memory-like CD8 T cells. *J. Leukoc. Biol.* (2020). doi:10.1002/jlb.2ab0420-646r
578. Akue, A. D., Lee, J. & Jameson, S. C. Derivation and Maintenance of Virtual Memory CD8 T Cells. *J. Immunol.* **188**, 2516–2523 (2012).
579. Lee, J.-Y., Hamilton, S. E., Akue, A. D., Hogquist, K. A. & Jameson, S. C. Virtual memory CD8 T cells display unique functional properties. *Proc. Natl. Acad. Sci.* **110**, 13498–13503 (2013).
580. White, J. T. *et al.* Virtual memory T cells develop and mediate bystander protective immunity in an IL-15-dependent manner. *Nat. Commun.* **7**, 11291 (2016).
581. Thompson, A. & Orr, S. J. Emerging IL-12 family cytokines in the fight against fungal infections. *Cytokine* **111**, 398–407 (2018).
582. Murphy, C. A. *et al.* Divergent Pro- and Antiinflammatory Roles for IL-23 and IL-12 in Joint Autoimmune Inflammation. *J. Exp. Med.* **198**, 1951–1957 (2003).
583. Presky, D. H. *et al.* A functional interleukin 12 receptor complex is composed of two β -type cytokine receptor subunits. *Proc. Natl. Acad. Sci. U. S. A.* **93**, 14002–14007 (1996).
584. Wu, C. *et al.* IL-12 Receptor β 2 (IL-12R β 2)-Deficient Mice Are Defective in IL-12-Mediated Signaling Despite the Presence of High Affinity IL-12 Binding Sites. *J. Immunol.* **165**, 6221–6228 (2000).
585. Kusaba, H. *et al.* Interleukin-12-induced interferon- γ production by human peripheral blood T cells is regulated by mammalian target of rapamycin (mTOR). *J. Biol. Chem.* **280**, 1037–1043 (2005).
586. Jacobson, N. G. *et al.* Interleukin 12 signaling in T helper type 1 (Th1) cells involves tyrosine phosphorylation of signal transducer and activator of transcription (Stat)3 and Stat4. *J. Exp. Med.* **181**, 1755–1762 (1995).
587. Thierfelder, W. E. *et al.* Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. *Nature* **382**, 171–174 (1996).
588. Galon, J., Sudarshan, C., Ito, S., Finbloom, D. & O’Shea, J. J. IL-12 induces IFN regulating factor-1 (IRF-1) gene expression in human NK and T cells. *J. Immunol.* **162**, 7256–7262 (1999).
589. Lehtonen, A. *et al.* IFN- α and IL-12 activate IFN regulatory factor 1 (IRF-1), IRF-4, and IRF-8 gene expression in human NK and T cells. *Cytokine* **24**, 81–90 (2003).
590. Foukas, L. C., Panayotou, G. & Shepherd, P. R. Direct Interaction of Major Histocompatibility Complex Class II-derived Peptides with Class Ia Phosphoinositide 3-Kinase Results in Dose-dependent Stimulatory Effects. *J. Biol. Chem.* **279**, 7505–7511 (2004).
591. Yanagida, T., Kato, T., Igarashi, O., Inoue, T. & Nariuchi, H. Second signal activity of IL-12 on the proliferation and IL-2R expression of T helper cell-1 clone. *J. Immunol.* **152**, 4919–4928 (1994).
592. Lim, Y. C. *et al.* Expression of functional selectin ligands on Th cells is differentially regulated by IL-12 and IL-4. *J. Immunol.* **162**, 3193–3193201 (1999).
593. Lim, Y.-C. *et al.* IL-12, STAT4-Dependent Up-Regulation of CD4 + T Cell Core 2 β -1,6- n - Acetylglucosaminyltransferase, an Enzyme Essential for Biosynthesis of P-Selectin Ligands. *J. Immunol.* **167**, 4476–4484 (2001).
594. Underhill, G. H. *et al.* A crucial role for T-bet in selectin ligand expression in T helper 1 (Th1) cells. *Blood* **106**, 3867–3873 (2005).
595. White, S. J., Underhill, G. H., Kaplan, M. H. & Kansas, G. S. Cutting Edge: Differential Requirements for Stat4 in Expression of Glycosyltransferases Responsible for Selectin Ligand Formation in Th1 Cells. *J. Immunol.* **167**, 628–631 (2001).
