

Neuroimmune responses in viral infection

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Abstract

The regulation of the immune responses is important in maintaining good health. Interactions between the nervous and immune systems are increasingly studied and widely appreciated to be influential in orchestrating immune responses. T cells express adrenergic receptors (AR) that enable them to respond to neurotransmitters produced by the sympathetic nervous system (SNS), noradrenaline (NA) and adrenaline, inducing downstream signalling and modulating cell functions, although whether this is stimulatory or inhibitory in T cell antiviral responses is unclear. In this thesis, I examine the effects of SNS in various models of viral infection through chemical sympathectomy and treatment with AR agonists. Modulation of sympathetic signals in systemic infections with LCMV had minor influences on T cell responses but resulted in increased viral loads. Notably, the infection results in a loss of tyrosine hydroxylase (TH) positive sympathetic fibres in the spleen as early as day 3 post infection and is reflected by decreased NA splenic NA. The immune response may play a role with interferon γ partially contributing to the depletion. Additionally, this thesis also investigates the capability of long-lasting resident memory T cell (T_{RM}) responses in the highly innervated, immune-privileged cornea. Using a model of herpes infection of the cornea, I showed that T cells are effectively recruited to the cornea with a small heterogeneous population able to persist following cessation of immune responses. These cells express CD69 and CD103, canonical markers of tissue residency, to varying degrees. Persistence, but not recruitment, of these cells is dependent on antigen availability at the cornea. These memory cells are capable of responding to secondary encounters with antigen. Moreover, circulating memory cells are also able to infiltrate the immune-privileged cornea more efficiently following infection. Together, these results highlight the important nuances in the regulation of immune responses by the nervous system.

Declaration

The work presented in this thesis was conducted at The University of Melbourne in the laboratory of Prof. Scott Mueller and at Monash University (Parkville) in the laboratory of A/Prof. Erica Sloan.

This is to certify that:

- (i) The these comprises only my original work except where indicated in the preface
- (ii) Due acknowledgement has been made in the text to all other materials used
- (iii) The thesis is less than 100,000 words in length, exclusive of tables, figures and references

Joon Keit Loi

Preface

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My contribution to experiments in each of the chapters were as follows:

Chapter 3: 95%

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Abbreviations

6-OHDA	6-hydroxydopamine
ACAID	Anterior chamber associated immune deviation
ACh	Acetylcholine
APC	Antigen presenting cell
ASC	Antibody secreting cells
ATP	Adenosine triphosphate
BDNF	Brain derived nerve growth factor
BEC	Blood endothelial cell
BMP2	Bone morphogenic protein 2
cAMP	Cyclic adenosine monophosphate
cDC	Conventional dendritic cell
cfu	Colony forming units
ChAT	Choline acetyltransferase
CNS	Central nervous system
CSF	Colony stimulating factors
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte-associated protein 4
Dbh	Dopamine hydroxylase
DC	Dendritic cell
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay

Erk	Extracellular signal-regulated kinase
FDC	Follicular dendritic cell
FRC	Fibroblastic reticular cell
GC	Germinal centre
GFP	Green fluorescent protein
GPCR	G protein coupled receptor
HPA	Hypothalamic-pituitary-adrenal
HSK	Herpes stromal keratitis
HSV	Herpes simplex virus
i.p.	Intraperitoneal
i.v.	Intravenous
IAV	Influenza A virus
IFN	Interferon
IL	Interleukin
ILC	Innate lymphoid cell
iRBC	Infected red blood cells
KLF2	Krüppel-like factor-2
KLRG1	Killer cell lectin-like receptor 1
LCMV	Lymphocytic choriomeningitis virus
LCMV CL13	Lymphocytic choriomeningitis virus Clone 13
LEC	Lymphatic endothelial cell
LESC	Limbal epithelial stem cells
LN	Lymph node
LPS	Lipopolysaccharide

MAPK	Mitogen-activated protein kinases
MDCK	Madin Darby canine kidney
MHC	Major histocompatibility complex
MPEC	Memory precursor effector cell
mRNA	Messenger ribonucleic acid
NA	Noradrenaline
NGF	Nerve growth factor
NK	Natural killer
NPY	Neuropeptide Y
NT3	Neurotrophin factor 3
PAMP	Pathogen associated molecular pattern
PaNS	Parasympathetic nervous system
PBS	Phosphate buffered saline
PD-1	Programmed cell death-1
pDC	Plasmacytoid dendritic cell
pfu	Plaque forming units
PKA	Protein kinase A
PNS	Peripheral nervous system
PRR	Pattern recognition receptor
qPCR	Quantitative real time polymerase chain reaction
RNA	Ribonucleic acid
RP	Red pulp
S1PR1	sphingosine-1-phosphate receptor 1
SHG	Second harmonic generation

SLEC	Short lived effector cell
SLO	Secondary lymphoid organ
SNS	Sympathetic nervous system
SPG	Sucrose-phosphate-glyoxylic acid
TCM	Circulating memory T cell
TCR	T cell receptor
TCZ	T cell zone
TEM	Effector memory T cell
Tfh	T follicular helper
TGF β	Transforming growth factor β
Th	T helper
TH	Tyrosine hydroxylase
TNF	Tumour necrosis factor
Treg	Regulatory T
TRM	Resident memory T cell
VEGF	Vascular endothelial growth factor
VSV	Vesicular stomatitis virus
VV-OVA	Vaccinia-OVA
WP	White pulp
WT	Wild type

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[Chapter 4:](#)

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Chapter 1 – Literature Review

1.1 The immune system

Humans are frequently exposed to microorganisms which have the potential to cause damage to the body. The immune system has evolved as a mean of protecting the body from infection by pathogens such as bacteria, viruses, and parasites while tolerating those that exist as part of the natural microenvironment. This system involves a complex array of molecules and cells that act in tandem to produce an effective immune response that is able to recognise foreign particles from invading pathogens and remove them from the body. The system also has to be able to distinguish non-infected and 'self' cells in order to mitigate any potential damage and killing of these cells. Thus, there are multiple tightly regulated mechanisms in limiting these responses. Failure or dysregulation of these mechanisms can result in immunopathology or autoimmune diseases which can negatively impact health.

The immune system can be divided into two major components – the innate immune system and the adaptive immune system. The innate arm acts as a rapid line of defence whilst the adaptive arm is capable of more specific and higher magnitude responses. While cells of the immune system can be broadly categorised to belong to either one of these arms, there is significant evidence of crosstalk between these two components which is required for the most effective immune responses with some cells playing a role in both components.

The innate immune system, consisting of acellular and cellular components, provides protection against pathogens by acting in a broad and non-specific manner. Physical barriers, such as the epithelium and mucosal lining, and chemical barriers, such as pH levels, of organs that are in contact with the environment, prevent foreign particles from entering the body whereas the complement system which is made up of a variety of proteins that can bind to pathogens and aid in their removal or play a role in enhancing adaptive immunity [1, 2]. Cells of the innate response include phagocytes such as macrophages, neutrophils, and dendritic

cells (DCs) that are capable of engulfing and removing foreign pathogen and cellular debris through phagocytosis. Following phagocytosis, these particles are broken down into smaller compounds via enzymatic processes. Macrophages and DCs also function as antigen presenting cells (APCs) and are capable of displaying peptide antigen loaded onto major histocompatibility (MHC) molecules on the surface for recognition by cells in the adaptive immune system [3]. These cells also express pattern recognition receptors (PRR) that recognise pathogen associated molecular patterns (PAMPs) such as bacterial lipopolysaccharide (LPS). Recognition of these PAMPs result in the production of various pro-inflammatory cytokines such as type-I interferons, IL-12, and IL-6 as well as activation molecules that allow for more efficient adaptive responses [4]. Innate lymphoid cells (ILCs) are a group of cells that have been recently described to be able to respond in a similar fashion to counterparts in the adaptive immune system. ILC are present in all tissues and can induce inflammation that is capable of modulating immune responses [5, 6]. One of the most well-studied ILC is the natural killer (NK) cell, capable of both effective direct and indirect killing of infected and cancer cells [7].

Whilst innate immunity is crucial in the response to pathogens, it alone is usually insufficient to clear an infection that has progressed. Moreover, there is generally no additional increase in the response to subsequent encounters of the same pathogen. Conversely, the adaptive immune system is able to produce a more robust response with secondary exposure to the same pathogen resulting in a more rapid and effective immune response.

1.1.1 The adaptive immune system

The adaptive arm of the immune system can be further compartmentalised into the humoral and cellular components. Compared to the innate response, both components of the adaptive response require up to 5 days or longer before peak effector functions can be carried out. However, the resulting response is highly specific and can lead to efficient clearing of the pathogen. The humoral component is reliant on antibodies secretion by B cells that bind to pathogens and target them for removal. B cells mature in the bone marrow and express a B cell receptor which when bound to its cognate antigen will result in activation of the B cell and differentiation into antibody secreting cells. B cells have been documented to have regulatory roles in controlling the humoral response to prevent uncontrolled damage [8]. Importantly, antibodies are only effective against extracellular components of pathogens as they are unable to enter the cell membrane of infected cells. As such, T cells from the cellular component play the role of targeting these cells for protection against intracellular pathogens. B cells can be important in orchestrating T cell responses as B cells are capable of acting as an APC for T cells [9].

Precursor cells from the bone marrow enter the thymus where they mature into naïve T cells following a series of positive and negative selection. T cells express membrane bound T cell receptors (TCR) that recognise antigen processed by APCs and presented on major histocompatibility (MHC) molecules. There are two classification of T cells – “conventional” and “unconventional” T cells. Populations of “unconventional” T cells are also found in most tissues, including natural killer T cells, $\gamma\delta$ T cells, and mucosal associated invariant T cells express TCRs which bind lipid-based antigens and other metabolites loaded onto MHC-like molecules. These T cells have been documented to augment immune responses by rapidly producing pro-inflammatory cytokines [10-12]. Conventional T cells are typically expressing

the co-receptors CD4 or CD8 as well as expressing an $\alpha\beta$ -TCR that binds to peptide antigens loaded on MHC molecules. CD4 T cells are capable of recognising antigen loaded into MHC class II present on APCs while CD8 T cells recognise antigen on MHC class I present on all cells. Specific DCs and APCs are capable of cross-presentation which involves the ability to take up exogenous antigen and present them on MHC class I, allowing for effective priming of CD8 T cell responses [13]. Once the pathogen has been successfully cleared by effector responses of immune cells, a system to induce cessation of the response is initiated resulting in one of the defining features of adaptive responses, which is the capability of both B and T cells to form immunological memory. This pool of antigen-experienced cells is then capable of rapid and robust responses following secondary encounters of the same pathogen [14, 15].

1.1.2 The spleen as an important secondary lymphoid organ

The secondary lymphoid organs (SLO), namely the spleen and lymph nodes (LN), are important sites for the initiation of immune responses. The SLO architecture is maintained by a network of stromal cells that also play an important role in orchestrating the immune response. They line the lymphoid compartments of both the spleen and LN, producing cytokines and supporting the function of lymphocytes [16]. These include fibroblastic reticular cells (FRC), follicular dendritic cells (FDC), lymphatic endothelial cells (LEC), and blood endothelial cells (BEC) [17]. LEC and BEC are important in the establishment of vessels that supply the SLO while FDCs are important in the activation of B cells in the follicle and the establishment of germinal centres [18, 19]. Notably, FRCs produce IL-7 and other molecules that support naïve T cells as well as allowing for expansion of the SLO to accommodate rapidly dividing immune cells over the course of the infection [20]. The LN can be divided into the cortex and the medulla. Lymphocytes enter the LN via high endothelial venules found

supplying the cortex. Lymphatic vessels connect the LN to peripheral tissues, allowing APCs to transport antigen found in the peripheral sites to the LN. Lymphocytes home to specific areas within the LN to survey these APCs. T cells generally home to the paracortical and interfollicular areas whilst B cells migrate towards the follicles in the cortical regions based on the respective chemokine gradient [21]. The LN is an important site for immune responses as the priming of naïve cells occur here to initiate the activation of those cells.

The spleen consists of two main compartments – the red pulp (RP) and the white pulp (WP) [22] (Fig 1.1). RP within the spleen not only acts as a filtration system for the blood but also houses macrophages that clear particles in the blood and remove dying red blood cells [23]. Lymphoid compartments within the spleen are contained in the WP which can be subdivided into a T cell zone (TCZ), and B cell follicles where T cells and B cells are found respectively [24] (Figure 1.1). The T cell zone has a high concentration of CCL19 and CCL21 produced by T cell zone FRCs which is recognised by CCR7 expressed by both T cells and activated B cells, allowing for trafficking between the follicle and TCZ of the WP [25]. Similarly, the follicles have high levels of CXCL13 which binds to CXCR5 expressed by B cells [26]. The TCZ is also rich with blood vessels and conduit-like bridging channels, formed by EPCAM production of FRCs [27], acting as a channel to the marginal zone located at the edges of the WP [28]. A number of macrophage populations also reside in the marginal zone and surrounding areas. In the murine spleen, CD8 and CD4 T cells are compartmentalised in the TCZ with CD4 T cells distributed in the outer areas of the TCZ and CD8 T cells in the central area [29, 30]. Following activation, CD8 T cells are further compartmentalised and migrate

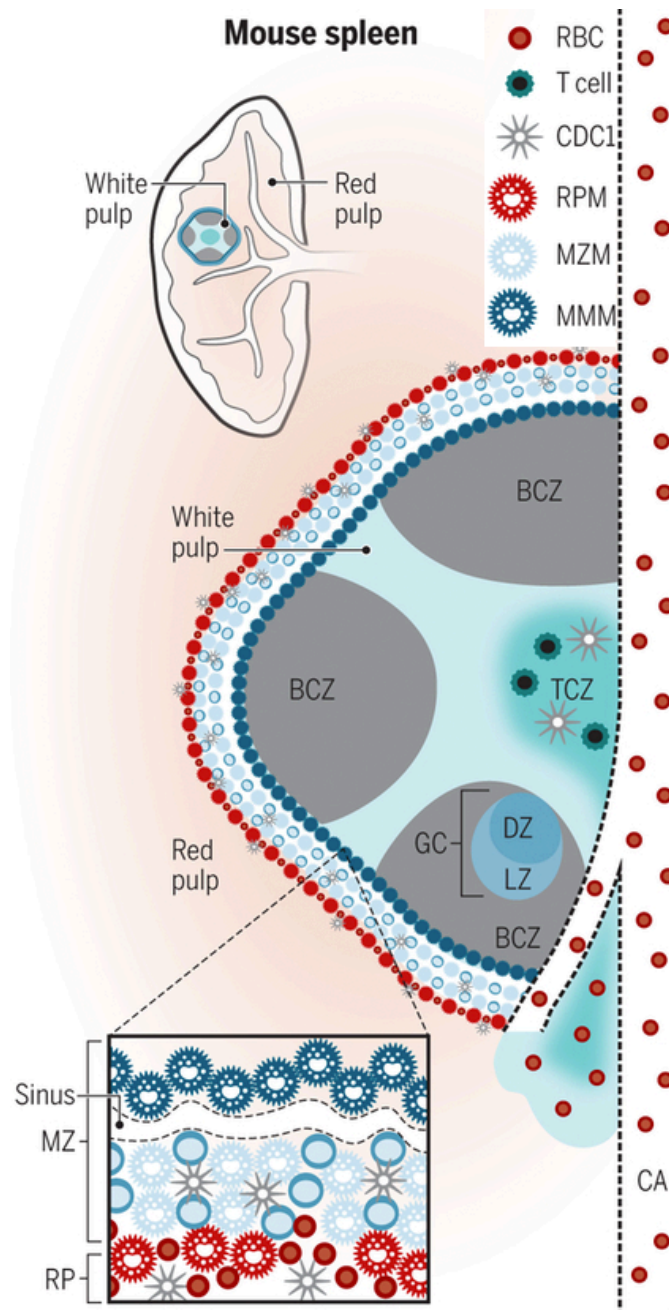


Figure 1.1 Architecture of the spleen

Schematic of the spleen depicting the white pulp and red pulp (RP) in the top left of the image. Close up view of a mouse white pulp. Inset shows the organisation of cells around the marginal zone (MZ). B cell zone (BCZ) located in the follicles contain germinal centres (GC) that can be split into the light zone (LZ) and dark zone (DZ). The follicles are located in the periphery of the white pulp with the T cell zone (TCZ), containing cDC and T cells, located more centrally. The central artery (CA) provides blood supply. Various macrophages also play an important role in the spleen. Adapted from [31]

out of the WP through the bridging channels [32]. Some CD4 T cells are also able to migrate into the follicles to interact with and provide help to B cells through upregulation of CXCR5 [33]. DCs in the spleen play an important role in the initiation of immune responses to systemic blood-borne antigen due to the lack of afferent lymphatics inhibiting DC trafficking from peripheral tissues. DCs are located throughout the spleen and are able to migrate into the WP and the TCZ through the upregulation of CCR7, allowing these DCs to come into contact with T cells to initiate T cell priming [34]. Thus, the spleen can act as a hub of activity for adaptive responses and the interactions that occur here are important in an effective adaptive immune response.

1.1.3 Role of T cells in an immune response

During steady state, mature naïve T cells circulate throughout the body via the blood and lymphatic systems. When T cells enter the LN, there is the opportunity for these cells to encounter their cognate antigens that may be presented by APCs, primarily by DCs, that process and present antigens locally in the LN or migrate to the draining LN after acquiring antigens at distal sites of infection [35]. Effective activation of T cells typically requires three signals. Interaction of T cells with their cognate antigen presented on APCs result in the production of signal 1. Signal 2 is the interaction of co-stimulatory molecules between T cells and APCs while signal 3 is the binding of cytokines in the microenvironment to their respective receptors expressed on the activated T cells [36-38]. Following these signals, CD69, a lectin molecule capable of down-regulating sphingosine-1-phosphate receptor 1 (S1PR1) expression resulting in reduced egress from LNs, is upregulated [39]. This reduced egress causes T cells to remain longer in the LN and cluster with APCs resulting in receiving further activation signals either via cytokines secreted into the LN microenvironment or co-

stimulatory molecules expressed on APCs thus promoting the differentiation into different effector T cells [40-42]. T cells are broadly categorised as CD8⁺ cytotoxic T lymphocytes (CTL) and CD4⁺ T helper (Th) cells due to their effector functions. Activated T cells can then leave the LN and preferentially home to non-lymphoid tissues to control the infection [43].

Once recruited to the site of inflammation, CTL and Th cells are able to carry out their effector functions. CTL are responsible for the killing of infected or damaged cells through the production of cytokines such as granzyme, perforin, and interferon gamma (IFN γ). These molecules have the capacity to damage surrounding cells and thus need to be regulated [44]. Activated CTL can be classified as short lived effector cells (SLEC) or memory precursor effector cell (MPEC) based on expression of the killer cell lectin-like receptor 1 (KLRG1) and IL7 receptor [45, 46]. Following cessation of CD8 T cell responses, MPECs are able to develop into memory CTL, poised for secondary encounters with the same pathogen though a small percentage of SLECs may also survive to form memory cells [47, 48].

Th cells provide support to CD8⁺ T cells and B cells during an immune response. Th cells play a different role depending on the type of immunogen involved [49]. The primary method of support provided by Th cells are in the form of cytokines and co-stimulatory molecules. Specialised T follicular helper cells (Tfh) are crucial in providing help for B cells and promote antibody responses [50]. Similarly, most Th subtypes such as Th1, Th2, Th17 also function to boost an immunological response. Th1 cells are important for cell-mediated immunity and clearance of intracellular pathogens whereas Th2 cells drive humoral immunity and are important in clearance of extracellular pathogens [51]. Th17 cells have been documented to be important for the response to some bacterial and fungal infections though they are also well known to be implicated in a variety of autoimmune conditions [52]. Regulatory T (Treg) cells act to dampen an immune response which are especially important

for the regulation and cessation of the immune response to avoid excessive cell damage due to inflammatory conditions [53].

The normal functioning of T cells is regulated by multiple systems to ensure a homeostatic balance is achieved. One widely discussed area is the crosstalk between the immune system and the nervous system which occur via the exchange of cytokines and neurotransmitters that can act on and influence cell types from the other system.

1.2 The nervous system

The nervous system is a complex network of cells and molecules that has developed to allow organisms to perceive and respond to the environment. One of the cell types within the nervous system responsible for this are called neurons which are cells with large cell bodies and long axons that connect one cell to another.

The connection between these cells is regulated by the release of neurotransmitters in the synaptic space between axons. In more evolved organisms such as mammals, the nervous system can be split into the central nervous system (CNS) and peripheral nervous system (PNS). The brain and spinal cord make up the CNS while the PNS are the nerves that connect the rest of the body to the CNS. The PNS can be further divided into the somatic nervous system, and the autonomic nervous system. Voluntary movements such as the contraction of skeletal muscle is governed by the somatic nervous system. Conversely, the autonomic nervous system is largely responsible for unconscious mode of actions, such as those involved in maintaining homeostasis and the regulation of bodily functions. There are three branches of the autonomic nervous system – the enteric nervous system, parasympathetic nervous system (PaNS), and sympathetic nervous system (SNS) [54]. One feature of the autonomic nervous system is a “two-step” process involving the differentiation of pre-ganglionic and

post-ganglionic neurons thus limiting the amount of direct impact of the CNS on the functioning of the signalling down the ganglia.

1.2.1 The parasympathetic nervous system

The PaNS is responsible for “rest and digest” responses such as those of reproductive or digestive nature. Ganglia associated with the PaNS are found distally from the spinal cord, usually close to the organ it supplies. As such, the preganglionic neurons tend to be longer than the postganglionic neurons. Signalling through the PaNS is dependent on the neurotransmitter acetylcholine (ACh) which is synthesised by the enzyme choline acetyltransferase (ChAT), characteristic of ACh producing fibres [55]. ACh is able to bind to both muscarinic and nicotinic receptors found in the ganglia and at neuromuscular junctions of various peripheral organs [56, 57]. Muscarinic receptors belong to the family of G protein coupled receptors and can be categorised into 5 different family subtypes (m1-m5) [58]. These muscarinic receptors, m2 and m4, are grouped as “even” while m1, m3, and m5 are grouped as “odd” subfamilies. The former group signals through increased intracellular Ca^{2+} signalling while the latter is dependent on decreased cyclic adenosine monophosphate (cAMP) production [59]. Inhibitory effects of muscarinic signalling is best attributed to the stimulation of “even” muscarinic receptors [60]. Nicotinic receptors differ from muscarinic receptors in that these receptors are capable of binding nicotinic compounds in addition to ACh. Similarly, there are also a number of nicotinic receptor subtypes which produces a range of functions depending on the location of the receptor [61].

1.2.2 The sympathetic nervous system

Signalling through the SNS results in “fight-or-flight” responses that function to ensure the survival of the organism during moments of danger. Sympathetic preganglionic neurons are typically shorter while postganglionic neurons are longer as the sympathetic ganglia are usually found closer to the spinal cord [62]. The catecholamines noradrenaline (NA) and adrenaline act as the neurotransmitter of the SNS. While ACh is typically used by the parasympathetic nervous system, preganglionic release of ACh and binding to nicotinic receptors in the ganglia is required for the production and subsequent release of NA from the postganglionic neurons [63]. These catecholamines are produced as a product of a series of enzymatic reactions involving the enzyme tyrosine hydroxylase (TH) as the rate-limiting step and so TH is reliably used as a surrogate marker for the identification of sympathetic fibres [64]. In addition to TH, there are several other markers that can be used to identify sympathetic fibres. Neuropeptide Y (NPY), a tyrosine rich peptide, has been found to co-localise with NA in postganglionic neurons [65, 66]. Dopamine hydroxylase (Dbh) is another enzyme involved in the synthesis of catecholamines and is therefore closely associated with sympathetic fibres. Using 6-hydroxydopamine (6-OHDA) as a means for chemical sympathectomy, serum levels of Dbh decreased compared to non-treated mice [67, 68].

NA and adrenaline are able to elicit responses by binding to α or β adrenergic receptors (adrenoceptors). Signalling through α_1 or α_2 -adrenoceptors (α_1 , α_2) and β_1 , β_2 or β_3 -adrenoceptors (β_1 , β_2 , β_3) can lead to different outcomes due to coupling with different down-stream pathways [69, 70]. α -adrenoceptors can be further subdivided into subtypes α_{1a} , α_{1b} and α_{2a} , α_{2b} , and α_{2c} . Adrenoceptors are G-protein coupled receptors (GPCR) that consist of an extracellular domain, seven transmembrane α -helices, and an intracellular domain [71]. Ligand binding to the extracellular domain of adrenoceptors induces conformational changes

that triggers signalling pathways via the G-proteins associated with the intracellular domain. Adrenoceptors largely utilise the $G\alpha$ proteins which can be categorised as $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$, and $G\alpha_{12}$ [72]. α_1 adrenoceptors typically signal through $G\alpha_{q/11}$, α_2 adrenoceptors through $G\alpha_{i/o}$, and β -adrenoceptors through $G\alpha_s$ and $G\alpha_i$ isoforms [73-75]. Synthetic compounds are also able to bind to these receptors in an antagonistic manner, preventing ligands to bind and inhibiting recruitment of these G proteins for downstream signalling.