596. Takatori, H. *et al.* Stat5a Inhibits IL-12-Induced Th1 Cell Differentiation through the Induction of Suppressor of Cytokine Signaling 3 Expression. *J. Immunol.* **174**, 4105–4112 (2005).
597. Yamamoto, K., Yamaguchi, M., Miyasaka, N. & Miura, O. SOCS-3 inhibits IL-12-induced STAT4 activation by binding through its SH2 domain to the STAT4 docking site in the IL-12 receptor β 2 subunit. *Biochem. Biophys. Res. Commun.* **310**, 1188–1193 (2003).
598. Ahn, H. J. *et al.* Requirement for distinct Janus kinases and STAT proteins in T cell proliferation versus IFN-

- γ, production following IL-12 stimulation. *J. Immunol.* **161**, 5893–5900 (1998).
599. Lee, S.-H., Fragoso, M. F. & Biron, C. A. Cutting Edge: A Novel Mechanism Bridging Innate and Adaptive Immunity: IL-12 Induction of CD25 To Form High-Affinity IL-2 Receptors on NK Cells. *J. Immunol.* **189**, 2712–2716 (2012).
 600. Waldmann, T. A. & Tagaya, Y. The multifaceted regulation of interleukin-15 expression and the role of this cytokine in NK cell differentiation and host response to intracellular pathogens. *Annu. Rev. Immunol.* **17**, 19–49 (1999).
 601. Waldmann, T. A. The biology of interleukin-2 and interleukin-15: Implications for cancer therapy and vaccine design. *Nat. Rev. Immunol.* **6**, 595–601 (2006).
 602. Mao, Y. *et al.* IL-15 activates mTOR and primes stress-activated gene expression leading to prolonged antitumor capacity of NK cells. *Blood* **128**, 1475–1489 (2016).
 603. Jabri, B. & Abadie, V. IL-15 functions as a danger signal to regulate tissue-resident T cells and tissue destruction. *Nat. Rev. Immunol.* **15**, 771–783 (2015).
 604. Richer, M. J. *et al.* Inflammatory IL-15 is required for optimal memory T cell responses. *J. Clin. Invest.* **125**, 3477–3490 (2015).
 605. Deshpande, P. *et al.* IL-7– and IL-15–Mediated TCR Sensitization Enables T Cell Responses to Self-Antigens. *J. Immunol.* **190**, 1416–1423 (2013).
 606. Liu, K., Catalfamo, M., Li, Y., Henkart, P. A. & Weng, N. P. IL-15 mimics T cell receptor crosslinking in the induction of cellular proliferation, gene expression, and cytotoxicity in CD8+ memory T cells. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 6192–6197 (2002).
 607. Correia, M. P. *et al.* Hepatocytes and IL-15: A Favorable Microenvironment for T Cell Survival and CD8+ T Cell Differentiation. *J. Immunol.* **182**, 6149–6159 (2009).
 608. Correia, M. P., Costa, A. V., Uhrberg, M., Cardoso, E. M. & Arosa, F. A. IL-15 induces CD8+ T cells to acquire functional NK receptors capable of modulating cytotoxicity and cytokine secretion. *Immunobiology* **216**, 604–612 (2011).
 609. Balin, S. J. *et al.* Human antimicrobial cytotoxic T lymphocytes, defined by NK receptors and antimicrobial proteins, kill intracellular bacteria. *Sci. Immunol.* **3**, (2018).
 610. Haluszczak, C. *et al.* The antigen-specific CD8+ T cell repertoire in unimmunized mice includes memory phenotype cells bearing markers of homeostatic expansion. *J. Exp. Med.* **206**, 435–448 (2009).
 611. Berg, L. J. Signalling through TEC kinases regulates conventional versus innate CD8+ T-cell development. *Nature Reviews Immunology* **7**, 479–485 (2007).
 612. Boyman, O., Cho, J.-H., Tan, J. T., Surh, C. D. & Sprent, J. A major histocompatibility complex class I–dependent subset of memory phenotype CD8+ cells. *J. Exp. Med.* **203**, 1817–1825 (2006).