1.2.3 Innervation of the secondary lymphoid organs

SLO have been found to be innervated by the SNS but not the PaNS [76, 77]. In many tissues, innervation of both the sympathetic and parasympathetic arms are crucial in maintaining a homeostatic balance [78]. LNs have been found to be innervated by sympathetic nerves which enter through the hilar region and continue into the medullary regions. These nerves also run along medullary cords and terminate throughout the paracortical region, coming into contact with T lymphocytes [79]. The sympathetic neurons enter the spleen through the hilar region closely associated with the vasculature and amongst the PALS. Some of the varicosities were found to extend out into the parenchyma where T cells are found. Additionally, there is little to no extension of these fibres into the follicular regions of the white pulp [80, 81]. This splenic sympathetic innervation was found to originate mostly from the celiac ganglia though a small percentage of neurons can be traced back to the superior mesenteric ganglia [82]. These sympathetic fibres provide a local source of catecholamines which could act on the surrounding cells in their proximity. Recent work with optical clearing of the SLO have shown the extensive network of TH^+ fibres which co-localise with synaptophysin, a generic marker of nerve fibres [83]

There are a range of factors which may alter the sympathetic innervation of SLO. Studies have shown age to have an effect on innervation [84-86] which can shift the homeostatic balance previously established. Interestingly, environmental factors such as stress appears to also induce an increase in the density of innervation of these organs [87-89]. This may be attributed to an increase in levels of various growth factors such as the nerve growth factor (NGF) which plays a major role in the maintenance of the SNS [90, 91]. The increase in nerve density may act as a mechanism used by the nervous system to modulate responses in the SLO since an increase in nerve fibres may translate to an increase in catecholamine release upon activation which can then lead to an increase in local catecholamine levels.

1.2.4 Physiological effects of sympathetic signalling

Catecholamine signalling results in multiple physiological changes throughout the body, particularly in having a degree of influence on the vasculature system. Expression of the adrenoceptor subtypes greatly influences the downstream effects that occur. High levels of receptor expression by cells of the cardiovascular and respiratory system, and skeletal muscle provides significant insight into the effects of the SNS on these signalling pathways [92-96]. Generally, activation of α_1 can lead to increased intracellular Ca^{2+} levels [97] while α_2 stimulation can result in decreased cAMP levels [98] and also has an effect on extracellular signal-regulated kinase (Erk) pathways [99]. As a consequence, there is reduced activation of transcription factors involved in the protein kinase A (PKA) pathway. β adrenoceptor activation can result in changes to downstream signalling pathways such as those involving mitogen-activated protein kinases (MAPK)/Erk activity. These pathways are important in a variety of processes including cell replication and migration [100, 101].

α -adrenoceptors are important in the control and modulation of the vasculature. The extent to which the vasculature is affected is dependent on the vessel and adrenoceptor involved. α_1 stimulation is able to elicit vasoconstriction of larger arterioles and venules while α_2 stimulation can constrict smaller precapillary arterioles [102-104]. There is also evidence that α_2 signalling may contribute to the modulation of vasculature contractility via indirect or protein-protein interactions [105]. Feedback loops exist within the SNS to regulate the level of NA and hence SNS signalling in the system with α_2 implicated to be responsible for this mechanism [106]. Lymphatic vessels are also capable of responding to adrenergic signalling with activation of β -adrenoceptors on lymphatic endothelial cells reducing the contractility while α -adrenoceptors increasing contractility of lymphatics [107]. Additionally, one study showed that SNS activity acting on non-hematopoietic cells resulted in changes in circadian rhythm control and affecting leucocyte distribution [108]. Together, these effects of adrenoceptor signalling provide a means which allows for the body to respond to scenarios which require immediate action to avoid scenarios that may be life-threatening.

1.3 Immune and nervous system crosstalk

It is well documented that there is significant interaction between the nervous and immune systems. Both systems are capable of producing cytokines and other compounds that can affect cells of the other system. One of the well-documented relationships is the hypothalamic-pituitary-adrenal (HPA) axis which involves the slow release of glucocorticoids due to a continuous stress stimulus to dampen inflammation [109]. Certain subsets of immune cells are also able to produce peptides and compounds characteristic of nervous systems as well as express receptors that can bind to these compounds, providing yet another method in which the two systems can communicate. The implications of this relationship are

not only important in ensuring homeostasis but can also dictate the outcome of clearing infections and cancer or affect how the body changes such as during aging. There has been developing interest in how the SNS can affect immune responses, but the mechanisms and effects are not clearly defined. The expression of adrenoceptors on immune cells of both the innate and adaptive immune system suggests significant potential for neuroimmune interactions [110, 111].

1.3.1 Sympathetic modulation of innate immune responses

It is evident that the SNS is able to modulate innate immune responses with high levels of adrenoceptors present on innate immune cells. SNS signalling often results in inhibition of innate immune responses resulting in anti-inflammatory microenvironments in both mouse and human models [112, 113] though there are also documented cases of pro-inflammatory responses [111]. The general view is that β -adrenoceptor activation inhibits while α -adrenoceptor activation promotes innate responses. Adrenergic signals augment neutrophil function through modulating trafficking and cytokine expression. mRNA expression of both α and β -adrenoceptors was found in neutrophils with β_3 and β_2 being the most highly expressed [114]. Prolonged treatment with NA suppressed neutrophil function by decreasing IFN γ production, IL-10, and neutrophil activation and phagocytosis [115]. This is likely a β -adrenoceptor dependent effect as activation of β_2 with increasing dosage of catecholamines in older studies was found to suppress chemotaxis, phagocytosis, and degranulation [116-118]. Moreover, β -adrenoceptor signalling also affects neutrophil migration by altering expression of adhesion molecules present on endothelial cells which allow neutrophils to adhere and complete trans-endothelial migration [108, 119]. Though there are limited studies investigating the role of α -adrenoceptor activation on neutrophils, there is evidence that α -

adrenoceptors are thought to enhance neutrophil migration and amplification of acute inflammation [120, 121] via direct or indirect methods. In cases of autoimmune disease, adrenoceptor signalling appears to increase the severity of these diseases by increasing neutrophil activity [122].

Studies of human NK cells showed expression of β_2 , α_1 , and α_2 expression with rodent studies validating this through functional studies [123, 124]. NK cells are particularly sensitive to the effects of SNS signalling which is dependent on the length of exposure [125]. Acute signals were found to increase NK cell mobilisation [126, 127] but continued expression can reduce numbers in the circulation [128]. The functionality of NK cells is impeded by β -adrenoceptor signalling through the decrease in essential cytokines for NK cell activity [129-131].

Adrenoceptor expression on monocytes and macrophages can differ as monocytes develop into macrophages. Monocytes express both β_1 and β_2 but as they mature into macrophages levels of β_2 increase and they are then also capable of expressing β_3 [132-134]. α -adrenoceptor expression is not well characterised but there is evidence that suggests that they may be present on monocytes and signalling through β -adrenoceptors may also induce α -adrenoceptor expression [135, 136]. While the effects of SNS signalling in monocytes and macrophages mimic that of neutrophils, there appears to be more variance in the recorded responses. β -adrenoceptor activation resulted in a decrease in IL-12 secretion by LPS-activated macrophages [129, 137, 138] and IFN γ -mediated killing [139, 140]. Further evidence of the anti-inflammatory effects of β -adrenoceptor activation is documented by the increase in IL-10 production by macrophages – a cytokine well known to dampen immune responses [141, 142]. Moreover TNF α , a regulator of the pro-inflammatory cytokine IL-1 β , release is inhibited following β_2 stimulation [143, 144]. Macrophages were also skewed towards an M2

anti-inflammatory phenotype when co-treated with LPS and NA or adrenaline [145]. Conversely, α -adrenoceptor signalling enhanced inflammatory responses in macrophages increased phagocytosis and TNF α secretion [146, 147]. Interestingly, enhanced responses were also documented through β -adrenergic signals in specific subsets of macrophages. CD64⁺CCR2⁺ macrophages in the cornea produced more pro-inflammatory cytokines while muscularis macrophages showed enhanced tissue-protective properties following β -adrenoceptor activation [148, 149].

Finally, the SNS is able to influence DC function through modulating antigen presentation, expression of activation molecules, and cytokine production, thus indirectly affecting adaptive immune responses [150]. Notable adrenoceptor expression on DCs is the expression of β_2 and α_1 with the possibility of α_2 as well [151, 152]. Studies have shown that treatment of skin derived DCs with NA hinder antigen presentation but can improve DC migration to LNs in a β -adrenoceptor and α -adrenoceptor dependent manner respectively as demonstrated using specific α or β blockers [153, 154]. β -adrenoceptors may also potentially play a role in DC migration as their activation is documented to improve migration to LNs in hypersensitivity responses [155]. With regards to antigen processing, NA was found to increase DC endocytosis via α -adrenoceptors but impedes cross-presentation via β -adrenoceptors [152, 156]. Treatment with β -adrenoceptor antagonists improves the production of pro-inflammatory cytokine by DCs suggesting a role for the β -adrenoceptors in creating a pro-inflammatory microenvironment [156]. Additionally, adrenoceptor activation on DCs is able to skew production of cytokines that influence the following adaptive immune response such as the preferential differentiation to favour Th2 or Th17 responses [157, 158].

1.3.2 Sympathetic modulation of adaptive immune responses

The effects of SNS signalling on T and B cell function is of particular interest as a target for manipulation of responses and therapeutics in cases of infection and autoimmunity. B and T cells predominantly express β_2 , with B cells having higher levels of the receptor with little to no evidence of α -adrenoceptor expression [159-162]. CD4 T cells were found to have lower levels of β_2 compared to CD8 T cells thus implying that there is less effect of adrenoceptor stimulation on these cells [163]. Given the higher expression of β -adrenoceptors on B and T cells, most studies have focused on the effects of stimulating these receptors.

Migration of T cells can be influenced by adrenoceptor signalling and can be attributed to MAPK pathways [164, 165]. Notably, treatment with β -adrenoceptor selective agonists resulted in a drop in T cell numbers in the blood and lymphatics and increased retention in LNs due to association of β_2 with chemokine receptors CXCR4 and CCR7 [166]. Additionally, adrenergic signalling is also capable of impacting normal physiological changes in the body by limiting the amount of T cells that are able to leave the LN during diurnal fluctuations [167]. This regulation of T cell migration may be altered depending on other external stimuli. Glucocorticoid release results in an overall decrease of T cells in the circulation following an initial increase [168]. Inflammation results in adrenoceptor agonists improving T cell recruitment to other organs such as the dorsal root ganglia [169]. There may be more intricate regulatory mechanisms of T cell migration by the SNS through the indirect effects of other molecular components. Adenosine triphosphate (ATP) release can occur following β_3 stimulation which can then act to reduce T cell motility by binding in a paracrine fashion [170, 171].

Adrenergic signal modulation of T cell effector responses is dependent on the state of the cells at the time of stimulation, leading to many conflicting reports. Stimulation of

adrenoceptors on naïve CD4 T cells is thought to drive differentiation towards the Th2 phenotype and suppress Th1 development [138, 172, 173]. This preferential development is attributed to decreased IFN γ production without affecting the amount of IL-4 production [174, 175]. Th1 cells that develop this way produce lower amounts of IFN γ but stimulation of β -adrenoceptors after formation of these Th1 cells potentially drives IFN γ production instead [176, 177]. *In vitro* stimulation appeared to increase Foxp3⁺ Treg differentiation through an upregulation of TGF β [178]. This increase in TGF β can also increase Th17 cells as β -adrenoceptor signalling results in IL-17A production – both important cytokines for Th17 differentiation [179]. Interestingly, *in vivo* SNS signalling under autoimmune conditions decreases Foxp3⁺ Treg cells [180, 181] but can mediate CNS autoimmunity by suppressing overall damage by CD4 T cells [182]. Moreover, in a model of collagen induced arthritis, Th17 cells in particular were found to produce TH which led to increased Treg cell numbers and anti-inflammatory effects [183]. Taking all this into consideration, it appears that SNS signalling functions to dampen immune responses through regulation of CD4 T cells.

Studies on the effects of SNS signalling on CTL are less defined but also suggest an inhibition of CTL function. Treatment with β -adrenoceptor agonists *in vitro* not only decreased IFN γ production but also suppressed the killing activity of CTLs [184]. Chemical sympathectomy prior to influenza infection *in vivo* resulted in increased IFN γ production implying that SNS signals reduce the potency of antiviral responses [185]. More recent work has validated these findings showing not only decreased IFN γ but also TNF α and IL-2 following *in vitro* stimulation. This defect in cytokine production, but not CTL priming and activation, was also observed following treatment with long-lasting β agonists following infection with vesicular stomatitis virus (VSV) [186]. Contrary to these results, another group have found that following chemical ablation of SNS fibres post-infection, there was a decreased antiviral

response to herpes simplex virus (HSV) [187, 188]. Further to this, under *in vitro* conditions, CTL have also been documented to assist other immune cells by switching to cytokine-producing profile and decreasing cytolytic capacity [189, 190]. This shift in CTL does not only impact cytokine production but also metabolic processes such as mitochondrial function to limit CD8 T cell activity [191]. β -adrenoceptor signalling can also impact CTL cell function in anti-tumour responses by decreasing the responsive of these cells to immunotherapy agents [192].

Modulation of B cell responses by SNS signalling may be attributed to indirect effects as a result of changes in Th function but there are still documented cases of altered B cell responses through direct adrenoceptor signalling. Notably, CD86 – a co-stimulatory molecule essential for germinal centre formation, is upregulated following β -adrenoceptor stimulation [193, 194]. This increase in CD86 expression results in increased IgG₁ production and switching, potentially leading to better humoral responses [195]. Adrenoceptor dependent changes of cAMP in B cells can be linked to changes in CD86 expression as it is modulated by cAMP levels [196]. CD86 binds the co-stimulatory molecule CD28 on T cells and so increased expression of CD86 can lead to augmentation of T-B cell interaction thus improving the ability for B cells to support T cell responses. While it seems like the SNS boosts B cell function and responses, stimulating β -adrenoceptors following pathological effects such as stroke can result in loss of B cell subsets and thus reduced humoral immunity [197]. Studies on the effects of α -adrenoceptor signals on B cell function is lacking. Both T and B cell function can also be modulated by β -adrenoceptor signal-induced cytokine changes in stromal cells of the SLO [198].

1.3.3 Adrenoceptor expression on immune cells can be modified

An interesting aspect of adrenoceptor expression in immune cells is the ability to modulate surface adrenoceptor which is dependent on both extrinsic and intrinsic factors. Adrenoceptors on innate cells can be affected by broad changes in the body such as following periods of strenuous exercise or in various pathological conditions [199-204]. A specific example of this is the documented increase in β -adrenoceptor levels in neutrophils of individuals with post-traumatic stress disorder [205]. In addition to these changes from steady state conditions, adrenoceptor expression on adaptive immune cells can also be impacted by more complex factors [206, 207]. Analysis of peripheral blood mononuclear cells (PBMC) showed a decrease in β -adrenoceptor density in patients with autoimmune diseases such as arthritis [208]. Interestingly, adrenoceptor activation of other cell types may result in feedback loops that impact adrenoceptor levels on lymphocytes [209]. While there is a lack of studies on the effects of differentiation on adrenoceptor expression in B cells, the differentiation and maturation of T cells resulted in altered adrenoceptor expression. In the same study, it was also found that irradiation of T cells *ex vivo* drastically increased β -adrenoceptor expression [210]. Additionally, antigen stimulation and activation of T cells decreases surface β -adrenoceptor expression [211]. Certain signals from the activation of B cells can also result in the downregulation of surface β -adrenoceptors [212]. Formation of memory CD8 T cells is able to impact the levels of adrenoceptor expression on these cells with effector memory cells having higher levels of expression compared to naïve or central memory T cells [186]. Cytokine-dependent differentiation of murine Th cells into the Th1 subtype maintains β 2 expression but differentiation into the Th2 subtype results in a loss of expression which can be attributed to differences in histone modifications [175, 213]. However, the various mechanisms and pathways that contribute to this augmentation of

adrenoceptor expression on lymphocytes are not clearly defined. A better understanding of this is important in highlighting how the microenvironment regulates the capacity for immune cells to respond to SNS signals.

1.4 T cell memory

Following the expansion and contraction of a T cell response is the generation of a pool of memory T cells [214]. These cells exhibit characteristics of both naïve and effector T cells. Memory cell formation results in an expanded pool of cells that are capable of initiating potent responses to secondary infection through having increased chromatin accessibility, rapid production of cytokines, and proliferation potential [215-217]. There is debate as to when in the process of activation and differentiation that the fate of memory cell formation is decided [218-220]. Recent work demonstrated the ability of effector T cells to de-differentiate into a more naïve-like state which are considered to be memory T cells [221]. Memory T cells can be divided into three distinct subsets: effector memory (T_{EM}), central memory (T_{CM}), and tissue resident (T_{RM}), based on expression of various surface markers and their ability for recirculation [222]. CD8 T cell memory formation was found to be dependent on CD4 T cell help [223, 224]. The role for B cells in CD8 T cell formation is required following bacterial infections but not viral infections [225, 226]. The scope of this literature review will focus on CD8 T cell memory though many features are shared with CD4 T cells.

1.4.1 Effector and central memory T cells

T_{EM} and T_{CM} cells make up what is known as the circulating memory cells as they are capable of circulating throughout the body and enter peripheral organs to potentiate immune responses following antigen stimulation. The first study to describe T_{EM} and T_{CM} cells utilised

expression of the chemokine receptor CCR7 [227]. T_{CM} cells expressing CCR7 and CD62L are typically found in secondary lymphoid organs and in the circulation, whereas T_{EM} cells are found circulating throughout the peripheral tissues and lack CCR7 and CD62L expression [228, 229]. CD62L is critical for the homing of T cells to LNs [230]. Studies suggest that repeated antigen contact or antigen induced cytokines are not required for the initial development of memory cells from the activated pool [231]. Conversely, one study has shown that generation and maintenance of the T_{CM} pool depends on a variety of factors such as the level of antigen stimulation, the strength of the initial stimulation, and clonal competition [232]. Upon reactivation, T_{CM} cells have higher proliferation and differentiation potential but do not produce cytokines and effector functions as rapidly [227, 233]. On the other hand, T_{EM} cells are capable of immediately responding to secondary re-infections due to potent cytokine production and ease of access to peripheral tissues, potentially due to the lack of CD62L expression and LN homing. However, T_{EM} cells have limited proliferation capacity and are typically terminally differentiated [234]. Both T_{EM} and T_{CM} cells work in tandem to complement the functioning of the other such as rapid IL-2 production by T_{CM} cells to induce proliferation and continue the high magnitude responses of T_{EM} cells over time after reactivation. Interestingly, one study found that circulating memory T cells that have been established for longer have an expression profile that is more similar to naïve cells resulting in increased proliferation, metabolism, and magnitude of response [47].

While circulating memory cells definitely improve immune responses to subsequent antigen encounters, it is less likely that these cells are able to control and eliminate the pathogen at the site of entry before the infection takes hold [235]. Thus, the establishment of a population of T cells that are maintained at the site of infection is beneficial.

1.4.2 Tissue resident memory T cells

Immediate removal of invading pathogens is preferred to avoid any sequelae of immune responses. T_{RM} cells are memory T cells that reside in the tissue in which they were formed and do not return to the circulation [236, 237]. T_{RM} cells are the most recent population of memory cells to have been described and characterised [238, 239] and were originally thought to be equivalent to blood T_{EM} cells that traffic to peripheral tissues [240, 241]. These cells have now been identified as a distinct population of memory cells with a core gene expression profile that is different to T_{CM} and T_{EM} cells [242]. Researchers have used multiple techniques to distinguish residency of these cells and their lack of interaction with the circulation such as using intravascular labelling, parabiosis, tissue transplantation, and depletion of circulating cells [238, 243-245]. Parabiosis, the process of joining the vasculature in the skin flap of two mice, in particular has been instrumental in establishing the residency of cells as immune cells in the circulation will equilibrate but those in the tissue of interest would be skewed towards host congenic markers [246, 247]. As previously mentioned, T_{RM} cells have a unique expression profile that includes genes involved in cell adhesion and migration, immune regulation, and transcription factors [242, 248]. Of note are the transcription factors Runx3 and Hobit (and its homolog Blimp1) which have been documented as master regulators for tissue residency [249, 250].

T_{RM} cells are found in almost every organ including lymphoid tissues and immune privileged sites such as the brain in both mice and humans (Figure 1.2). These cells are typically identified by the expression of CD69 and CD103 though other surface molecules such

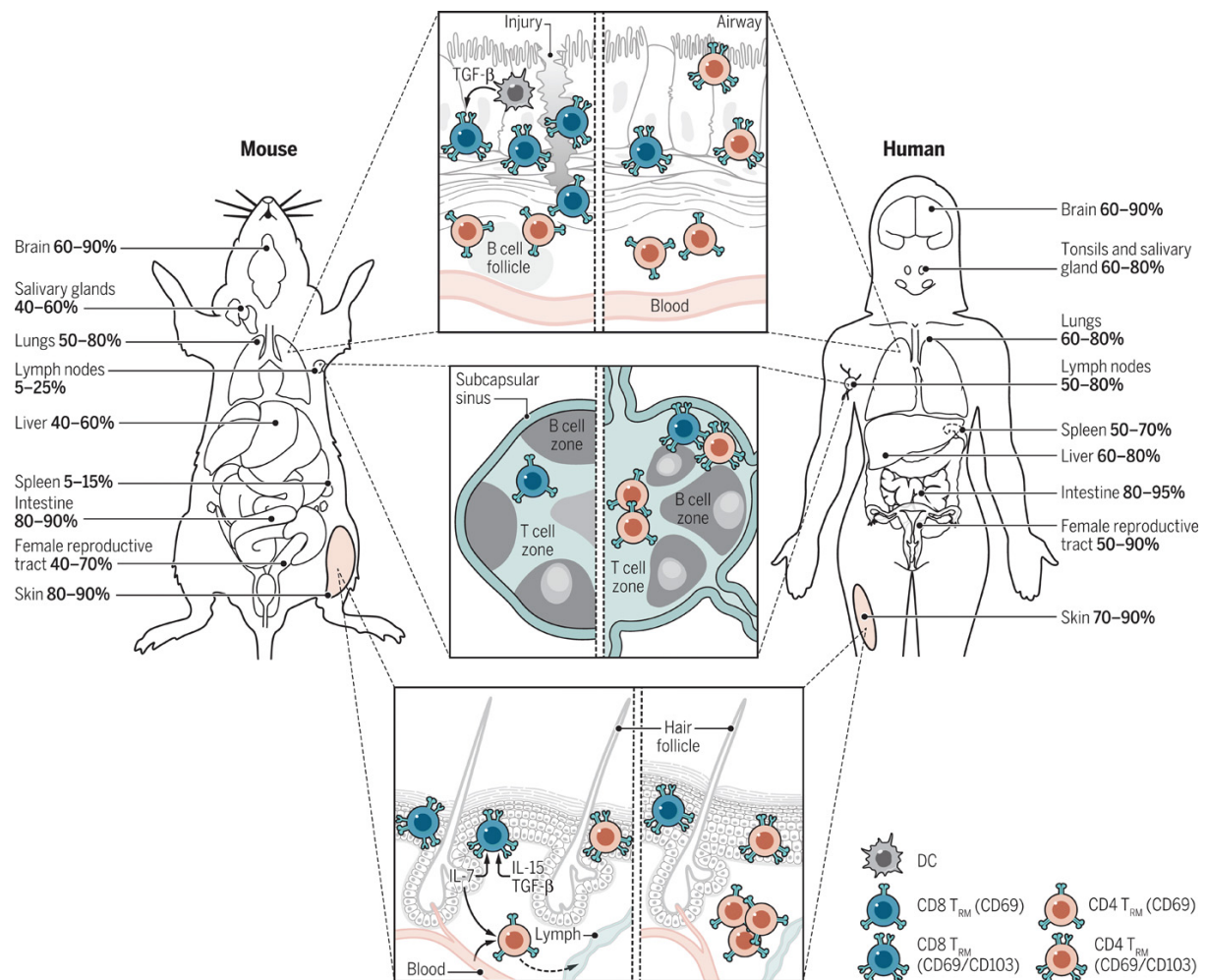


Figure 1.2 Distribution of T_{RM} cells in the organs of mice and humans.

Schematic representation of the distribution of non-circulating cells in organs of mice and humans.

Percentages show the frequency of (CD69⁺CD103^{+/−}) T cells in the organ. Modified from [236]

as CXCR6 and CD49a are also used to distinguish T_{RM} cells from circulating memory cells [238, 243, 248]. CD103 expression is usually linked to epithelial sites and thus there exists populations of T_{RM} cells, such as in the liver, that do not express CD103 [251, 252]. As such, surface expression of the different molecules can vary depending on location of the cells [236]. Functionally, T_{RM} cells are thought to express high affinity T cell receptors [253] and are able provide superior protection compared to the circulating pool of memory T cells [243, 254, 255]. This property of T_{RM} cells has been successfully utilised in various experimental vaccination strategies where establishment of these cells results in protection from pathogen challenge [251, 256-258]. Notably, T_{RM} cells have also been shown to promote an antiviral state in the residing tissue through rapid production of cytokines and innate-like signals [259-261]. These signals can result in sizeable recruitment of immune cells, including circulating memory T cells, to the site through the modulation of various chemokines, contributing to the overall immune response [260, 262]. Protection may also occur via direct cell killing orchestrated by T_{RM} cells through elevated levels of preformed granzyme B [263-265]. Finally, T_{RM} cells are also key players in anti-tumour responses, maintaining tissue homeostasis, and the pathology of autoimmune responses [266-268].

1.4.3 Formation, retention, and maintenance of resident memory T cells

There is a requirement for signalling from multiple sources before T_{RM} cell formation can occur. Given the requirement for CD4 T cell help in CD8 memory T cell formation, it is hypothesised that CD4 T cell involvement is also important in CD8 T_{RM} cell formation [269]. Upregulation of CXCR6 by T_{RM} cell precursors is needed for the early accumulation of the T_{RM} cells [270]. Dissemination of T_{RM} cells can occur in strong pro-inflammatory conditions without the need for antigen stimulation, although antigen stimulation in the tissues can

enhance T_{RM} formation [258, 271]. Another key factor are signals from transforming growth factor (TGF)- β [272]. TGF β signalling, together with IL-15, plays an important role in the upregulation of adhesion molecules such as CD103 and CD49a [273]. TGF β levels are regulated by the transcription factor Runx3 [274]. The lack of TGF β signalling or Runx3 expression results no upregulation of CD103 leading to no T_{RM} cells in the epithelial layer of various organs [242, 249, 275]. The origin of T_{RM} precursors is an area of large interest though there has been no definitive identification of such a specialised effector T cell lineage to date. Downregulation of migratory factors such as CCR7 and S1P1 is important in the retention of memory T cells in peripheral tissues. These molecules are regulated by the expression of Krüppel-like factor-2 (KLF2) [276]. Continued expression of CD69 in T_{RM} cells suppresses S1P1 expression, as opposed to the downregulation of CD69 in circulating memory T cells, and thus inhibits egress from the peripheral tissues back into the circulation [277, 278]. In addition to this, the inhibition of S1P1 is supported the intermediate expression of the transcription factor Hobit which represses KLF2 [250]. KLF2 is further repressed by the low levels of Eomes and Tbet expression in T_{RM} cells [279]. Eomes and Tbet also modulate levels of IL-15 and TGF β receptors respectively to sustain expression on the cell surface. Taken all together, these changes in migration capability of these cells allow for efficient retention at the site of recruitment.

Maintenance of these T_{RM} appears to be highly dependent on the niche in which the cells occupy. T_{RM} cells in the epidermis of the skin are capable of persisting for long periods of time despite limited physical space in the tissue [280]. Additional development of T_{RM} cells in this space does not displace previously formed cells but rather increases the capacity for the formation of these new cells [281]. On the other hand, T_{RM} cells in the lung experience high attrition rates leading to poor persistence and thus may require constant

replenishment from the circulation [282, 283]. IL-15 and IL-7 are important for the homeostatic proliferation of memory CD8⁺ T cells [284]. T_{RM} cells appear to be even more dependent on external microenvironment factors such as requiring IL-15 for regulating cell survival and turnover through expression of pro-survival molecules like Bcl2 and Bcl6 [242, 256, 265, 285]. This is tissue dependent as T_{RM} cells fail to persist in the skin in the absence of IL-15 but T_{RM} cell persistence is not affected in secondary lymphoid organs [242, 286]. It is evident that there are a series of complex and intricate regulatory systems in place to support T_{RM} cells at their site of development. Notably, these cells also have the potential to proliferate following secondary encounter with antigens thus replenishing the pool of T_{RM} cells at the site [287, 288].

The formation and maintenance of T_{RM} cells in many peripheral tissues have been well studied. However, the extent in which the properties of the tissue is able to support the presence of T_{RM} cells remains an area that requires further investigation. In particular, tissues such as the brain and ganglia are “immune privileged” tissues where any excessive damage from inflammation can result in dire consequences. Interestingly, T_{RM} cells in the brain appear to express the immune checkpoint markers programmed cell death-1 (PD-1) and cytotoxic T lymphocyte-associated protein 4 (CTLA-4) suggesting that this may be a method of controlling the inflammatory activities of these cells in the immune regulated tissue [289]. Additionally, not only is the establishment of T_{RM} cells in these tissues heavily dependent on antigen exposure, these T_{RM} cells also appear to be highly sensitive to changes in their environment [264]. Thus, it would seem that the properties of the tissues T_{RM} cells reside dictates the conditions in which these cells are able to persist in and that more highly regulated tissues may impose stricter conditions for the formation of these cells.

1.5 The cornea

The cornea is the most anterior compartment of the eye. It is divided into five layers: an epithelium, Bowman's layer, stroma, Descemet's membrane, and endothelium, though the presence of the Bowman's layer in mice has been disputed due to the lack of reliable identification [290] (Figure 1.3). Transparency of the cornea plays a big role in dictating the clarity of vision. The stromal layer makes up approximately two thirds of the corneal thickness and the epithelium making up the other third [291]. There is also a decreasing thickness towards the periphery of the cornea. Epithelial cells in the mouse cornea are tightly packed, showing no obvious space between cells thus providing a solid barrier to prevent foreign material from entering the eye [291]. The mouse corneal stroma is made of a heterologous population of keratocytes (cells expressing keratocan) that also have differential expression of a variety of other markers [292, 293]. Arrangement of the collagen fibres that support the keratocytes is in such a way that gives the cornea its transparency [290]. The endothelium comprises of a single layer of endothelial cells held together by a specialised basement membrane, forming a hexagonal lattice structure made of various collagenous components, known as the Descemet's membrane [294, 295]. The endothelium is important in regulating the transport of fluid and other substances out of the cornea thus maintaining corneal dehydration and transparency [290]. The cornea is also lacking of blood and lymphatic vessels at steady state which also contributes to the transparency of the cornea [296]. The corneoscleral junction, also known as the limbus, demarcates the cornea from the sclera, highlighting the boundaries of the cornea.

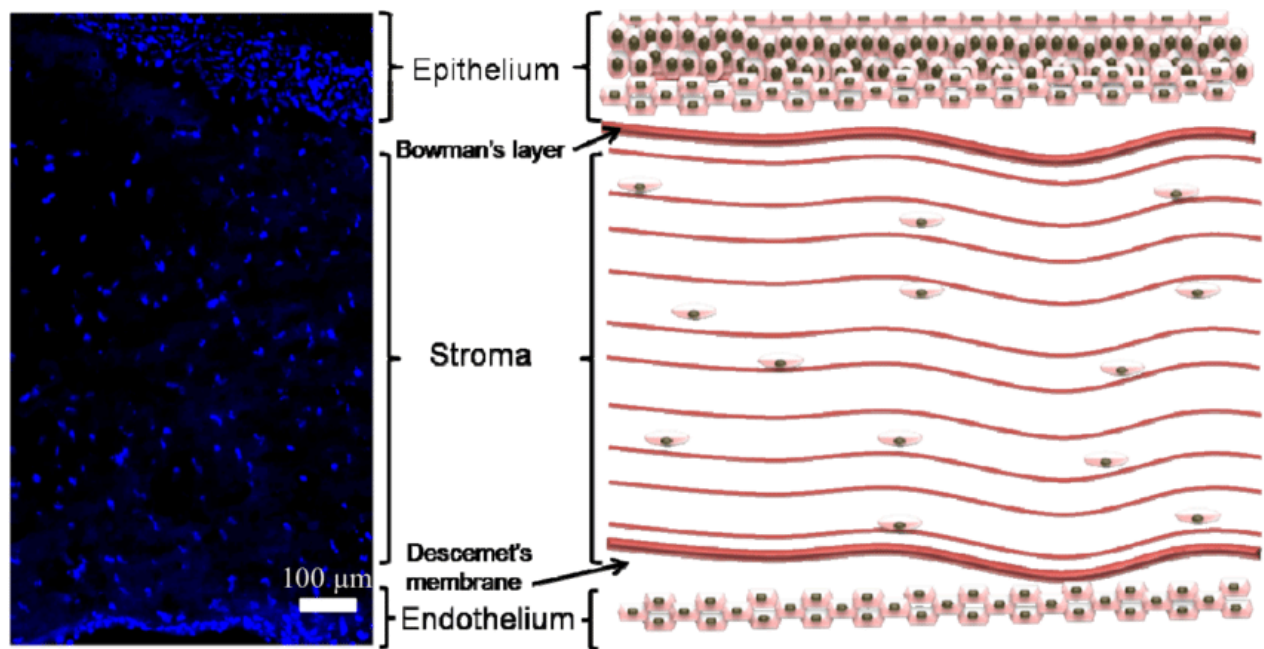


Figure 1.3 Layers of the cornea

Cross-section of a cornea stained with DAPI and a schematic representation of the cells in the cornea. A tightly packed epithelium can be seen above the stromal layer with the endothelium at the bottom. [297]

The cornea is densely innervated, mostly by sensory nerves with variable innervation by the sympathetic and parasympathetic nervous system depending on the species. In mice, sympathetic nerve fibres are incredibly scarce or found only in the limbus of the cornea [298-300]. Nerve bundles enter the cornea in the periphery through the limbus and subdivide into branches. These fibres lose their myelin sheath to retain corneal transparency. The branches continue along the stromal network, coming into close contact with keratocytes before moving up through the stroma towards the epithelium. The branches divide into smaller bundles and then continue to spread through the corneal surface, parallel and under the epithelial cell layer, forming distinctive whorl-like innervation patterns [301, 302]. These nerves can release a variety of neurotransmitters and soluble trophic substances that can influence multiple processes. For example, substance P, found to be released by termini in the corneal epithelium promotes epithelial cell adhesion by modulating cytoskeletal proteins and adhesion molecules such as E-cadherin [303].

1.5.1 Immune privilege of the cornea

The cornea is regarded as being immune privileged where immune responses, under normal circumstances, do not disrupt the normal functioning of the tissue [304, 305]. The lack of vasculature in the central cornea under steady state conditions may restrict the migration of lymphocytes into the cornea. While there are no cells of the adaptive immune system in the cornea at steady state, populations of dendritic cells and monocytes reside under the epithelium and in the stroma respectively [306, 307]. The corneal endothelium also acts to suppress lymphocyte activation and inflammation via secretion of anti-inflammatory molecules and expression of Fas-ligand to induce apoptosis in lymphocytes [308, 309]. Additionally, the endothelial layer is important in conferring immune privilege via the

expression of CD95 [310]. The corneal microenvironment is anti-inflammatory and immunosuppressive [296]. One of the molecules that is responsible for this is TGF- β which is readily available in the corneal microenvironment. TGF β isoform expression (TGF β 1,2,3) and their receptors have been found in both human and mouse corneal epithelial cells [311]. Though largely found in the epithelium of healthy intact corneas, corneal injury can result in the upregulation and secretion of various TGF β isoforms [312, 313]. In the cornea, TGF- β has been shown to play a role in preventing local DCs from fully maturing [314, 315]. Despite this, under sufficient inflammatory conditions these DCs can mature and migrate out of the cornea to the draining LN [316, 317]. Moreover, TGF- β is well known to be involved in the generation of immunosuppressive Treg cells in the periphery which may translate to a similar skewing in the cornea [318]. There is also mounting evidence that nerves and neuropeptides have a role in maintaining immune privilege in the cornea [319].

Despite the anti-inflammatory microenvironment of the cornea, there are studies that have shown a pro-inflammatory microenvironment may be possible in the cornea. Incubation of active T cells with corneal stroma decreased the production of anti-inflammatory cytokines by the T cells but did not change levels of protective cytokines [320]. Not only that, studies of human corneal cells have revealed that corneal epithelial and stromal cells are also capable of producing cytokines such as IL-1 α and IL-18 which are involved in inflammation and mediation of IFN γ responses [321, 322]. Additionally, a range of innate factors help facilitate removal of harmful pathogens without resulting in extensive damage to the cornea. These include antibodies found in the tears and complement molecules in the cornea itself [323, 324]. Taken together, while immune privilege of the cornea is essential in controlling inflammation to preserve corneal transparency and prevent any loss of vision, several

mechanisms are also in place to allow for some degree of protection against invading pathogens.

1.5.2 Corneal responses to injury

The wound healing response of the cornea varies depending on the layers that are injured [325]. Epithelial damage is the least severe and is resolved through the deployment and differentiation of limbal epithelial stem cells (LESC) in the limbus region [326]. LESC activity is apparent even under homeostatic conditions but injury increases the rate of proliferation and growth [327, 328]. In a study with diabetic mice, there was increased expression of TGF β 1 and TGF β 3 in response to wounding where removal of TGF β 3 resulted in slower closing of corneal wounds [329]. Corneal damage that extends into the stromal layer can result in potential fibrosis if a large replacement of keratocytes is required [330, 331]. The fate of these cells following resolution of wound healing is not well known but is likely to be removed through apoptosis or conversion to scar tissue [332]. Wound healing of both corneal epithelia and stroma also involve the remodelling of extracellular matrix (ECM) [333, 334]. Remodelling of the stromal ECM can lead to increased opacity of the cornea due to altered structures and production of irregular proteins [335].

Corneal injury involves the production of cytokines and recruitment of immune cells. Immune cells can originate from the limbus or from the circulation through neovascularisation of the cornea [336]. Neovascularisation can occur when there is sustained damage in the cornea that results in the dysfunctional regulation of angiogenic and anti-angiogenic factors in the cornea [337]. Release of IL-1 from damaged corneal cells can lead to increased vascularisation of the cornea [338]. Th17 cells that get recruited to the cornea can promote angiogenesis through the stimulation of IL-1 β production [339]. Macrophages also

support vascularisation of the cornea through interactions with blood vessels and the release of vascular endothelial growth factors (VEGF) that drive the formation of lymph and blood vessels [340-342]. Many checks and mechanisms are in place in the cornea to limit damage and opacity caused by injury. However, in some cases, these mechanisms are dysregulated leading to pathologies such as blindness.

Constitutive low levels of IL-6 are produced by corneal endothelium and keratocytes while expression of IL-10 was recorded in cultured corneal epithelial cells and fibroblasts [343, 344]. These cytokines have been documented to be involved with healing of corneal epithelial injuries [345, 346]. Immune cells are known to be necessary for wound healing by increasing levels of growth factors and the removal of cellular debris from damaged cells [347-349]. Neutrophils are generally the first immune cells to be recruited to the cornea and are important in re-epithelialisation following corneal abrasion [350]. Secretion of VEGF-A and IL-17 by neutrophils are also important for corneal nerve regeneration [351]. In addition to contributing to neovascularisation, macrophages are also recruited to the cornea to remove cellular debris and apoptotic cells at the site of injury [352]. NK cells also play a role in orchestrating the corneal wound healing through modulation of inflammatory responses [353]. There is also an increased amount of Langerhans cell in the corneal epithelium that are recruited following corneal damage [354]. While the function of these DCs are important in healing of the cornea [348], their exact role is not well characterised. CCL20 expression in the cornea recruits $\gamma\delta$ T cells which produces IL-22 to promote wound healing [355]. The role for conventional T cells in recovery following corneal injury has not been documented.

1.5.3 Corneal responses to infection

Infection of the cornea can result in many potential complications and consequences. A leading cause of blindness is herpes simplex virus (HSV) infection of the cornea resulting in inflammation of the corneal stroma known as herpes stromal keratitis (HSK) [356]. HSK can be recapitulated using a mouse model of infection with HSV-1 [357, 358] and is thought to be a sequelae of the immune response [359, 360]. Indeed, in mice that are lacking T cells, the symptoms associated with HSK do not manifest in mice with HSV-infected corneas [361].

Infection with HSV in the cornea initially results in increased production of IL-1 and IL-6 that generate the pro-inflammatory microenvironment [362]. This results in the recruitment of other immune cells from the limbus and the circulation, majority of them being neutrophils [363]. These neutrophils can function to clear virus and infected cells in the early stages of the infection [364]. The infiltrating neutrophils are also a source of other pro-inflammatory cytokines such as IL-1 β , IL-12, and TNF α as well as growth factors such as VEGF [365, 366]. It should be noted, however, that in other models of HSV infection, neutrophils do not appear to play a role in potentiating the adaptive response to HSV-1 [367, 368]. Other innate immune cells, namely macrophages and DCs are also recruited into the cornea much like in the response to injury discussed above [369, 370]. Depletion of corneal macrophages prior to HSV-1 infection resulted in increased viral titres though they do not appear to be important in early removal of the virus and are instead thought to potentiate adaptive responses [371]. Antigen presentation by infiltrating DCs with antigen from the infected cornea is important for the priming of CD4 T cells and the eventual control of the infection [372, 373]. Priming by resident Langerhan cells (a subset of DC) is not required for efficient CD8 T cell responses but does mediate CD4 T cell function in the cornea [374].

As previously alluded to, CD4 T cell responses are heavily involved in the immune response to corneal HSV-1 infection with Th1 cells being the ones implicated [375]. CD4 T cells may create a microenvironment that leads to increased recruitment of innate immune cells into the cornea 7-14 days post infection when most of the virus is already cleared. Depletion of CD4 T cells abrogates this response [376]. This increased recruitment of immune cells to the cornea results in an increase in pro-inflammatory and pro-angiogenesis molecules. Functional experiments have showed Treg cells to be effective at controlling corneal immune responses via IL-10 and TGF β [377-380]. Interestingly, a study using an attenuated strain of HSV-I that did not result in excessive corneal still showed an immune response that is dependent on CD4 T cells to provide enhanced innate responses to further infections [381].

There is also effective generation of CD8 T cell responses to corneal HSV infection. Even so, the proportion of T cells responding to HSV lesions in the cornea is skewed towards CD4 T cells when comparing against CD8 T cells though the CD8 T cells are capable of responding in organs such as the trigeminal ganglia [382, 383]. The reason for this remains unclear but studies have shown that CD8 T cells are required for viral control in the cornea [384] and form a pool of memory T cells that reside in the ganglia to prevent re-activation of the virus [385]. Another study suggests that this may not be the case as mice lacking CD8 T cells in their experiments did not appear to have increased viral loads [386]. Whether or not this pool of memory cells can be found at the initial site of infection in the cornea and what their function may be is unclear.

1.6 Models of infection

1.6.1 Mouse model of herpes simplex virus

Herpes simplex virus (HSV) is a virus in the herpesviridae family and can be classified as HSV-1 or HSV-2 based on the serotype. HSV-1 infects the oral-facial region and manifests as cold sores though severe cases can lead to inflammation and damage of the nervous system. HSV-2 on the other hand is associated with genital infections which can lead to complications if active during childbirth or in immunocompromised individuals [387, 388]. HSV infection begins with viral replication in epithelial cells at the initial site of entry. Following this, the virus is able to travel to the innervating ganglion where it can remain latent [389, 390]. This latency is maintained via the expression of the latency-associated transcript [391]. Under the right circumstances, reactivation is possible leading to manifestation of symptoms at the initial site of infection.

The HSV virion consists of a double-stranded DNA genome contained in an icosapentahedral capsid and protein layer wrapped in an envelope. Transcription occurs in three stages: immediate early, early, and late proteins which consist of proteins that control replication, packaging and synthesis, and virion particles respectively [392]. The viral glycoprotein B (gB) is required for the infection of epithelial cells by facilitating cell entry via heparan sulphate moieties [393]. Fusion of the membrane to complete cell entry involves the binding of gD to TNF receptor superfamily member 14 (TNFRSF-14). Following entry, HSV is capable of replicating in a lytic manner causing cell death. HSV-1 and HSV-2 have co-evolved with human hosts and adapted to ensure that the virus is able to replicate and produce progeny without rapidly killing its host and so primary infections in humans are usually asymptomatic [394]. Multiple different strains of HSV-1 and HSV-2 are used in mouse models of HSV which result in varying levels of pathology, virulence, and the resulting immune response [395].

Both the innate and adaptive immune system play a role in the clearance and protection against HSV infection. Production of type I interferon such as IFN α and IFN β by innate immune cells is important in controlling viral burden and disease severity as evidenced by studies in type I interferon knockout mice [396-398]. Cellular immunity is documented to be the most important in the immune response to HSV. In immunosuppressed patients, HSV reactivation occurs at a higher frequency than normal [399, 400]. In mice a lack of T cells results in paralysis and death due to uncontrolled HSV infection [401, 402]. CD4 T cells are crucial for protection against both primary and secondary HSV infections. Depletion of CD4 T cells results in delayed viral clearance whilst adoptive transfer into T cell deficient mice enables viral clearance [403, 404]. DCs that process and display antigen from the site of infection migrate to the draining LN to prime naïve T cells. Following this, activated T cells migrate to the spleen where expansion and differentiation occur. These cells are then able to move to the site of infection to eliminate the HSV-infected cells. Recent work found CD4 T cells to be primed before CD8 T cells which results in rapid CD4 responses that can further drive CD8 responses [405]. This may be linked to efficient IFN γ and IL-2 production by CD4 T cells which provide an pro-inflammatory microenvironment and attracting CTL to the site of infection [406]. CTL are important in the clearance of HSV-infected cells at the site of infection through the action of cytokines such as IFN γ and effector molecules such as perforin and granzyme [407]. While CTL are unable to prevent the infection of ganglia by HSV, these CD8 T cells are capable of persisting for long periods in the ganglia and work to control latency within the ganglia [408-410]. Following the resolution and clearance of HSV infection, both CD8 and CD4 T cells have been demonstrated to form long-lasting memory T cells that reside at epithelial sites such as the skin though this has not been reported in the cornea [238, 242, 410]. These cells provide a rapid response to secondary HSV infections close to the site of the

primary infection through rapid cytokine release and/or cell recruitment. Infection with HSV can induce antibody production and class switching of B cells. While humoral responses to HSV are important in limiting the spread of HSV, antibodies alone are insufficient to clear the infection [411, 412].

1.6.2 Mouse model of lymphocytic choriomeningitis virus

Lymphocytic choriomeningitis virus (LCMV) is a negative sense RNA virus of the *Arenaviridae* family, capable of infecting many mammals including humans but has a natural reservoir in mice [413]. Many aspects of LCMV infection and the subsequent immune responses are well-characterised, making it a very useful and suitable model for studying how immune responses can be modulated.

The LCMV glycoprotein binds to α -dystroglycan to facilitate entry of the virus into cells [414]. α -dystroglycan is ubiquitously expressed [415, 416] thus allowing for LCMV to cause a systemic infection of a wide range of cells including stromal cells and DCs [417, 418]. The route of infection is also important when using LCMV in a mouse model. Intracerebral injection of LCMV even at very low titres can result in severe infection and pathology of the central nervous system that would not occur with other routes of infection [413, 419]. Viral replication in infected cells usually do not result in cytopathic effects and pathology observed can be attributed to actions of the immune response. For example, there is increased cachexia in mice chronically infected with LCMV that is maintained by responding CD8 T cells resulting in increased morbidity [420]. However, there are also cases where persistent LCMV infection can alter the function of an infected cell leading to cell death from dysregulation [421, 422]. Two strains of LCMV, Armstrong (Arm) and the variant Clone 13 (CL13), are widely used in

mouse models. In mice, LCMV Arm is cleared by the immune system within 8 days post infection while LCMV CL13 suppresses immunity and persists [423, 424].

These two strains differ by only two amino acid substitutes – one in the glycoprotein that increases the binding affinity to α -dystroglycan and augments entry into cells and the other in the viral polymerase that promotes viral replication [425]. Given that the similarities in the biology of different LCMV strains are able to generate different immune responses, LCMV is an important tool to study immune responses in chronic and acute systemic infections.

While CD8 T cells are the main players in the clearance of LCMV, other cell types and arms also contribute to protection [426]. Type-I interferon is upregulated in the early phases of LCMV infection [427]. Type-I interferon is crucial in the early response for LCMV clearance with IFNAR mice (mice lacking the receptor for type-I interferon) experiencing higher viral burden which can manifest as persistent infection [428]. In an acute infection of LCMV, blockade of type-I interferon resulted in decreased CD8 T cell responses and proliferation [429]. Conversely, in chronic infections, the absence of type-I interferon signalling is able to facilitate viral clearance through modulating suppression of cellular adaptive immunity [430, 431]. DCs were found to express higher levels of IL-10 when mice were infected with LCMV CL13 compared to LCMV Arm [432]. IL-10 was found to inhibit CD4 T cell responses but not CD8 [433]. Ablation of the IL-10 response led to effective clearance of LCMV CL13 implying a role for IL-10 in viral persistence [432]. IFN γ has been well-established to be important in the clearance of LCMV [434, 435] likely through the modulation of CTL responses against the virus [436]. As a result, different strains of LCMV are able to produce persistent infection based on IFN γ resistance [437]. Importantly, the lack of IFN γ signalling resulted in death following infection with different strains of LCMV presumably attributed to early dampening of CTL

responses [428]. The exact mechanism in which IFN γ elicits protection in LCMV infection remains uncertain.