 613. Chiu, B.-C., Martin, B. E., Stolberg, V. R. & Chensue, S. W. Cutting Edge: Central Memory CD8 T Cells in Aged Mice Are Virtual Memory Cells. *J. Immunol.* **191**, 5793–5796 (2013).
 614. White, J. T., Cross, E. W. & Kedl, R. M. Antigen-inexperienced memory CD8+ T cells: where they come from and why we need them. *Nat. Rev. Immunol.* **17**, 391–400 (2017).
 615. Hussain, T. & Quinn, K. M. Similar but different: virtual memory CD8 T cells as a memory-like cell population. *Immunol. Cell Biol.* **97**, 675–684 (2019).
 616. Quinn, K. M. *et al.* Age-Related Decline in Primary CD8+ T Cell Responses Is Associated with the Development of Senescence in Virtual Memory CD8+ T Cells. *Cell Rep.* **23**, 3512–3524 (2018).
 617. Renkema, K. R. *et al.* Two Separate Defects Affecting True Naive or Virtual Memory T Cell Precursors Combine To Reduce Naive T Cell Responses with Aging. *J. Immunol.* **192**, 151–159 (2014).
 618. Smith, N. L. *et al.* Developmental Origin Governs CD8+ T Cell Fate Decisions during Infection. *Cell* **174**, 117–130.e14 (2018).
 619. Darrah, P. A. *et al.* Multifunctional TH1 cells define a correlate of vaccine-mediated protection against *Leishmania major*. *Nat. Med.* **13**, 843–850 (2007).
 620. Rolot, M. *et al.* Helminth-induced IL-4 expands bystander memory CD8+ T cells for early control of viral infection. *Nat. Commun.* **9**, (2018).
 621. Lin, J. S. *et al.* Virtual memory CD8 T cells expanded by helminth infection confer broad protection against bacterial infection. *Mucosal Immunol.* **12**, 258–264 (2019).
 622. Lanzer, K. G., Cookenham, T., Reiley, W. W. & Blackman, M. A. Virtual memory cells make a major contribution to the response of aged influenza-naïve mice to influenza virus infection. *Immun. Ageing* **15**, 1–13 (2018).
 623. Jameson, S. C. T cell homeostasis: Keeping useful T cells alive and live T cells useful. *Semin. Immunol.* **17**, 231–237 (2005).
 624. Surh, C. D. & Sprent, J. Regulation of mature T cell homeostasis. *Semin. Immunol.* **17**, 183–191 (2005).

625. Le Champion, A. *et al.* Naive T cells proliferate strongly in neonatal mice in response to self-peptide/self-MHC complexes. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 4538–4543 (2002).
626. Min, B. *et al.* Neonates support lymphopenia-induced proliferation. *Immunity* **18**, 131–140 (2003).
627. Schüler, T., Hämmerling, G. J. & Arnold, B. Cutting Edge: IL-7-Dependent Homeostatic Proliferation of CD8⁺ T Cells in Neonatal Mice Allows the Generation of Long-Lived Natural Memory T Cells. *J. Immunol.* **172**, 15–19 (2004).
628. Goldrath, A. W., Bogatzki, L. Y. & Bevan, M. J. Naive T Cells Transiently Acquire a Memory-like Phenotype during Homeostasis-Driven Proliferation. *J. Exp. Med.* **192**, 557–564 (2000).
629. Tan, J. T. *et al.* IL-7 is critical for homeostatic proliferation and survival of naïve T cells. *Proc. Natl. Acad. Sci. U. S. A.* **98**, 8732–8737 (2001).
630. Cho, J. H. *et al.* An intense form of homeostatic proliferation of naive CD8⁺ cells driven by IL-2. *J. Exp. Med.* **204**, 1787–1801 (2007).
631. Kieper, W. C. *et al.* IL-12 Enhances CD8 T Cell Homeostatic Expansion. *J. Immunol.* **166**, 5515–5521 (2001).
632. Sandau, M. M., Winstead, C. J. & Jameson, S. C. IL-15 Is Required for Sustained Lymphopenia-Driven Proliferation and Accumulation of CD8 T Cells. *J. Immunol.* **179**, 120–125 (2007).
633. Sosinowski, T. *et al.* CD8 α ⁺ Dendritic Cell Trans Presentation of IL-15 to Naive CD8⁺ T Cells Produces Antigen-Inexperienced T Cells in the Periphery with Memory Phenotype and Function. *J. Immunol.* **190**, 1936–1947 (2013).
634. Martinet, V. *et al.* Type I interferons regulate eomesodermin expression and the development of unconventional memory CD8⁺ T cells. *Nat. Commun.* **6**, 7089 (2015).
635. Miller, C. H. *et al.* Eomes identifies thymic precursors of self-specific memory-phenotype CD8⁺ T cells. *Nat. Immunol.* 1–11 (2020). doi:10.1038/s41590-020-0653-1
636. Welsh, R. M. & Selin, L. K. No one is naive: The significance of heterologous T-cell immunity. *Nature Reviews Immunology* **2**, 417–426 (2002).
637. Rudd, B. D. *et al.* Nonrandom attrition of the naive CD8⁺ T-cell pool with aging governed by T-cell receptor: pMHC interactions. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 13694–13699 (2011).
638. Drobek, A. *et al.* Strong homeostatic TCR signals induce formation of self-tolerant virtual memory CD8 T cells. *EMBO J.* **37**, (2018).
639. Burchill, M. A. *et al.* Linked T Cell Receptor and Cytokine Signaling Govern the Development of the Regulatory T Cell Repertoire. *Immunity* **28**, 112–121 (2008).
640. Jacomet, F. *et al.* Evidence for eomesodermin-expressing innate-like CD8⁺ KIR/NKG2A⁺ T cells in human adults and cord blood samples. *Eur. J. Immunol.* **45**, 1926–1933 (2015).
641. Jin, J. H. *et al.* Virtual memory CD8⁺ T cells restrain the viral reservoir in HIV-1-infected patients with antiretroviral therapy through derepressing KIR-mediated inhibition. *Cell. Mol. Immunol.* **17**, 1257–1265 (2020).
642. Pribikova, M., Moudra, A. & Stepanek, O. Opinion: Virtual memory CD8 T cells and lymphopenia-induced memory CD8 T cells represent a single subset: homeostatic memory T cells. *Immunol. Lett.* **203**, 57–61 (2018).
643. Ponta, H., Sherman, L. & Herrlich, P. A. CD44: From adhesion molecules to signalling regulators. *Nat. Rev. Mol. Cell Biol.* **4**, 33–45 (2003).
644. English, N. M., Lesley, J. F. & Hyman, R. Site-specific de-N-glycosylation of CD44 can activate hyaluronan binding, and CD44 activation states show distinct threshold densities for hyaluronan binding. *Cancer Res.* **58**, 3736–42 (1998).
645. Baaten, B. J. G., Tinoco, R., Chen, A. T. & Bradley, L. M. Regulation of Antigen-Experienced T Cells: Lessons from the Quintessential Memory Marker CD44. *Front. Immunol.* **3**, 23 (2012).
646. Pacheco, K. A., Tarkowski, M., Klemm, J. & Rosenwasser, L. J. CD49d expression and function on allergen-stimulated T cells from blood and airway. *Am. J. Respir. Cell Mol. Biol.* **18**, 286–293 (1998).
647. Andersson, E. C., Christensen, J. P., Marker, O. & Thomsen, A. R. Changes in cell adhesion molecule expression on T cells associated with systemic virus infection. *J. Immunol.* **152**, 1237–45 (1994).
648. Christensen, J. P., Andersson, E. C., Scheynius, A., Marker, O. & Thomsen, A. R. α 4 Integrin directs virus-activated CD8⁺ T cells to sites of infection. *J. Immunol.* **154**, 5293–5301 (1995).
649. Hamann, D. *et al.* Phenotypic and functional separation of memory and effector human CD8⁺ T cells. *J. Exp. Med.* **186**, 1407–1418 (1997).
650. He, R. *et al.* Follicular CXCR5-expressing CD8⁺ T cells curtail chronic viral infection. *Nature* **537**, 412–416 (2016).
651. Im, S. J. *et al.* Defining CD8⁺ T cells that provide the proliferative burst after PD-1 therapy. *Nature* **537**, 417–421 (2016).