The T cell response to LCMV has been studied extensively. LCMV Armstrong infection produces CD8 T cells that have a high capacity to produce a variety of cytokines that boosts the immune response following gp33 stimulation *ex vivo*. This ability to produce cytokines is progressively lost in CD8 T cells that are generated from LCMV CL13 infection [423]. This canonical feature of immune response to acute versus chronic LCMV infection arises due to the immunosuppression termed 'exhaustion' due to the expression of various inhibitory markers such as PD-1, and altered genetic expression profile [438]. Chronic LCMV infections can also lead to a change in the immunodominant CD8 epitope response [439]. In acute LCMV infection, the kinetics of T cell expansion between the two is comparable but the magnitude of CD8 responses are higher than CD4 responses [440]. A dysregulation of CD4 T cell function coincides with CD8 T cell exhaustion occurs with chronic LCMV infection suggesting the need for an intact CD4 response to support CD8 responses [441]. Vaccination with a CD4 epitope-based vaccine enabled effective clearance of chronic LCMV infection by increasing CD8 T cell responses. This response was further boosted using anti-PD-1 treatment [442]. However, CD4 T cells alone do not efficiently clear LCMV infections and can lead to pathologies that are not observed when there is efficient clearing by CD8 T cells [443, 444]. T cells responding to LCMV are able to form memory populations in mucosal and non-mucosal sites following both acute and chronic infections. However, there are differences in the functionality and core gene expression between the cells generated by the different infections suggesting that viral persistence can augment the formation of these memory cells [438, 445]. Neutralising antibodies can be produced by LCMV-specific B cells following LCMV infection but these B cells are removed by the action of type-I interferon and inflammatory monocytes generated

by the infection [446]. Taken together, LCMV infection elicits a potent immune response that is predominantly T cell dependent though the role of other immune cells is also important for the efficient functioning of T cells and assisting in the clearance of the virus.

1.7 Thesis aims

The interaction between the SNS and immune system is beginning to be appreciated leading to the potential for the development of new interventions for improving health outcomes. This thesis aims to elucidate the influence of sympathetic nervous system signalling on T cell responses following both local and systemic viral infections. Additionally, given that the crosstalk between the systems is bi-directional, the effects of immune responses on the splenic sympathetic nervous system will also be investigated. Finally, this thesis will study T cell responses in the densely innervated, immune privileged cornea following HSV infection to increase the understanding of memory responses in the cornea. The potential for T_{RM} formation in the cornea and potential strategies to promote T_{RM} numbers will also be investigated, potentially leading to better antiviral responses and protection in the cornea.

Chapter 2 – Materials and

methods

2.1 Materials

2.1.1 General reagents

Commercially sourced reagents used in this project are listed in Table 1.

Table 1. Commercially sourced reagents and their supplier

Reagent	Supplier
Hank's Balanced Salt Solution (HBSS)	Media Preparation Unit, University of Melbourne
Roswell Park Memorial Institute Medium (RPMI)	Media Preparation Unit, University of Melbourne
1 x Phosphate Buffered Saline (PBS)	Media Preparation Unit, University of Melbourne
Baxter's Water for Irrigation	Baxter Healthcare
Bovine Serum Albumin (BSA)	Sigma Aldrich
Foetal Calf Serum (FCS)	Gibco BRL
Ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich
Red Blood Cell Lysis Buffer	Sigma Aldrich
70µm MACS SmartStrainer	Miltenyi Biotec
Liberase	Roche
Type I collagenase	Worthington
DNase	Sigma Aldrich
Collagenase D	Roche
Dispase II	Sigma Aldrich
gB ₄₉₈₋₅₀₅ (SSIEFARL) peptide	Auspep
gp33-42(KAVYNFATC) peptide	Auspep
gp61-80 (GLKGPDIYKGVYQFKSVEFD) peptide	Auspep
SIINFEKL peptide	Auspep
Ketamine	Parnell laboratories
Ilium Xylazil	Troy laboratories
Atropine	Alcaine

GolgiPlug	BD Biosciences
Cytofix/Cytoperm kit	BD Biosciences
6-hydroxydopamine	Sigma Aldrich
Ascorbic acid	Sigma Aldrich
Isoprotenerol (Isoprenaline)	Sigma Aldrich
Dimethylsulfoxide (DMSO)	Sigma Aldrich
Agarose	Sigma Aldrich
Trypsin	Sigma Aldrich
Noradrenaline Research ELISA	LDN
Isoflurane	Cenvet
Powdered medium 199	Sigma Aldrich
Powdered Neutral Red	Sigma Aldrich
Phenol Red	Media Preparation Unit, University of Melbourne
LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit	Life Technologies
Recombinant human IL-2	Peprtech Inc.
Trypan blue	Sigma Aldrich
Sphero blank calibration beads (6.0-6.4µm)	BD Pharmingen
Ultracomp eBeads compensation beads	Life Technologies
Micropore™ and transpore™ surgical tape	3M
Veet™	Reckitt Benckiser
GenTeal gel	Alcon

2.1.2 Histology reagents

Reagents used for histology in this project are listed in Table 2.

Table 2. Histology reagents and their supplier

Reagent	Supplier
Sucrose	Sigma Aldrich
Paraformaldehyde (PFA)	Electron Microscopy Science
Tissue-Tek Optimal Cutting Temperature (OCT) Compound	Sakura Finetek
Acetone	Chem-Supply
DAKO Serum-Free Protein Block	DAKO
Normal Donkey Serum (NDS)	Abcam
PAP hydrophobic pen	Daido Sangyo Co.
ProLong [®] Gold Antifade Reagent	Life Technologies
Glyoxylic acid	Sigma Aldrich
Monobasic potassium phosphate	Sigma Aldrich
Malachite green	Sigma Aldrich
Immersion oil	Sigma Aldrich
Glucose	Sigma Aldrich
Glass slides	Lomb
Coverslips	ProSciTech
Fructose	Sigma Aldrich

2.1.3 Molecular biology reagents

Reagents used for molecular work in this project are listed in Table 3.

Table 3. qPCR reagents and their supplier

Reagent	Supplier
RNeasy Mini Kit Plus	Qiagen
QIAzol	Qiagen

RNALater	Sigma Aldrich
High-Capacity cDNA Reverse Transcription Kit	Thermo Fisher Scientific
TaqMan Fast Advanced Master Mix	Thermo Fisher Scientific
TaqMan PreAmp Master Mix	Thermo Fisher Scientific
Fast SYBR Green Master Mix	Thermo Fisher Scientific

2.1.4 Molecular biology primers

Primers used with SYBR Green for qPCR in this project are listed in Table 4. Primers used with Taqman probes are listed in Table 5.

Table 4. SYBR Green primers

Gene	Forward primer (5'→3')	Reverse primer (3'→5')
<i>Th</i>	CAGGAAGTATGCCTCTCGTATC	GAGGACTGTCCAGTACATCAAT
<i>Dbh</i>	GATCTCATCATGCTCTGGACTG	CTGGTAGTCTTGCTGGGAATC
<i>Ntrk1</i>	AGAGTGGCCTCCGCTTTGT	CGCATTGGAGGACAGATTCA
<i>Ntrk2</i>	CCGGCTTAAAGTTTGTGGCTTAC	GGATCAGGTCAGACAAAGTCAAG
<i>Ntrk3</i>	GGATGCCCCCTGAAAGCATA	TGACCTCCGTGTTGGAAAGC
<i>Ngfb</i>	ACACTCTGATCACTGCGTTTTTG	CCTTCTGGGACATTGCTATCTGT
<i>Bdnf</i>	CACTGGCTGACACTTTTGAGCAC	GCTGTGACCCACTCGCTAATACTG
<i>Ntf3</i>	GGAGTTTGCCGGAAGACTCTC	GGGTGCTCTGGTAATTTTCCTTA
<i>Gfra3</i>	CTTGGTGACTACGAGTTGGATGTC	AGATTCATTTTCCAGGGTTTGC
<i>Tubb3</i>	AGTTTGCCGGAAGACTCTCTC	TAAGGAAAATTACCAGAGCACCC
<i>Uchl1</i>	ACGGCCCAGCATGAAAAC	GACGGATCCATCCTCAAATTCC
<i>Cgrp</i>	TCAGCATCTTGCTCCTGTACCA	CTGGGCTGCTTCCAAGATT
<i>Tac1</i>	AAGAAGCGCGATGCAGAACT	ACAGATGAAGATGAATAGATAGTGCGTT
<i>Artn</i>	GGCCAACCCTAGCTGTTCT	TGGGTCCAGGGAAGCTT
<i>Npy</i>	AGGCTTGAAGACCCTTCCAT	ACAGGCAGACTGGTTTCAGG
<i>Snap25</i>	AGGCATGAACCATATCAACCA	GATTATTGCCCCAGGCTTTT
<i>Vacht</i>	GAGAGTACTTTGCCTGGGAGGA	GGCCACAGTAAGACCTCCCTTG

<i>Chat</i>	CCTGGATGGTCCAGGCACT	GTCATACCAACGATTCGCTCC
<i>Hprt</i>	TCAGTCAACGGGGGACATAAA	GGGGCTGTACTGCTTAACCAG
<i>IFNγ</i>	ATGAACGCTACACACTGCATC	CCATCCTTTTGCCAGTTCCTC
<i>Tnfα</i>	ATAGCTCCCAGAAAAGCAAGC	CACCCCGAAGTTCAGTAGACA
<i>CXCL13</i>	TGGCCAGCTGCCTCTCTC	TTGAAATCACTCCGAACACCTACA
<i>CCL19</i>	CTGCCTCAGATTATCTGCCAT	AGGTAGCGGAAGGCTTTCAC
<i>CCL21</i>	ATCCCGGCAATCCTGTTCTC	GGTTCTGCACCCAGCCTTC
<i>TGFb1</i>	CCTGAGTGGCTGTCTTTTGA	GTGGAGTACATTATCTTTGCTG

Table 5. Taqman probes and their supplier

Gene target	Assay ID	Supplier
<i>Th</i>	Mm00447557_m1	Thermo Fisher Scientific
<i>Chat</i>	Mm01221880_m1	Thermo Fisher Scientific
<i>Ifng</i>	Mm01168134_m1	Thermo Fisher Scientific
<i>Hprt</i>	Mm03024075_m1	Thermo Fisher Scientific

2.1.5 Media and Buffers

FACS Buffer: 1x PBS with 1% BSA (w/v), 5mM EDTA

Supplementum Complementum (SC): dH₂O containing 2 x 10⁶ U/L benzylpenicillin, 23.83g/L

HEPES, 2g/L streptomycin, 6g/L L-glutamine, 70 uL 2-mercaptoethanol

RP-10: RPMI containing 10% FCS (v/v) and 5% SC (v/v)

RP2: RPMI containing 2% FCS (v/v)

D-10: DMEM containing 10% FCS (v/v) and 5% SC (v/v)

SPG solution 2: MilliQ H₂O containing 1% glyoxylic acid, 0.236 M monobasic potassium phosphate, 0.2 M sucrose

SPG solution 1: SPG solution 2 containing 1.75mg/ml malachite green

2x 199 medium: Baxter's Water containing 1.96% (w/v) powdered medium 199, 0.44% (w/v) sodium bicarbonate, 10% FCS, 10% SC, 11mg/mL phenol red

Neutral Red Solution: MilliQ water containing 1% neutral red powder (w/v)

Special wash buffer (SPW): 1g BSA, 50mL 10x PBS, 500mL dH₂O, 1 ml Tween, 1 ml Triton X-100, 1 ml 10% SDS

Washing buffer (WB): 1g BSA, 50mL 10x PBS, 500mL dH₂O, 1 ml Tween, 1 ml Triton X-100, 1 ml 10% SDS

FUnGI clearing solution: 330 mL glycerol, 70 mL dH₂O, 300 g fructose, 100 g Urea

2.1.6 Mice

Mouse strains used in this project are listed in Table 6.

Table 6. Description of mouse strains

Strain	Description
C57BL/6 (B6)	"wild-type" strain expressing MHC class I H-2 ^b and MHC class II I-Ab, the haematopoietic marker CD45.2/Ly5.2, and surface marker CD90.2/Thy1.2
B6.P14/Ly5.1	B6 mice with TCR transgenic cells resulting in CD8 T cells capable of recognising gp33 of LCMV and expressing the haematopoietic marker CD45.1/Ly5.1
B6.SMARTA/uGFP	B6 mice with TCR transgenic cells resulting in CD4 T cells capable of recognising gp61 of LCMV and GFP expression of all cells regulated by the ubiquitin promoter.

B6.SMARTA/Thy 1.1	B6 mice with TCR transgenic cells resulting in CD4 T cells capable of recognising gp61 of LCMV and expressing the surface marker CD90.1/Thy1.1
B6.gBT-I/Ly5.1	B6 mice with TCR transgenic cells resulting in CD8 T cells capable of recognising gB of HSV and expressing the haematopoietic marker CD45.1/Ly5.1
B6.gBT-1/uGFP	B6 mice with TCR transgenic cells resulting in CD8 T cells capable of recognising gB of HSV and GFP expression of all cells regulated by the ubiquitin promoter.
B6.gBT/dsRed	B6 mice with TCR transgenic cells resulting in CD8 T cells capable of recognising gB of HSV and dsRed (RFP variant) expression of cells
B6.gDT-II/Ly5.1	B6 mice with TCR transgenic cells resulting in CD4 T cells capable of recognising gD of HSV and expressing the haematopoietic marker CD45.1/Ly5.1
B6.gDT-II/uGFP	B6 mice with TCR transgenic cells resulting in CD4 T cells capable of recognising gD of HSV and GFP expression of all cells regulated by the ubiquitin promoter.
B6.gDT-II/dsRed	B6 mice with TCR transgenic cells resulting in CD4 T cells capable of recognising gD of HSV and dsRed (RFP variant) expression of cells
B6.OT-I/Ly5.1	B6 mice with TCR transgenic cells resulting in CD8 T cells capable of recognising SIINFEKL of ovalbumin and expressing the haematopoietic marker CD45.1/Ly5.1

B6.IFNAR1 ^{-/-}	B6 mice with cells lacking the type I interferon receptor
B6.IFN γ ^{-/-}	B6 mice with cells lacking the capability of producing IFN γ
ROSA ^{mT/mG}	B6 mice expressing tdTomato on the surface of cells under the ROSA26 promoter
B6.OT-I/ Ly5.1/TGF β RII ^{-/-}	B6 mice with TCR transgenic cells resulting in CD8 T cells capable of recognising SIINFEKL of ovalbumin, expressing the haematopoietic marker CD45.1/Ly5.1 with the deletion of TGF β RII.

Mice were bred at the Bioresource Facility of the Department of Microbiology and Immunology, The University of Melbourne. Work was carried out according to the guidelines set by The University of Melbourne Animal Ethics Committee.

2.1.7 Antibodies for flow cytometry

Antibodies for flow cytometry used in this project are listed in Table 7.

Table 7. List of antibodies used for flow cytometry

Antigen-conjugate	Clone	Supplier
CD4-BV711	RM4-5	BioLegend
CD4-FITC	RM4-5	BioLegend
CD4-A700	RM4-5	BioLegend
CD8 α -PE-Cy7	53-6.7	BioLegend
CD8 α -AF700	53-6.7	BioLegend
CD8 β -PE-Cy7	YTS156.7.7	BioLegend
CD8 β -A700	YTS156.7.7	BioLegend
CD45R/B220-Pacific Blue	RA3-6B2	BioLegend
CD19-BV605	6D5	BioLegend
CD19-FITC	1D3	BD Biosciences
CD138-PE	MI15 (ASR)	BD Biosciences

CD38-PE-Cy7	90	BioLegend
GL7-APC	GL7	BioLegend
NK1.1-PerCP-Cy5.5	PK136	BD Biosciences
CD45.1-BV785	A20	BioLegend
CD45.1-FITC	A20	BD Biosciences
CD45.2-AF700	104	BD Biosciences
Thy1.1-AF488	OX-7	BioLegend
CD44-BV510	IM7	BD Biosciences
CD44-PerCP-Cy5.5	IM7	BD Biosciences
PD1-PerCP-Cy5.5	29F.1A12	BioLegend
CD62L-PE	MEL-14	BD Biosciences
KLRG1-APC	2F1	eBioscience
CD69-APC	H1.2F3	eBioscience
CD103-PerCP-Cy5.5	2E7	BioLegend
CXCR6-PE	SA051D1	BioLegend
F480-AF647	BM8	BioLegend
CD11c-BV605	N418	BioLegend
CD11b-BV711	M1/70	BioLegend
Ly6c-BV785	HK1.4	BioLegend
TCR β -BV421	H57-597	BioLegend
XCR1-BV510	ZET	BioLegend
IA/IE-AF700	M5/114.15.2	Life Technologies
IL2-PE	JES6-5H4	BioLegend
IFN γ -PE-Cy7	XMG1.2	BD Biosciences
TNF α -APC	MP6-XT22	BD Biosciences
IL-10-PE-Cy7	JES5-16E3	BioLegend

2.1.8 Antibodies for histology

Antibodies for histology used in this project are listed in Table 8.

Table 8. List of antibodies used for histology

Antigen-conjugate	Host	Clone	Supplier
CD31-BV421	Rat	390	BioLegend

CD31-AF647	Rat	MEC13.3	BioLegend
CD3-e660	Rat	17A2	eBiosciences
B220/CD45R-Pacific Blue	Rat	RA3-6B2	BioLegend
Ter119-AF700	Rat	TER-119	BioLegend
TH (Purified)	Goat	Polyclonal	Merck
TH (Purified)	Chicken	Polyclonal	Abcam
β III-Tubulin (Purified)	Rabbit	Polyclonal	Abcam
Goat IgG ₂ -AF488	Donkey	Polyclonal	Life Technologies
Chicken IgG ₂ -AF488	Goat	Polyclonal	Life Technologies
Rabbit IgG ₂ -AF594	Donkey	Polyclonal	Life Technologies
Goat IgG ₂ -AF568	Donkey	Polyclonal	Life Technologies
Rabbit IgG ₂ -AF488	Donkey	Polyclonal	Life Technologies

2.1.9 Antibodies for in-vivo depletion

Antibodies for in-vivo depletion used in this project are listed in Table 9.

Table 9. List of antibodies used for in-vivo depletion

Antigen	Clone	Supplier
NK1.1	PK136	WEHI Antibody Facility
CD4	GK1.5	WEHI Antibody Facility
CD8	2.43	WEHI Antibody Facility

2.2 Methods

2.2.1 Cell suspensions from mouse tissues

Spleen and LN were harvested from mice and placed into HBSS kept on ice. Cell suspensions were prepared by straining the organs through a steel mesh then filtered through 75 μ m nylon mesh. Red blood cell lysis was then performed for spleens by resuspending the cell pellet in 2mL of RBC Lysis buffer for 5 minutes, washed then resuspended in media.

Cells from the skin were isolated by harvesting a 1cm x 2cm section of the skin into 0.3mg/mL type I collagenase. The sample was chopped into small pieces and incubated at 37°C for 90 minutes. The sample was then vigorously pipetted using a Pasteur pipette and filtered through a 75µm nylon mesh.

Cells were recovered from the cornea either by (i) incubating a single cornea in 0.25mg/mL of Liberase (Roche) with 0.1mg/mL DNase at 37°C for 30 minutes before chopping into small pieces and incubating for a further 60 minutes, and triturating samples through a 1mL pipette tip or (ii) two corneas from a mouse were incubated in 1 mg/mL Collagenase D and 0.1mg/mL DNase at 37°C for 25 minutes, triturated, supernatant removed and placed into FACS buffer, and corneal pieces resuspended in collagenase solution with 0.8mg/mL Dispase added. Samples were incubated at 37°C, triturated, supernatant removed, and placed into FACS buffer. This was repeated once more for complete digestion of corneal pieces.

2.2.2 Adoptive T cell transfer

Naïve T cell transfer

Spleen and LN from transgenic mice were isolated and injected into mice intravenously via tail vein to allow cells to distribute to the secondary lymphoid organs. Number of cells injected are described in figure legends.

Activated T cell transfer

Single cell suspensions of spleen from wild type and transgenic gBT-I or OT-I cells were obtained. Cells from wild type spleens were incubated at 37°C with either 1µg/mL gB peptide or 0.5mg/mL of SIINFEKL peptide for one hour. The transgenic cells were then incubated with the pulsed wild type cells at 37°C, 5% CO₂. 4 days later, cells were injected into mice as described in the text.

T cell co-transfer

Lymph nodes from wild type OT-I mice and TGF β R-II KO mice or gBT-I and P14 mice were isolated injected into mice intravenously in a 1:1 ratio.

2.2.3 Infection models

Lymphocytic choriomeningitis virus

Mice were infected with 200 μ L RPMI containing 2×10^5 pfu LCMV Armstrong intraperitoneally (i.p.) or 2×10^6 LCMV CL13 intravenously (i.v.).

Herpes Simplex Virus (Skin)

HSV infections of the skin were performed by flank scarification as previously described [238]. Briefly, mice were anaesthetised with a mixture of ketamine/xylazine at 100mg/kg and 15mg/kg, respectively. Skin on the left flank was shaved and depilated then lightly abraded with a power tool to remove the epidermal layer of the skin. 10 μ L of PBS containing 10^6 PFU HSV-KOS strain was then applied to the Dremel site. Opsite was placed over the site and mice bandaged for 2 days.

Influenza A virus

Mice were infected intraperitoneally at 1.5×10^7 pfu with Influenza A virus (IAV) (strain A/Puerto Rico/8/1934 H1N1).

Listeria monocytogenes

Mice were starved for 4 hours and fed 1cm³ bread pieces containing 10^9 cfu *Listeria monocytogenes*.

Plasmodium bergii ANKA

Mice were injected iv. with 5×10^4 red blood cells containing *Plasmodium bergii* ANKA.

Corneal Infections and application of immunomodulatory compounds

Mice were anaesthetised with a mixture of ketamine/xylazine at 100mg/kg and 15mg/kg, respectively. A drop of Atropine (Alcaine) was placed onto each eye for 5 minutes and then wicked off using a cotton swab. The corneas were then lightly scratched using a 30G needle and 10µL of PBS containing 10^6 pfu of HSV KOS, HSV-OVA, VV-OVA or LCMV Armstrong was placed onto the eye. For the use of immunomodulatory compounds, PBS containing 50µg gB or SIINFEKL peptide, 2nM CpG 1668 or CpG Combo (modified CpG consisting of CpG B and CpG P) or 10µg LPS was used instead. Peptides were initially made up in DMSO and diluted with PBS to 10% v/v. Mice were then allowed to recover in a cage on a heat pad.

2.2.4 Flow cytometry

Cells from single cells suspensions were washed with FACS buffer and resuspended in FACS buffer containing fluorescently labelled antibodies. The cells were incubated on ice in the dark for 30 minutes and washed with FACS buffer before being resuspended in FACS buffer. 20µL of predetermined concentration of counting beads were added to the samples before acquisition.

Intracellular cytokine staining

Single cell suspensions were resuspended in RP-10 and 1:1000 dilution of GolgiPlug (BD) containing Brefeldin A was added as per manufacturer's instructions to stop the release of vesicles from the Golgi apparatus. The cells were then stimulated with or without the relevant peptide and incubated at 37°C, 6.5% CO₂ for 5 hours. A Cytofix/Cytoperm kit (BD) was then used as per manufacturer's instructions. Samples were acquired using BD FACS Canto II or BD FACS Fortessa and analysed using FlowJo X (Treestar).

2.2.5 Depletion of sympathetic nerves

6-OHDA was made up in 0.9% NaCl solution containing 10^{-7} M ascorbic acid as an antioxidant. 6-OHDA was injected intraperitoneally (i.p.) at 100mg/kg, 7 and 5 days prior to infection, and 200mg/kg on 3 days prior to infection. Multiple treatments were used to ensure full depletion of the spleen. Mice were only infected 3 days post treatment to ensure there is no residual catecholamine release following depletion of the sympathetic fibres.

2.2.6 Agonist treatments

Isoprenaline was weighed out and dissolved in PBS. Isoprenaline was given intraperitoneally daily at a dose of 0.1mg per mouse from day 0 to day 7 of infection.

Salmeterol was made up in DMSO and diluted with PBS. Mice were treated intraperitoneally daily with 0.2mg salmeterol per mouse from day 0 to day 7 of infection.