652. Leong, Y. A. *et al.* CXCR5+ follicular cytotoxic T cells control viral infection in B cell follicles. *Nat. Immunol.* **17**, 1187–1196 (2016).
653. Hou, S. *et al.* Virtual memory T cells orchestrate extralymphoid responses conducive to resident memory. *Sci. Immunol.* **6**, (2021).
654. Seyda, M., Elkhail, A., Quante, M., Falk, C. S. & Tullius, S. G. T Cells Going Innate. *Trends Immunol.* **37**, 546–556 (2016).
655. Zingoni, A. *et al.* NKG2D and its ligands: ‘One for all, all for one’. *Front. Immunol.* **9**, (2018).
656. Tieng, V. *et al.* Binding of Escherichia coli adhesin AfaE to CD55 triggers cell-surface expression of the MHC class I-related molecule MICA. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 2977–2982 (2002).
657. Dhanji, S. & Teh, H.-S. IL-2-Activated CD8 + CD44 high Cells Express Both Adaptive and Innate Immune System Receptors and Demonstrate Specificity for Syngeneic Tumor Cells . *J. Immunol.* **171**, 3442–3450 (2003).
658. Maasho, K., Opoku-Anane, J., Marusina, A. I., Coligan, J. E. & Borrego, F. Cutting Edge: NKG2D Is a Costimulatory Receptor for Human Naive CD8 + T Cells . *J. Immunol.* **174**, 4480–4484 (2005).
659. Meresse, B. *et al.* Coordinated induction by IL15 of a TCR-independent NKG2D signaling pathway converts CTL into lymphokine-activated killer cells in celiac disease. *Immunity* **21**, 357–366 (2004).
660. Groh, V. *et al.* Costimulation of CD8 $\alpha\beta$ T cells by NKG2D via engagement by MIC induced on virus-infected cells. *Nat. Immunol.* **2**, 255–260 (2001).
661. Jamieson, A. M. *et al.* The role of the NKG2D immunoreceptor in immune cell activation and natural killing. *Immunity* **17**, 19–29 (2002).
662. Roberts, A. I. *et al.* Cutting Edge: NKG2D Receptors Induced by IL-15 Costimulate CD28-Negative Effector CTL in the Tissue Microenvironment. *J. Immunol.* **167**, 5527–5530 (2001).
663. Kim, T.-S. & Shin, E.-C. The activation of bystander CD8+ T cells and their roles in viral infection. *Exp. & Mol. Med.* **51**, 154 (2019).
664. Zloza, A. *et al.* NKG2D signaling on CD8 + T cells represses T-bet and rescues CD4-unhelped CD8 + T cell memory recall but not effector responses. *Nat. Med.* **18**, 422–428 (2012).
665. Lanier, L. L. NKG2D receptor and its ligands in host defense. *Cancer Immunol. Res.* **3**, 575–582 (2015).
666. Grau, M. *et al.* Antigen-Induced but Not Innate Memory CD8 T Cells Express NKG2D and Are Recruited to the Lung Parenchyma upon Viral Infection. *J. Immunol.* **200**, 3635–3646 (2018).
667. Li, D., Molldrem, J. J. & Ma, Q. LFA-1 Regulates CD8+ T Cell Activation via T Cell Receptor-mediated and LFA-1-mediated Erk1/2 Signal Pathways. *J. Biol. Chem.* **284**, 21001–21010 (2009).
668. Sharma, A. *et al.* LFA-1 Ligation by High-Density ICAM-1 Is Sufficient To Activate IFN- γ Release by Innate T Lymphocytes. *J. Immunol.* **201**, 2452–2461 (2018).
669. Clark, J. T. *et al.* IL-33 promotes innate lymphoid cell-dependent IFN- γ production required for innate immunity to Toxoplasma gondii. *Elife* **10**, (2021).
670. DeGrendele, H. C., Kosfisz, M., Estess, P. & Siegelman, M. H. CD44 activation and associated primary adhesion is inducible via T cell receptor stimulation. *J. Immunol.* **159**, 2549–2553 (1997).
671. Garcia, S., DiSanto, J. & Stockinger, B. Following the development of a CD4 T cell response in vivo: From activation to memory formation. *Immunity* **11**, 163–171 (1999).