2.2.7 LCMV plaque assay

Confluent Vero cells were washed, trypsinised and diluted 1:20 and then plated in 6 well plates. The plates were incubated for 4 days until approximately 80% confluent. Plasma and spleens that were weighed were frozen and stored at -80°C . On the day of the assay, the samples were briefly thawed in a 37°C water bath and spleen homogenised with a Polytron homogeniser (Kinematica AG) for approximately 5-10 seconds (speed 19) then titrated in ten-fold dilutions. 200 μL of the titrated samples was overlaid onto the confluent Vero cells and incubated for one hour. 1.5% agarose and 2X 199 media mixed in a 1:1 ratio was then added to each well and incubated for 4 days. A solution containing 1:1 1.5% agarose, 2X 199 media, and 2% Neutral Red solution (v/v) was overlaid onto each well. Plaques were enumerated using a dissecting microscope the following day.

2.2.8 Sucrose-phosphate-glyoxylic acid staining (SPG)

20 μ M sections were cut using a cryostat (Leica CM3050S) and placed immediately in SPG solution 1 containing malachite green for 3 seconds to create contrast then washed in SPG solution 2 for 3 seconds to wash out the excess malachite green. The sections were then dried using a hair dryer. Sections were covered in immersion oil and placed in an oven kept between 90°C-95°C. Excess oil was removed before covering with a glass coverslip and sealed with nail polish. Images were acquired using an SP8 fluorescence microscope (Leica), capturing between 440-520nm and processed using Leica LAS X 1.9.

2.2.9 Immunofluorescence and confocal microscopy

Cryosections

Naïve or LCMV infected spleens were harvested and placed either in HBSS or fixed in 2% PFA for 2-4 hours at 4°C before being washed twice in PBS and incubated overnight in 30% sucrose (w/v) in PBS at 4°C. Naïve or LCMV infected celiac ganglia were harvested and fixed in 2% PFA for overnight at 4°C before being washed twice in PBS and incubated overnight in 30% sucrose (w/v) in PBS at 4°C. The tissues were then embedded in OCT, frozen on liquid nitrogen and stored in -20°C for until ready to be sectioned. 16-20 μ m splenic sections or 10-14 μ m ganglia sections were cut using a cryostat (Leica CM3050S), air dried and fixed in acetone before blocking with DAKO serum-free protein block. A hydrophobic barrier was applied using a PAP pen (Daido Sangyo Co. Ltd). Sections were then stained with relevant primary antibodies in a humid chamber in the dark at room temperature overnight. A secondary stain containing fluorescently labelled antibodies were then applied to each section for 1 hour. An additional tertiary stain was used if there were antibodies from the same host. Slides were then mounted with ProLong® Gold, coverslipped, left overnight to dry, and imaged using a confocal

microscope (Zeiss LSM 710). Post-acquisition adjustments and analysis was performed using Imaris (Bitplane).

Vibratome sectioning and clearing

Naïve or LCMV infected spleens were harvested and placed either in HBSS or fixed in 2% PFA for 2-4 hours at 4°C before being washed twice in PBS and embedded in 2% agarose. 150µm tissue slices were obtained using a VT1200 S vibratome (Leica Biosystems) as previously described [405]. Each slice was placed into 2mL SPW buffer overnight at 4°C with light agitation using a MR-1 Rocker-shaker (Fisher Biotec) before being transferred into washing buffer containing primary antibodies for 3 days at 4°C with light agitation. Slices were then washed for 2 hours in washing buffer and another 2 times for 30 minutes in washing buffer. Washing buffer containing secondary antibodies was used to stain slices for a further 2 days at 4°C with light agitation. Slices were washed again as before and placed into FunGi clearing solution overnight at room temperature while agitated.

A shallow well was created on glass slides using vacuum grease and slices mounted using FunGi clearing solution. Images were acquired using a confocal microscope (Zeiss LSM 710). Post-acquisition adjustments and analysis was performed using Imaris 8.4 (Bitplane).

Cornea wholemount preparation

Eyes from mice were removed and placed in 4% PFA for 15 minutes. The cornea was excised along with small parts of the limbus and returned to 4% PFA for a further 45 minutes. If antibody staining was required, corneas were washed in a 96-well plate for 3 x 10 minutes in PBS and placed in DAKO protein block containing 0.5% Triton-X 100 for 2 hours at room temperature. Corneas were then placed into an antibody cocktail made in 2% NDS and 0.5%

Triton X-100 in the dark for 24 hours on a plate rocker at room temperature. Corneas were washed for 3 x 10 minutes in PBS and if needed, placed into antibody cocktail with secondary antibodies in the dark overnight on a plate rocker at room temperature.

Samples were washed a further 3 x 10 minutes in PBS and mounted onto slides with ProLong[®] Gold, coverslipped, left overnight to dry, and imaged using a confocal microscope (Zeiss LSM 710). Post-acquisition adjustments and analysis was performed using Imaris 8.4 (Bitplane).

2.2.10 Image analysis

Co-localisation analysis and creation of a co-localisation channel was performed using Imaris 8.4 (Bitplane). The number of pixels in the co-localisation channel was enumerated using Fiji (ImageJ).

2.2.11 Noradrenaline ELISA

Spleens and plasma of LCMV infected and non-infected mice were collected. Spleens were weighed and homogenised with a Polytron homogeniser (Kinematica AG) for approximately 5-10 seconds (speed 19). Samples were assayed for noradrenaline using a Noradrenaline Research ELISA (LDN) as per manufacturer's instructions. Data were acquired using a Multiskan Ascent microplate reader (Thermo Fisher Scientific).

2.2.12 in vivo cell depletion

NK cell depletion

Mice were given 100µg anti-NK1.1 antibody intraperitoneally two days prior to infection.

CD4 T cell depletion

200µg anti-CD4 GK1.5 antibody was administered intraperitoneally 4 days prior to infection.

Another 100µg of anti-CD4 GK1.5 antibody was given 1 day prior to infection.

CD8 T cell depletion

200µg anti-CD8 2.43 antibody was administered intraperitoneally 4 days prior to infection.

Another 100µg of anti-CD8 2.43 antibody was given 1 day prior to infection.

2.2.13 RNA isolation and qPCR

Celiac ganglia, spleen or gut from LCMV infected and non-infected mice were homogenised with a Polytron homogeniser (Kinematica AG) for approximately 5-10 seconds (speed 19) in RLT buffer from the RNeasy Mini Kit (Qiagen) for spleen and gut, and Qiazol (Qiagen) for ganglia. RNA was isolated according the manufacturer's instructions using the RNeasy Mini Kit (Qiagen). Total RNA was quantified using a Nanodrop (ThermoFisher) and stored in -80°C. A standardised amount of RNA was used to generate cDNA using a cDNA Reverse Transcription Kit (Applied Biosystems). If required, cDNA was amplified using a TaqMan PreAmp Master Mix Kit (Applied Biosystems) with the relevant primers for 10 cycles.

SYBR Green Mastermix and relevant primers (Table 4) or Taqman probes (Table 5) were used for qPCR and acquired using OneStepPlus Real-Time PCR system (Life Technologies) or QuantStudio 6 and 7 Flex Real-Time PCR System (Applied Biosystems). The cycling threshold (Ct) of gene transcripts for each sample was determined by qPCR and normalised to the geometric mean of the Ct values of housekeeping gene *Hprt* for the calculation of the ΔCt value. The expression of each gene from LCMV infected samples were compared to the non-infected samples using the $2^{-\Delta\Delta Ct}$ method.

2.2.14 Secondary challenge of the cornea

Mice previously infected in the cornea with LCMV Armstrong or HSV were anaesthetised with a mixture of ketamine/xylazine at 6.6mg/kg and 100mg/kg, respectively 28-35 days post infection. gB peptide or HSV was applied to the cornea as previously described above. Mice were then allowed to recover on a heat pad.

2.2.15 Two-photon microscopy of the cornea

Isoflurane was used to anaesthetise mice (3.0% induction, maintained at ~1.5%, vaporised in a mixture of 80:20 oxygen and air) using a Tech 3 vaporizer (Surgivet). The whiskers on the side of imaging were shaved off and mouse is placed in a head holder (Narashige). The non-imaged eye was covered with artificial tear gel (GenTeal) and the imaged eye was anaesthetised using Atropine (Alcaine) for 5 minutes then wicked away. The eye was gently exposed from the socket and secured using a plastic loop, a cone placed around the eye, sealed with vacuum grease, and filled with PBS. Intravital imaging was performed in a custom-made environmental chamber (Precision Plastics) maintained at 35°C using heated air. Image acquisition was performed with a LSM710 NLO multiphoton microscope (Carl Zeiss Microimaging) using a x20/1.0 NA water immersion objective. The tissue was excited at 925nm using a two-photon Ti:sapphire femto-second pulsed laser (Chameleon Vision II, Coherent Scientific). Three dimensional stacks were acquired at 30-40 second intervals for 30-40 minutes. Data and movies were processed using Imaris 8.3.1 (Bitplane).

2.2.16 Statistical analysis

Student's t-test was used to compare the means of two populations following confirmation of population normalcy using a Kolmogorov-Smirnov or Shapiro-Wilk test. One way ANOVA and Tukey's multiple comparisons test was used when comparing between multiple populations. Statistical analysis was performed using Prism 7.0 (Graphpad).

Chapter 3 – Investigating the

role of the SNS in viral

infection

3.1 Introduction

The sympathetic nervous system (SNS) is important in the regulation of multiple physiological systems such as blood circulation and muscle contraction to maintain normal function and homeostasis of the body. Interestingly, there is increasing evidence of crosstalk between the SNS and the immune system [447]. The immune system protects the body from pathogens and disease through a series of regulated mechanisms. These mechanisms can be modulated by signals from the SNS through adrenergic receptors that are present on immune cells [110, 111]. The SNS has been documented to innervate secondary lymphoid organs and thus provides an interface for the crosstalk between the nervous and immune systems [80, 81]. Since the discovery of this relationship between the two systems, work has been done to understand the effects of SNS signalling on immune responses. These studies have found that whether SNS modulation is stimulatory or inhibitory is dependent on the duration of SNS signalling and the type of immune response [448]. One aspect of interest in SNS modulation of the immune system is the effect on T cells, an important cell type of the adaptive immune system that plays a large role in immune responses and clearing pathogens. Several studies have investigated the effects of removing sympathetic signals on antiviral responses and shown improved effector T cell responses following viral challenge [185, 188]. This chapter focuses utilising both systemic and local infection models to investigate how SNS signalling can affect the antiviral T cell responses to different models of infection. Additionally, as most previous work focuses on the extent to which the SNS affects antiviral responses, the possibility of antiviral responses affecting SNS activity will be further explored. Notably, results from this chapter showed manipulating the SNS modulation of immune responses to acute and chronic LCMV infection resulted in not only in changes to the effector T cell response but also in viral control and the sympathetic innervation of the spleen.

3.2 Results

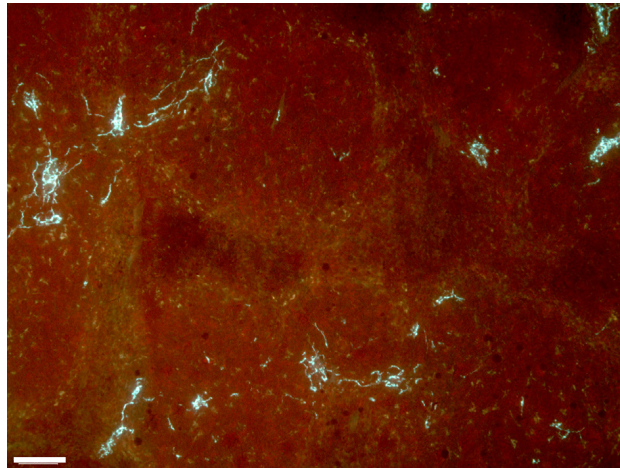
3.2.1 Depletion of splenic SNS fibres using chemical sympathectomy

Chemical sympathectomy with 6-hydroxydopamine (6-OHDA) has been well characterised as a method to deplete sympathetic fibres in the periphery when injected intraperitoneally [449]. Here, 6-OHDA was used to deplete peripheral sympathetic fibres, including that of the spleen. To confirm depletion, thin sections of frozen spleen from mice were used to investigate the density of sympathetic innervation following treatment with vehicle or 6-OHDA. Through sucrose-phosphate-glyoxylic acid (SPG) staining, catecholamines within nerve fibres interact with glyoxylic acid within nerve fibres and can be detected by fluorescence microscopy (cyan staining) in the vehicle treated spleens (Fig. 3.1a). Following administration of 6-OHDA, no fibres containing catecholamines were detected in the section indicating successful depletion of sympathetic fibres from the spleen (Fig. 3.2b).

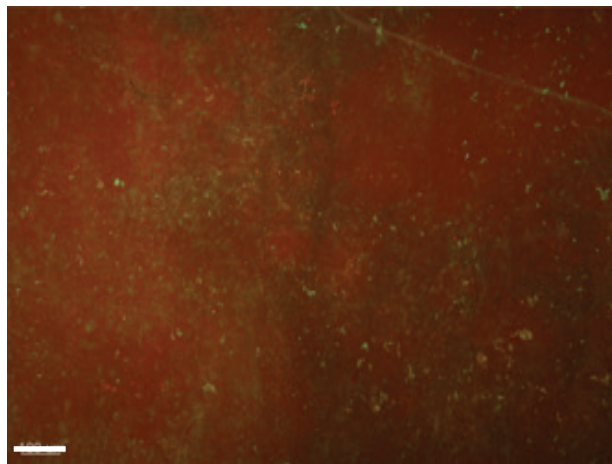
3.2.2 Investigating the T cell response to influenza A challenge following 6-OHDA treatment

To investigate the effects of the SNS on the T cell responses to non-productive infection, mice were challenged with influenza A PR8 strain (IAV PR8) intraperitoneally 3 days following treatment with 6-OHDA (Fig. 3.2a). Tetramers containing the immunodominant epitopes PA224 and NP366 were used to identify the IAV PR8-specific CD8 T cells from the splenocytes at the peak of the response [450]. The proportion and number of CD8 T cells that bound either tetramer was comparable between the treated and non-treated groups suggesting that the expansion of these epitope specific T cells was not impacted by chemical sympathectomy at this effector phase (Fig. 3.2b). The ability of these cells to produce $\text{TNF}\alpha$ or $\text{IFN}\gamma$ was then tested by stimulating the cells with PA224 or NP366 peptide *ex vivo*. 6-OHDA treatment did not impact the production of either $\text{TNF}\alpha$ or $\text{IFN}\gamma$ with similar proportions of CD8 T cells

a)



b)



SPG staining

Counterstain

Figure 3.1 Chemical sympathectomy of the spleen **a)** Sympathic fibres in splenic sections of naïve B6 mice following SPG staining. Nerve fibres containing catecholamines are shown in blue. **b)** B6 mice were treated with 200mg/kg 6-OHDA on D0 and D2, and 100mg/kg 6-OHDA on D4. Loss of SPG staining in splenic sections were then determined on D7. Images are representative of 2 independent experiments with n=6-8 mice. Scale bar, 100µm

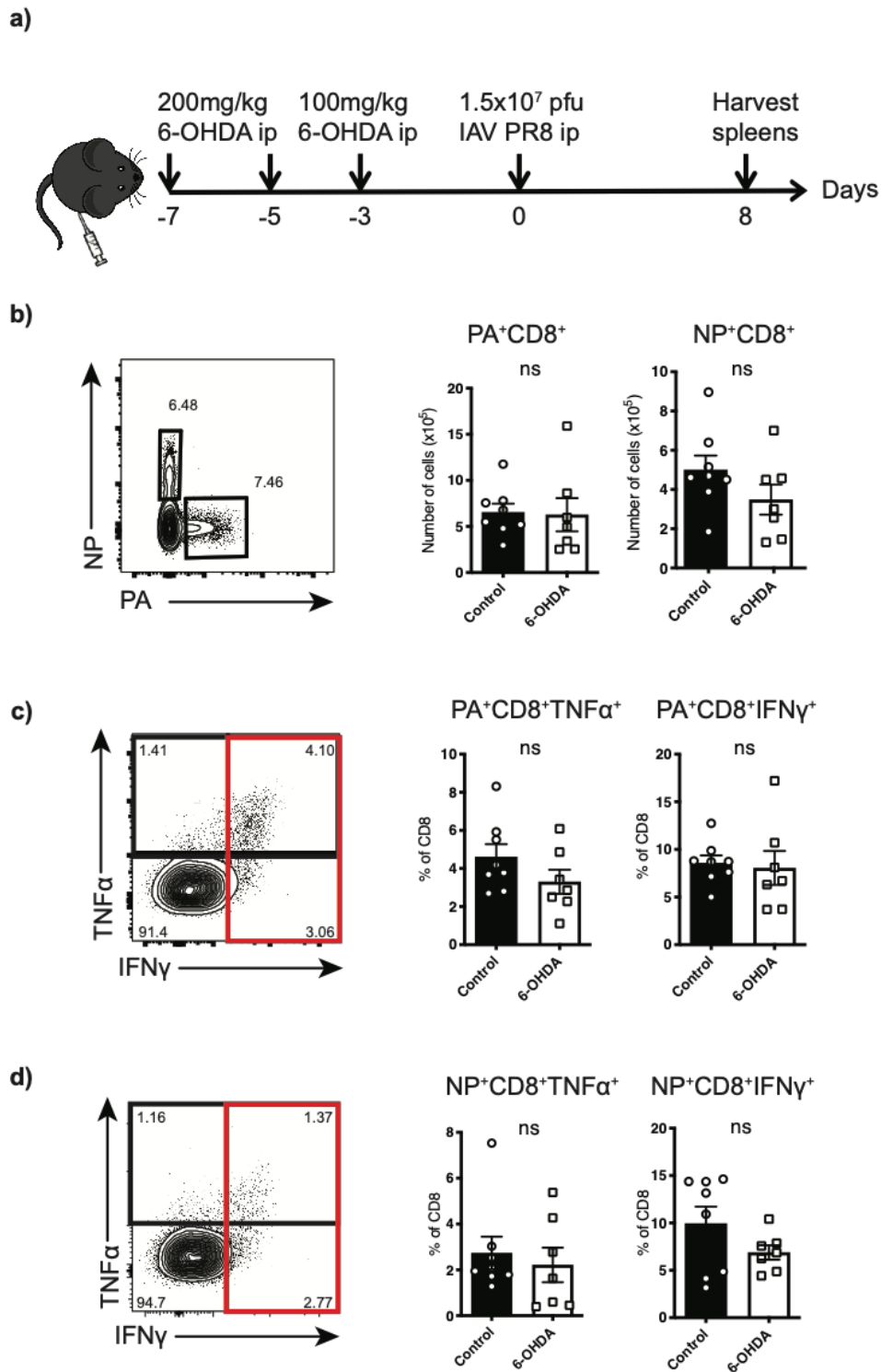


Figure 3.2 T cell responses to IAV PR8 following 6-OHDA treatment **a)** Schematic of experiment outline. Mice were treated with 6-OHDA prior to ip challenge with IAV PR8. **b)** NP and PA tetramers were used to identify IAV PR8 specific T cells. Cells were gated on splenic live CD8⁺ cells and absolute numbers quantified. Splenic cells were stimulated *ex vivo* with **c)** PA peptide or **d)** NP peptide. Percentages of TNFα (black box) and IFNγ (red box) production by CD8 T cells in response to the peptides was measured. Example gating on live CD8⁺ cells. Data is pooled from two independent experiments. n=7-8 per group. Error bars represent the SEM.

producing either cytokine in response to either PA224 or NP366 (Fig. 3.2c). While the unchanged levels of TNF α is consistent with the previous findings, 6-OHDA treatment did not result in improved IFN γ production by PA224-specific CD8 $^{+}$ T cells, as previously reported by Grebe *et al* [185]. Overall, it would appear that the SNS does not play a major role in the immune response to intraperitoneal influenza challenge.

3.2.3 Investigating the T cell response to local herpes simplex virus challenge following chemical sympathectomy

SNS signals have been found to impact lymphocyte recirculation and trafficking [166]. A model of flank infection with herpes simplex virus KOS strain (HSV-KOS) was used to investigate if peripheral SNS nerves have an impact on T cell responses to localised infection. Mice injected with HSV-specific TCR transgenic gBT-I CD8 $^{+}$ T cells and gDT-II CD4 $^{+}$ T cells were treated with 6-OHDA prior to infection via flank scarification which results in lesions on the skin at the site of infection (Fig. 3.3a). Following establishment of infection, viral antigen first drains to the draining LN. The number of gBT-I and gDT-II cells in the draining LN were enumerated and found unchanged following 6-OHDA treatment (Fig 3.3b). This pattern was consistent with the number of gBT-I and gDT-II in the spleen (Fig 3.3c). Skin containing lesions from the HSV infection was taken and the number of gBT-I and gDT-II cells were also enumerated. 6-OHDA treatment also did not affect the number of gBT-I or gDT-II cells in the skin (Fig 3.3d). Taken together, depletion of SNS nerve fibres by 6OHDA did not appear to modulate the ability of responding T cells to mobilise or traffic to different sites following HSV-KOS infection. To determine if TNF α and IFN γ production by these cells was impacted by chemical sympathectomy, an *ex vivo* stimulation of the gBT-I and gDT-II cells, with gB and gD peptide respectively, was performed. Splenic gBT-I cells in both groups were able to produce

both cytokines to the same extent whereas 6-OHDA treatment resulted in a lower proportion of splenic gDT-II cells producing either TNF α or IFN γ (Fig. 3.3e). However, when gBT-I and gDT-II cells from the skin were stimulated, there was no significant change in the production of TNF α or IFN γ in either groups (Fig. 3.3f). While the results suggest that the SNS plays a small role in modulating the splenic immune response to local infection, there appeared to be no influence at the localised site of infection.

3.2.4 Investigating T cell responses to systemic LCMV infection following 6-OHDA treatment

Given the minor effect of chemical sympathectomy on local infections, I wanted to explore if the SNS would augment systemic infections differently. Systemic infections can result in immune responses of different magnitudes and nature compared to that generated by localised infections [451]. Lymphocytic choriomeningitis (LCMV) infection results in infection of multiple organs in the body. Mice treated with 6-OHDA were infected with the Armstrong strain of LCMV that causes an acute infection and the spleen and blood of these mice recovered for T cell analysis and measurement of viral titres (Fig. 3.4a). LCMV-specific TCR transgenic P14 CD8⁺ T cells and SMARTA CD4⁺ T cells were enumerated in the spleen. There was no significant difference in the numbers of P14 or SMARTA T cells following 6-OHDA treatment (Fig 3.4b). Additionally, production of the cytokines TNF α and IFN γ was also unchanged following chemical sympathectomy indicating minimal regulation of effector T cell function to LCMV Armstrong by the SNS (Fig 3.4c). The effect of SNS signalling on viral titres was then determined by plaque assay. Despite a trend towards increased virus titres in the spleen, 6-OHDA treatment did not significantly alter viral load (Fig. 3.4d). Similarly, viral load in the plasma also remained unchanged with treatment (Fig 3.4e).

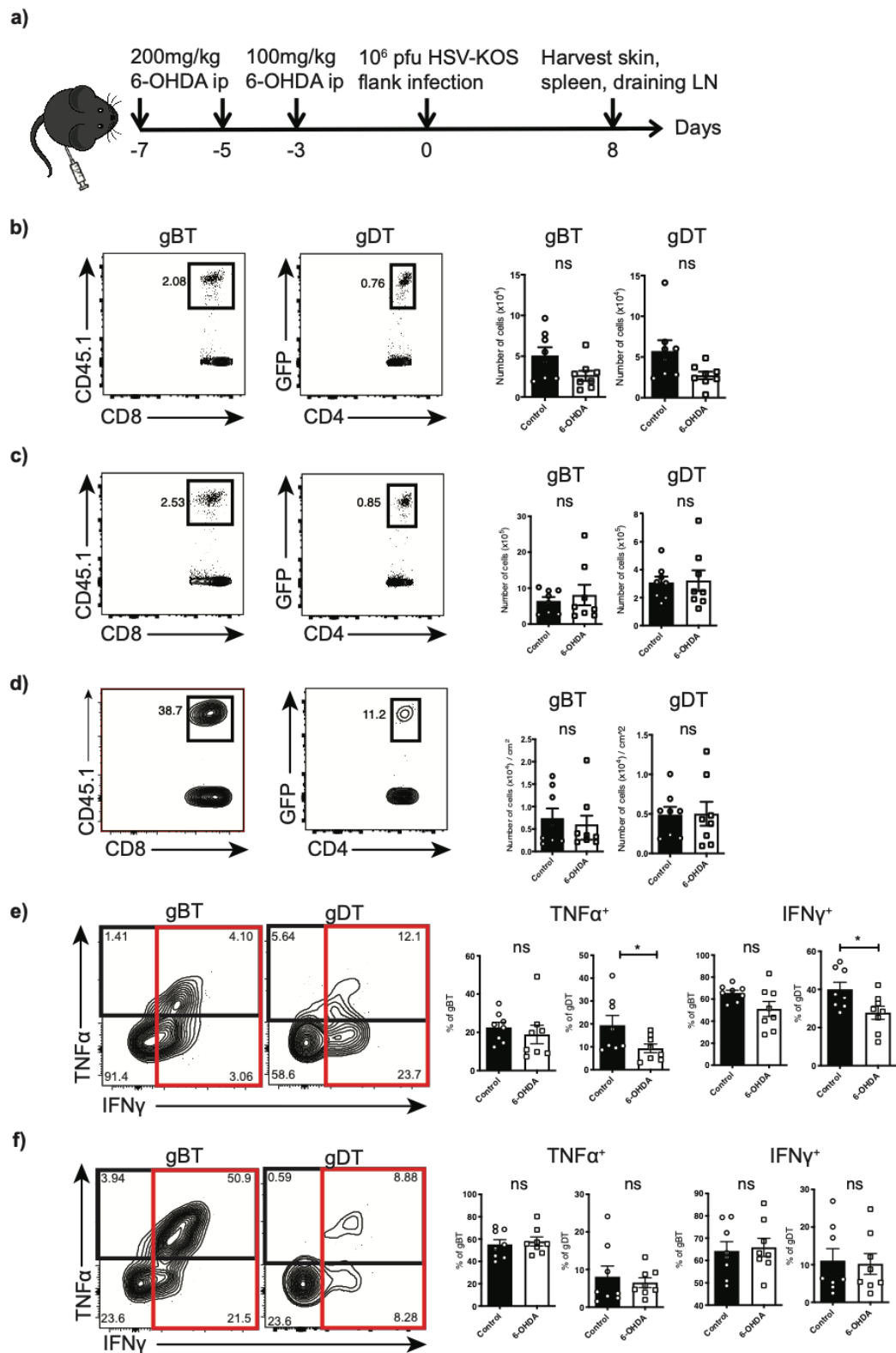


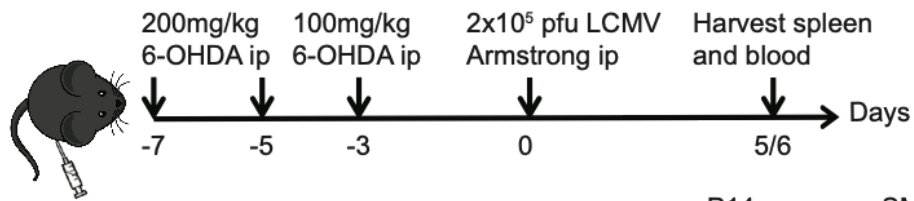
Figure 3.3 T cell responses to HSV-KOS following 6-OHDA treatment **a)** Schematic of experiment outline. Mice seeded with 5×10^4 gBT-I cells and 1×10^4 gDT-II cells were treated with 6-OHDA prior to flank challenge with HSV-KOS. gBT-I and gDT-II cells in the **b)** draining lymph node, **c)** spleen, and **d)** infected skin were identified and enumerated. Gating for gBT-I and gDT-II cells was performed on live CD8⁺ or CD4⁺ lymphocyte populations respectively. gBT-I and gDT-II cells from the **c)** spleen and **d)** skin were stimulated *ex vivo* with gB or gD peptide respectively. Percentages of TNFα (black box) and IFNγ (red box) production in response to the peptides was measured. Data is pooled from two independent experiments. $n=4$ per group per experiment. Error bars represent the SEM. * $p < 0.05$

Whilst LCMV Armstrong is an acute infection which can be cleared easily by day 8 post-infection, LCMV Clone 13 (CL13) is a strain of LCMV that is genetically very similar to LCMV Armstrong aside from two amino acid changes that increase infectivity and replication of CL13 and lead to persistence in C57BL/6 mice [425]. LCMV CL13 is able to persist up to 30 days in the spleen - and longer in other organs – in part by dampening the immune response [423, 424]. To investigate if sympathetic fibres modulate the T cell response during LCMV CL13 infection, mice were subjected to chemical sympathectomy and the T cell response measured (Fig. 3.4f). Similar to LCMV Armstrong infection, there were no significant changes in the number of responding cells with chemical sympathectomy at 7 days post-infection (Fig 3.4g). Following *ex vivo* peptide stimulation, TNF α production by P14 cells but not SMARTA cells was lower with 6-OHDA treatment though IFN γ production was not impacted (Fig 3.4h). Interestingly, viral load in the treated mice was higher in both the spleen and the plasma indicating poorer control of the infection (Fig. 3.4i & j). These results indicate that the SNS does not influence the effector T cell response to LCMV Armstrong or CL13 infection, but may influence the progression of chronic LCMV infection, though this is likely through a T cell-independent mechanism.

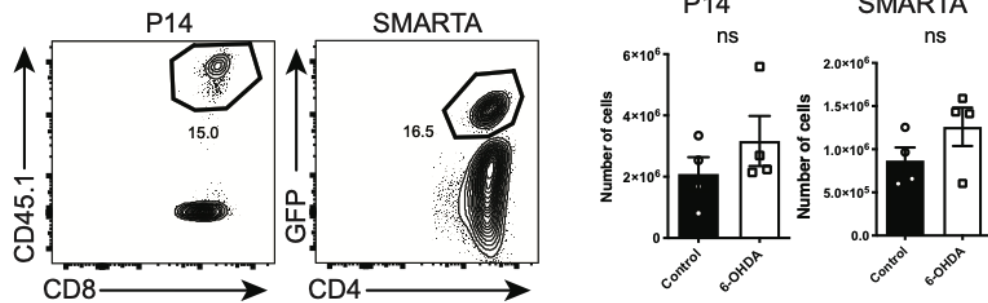
3.2.5 Catecholamine levels in the spleen following LCMV Armstrong infection

Spleen sections from HSV-infected mice treated with 6-OHDA did not stain positive for any catecholamine positive nerve fibres (Fig 3.5a) while nerve fibres were still present in those treated with the vehicle (Fig 3.5b). Spleen sections from LCMV-infected mice treated with 6-OHDA also did not show positive staining for catecholamines (Fig 3.5c). Surprisingly, SPG staining was also absent from the spleen sections of LCMV-infected mice treated with

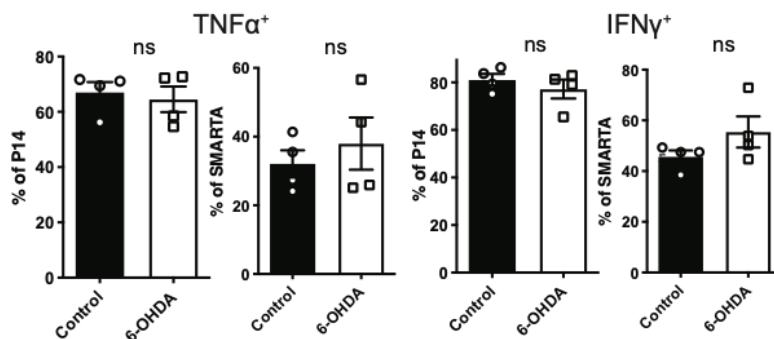
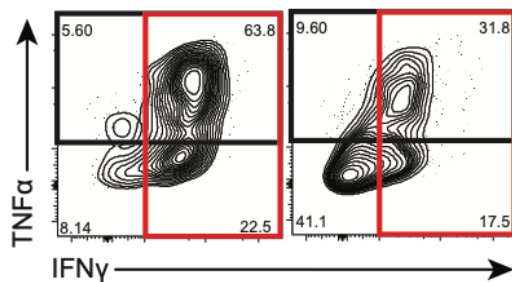
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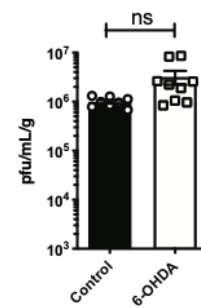
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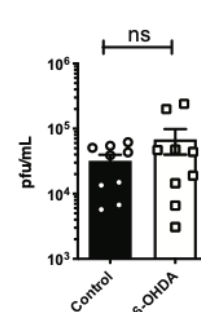
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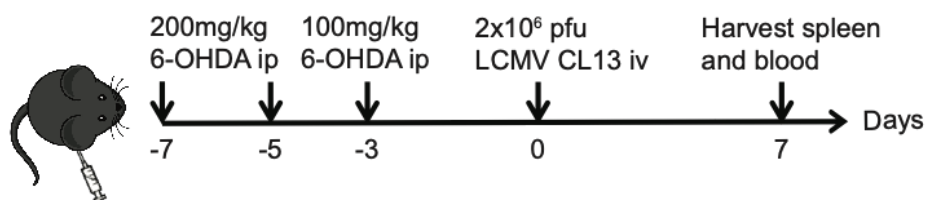
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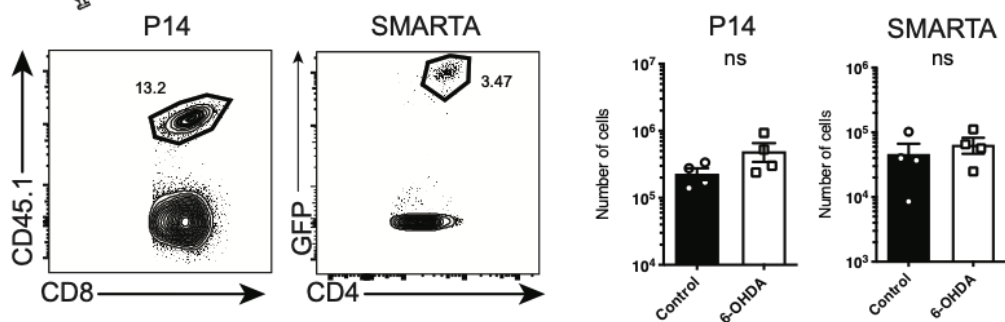
e)



f)



g)



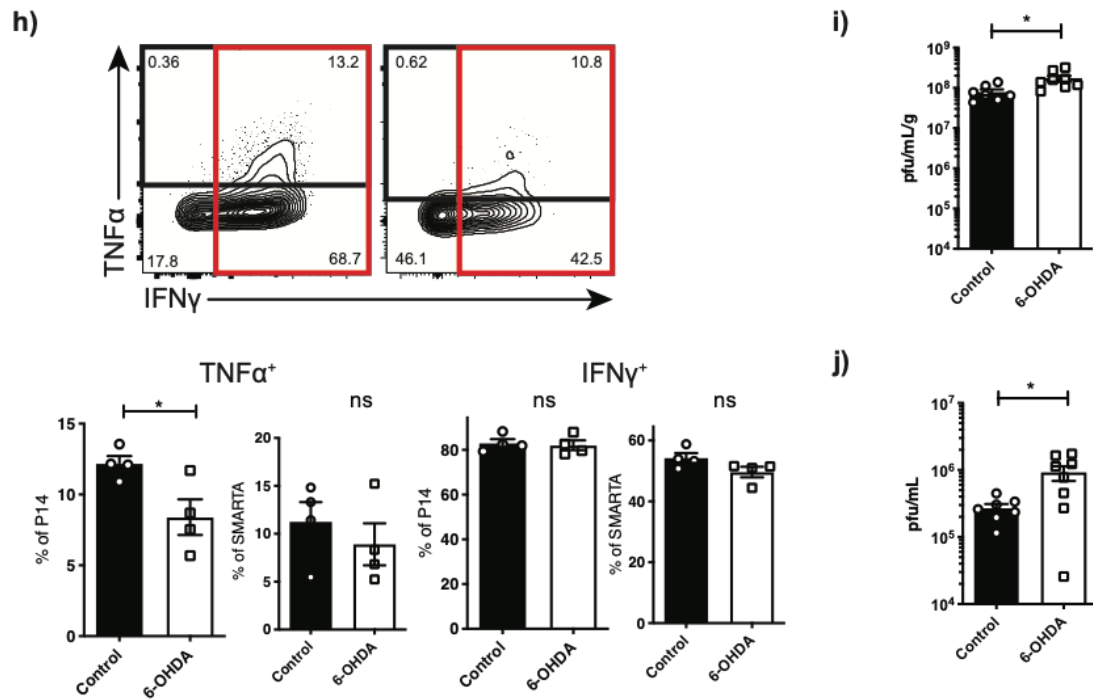


Figure 3.4 T cell responses to systemic LCMV infection following 6-OHDA treatment

a) Schematic of experiment outline. Mice seeded with 1×10^4 P14 cells and 1×10^4 SMARTA cells were treated with 6-OHDA prior to ip challenge with LCMV Armstrong. **b)** P14 and SMARTA cells in the spleen were identified and enumerated 6 days post infection. Gating for P14 and SMARTA cells was performed on live CD8⁺ or CD4⁺ lymphocyte populations respectively. **c)** P14 and SMARTA cells were stimulated *ex vivo* with gp33 or gp61 peptide respectively. Percentages of TNFα (black box) and IFNγ (red box) production in response to the peptides was measured. Virus from **d)** spleen and **e)** blood of mice 5 days post infection was determined using a plaque assay. **f)** Schematic of experiment outline. Mice seeded with 1×10^4 P14 cells and 1×10^4 SMARTA cells were treated with 6-OHDA prior to iv challenge with LCMV CL13. **g)** P14 and SMARTA cells in the spleen were identified and enumerated 7 days post infection. Gating for P14 and SMARTA cells was performed on live CD8⁺ or CD4⁺ lymphocyte populations respectively. **h)** P14 and SMARTA cells were stimulated *ex vivo* with gp33 or gp61 peptide respectively. Percentages of TNFα (black box) and IFNγ (red box) production in response to the peptides was measured. Virus from **i)** spleen and **j)** blood of mice 7 days post infection was determined using a plaque assay. **(b,c)** Data representative of two independent experiments. $n=4-5$ per group per experiment. **(d,e)** Data pooled from two independent experiments. $n=4-5$ per group per experiment. **(g,h)** Data representative of two independent experiments. $n=4-5$ per group per experiment. **(i,j)** Data pooled from two independent experiments. $n=3-4$ per group per experiment. Error bars represent the SEM. * $p < 0.05$

vehicle (Fig. 3.5d). Thus, LCMV infection appeared to result in a reduction in sympathetic innervation in the spleen.

Given these results, the question of how LCMV infection could impact SNS signalling in the spleen needed to be clarified. To do this, noradrenaline (NA) was quantified in spleen and blood samples from naïve and LCMV Armstrong-infected mice by enzyme-linked immunosorbent assay (ELISA). In the spleen, NA levels dropped from approximately 9.72 ± 0.67 ng/spleen to approximately 2.67 ± 0.52 ng/spleen as early as day 3 post-infection ($p < 0.0001$) with a further drop observed at 8 days post-infection (approximately 1.88 ± 0.36 ng/spleen, $p < 0.0001$) (Fig. 3.5e). By 28 days post-infection, NA levels recovered to approximately 7.74 ± 0.41 ng/spleen though not to the same extent as in the naïve state (Fig 3.5e). Notably, circulating levels of NA in the plasma remained unchanged throughout the course of LCMV Armstrong infection (Fig 3.5f). This effect on splenic sympathetic fibres and NA levels was also observed in LCMV CL13-infected mice (data not shown). These results suggest that LCMV infection produces a localised loss of NA and thus may reduce the impact of SNS signals on immune cells in the spleen.

3.2.6 Sympathetic innervation of the spleen following LCMV Armstrong infection

Together with the results from the above experiments revealing a change in NA levels in the spleen following LCMV infection, I wanted to determine if splenic innervation was altered by infection. Previous findings showed that viral infection can affect sympathetic innervation in LN in primates [452]. Immunofluorescence detection of the enzyme tyrosine hydroxylase (TH) and the nerve marker β III-tubulin revealed sympathetic fibres in the spleens of uninfected mice. The highest density of innervation was observed in the white pulp

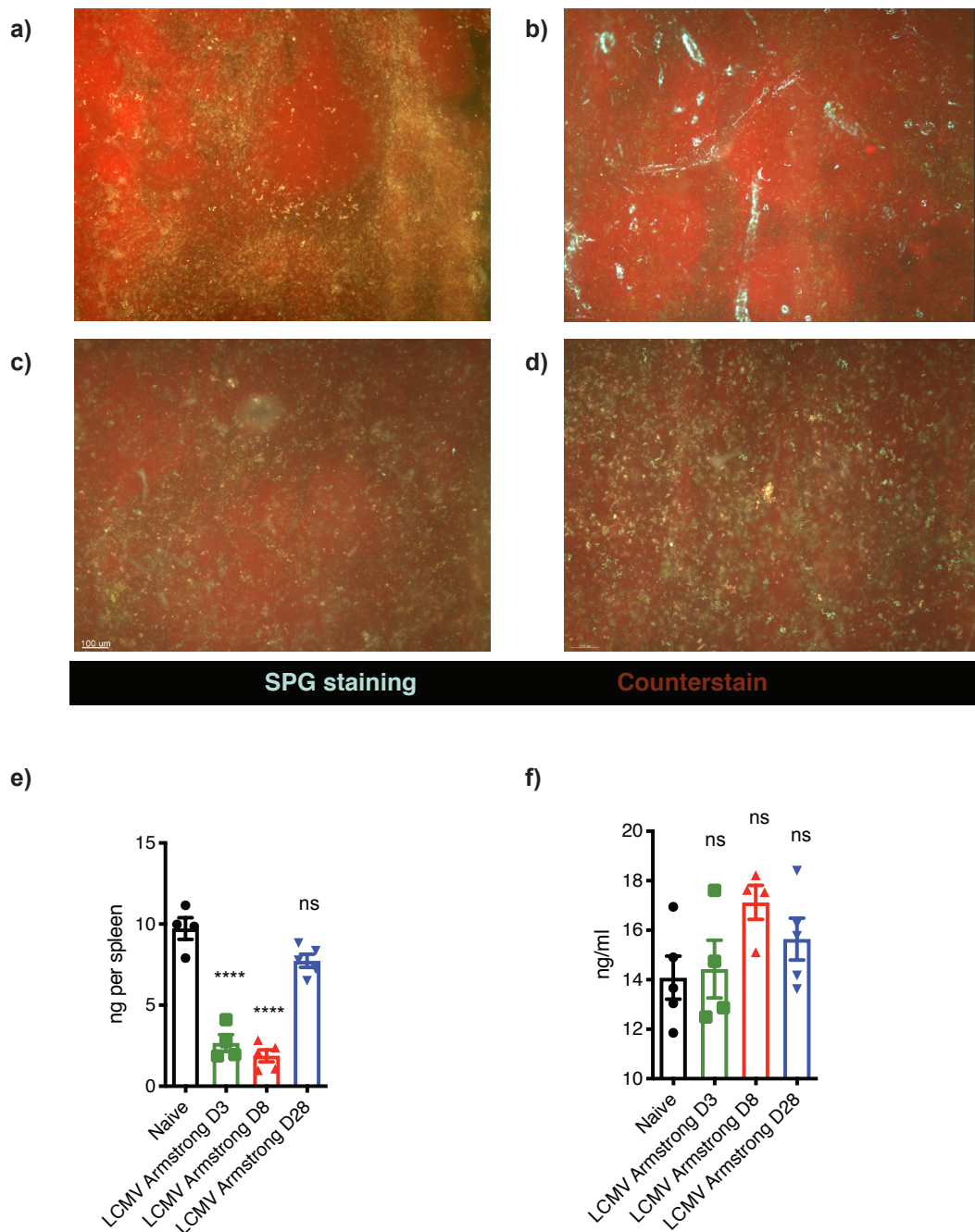


Figure 3.5 Catecholamine levels in the spleen following LCMV Armstrong infection

Spleen from naive and infected were frozen fresh and sectioned for SPG staining. Catecholamines are depicted by cyan staining. **a)** SPG staining of spleen from HSV flank infected mice treated with 6-OHDA. **b)** SPG staining of spleen from HSV flank infected mice treated with vehicle only. **c)** SPG staining of spleen from LCMV Armstrong infected mice treated with 6-OHDA. **d)** SPG staining of spleen from LCMV Armstrong-infected mice treated with vehicle only. **e)** Quantification of noradrenaline in the spleen using ELISA. **f)** Quantification of noradrenaline in the plasma using ELISA (**a-d**) Images representative of two independent experiments. $n=3-5$ per group per experiment. **(e)** Data representative of three independent experiments. $n=4-5$ per group per experiment. Error bars represent the SEM. **** $p<0.0001$

associated with blood vessels and extending out into the parenchyma of the white pulp (Fig 3.6a). There was a loss of detectable TH⁺ βIII-tubulin⁺ fibres in the spleen as early as 3 days post-infection with LCMV. By 8 days post-infection, a majority of the fibres were no longer detected by immunofluorescence. Following clearance of the virus and cessation of immune responses many sympathetic fibres were detected once more (Fig 3.6a). The reduction in sympathetic innervation correlated with altered NA levels in the spleen, with the largest decrease observed 8 days post LCMV-Armstrong infection (Fig 3.6b). Using thicker vibratome sections to visualise the splenic sympathetic network, the difference in innervation following LCMV Armstrong becomes more apparent. Sympathetic fibres were observed wrapped around blood vessels (in white) and extending out to come in contact with other cells and architecture of the white pulp (orange arrows) (Fig 3.6c). However, at 8 days post LCMV infection, far fewer fibres were observed branching out from the vessel-associated fibres (Fig 3.6c). Thus, LCMV Armstrong infection decreased the amount of TH⁺ innervation in the spleen.

3.2.7 The role of immune cells in the loss of splenic TH⁺ fibres.

To determine if immune cells play a role in the loss of splenic TH⁺ fibres, various subsets of immune cells were depleted prior to LCMV Armstrong infection. Given that natural killer (NK) cells were found to be rapidly recruited following LCMV infection [453], mice were depleted of NK cells prior to LCMV infection to determine if NK cells contributed to altered innervation in the spleen. Spleens of NK-depleted mice infected with LCMV showed reduced sympathetic innervation when compared to naïve mice, similar to what was observed in infected non-NK depleted mice (Fig. 3.7a and Fig. 3.7b). However, the depletion of NK cells partially rescued

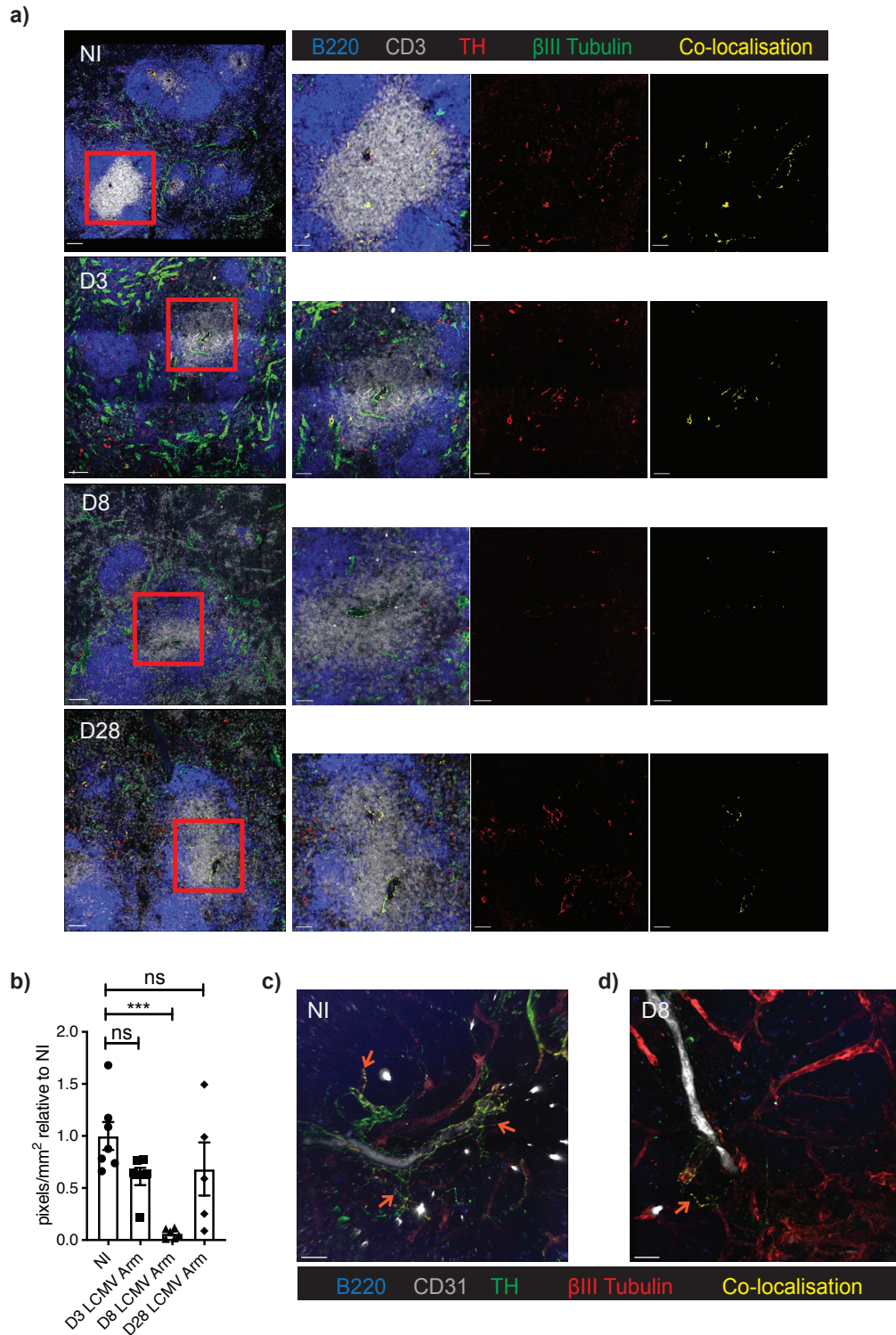


Figure 3.6 Sympathetic innervation of the spleen following LCMV Armstrong infection

Spleen from non-infected (NI) mice and mice injected with LCMV Armstrong ip were fixed frozen and sectioned for immunofluorescence. **a)** Tile scan images of sympathetic fibres in the spleen of NI mice, LCMV-infected mice 3 days (D3), 8 days (D8), and 28 days (D28) post infection are depicted by the colocalisation (yellow) of tyrosine hydroxylase (TH) (red) and β III tubulin (green) staining. Close up view of a white pulp region in the red box is shown next to tile scans. **b)** Quantification of colocalised pixels in splenic tile scan images. Visualisation of the sympathetic network in the spleen in thick vibratome slices of spleen from **c)** naive and **d)** 8 days post LCMV infection. Orange arrows show extension of sympathetic fibres. **(a)** Images representative of two independent experiments. $n=2-4$ per group per experiment. Scale bar 100 μ m. Scale bar on closeups 40 μ m **(b)** Data pooled from two independent experiments. $n=2-4$ per group per experiment. **(c,d)** Images representative of two independent experiments. $n=3-4$ per group per experiment. Scale bar 40 μ m. Error bars represent the SEM. *** $p<0.001$

the loss of TH⁺ fibres when comparing to non-depleted infected spleens (Fig 3.7e). While CD4⁺ T cells are not crucial for the clearance of LCMV Armstrong, they are capable of producing various cytokines that can stimulate other immune cells. Following depletion of CD4⁺ T cells, mice infected with LCMV Armstrong also showed reduced TH⁺ innervation in the spleen (Fig 3.7c, f). I also examined a role for CD8⁺ T cells, which produce cytokines such as IFN γ and kill virus infected cells [426]. Infection with LCMV Armstrong in mice depleted of CD8⁺ T cells resulted in substantial loss of splenic sympathetic innervation (Fig 3.7d and Fig 3.7g). These results suggest that NK cells and CD4⁺ or CD8⁺ T cells alone are not responsible for the decrease in splenic TH⁺ fibres.