672. Yang, C.-P., Sparshott, S. M., Duffy, D., Garside, P. & Bell, E. B. The phenotype and survival of antigen-stimulated transgenic CD4 T cells in vivo: the influence of persisting antigen. *Int. Immunol.* **18**, 515–523 (2006).
673. McDermott, D. S. & Varga, S. M. Quantifying Antigen-Specific CD4 T Cells during a Viral Infection: CD4 T Cell Responses Are Larger Than We Think. *J. Immunol.* **187**, 5568–5576 (2011).
674. Whitmire, J. K., Benning, N. & Whitton, J. L. Precursor Frequency, Nonlinear Proliferation, and Functional Maturation of Virus-Specific CD4 + T Cells . *J. Immunol.* **176**, 3028–3036 (2006).
675. Whitmire, J. K. Induction and function of virus-specific CD4+ T cell responses. *Virology* **411**, 216–228 (2011).
676. Moran, A. E. *et al.* T cell receptor signal strength in Treg and iNKT cell development demonstrated by a novel fluorescent reporter mouse. *J. Exp. Med.* **208**, 1279–1289 (2011).
677. Rifa’i, M., Kawamoto, Y., Nakashima, I. & Suzuki, H. Essential Roles of CD8+CD122+ Regulatory T Cells in the Maintenance of T Cell Homeostasis. *J. Exp. Med.* **200**, 1123–1134 (2004).
678. Johnson, L. D. S. & Jameson, S. C. Self-Specific CD8 + T Cells Maintain a Semi-Naive State Following Lymphopenia-Induced Proliferation . *J. Immunol.* **184**, 5604–5611 (2010).
679. Smith, N. L. *et al.* Developmental Origin Governs CD8+ T Cell Fate Decisions during Infection. *Cell* **174**, 117–130.e14 (2018).
680. Gordon, S. M. *et al.* Requirements for Eomesodermin and Promyelocytic Leukemia Zinc Finger in the

- Development of Innate-Like CD8⁺ T Cells. *J. Immunol.* **186**, 4573–4578 (2011).
681. Hu, G. & Chen, J. A genome-wide regulatory network identifies key transcription factors for memory CD8⁺ T-cell development. *Nat. Commun.* **4**, 2830 (2013).
 682. Nolz, J. C. & Harty, J. T. Protective Capacity of Memory CD8⁺ T Cells Is Dictated by Antigen Exposure History and Nature of the Infection. *Immunity* **34**, 781–793 (2011).
 683. Freeman, B. E., Hammarlund, E., Raué, H. P. & Slifka, M. K. Regulation of innate CD8⁺ T-cell activation mediated by cytokines. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 9971–9976 (2012).
 684. Cottalorda, A. *et al.* TLR2 engagement on CD8 T cells lowers the threshold for optimal antigen-induced T cell activation. *Eur. J. Immunol.* **36**, 1684–1693 (2006).
 685. Tabiasco, J. *et al.* Human Effector CD8⁺ T Lymphocytes Express TLR3 as a Functional Coreceptor. *J. Immunol.* **177**, 8708–8713 (2006).
 686. Salerno, F., Freen-van Heeren, J. J., Guislain, A., Nicolet, B. P. & Wolkers, M. C. Costimulation through TLR2 Drives Polyfunctional CD8⁺ T Cell Responses. *J. Immunol.* **202**, 714–723 (2019).
 687. Yoshimoto, T. *et al.* IL-12 up-regulates IL-18 receptor expression on T cells, Th1 cells, and B cells: synergism with IL-18 for IFN- γ production. *J. Immunol.* **161**, 3400–3407 (1998).
 688. Xu, D. *et al.* Selective Expression and Functions of Interleukin 18 Receptor on T Helper (Th) Type 1 but not Th2 Cells. *J. Exp. Med.* **188**, 1485–1492 (1998).
 689. Chang, J. T., Segal, B. M., Nakanishi, K., Okamura, H. & Shevach, E. M. The costimulatory effect of IL-18 on the induction of antigen-specific IFN- γ production by resting T cells is IL-12 dependent and is mediated by up-regulation of the IL-12 receptor β 2 subunit. *Eur. J. Immunol.* **30**, 1113–1119 (2000).