3.2.8 The role of interferon in the loss of splenic TH⁺ fibres

Next, other components of the immune response to LCMV that might contribute to the change in nerve fibres in the spleen were examined. Type I and type II interferons have both been implicated in the immune responses to LCMV [430, 431, 454]. IFNAR mice with cells lacking the receptor for type I interferon were infected with LCMV Armstrong and spleens examined for innervation. Splenic sympathetic nerve fibres were not retained in infected IFNAR mice (Fig 3.8a, b and d). Similarly, spleens of IFN γ ^{-/-} mice infected with LCMV had an overall decrease in TH⁺ fibres (Fig 3.8c). Interestingly, the drop in TH⁺ nerve fibres in IFN γ ^{-/-} mice was not to the same extent as wild type mice suggesting that IFN γ may contribute to the loss of TH⁺ nerve fibres in the spleen (Fig 3.8e). While not entirely responsible for the decrease in nerve fibres of the spleen, these results suggest that IFN γ can influence TH⁺ innervation in the spleen.

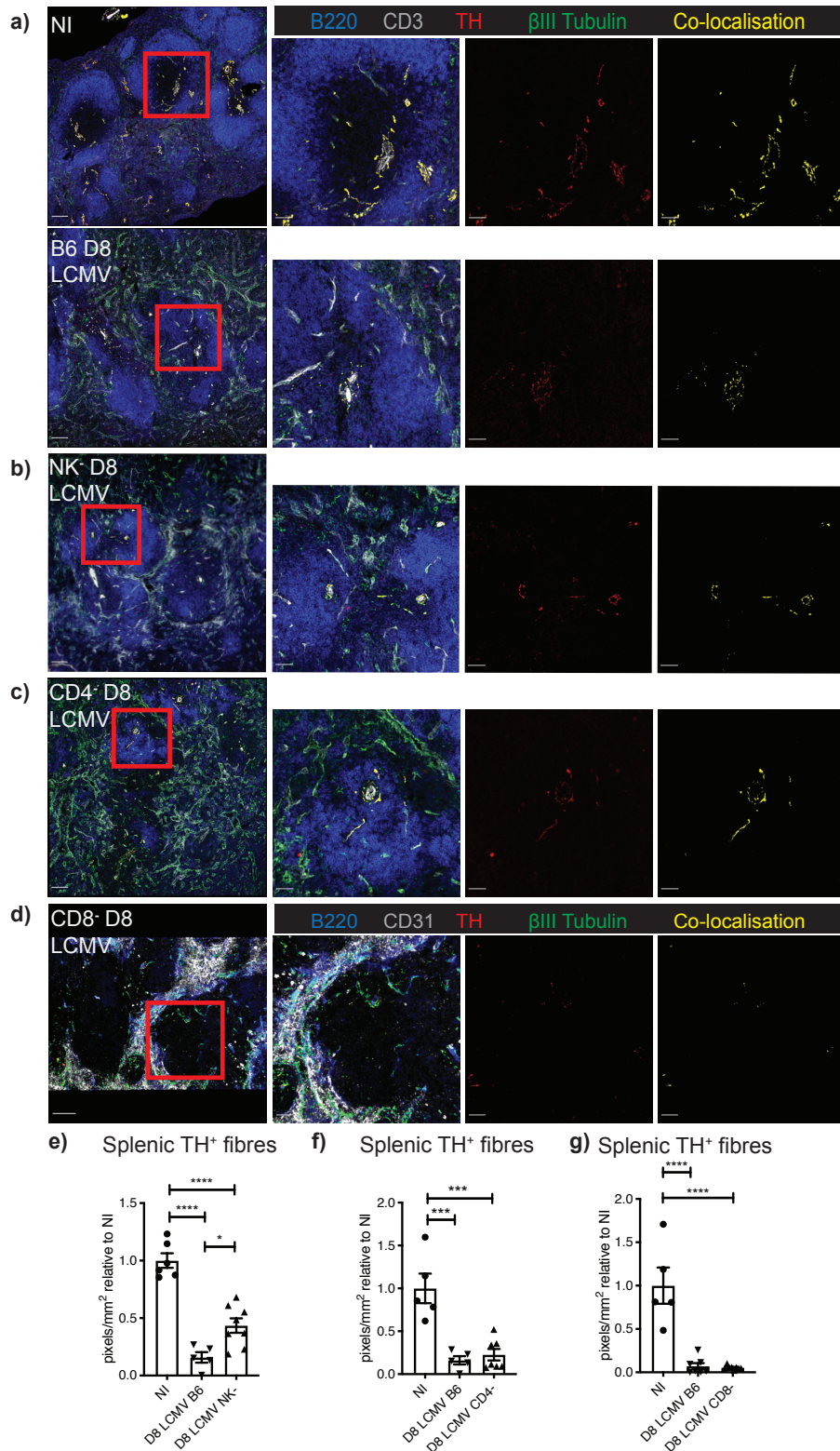


Figure 3.7 The role of immune cells in the loss of splenic TH⁺ fibres. Spleen from non-infected (NI) and infected mice were fixed frozen and sectioned for immunofluorescence. **a)** Tile scan images of sympathetic fibres in the spleen of NI mice and LCMV-infected mice 8 days (D8) post infection are depicted by the colocalisation (yellow) of tyrosine hydroxylase (TH) (red) and βIII tubulin (green) staining. Close up view of a white pulp region in the red box is shown next to tile scans. **b)** Tile scan images of spleen in mice where NK cells were depleted *in vivo* prior to LCMV infection. **c)** Tile scan images of spleen in mice where CD4 T cells were depleted *in vivo* prior to LCMV infection. **d)** Tile scan images of spleen in mice where CD8 T cells were depleted *in vivo* prior to LCMV infection. **e)** Quantification of colocalised pixels in NK⁺ splenic tile scan images. **f)** Quantification of colocalised pixels in CD4 T cell⁺ splenic tile scan images. **g)** Quantification of colocalised pixels in CD8 T cell⁺ splenic tile scan images. **(a-d)** Images representative of two independent experiments. n=2-4 per group per experiment. **(e-g)** Data pooled from two independent experiments expressed as a ratio to NI. n=2-4 per group per experiment. Scale bar 100 μm. Scale bar on closeups 40 μm. Error bars represent the SEM. * $p < 0.05$ *** $p < 0.001$ **** $p < 0.0001$

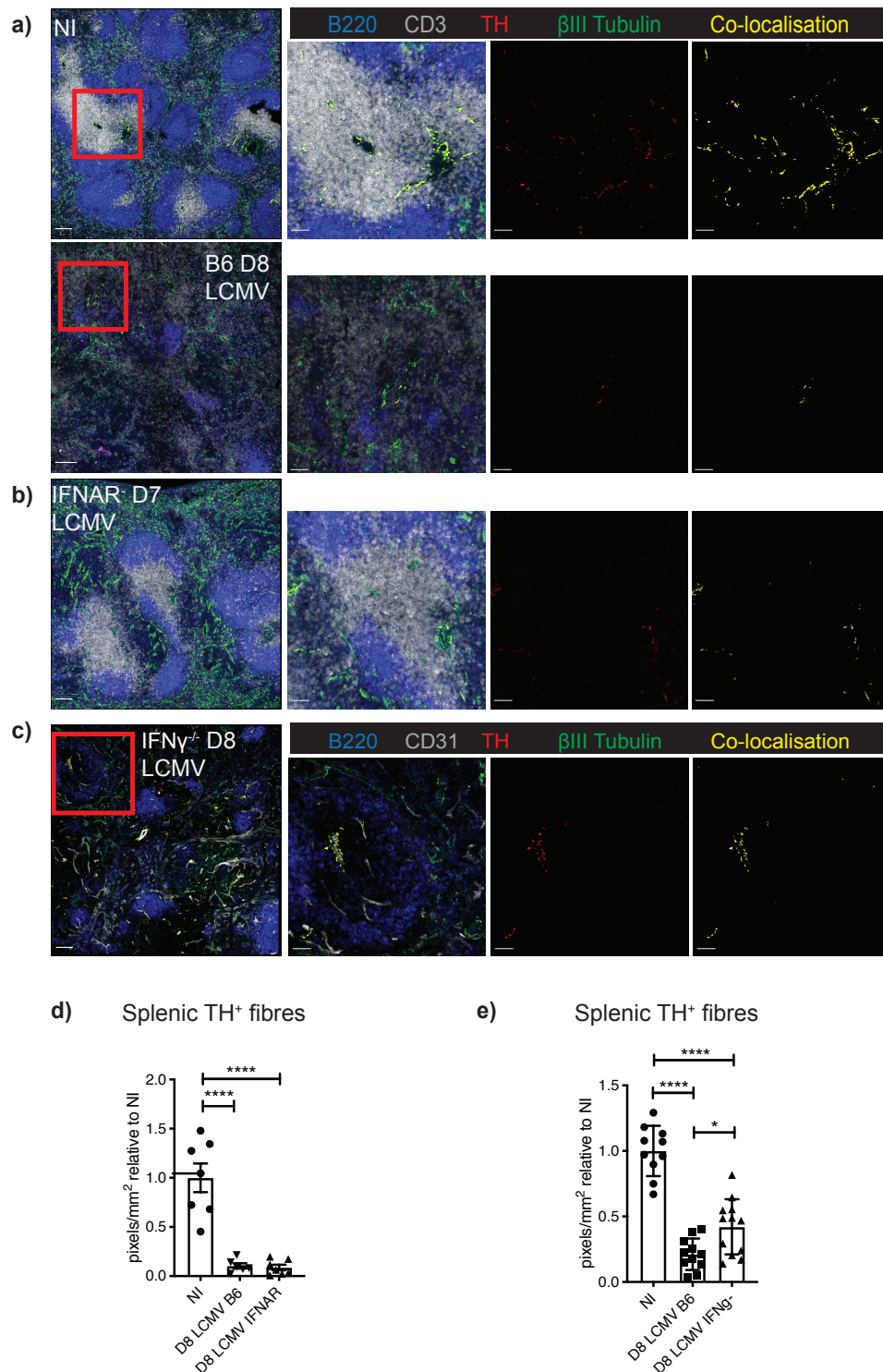


Figure 3.8 The role of interferon in the loss of splenic TH⁺ fibres. Spleen from non-infected (NI) and infected mice were fixed frozen and sectioned for immunofluorescence. **a)** Tile scan images of sympathetic fibres in the spleen of naive mice and LCMV-infected mice 8 days post infection are depicted by the colocalisation (yellow) of tyrosine hydroxylase (TH) (red) and β III tubulin (green) staining. Close up view of a white pulp region in the red box is shown next to tile scans. **b)** Tile scan images of spleen in IFNAR mice 7 days (D7) post LCMV infection. **c)** Tile scan images of spleen in IFN γ ^{-/-} mice 8 days (D8) post LCMV infection. **d)** Quantification of colocalised pixels in IFNAR splenic tile scan images. **e)** Quantification of colocalised pixels in IFN γ ^{-/-} splenic tile scan images. **(a,b)** Images representative of two independent experiments. n=2-4 per group per experiment. **(c)** Images representative of three independent experiments. n=3-5 per group per experiment. **(d)** Data pooled from two independent experiments expressed as a ratio to NI. n=2-4 per group per experiment. **(e)** Data pooled from three independent experiments expressed as a ratio to NI. n=3-5 per group per experiment. Scale bar 100 μ m. Scale bar on closeups 40 μ m. Error bars represent the SEM. * $p < 0.05$ **** $p < 0.0001$

3.2.9 Transcriptional changes in the ganglia and spleen following LCMV Armstrong infection

Sympathetic fibres in the spleen originate from the celiac ganglia (CG) and changes in the spleen may be the result of changes in the ganglia. To investigate if this is the case, CG of infected mice were prepared for immunohistochemistry or qPCR. Nerve cell bodies in the CG of LCMV-infected mice showed reduced intensity of TH staining by immunofluorescence (Fig. 3.9a and Fig 3.9b). Expression levels, relative to the housekeeper gene *Hprt*, of various genes that are associated with nerve growth and survival were sampled in the tissues of LCMV-infected and non-infected mice to determine potential changes that could contribute to the decreased TH staining in the CG and spleen. Notably, *Th* and *Dbh* - which encode the enzymes TH and dopamine hydroxylase and are essential enzymes in the production of SNS catecholaminergic neurotransmitters - were downregulated in both the CG and spleen (Fig 3.10c and 3.10d). Transcription of genes encoding nerve growth factors including *Ngf*, *Bdnf*, *Vegf*, and *Ntf3* were highly upregulated in the CG but downregulated in the spleen (Fig 3.9c and 3.9d). Expression of growth factor receptors in the spleen, such as *Ngfr* and *Gfra3*, remained largely unchanged, although there was a decrease of *Ngfr* in the CG. Additionally, gene expression of *Npy*, coding for neuropeptide Y that can also be found in sympathetic neurons, was downregulated in the spleen but not the CG. *Chat* expression encoding for choline acetyltransferase (Chat), the enzyme responsible for the synthesis of the neurotransmitter acetylcholine, was increased in the CG and decreased in the spleen (Fig 3.9c and 3.9d). CG neurons are predominantly sympathetic in nature and do not typically express Chat [455]. A separate assay using different primers for *Th* was performed to validate the TH results, revealing a similar decrease in *Th* gene expression in both the CG and spleen (Fig. 3.9e). Overall, LCMV infection impacts expression of various neural associated genes in both the CG and the spleen resulting in altered functions of nerve fibres that innervate the spleen.

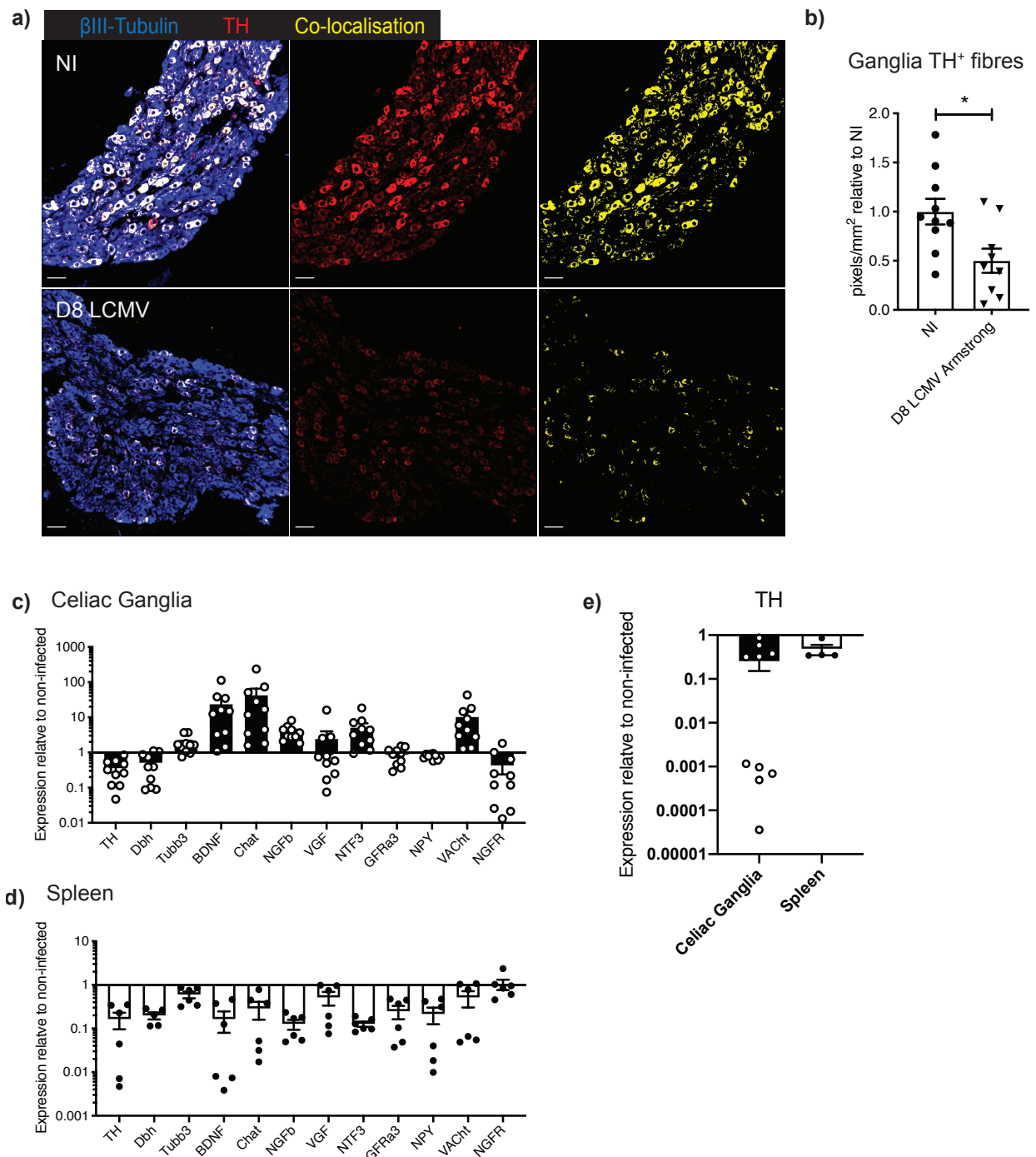


Figure 3.9 Transcriptional changes in the ganglia and spleen following LCMV Armstrong infection
 Celiac ganglia from naive and infected mice were fixed frozen and sectioned for immunofluorescence. **a)** Tile scan images of sympathetic neurons in the celiac ganglia of naive mice and LCMV-infected mice 8 days post infection are depicted by the colocalisation (yellow) of TH (red) and β III tubulin (blue) staining. **b)** Quantification of colocalised pixels in LCMV-infected celiac ganglia tile scan images. **c)** Relative gene expression of various genes determined using SYBR Green qPCR in the celiac ganglia following LCMV infection. **d)** Relative gene expression of various genes determined using SYBR Green qPCR in the spleen following LCMV infection. **e)** Relative gene expression of TH determined using Taqman qPCR in the celiac ganglia and spleen following LCMV infection. **(a)** Images representative of two independent experiments. $n=4-5$ per group per experiment. **(b)** Data pooled from two independent experiments. $n=4-5$ per group per experiment. **(c-e)** Data pooled from the average of two independent experiments. $n=2-5$ per group per experiment. Scale bar 100 μ m. Error bars represent the SEM. * $p<0.05$

3.2.10 Sympathetic innervation of the spleen in other models of systemic infection

To determine if the decrease in sympathetic innervation of the spleen is specific to LCMV infection, I investigated the effect of other models of systemic infection that also affect the spleen. *Listeria monocytogenes* is a bacterial infection that initially infects gut tissue but can then disseminate to other organs and become systemic [456]. Sections of spleen from *Listeria*-infected mice showed unchanged sympathetic innervation of the splenic white pulp both in terms of pattern and amount of innervation (Fig. 3.10a and 3.10c).

Plasmodium berghei ANKA was also used as an alternative model of systemic infection. Injection of mice with infected red blood cells (iRBC) containing *Plasmodium berghei* ANKA causes a systemic infection resulting in the symptoms of malaria [457, 458]. Similar to infection with *Listeria*, infection with *Plasmodium berghei* ANKA appeared to have no influence on the sympathetic innervation of the spleen (Fig. 3.10b and 3.10c). Nerve fibres were still seen associated with blood vessels and branching into the parenchyma. Taken together, the results suggest that sympathetic innervation of the spleen is impacted by LCMV infection but not *Listeria* or malaria infection.