 690. Ghanem, E. N. B., Nelson, C. C. & D’Orazio, S. E. F. T Cell-Intrinsic Factors Contribute to the Differential Ability of CD8⁺ T Cells To Rapidly Secrete IFN- γ in the Absence of Antigen. *J. Immunol.* **186**, 1703–1712 (2011).
 691. Smeltz, R. B. Profound Enhancement of the IL-12/IL-18 Pathway of IFN- γ Secretion in Human CD8⁺ Memory T Cell Subsets via IL-15. *J. Immunol.* **178**, 4786–4792 (2007).
 692. Strengell, M., Sareneva, T., Foster, D., Julkunen, I. & Matikainen, S. IL-21 Up-Regulates the Expression of Genes Associated with Innate Immunity and Th1 Response. *J. Immunol.* **169**, 3600–3605 (2002).
 693. Olesin, E. *et al.* The Transcription Factor Runx2 Is Required for Long-Term Persistence of Antiviral CD8⁺ Memory T Cells. *ImmunoHorizons* **2**, 251–261 (2018).
 694. Ganapamo, F., Dennis, V. A. & Philipp, M. T. CD19⁺ cells produce IFN- γ in mice infected with *Borrelia burgdorferi*. *Eur. J. Immunol.* **31**, 3460–3468 (2001).
 695. Lawless, V. A. *et al.* Stat4 Regulates Multiple Components of IFN- γ -Inducing Signaling Pathways. *J. Immunol.* **165**, 6803–6808 (2000).
 696. Güler, M. L., Jacobson, N. G., Gubler, U. & Murphy, K. M. T Cell Genetic Background Determines Maintenance of IL-2 Signaling: Effects on BALB/c and B10.D2 T Helper Cell Type 1 Phenotype Development. *J. Immunol.* **159**, 1767–1774 (1997).
 697. Fenimore, J. & Young, H. A. Regulation of IFN- γ expression. *Adv. Exp. Med. Biol.* **941**, 1–19 (2016).
 698. Altan-Bonnet, G. & Mukherjee, R. Cytokine-mediated communication: a quantitative appraisal of immune complexity. *Nat. Rev. Immunol.* **19**, 205–217 (2019).
 699. Kim, J. *et al.* Innate-like Cytotoxic Function of Bystander-Activated CD8⁺ T Cells Is Associated with Liver Injury in Acute Hepatitis A. *Immunity* **48**, 161–173.e5 (2018).
 700. Muntasell, A., Magri, G., Pende, D., Angulo, A. & López-Botet, M. Inhibition of NKG2D expression in NK cells by cytokines secreted in response to human cytomegalovirus infection. *Blood* **115**, 5170–5179 (2010).
 701. Fujinami, R. S. & Oldstone, M. B. A. Amino acid homology between the encephalitogenic site of myelin basic protein and virus: Mechanism for autoimmunity. *Science (80-.)*. **230**, 1043–1045 (1985).
 702. Bachem, A. *et al.* Superior antigen cross-presentation and XCR1 expression define human CD11c⁺CD141⁺ cells as homologues of mouse CD8⁺ dendritic cells. *J. Exp. Med.* **207**, 1273–1281 (2010).
 703. Brewitz, A. *et al.* CD8⁺ T Cells Orchestrate pDC-XCR1⁺ Dendritic Cell Spatial and Functional Cooperativity to Optimize Priming. *Immunity* **46**, 205–219 (2017).
 704. Eickhoff, S. *et al.* Robust Anti-viral Immunity Requires Multiple Distinct T Cell-Dendritic Cell Interactions. *Cell* **162**, 1322–1337 (2015).
 705. Rutishauser, R. L. & Kaech, S. M. Generating diversity: Transcriptional regulation of effector and memory CD8⁺ T-cell differentiation. *Immunol. Rev.* **235**, 219–233 (2010).
 706. Richer, M. J., Nolz, J. C. & Harty, J. T. Pathogen-Specific Inflammatory Milieu Tune the Antigen Sensitivity of CD8⁺ T Cells by Enhancing T Cell Receptor Signaling. *Immunity* **38**, 140–152 (2013).
 707. Schoenborn, J. R. & Wilson, C. B. Regulation of Interferon- γ During Innate and Adaptive Immune

- Responses. *Adv. Immunol.* **96**, 41–101 (2007).
708. Araki, K. *et al.* mTOR regulates memory CD8 T cell differentiation. *Nature* **460**, 108–112 (2009).
 709. Powell, J. D. & Delgoffe, G. M. The Mammalian Target of Rapamycin: Linking T Cell Differentiation, Function, and Metabolism. *Immunity* **33**, 301–311 (2010).
 710. Waickman, A. T. & Powell, J. D. Mammalian Target of Rapamycin Integrates Diverse Inputs To Guide the Outcome of Antigen Recognition in T Cells. *J. Immunol.* **188**, 4721–4729 (2012).
 711. Lekmine, F. *et al.* Interferon- γ engages the p70 S6 kinase to regulate phosphorylation of the 40S S6 ribosomal protein. *Exp. Cell Res.* **295**, 173–182 (2004).
 712. Kaur, S. *et al.* Regulatory effects of mammalian target of rapamycin-activated pathways in type I and II interferon signaling. *J. Biol. Chem.* **282**, 1757–1768 (2007).
 713. Kaur, S. *et al.* Role of the Akt pathway in mRNA translation of interferon-stimulated genes. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 4808–4813 (2008).
 714. Noto, A., Ngauv, P. & Trautmann, L. Cell-based flow cytometry assay to measure cytotoxic activity. *J. Vis. Exp.* 6–11 (2013). doi:10.3791/51105
 715. Varadarajan, N. *et al.* A high-throughput single-cell analysis of human CD8 + T cell functions reveals discordance for cytokine secretion and cytotoxicity. *J. Clin. Invest.* **121**, 4322–4331 (2011).
 716. Setoguchi, R. IL-15 boosts the function and migration of human terminally differentiated CD8+ T cells by inducing a unique gene signature. *Int. Immunol.* **28**, 293–305 (2016).
 717. Geginat, J. *et al.* Proliferation and differentiation potential of human CD8+ memory T-cell subsets in response to antigen or homeostatic cytokines. *Blood* **101**, 4260–4266 (2003).
 718. Beura, L. K. *et al.* Normalizing the environment recapitulates adult human immune traits in laboratory mice. *Nature* **532**, 512–516 (2016).
 719. Fulton, R. B. *et al.* The TCR's sensitivity to self peptide-MHC dictates the ability of naive CD8 + T cells to respond to foreign antigens. *Nat. Immunol.* **16**, 107–117 (2015).
 720. Hogquist, K. A. & Jameson, S. C. The self-obsession of T cells: How TCR signaling thresholds affect fate 'decisions' and effector function. *Nat. Immunol.* **15**, 815–823 (2014).
 721. Kim, H. J. *et al.* CD8+ T regulatory cells express the Ly49 class I MHC receptor and are defective in autoimmune prone B6-Yaa mice. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 2010–2015 (2011).
 722. Sakuraba, K., Shibata, K., Iwamoto, Y., Yoshikai, Y. & Yamada, H. Naturally Occurring PD-1+ Memory Phenotype CD8 T Cells Belong to Nonconventional CD8 T Cells and Are Cyclophosphamide-Sensitive Regulatory T Cells. *J. Immunol.* **190**, 1560–1566 (2013).
 723. Lin, S., Peacock, C. D., Bahl, K. & Welsh, R. M. Programmed death-1 (PD-1) defines a transient and dysfunctional oligoclonal T cell population in acute homeostatic proliferation. *J. Exp. Med.* **204**, 2321–2333 (2007).
 724. Kim, H.-J., Verbinnen, B., Tang, X., Lu, L. & Cantor, H. Inhibition of follicular T helper cells by CD8+ Treg is essential for self tolerance. *Nature* **467**, 328–332 (2010).
 725. Dai, H. *et al.* Cutting Edge: Programmed Death-1 Defines CD8 + CD122 + T Cells as Regulatory versus Memory T Cells. *J. Immunol.* **185**, 803–807 (2010).
 726. Boyden, A. W., Brate, A. A., Stephens, L. M. & Karandikar, N. J. Immune Autoregulatory CD8 T Cells Require IFN- γ Responsiveness to Optimally Suppress Central Nervous System Autoimmunity. *J. Immunol.* **205**, 359–368 (2020).