3.2.11 T cell responses to LCMV Armstrong following isoproterenol treatment

The early decrease in TH⁺ fibres and catecholamines within the spleen following LCMV infection raised the question of how the immune response might be impacted by reduced local catecholamine availability. To test if the loss of NA had an impact on T cell responses, mice infected with LCMV Armstrong were treated daily with isoproterenol, a β -adrenoceptor agonist to supplement the reduced sympathetic signalling as a result of LCMV infection (Fig 3.11a). P14 and SMARTA T cells from the spleen of these mice were enumerated and found

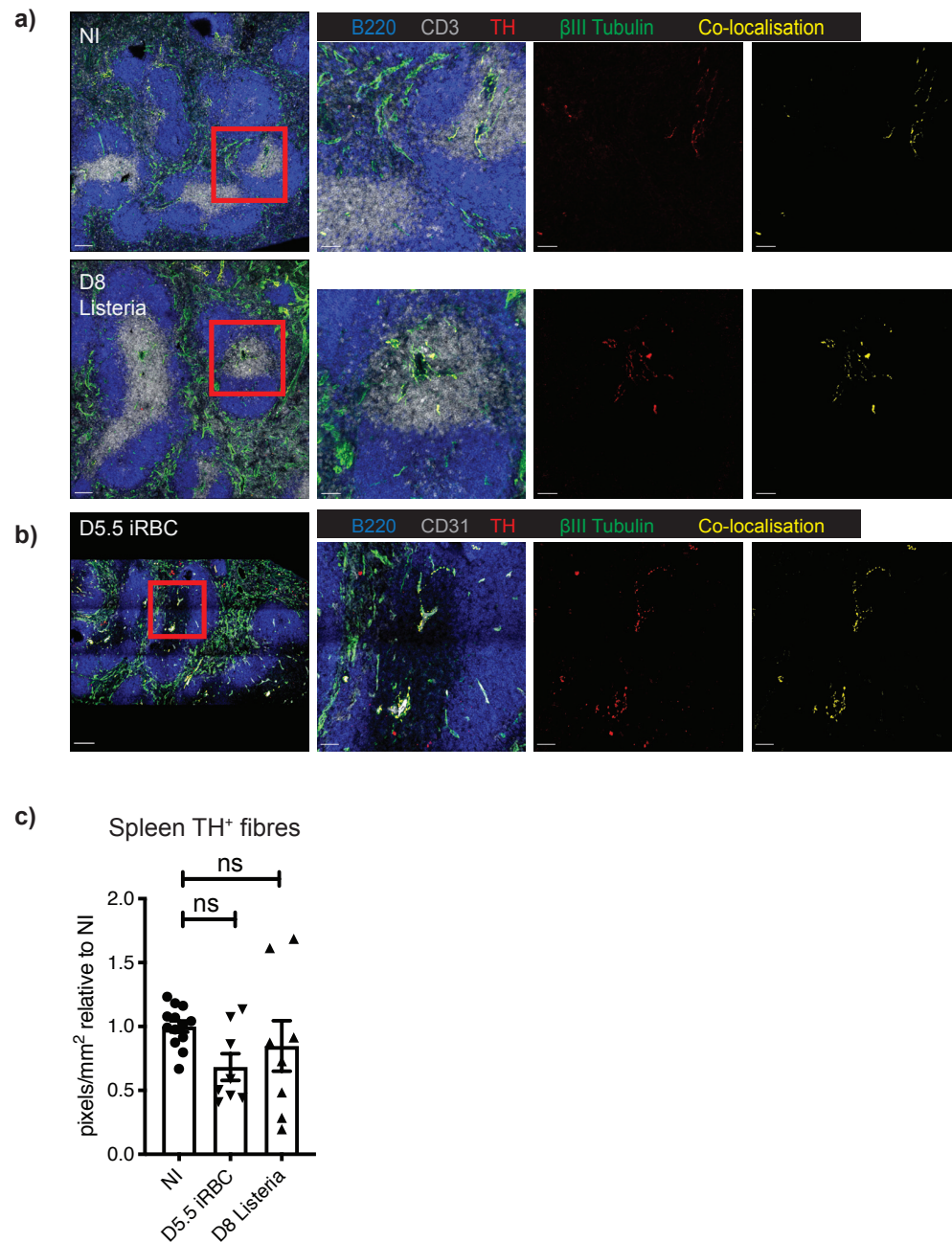


Figure 3.10 Sympathetic innervation of the spleen in other models of systemic infection

Spleen from noon-infected (NI) and infected mice were fixed frozen and sectioned for immunofluorescence. **a)** Tile scan images of sympathetic fibres in the spleen of NI mice and *Listeria monocytogenes*-infected mice 8 days (D8) post infection are depicted by the colocalisation (yellow) of tyrosine hydroxylase (TH) (red) and β III tubulin (green) staining. Close up view of a white pulp region in the red box is shown next to tile scans. **b)** Tile scan images of sympathetic fibres in the spleen of *Plasmodium berghei* ANKA-infected mice 5.5 days (D5.5) post injection of infected red blood cells. **c)** Quantification of colocalised pixels in *Listeria monocytogenes*-infected or *Plasmodium berghei* ANKA-infected splenic tile scan images. **(a)** Images representative of two independent experiments. $n=3-4$ per group per experiment. **(b)** Images representative of two independent experiments. $n=2-5$ per group per experiment. **(c)** Data pooled from two independent experiments. $n=3-4$ per group per experiment. Scale bar on closeups 40 μ m. Error bars represent the SEM.

to be no different between the isoprotenerol treated and PBS treated groups with regards to cell numbers (Fig 3.11b). $\text{TNF}\alpha$ and $\text{IFN}\gamma$ production by these T cells was also measured following *ex vivo* peptide stimulation. Isoprotenerol treatment had no effect on P14 T cell production of $\text{TNF}\alpha$ and $\text{IFN}\gamma$. While SMARTA T cells also produce similar levels of $\text{TNF}\alpha$ following isoprotenerol treatment, they were observed to produce less $\text{IFN}\gamma$ following restimulation (Fig 3.11c). Plaque assays of spleen and blood samples to determine viral load were also performed but both groups successfully cleared the virus 8 days post-infection (data not shown) indicating that the effects of isoprotenerol did not significantly delay viral control. This is consistent with observation that isoprotenerol treatment did not influence T cell responses and thus imply that increasing SNS signalling following the reduction in endogenous NA with LCMV Armstrong infection does not affect the antiviral response.

3.2.12 Immune responses to LCMV CL13 following isoprotenerol treatment

LCMV CL13 infection also resulted in loss of splenic TH^+ fibres. LCMV CL13-infected mice were treated with isoprotenerol from day 1 to 7 (Fig. 3.12a). Comparable numbers of P14 and SMARTA T cells were recovered from the spleen, although a non-significant trend towards lower numbers of cells was observed in the treated group (Fig. 3.12b). $\text{TNF}\alpha$ and $\text{IFN}\gamma$ production by P14 T cells was modulated by the isoprotenerol treatment with a decrease in $\text{TNF}\alpha$ and a slight increase in $\text{IFN}\gamma$ levels (Fig 3.12c). SMARTA T cells were less affected by treatment resulting in a slight increase in $\text{IFN}\gamma$ production (Fig 3.12c and Fig. 3.11c). Treatment with isoprotenerol also resulted in an increased viral load in both the spleen and plasma (Fig. 3.12d and Fig. 3.12e). Additionally, mice treated with isoprotenerol generally had poorer body condition scores compared to the untreated counterparts suggesting that isoprotenerol treatment led to impaired health outcomes.

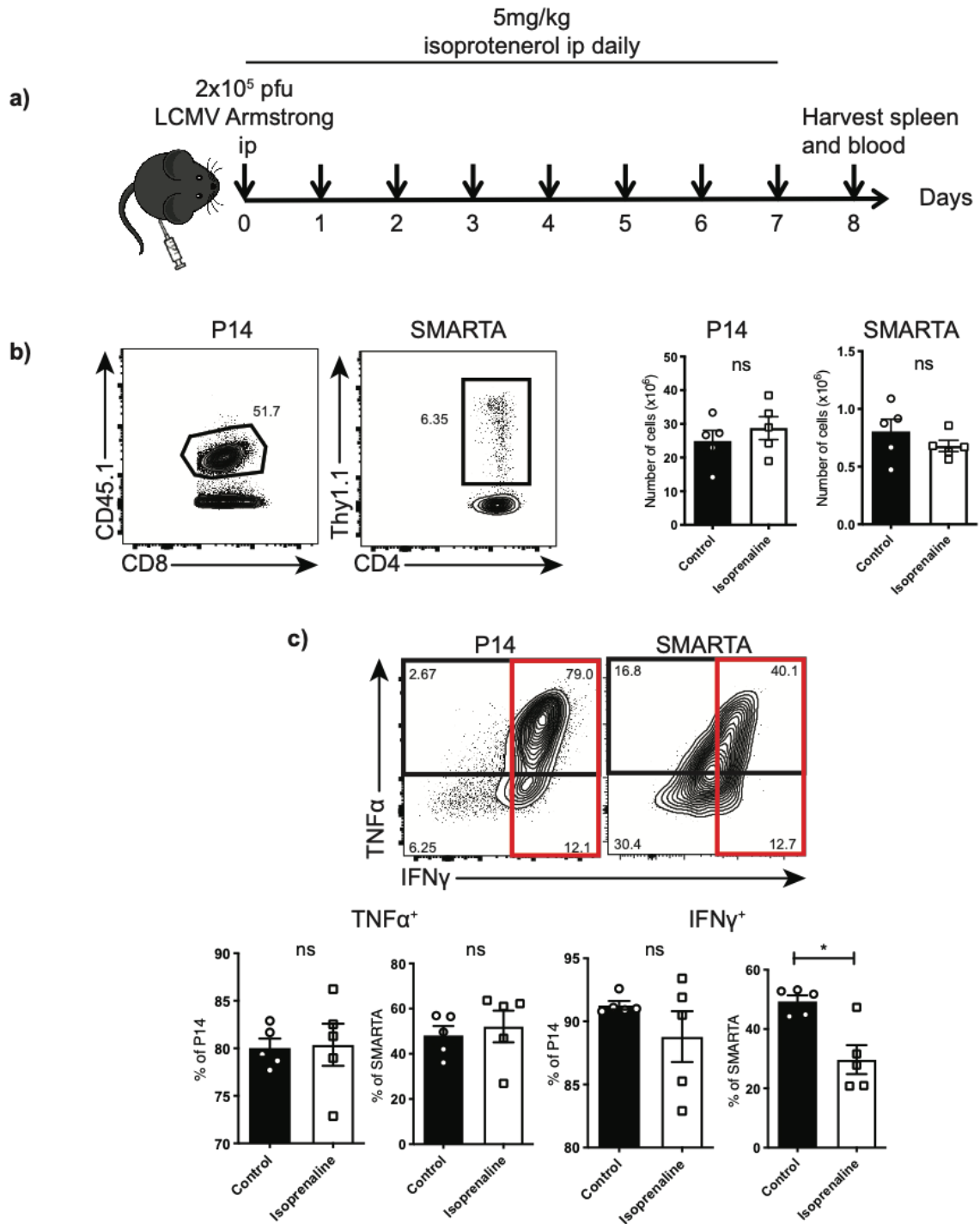
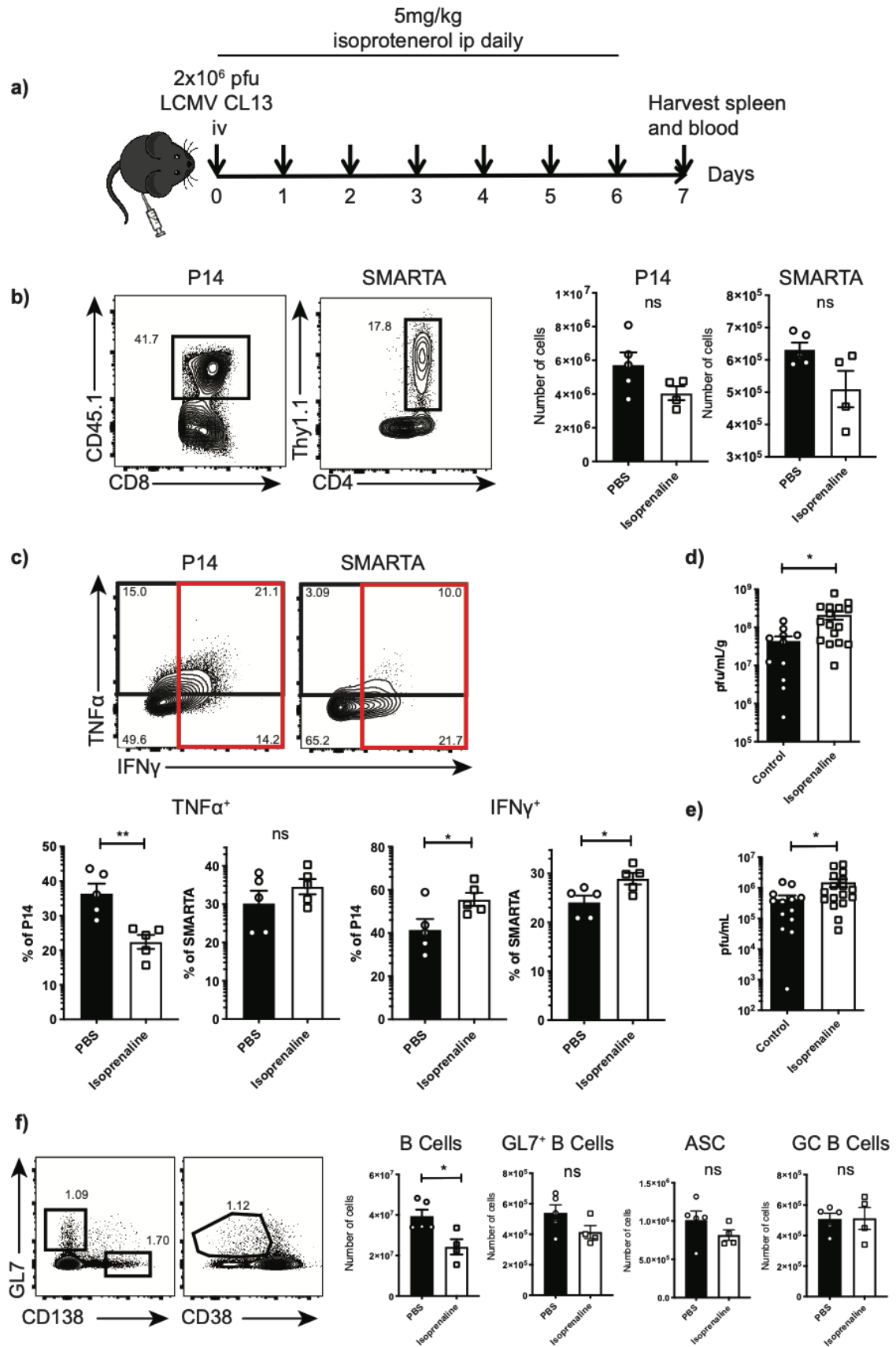


Figure 3.11 T cell responses to LCMV Armstrong following isoprotenerol treatment

a) Schematic of experiment outline. Mice seeded with 1×10^4 P14 cells and 1×10^4 SMARTA cells were treated daily with 5mg/kg isoprotenerol following ip challenge with LCMV Armstrong. **b)** P14 and SMARTA cells in the spleen were identified and enumerated 8 days post infection. Gating for P14 and SMARTA cells was performed on live CD8⁺ or CD4⁺ lymphocyte populations respectively. **c)** P14 and SMARTA cells were stimulated *ex vivo* with gp33 or gp61 peptide respectively. Percentages of TNF α (black box) and IFN γ (red box) production in response to the peptides was measured. **(b,c)** Data representative of two independent experiments. $n=4-5$ per group per experiment. Error bars represent the SEM. * $p < 0.05$

To determine if the poorer disease outcome following isoprotenerol treatment might be related to the immune response, other immune cell types were also examined to discover if they were affected by the increased SNS signalling activity. There were fewer B cells in the isoproterenol-treated group, although when looking further into a few B cell subtypes such as activated GL7⁺ B cells, antibody secreting cells (ASC), and germinal centre (GC) B cells, there were no significant differences in the number of cells recovered (Fig. 3.12f). There were equal numbers of monocytes and neutrophils between the PBS and treated groups (Fig. 3.12g). Similarly, there was no significant change in the number of F480⁺ macrophages with isoprotenerol treatment. Next, subsets of dendritic cells (DCs) were examined. Plasmacytoid DCs (pDCs) are important for successful clearing of LCMV [459] and so may be important in the altered outcomes seen in the treated group. However, pDC numbers were unaffected by isoprotenerol treatment (Fig. 3.12i). Conventional DCs 1 (cDC1) and conventional DCs 2 (cDC2) that are involved in antigen presentation both appeared to be lower in the treated mice, but this decrease was not significant (Fig. 3.12i). Finally, natural killer (NK) and natural killer T (NKT) cells were enumerated and found to not change with isoprotenerol treatment (Fig. 3.12j).

Increased IL-10 is associated with viral persistence of LCMV CL13 [432] and IL-10 production can be increased following β -adrenoceptor signalling [141]. Thus, the amount of IL-10 produced by the various cell types were analysed by flow cytometry. Approximately 4% of lymphocytes in the spleen were producing IL-10 with the highest proportion of cell types being neutrophils and NK T cells, both which are documented to be capable of IL-10 production though not in the context of LCMV CL13 [460, 461] (Fig. 3.12k). Of the cells that did produce IL-10, isoprotenerol treatment did not affect the amount of IL-10 that each cell type produced (Fig. 3.12l).



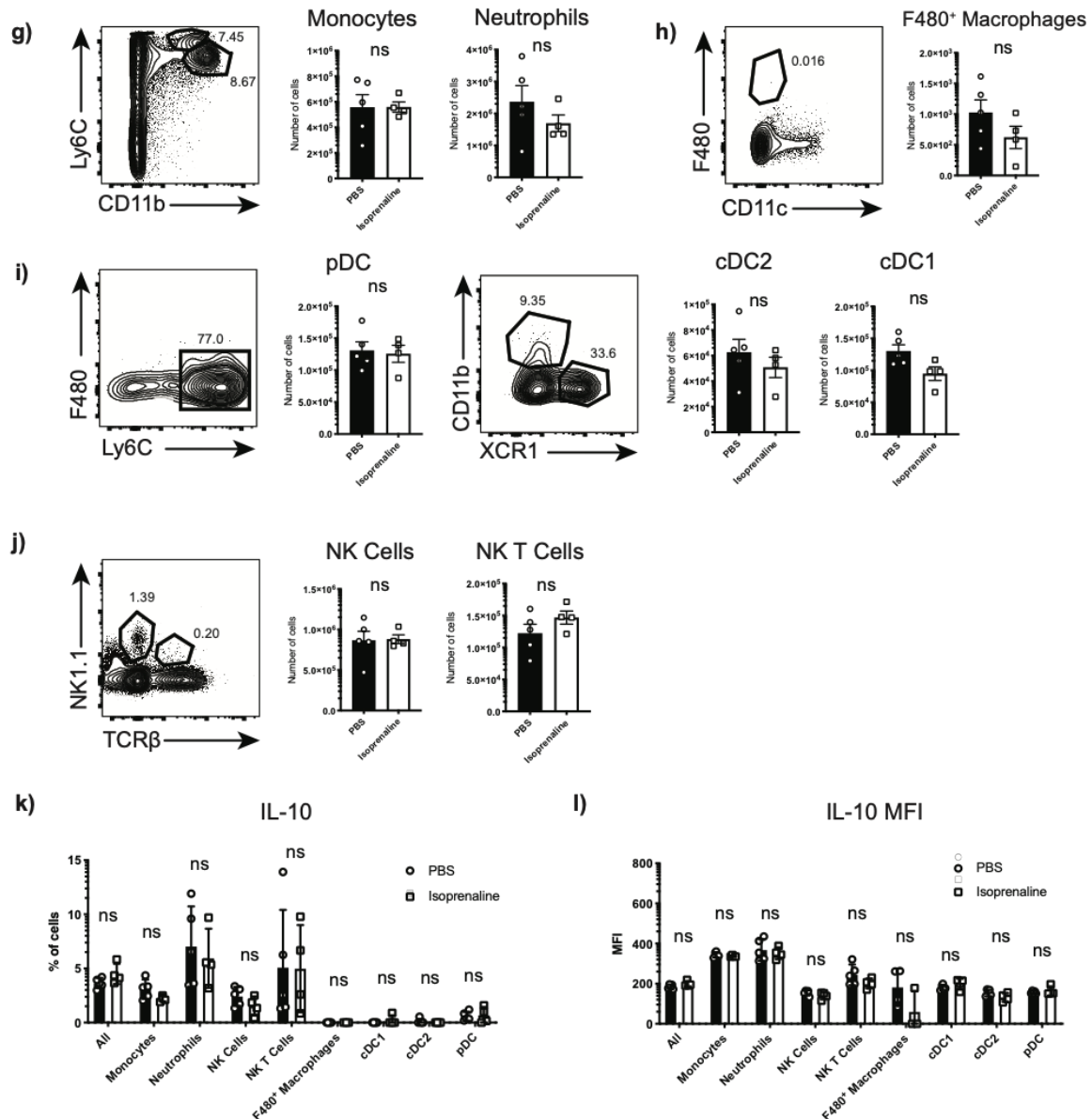


Figure 3.12 Immune responses to LCMV CL13 following isoprotenerol treatment

a) Schematic of experiment outline. Mice seeded with 2×10^4 P14 cells and 2×10^4 SMARTA cells were treated with 5mg/kg isoprotenerol following iv challenge with LCMV CL13. **b)** P14 and SMARTA cells in the spleen were identified and enumerated 7 days post infection. Gating for P14 and SMARTA cells was performed on live CD8⁺ or CD4⁺ lymphocyte populations respectively. **c)** P14 and SMARTA cells were stimulated *ex vivo* with gp33 or gp61 peptide respectively. Percentages of TNFα (black box) and IFNγ (red box) production in response to the peptides was measured. Virus from **d)** spleen and **e)** blood of mice 7 days post infection was determined using a plaque assay. **f)** Enumeration of B cells in spleens of LCMV CL13 infected mice with or without isoprotenerol treatment. B cells were gated on live NK1.1⁻ B220⁺ and/or CD19⁺ lymphocytes. B cell subsets gated from B cell population. **g)** Enumeration of monocytes and neutrophils in spleens of LCMV CL13 infected mice with or without isoprotenerol treatment. Gated on live NK1.1⁻ CD19⁻ TCRβ⁻ lymphocytes. **h)** Enumeration of F480⁺ macrophages in spleens of LCMV CL13 infected mice with or without isoprotenerol treatment. Gated on live NK1.1⁻ CD19⁻ TCRβ⁻ CD11b⁻ lymphocytes. **i)** Enumeration of dendritic cell subsets in spleens of LCMV CL13 infected mice with or without isoprotenerol treatment. cDC gated on live NK1.1⁻ CD19⁻ TCRβ⁻ MHC-II⁺ CD11c⁺ lymphocytes. pDC gated on live NK1.1⁻ CD19⁻ TCRβ⁻ MHC-II⁺ CD11c⁻ CD11b⁻ XCR1⁺ lymphocytes. **j)** Enumeration of NK cells and NK T cells in spleens of LCMV CL13 infected mice with or without isoprotenerol treatment. Gated on live lymphocytes. **k)** Proportion of immune cell types producing IL-10 **l)** MFI of IL-10 in various immune cell types (**b,c**) Data representative of three independent experiments. n=3-5 per group per experiment. (**d,e**) Data pooled from four independent experiments. n=3-4 per group per experiment. (**f-k**) Data from one experiment. n=4-5 per group per experiment. Error bars represent the SEM. * $p < 0.05$ ** $p < 0.01$

3.3 Discussion

This chapter examined the role of the SNS in the modulation of immune responses to viral infections. Disruption of peripheral SNS innervation by chemical sympathectomy resulted in altered control of LCMV infection. Replacing beta adrenergic signals during infection by treatment with the β -adrenoceptor agonist isoprotenerol was also found to impair control of LCMV CL13 infection, indicating that dysregulation of peripheral SNS signals can impact disease outcomes during viral infection. However, LCMV infection was also found to induce a disruption of sympathetic innervation in the spleen, which was partially attributed to NK cells and IFN γ . Taken together, these results suggest that the SNS neuroimmune crosstalk during LCMV infection contributes to viral control.

Chemical sympathectomy with 6-OHDA was used to deplete sympathetic fibres for the investigation of SNS input in modulating antiviral responses in the various infectious models used in this chapter. The experiments with 6-OHDA and IAV here showed that the SNS had no discernible influence on the effector T cell response to IAV. Previous work found that intraperitoneal challenge with IAV-PR8 resulted in increased IFN γ production by PA224-specific CD8⁺ T cells [185]. One point to note is that the infectious dose used between the previously published study and the experiments in this chapter were different. Grebe *et al* utilised an infectious dose that was based on the tissue culture 50% infective dose (TCID₅₀) in Madin Darby canine kidney (MDCK) cells whereas I used an infectious dose based on the plaque forming units. Measurements of infectious viral titres with TCID₅₀ have been found to be lower than when calculated via plaque assay [462]. In humans, influenza A infection has been thought to increase in severity as the exposure to infected individuals, and hence infectious particles, increased [463]. Similarly, other studies using mice have shown that changing the viral dose can alter T cell priming as well as the following antiviral responses at

both the acute and memory phases [464, 465]. The different infection dose of IAV-PR8 used between Grebe *et al* and in the experiments for this thesis could potentially affect the magnitude of T cell responses that were observed which may be influenced by SNS signalling to a different extent.

A model of HSV skin infection using HSV-1 infection allowed for investigation of the role of SNS signalling in local immune responses. Though there were no differences in the T cell response in the skin or draining LN, splenic gDT-II T cells had reduced TNF α and IFN γ production. Given that others have observed the influence of SNS signalling on lymphocyte migration into the LN [165, 166], it was surprising that there did not appear to be any defect in the numbers of effector cells in the LN. However, any likely defect as a result of impaired lymphocyte migration may have been more prominent at the start of the immune response during priming of the T cells due to reduced encounters with APCs. Effects on the number of LN effector cells at the peak of the response may have been masked by proliferation of the cells as the response is mounted. As the work of this thesis focuses on the relationship between effector function of T cells and the SNS, future work could investigate the effects of the SNS on early T cell activation and priming. Additionally, previous studies of T cell responses to HSV-1 and the SNS following intraperitoneal infection found that splenic CD8⁺ T cell function was suppressed at a primary effector and the memory phase [188]. While this was not observed here following skin infection, it is interesting that the splenic gDT-II responded in a similar fashion. CD4 T cells have been found to be preferentially primed in HSV-1 infections [405] and thus may be more sensitive to the lack of SNS input in the spleen following 6-OHDA treatment. Moreover, the effects of 6-OHDA in the earlier study [188] looked at responses 5 days post-infection whereas in the current experiments the T cell responses were analysed 7 days post-infection. There may have been sufficient time for the

observed defects in CD8 T cells at day 5 post-infection to have been lost following the extra two days of carrying out the immune response. Results indicate that T cells in the skin produced $\text{TNF}\alpha$ and $\text{IFN}\gamma$ at similar levels (see 3.2.3) suggesting that 6-OHDA treatment and the removal of SNS signals had no effect on T cells that infiltrate the site of infection.

The lack of effect of SNS signalling on immune response to LCMV Armstrong may be attributed to the strong and robust immune response that is generated thus potentially masking any modulation by the SNS. This is supported by the comparable viral load in both 6-OHDA denervated and control mice thus suggesting that the SNS has no influence on the immune response in clearing the virus. During chronic LCMV infection, the further decrease in $\text{TNF}\alpha$ production by P14 cells in addition to the natural suppression of $\text{TNF}\alpha$ by the virus resulting in already low levels of $\text{TNF}\alpha$ [423] may have contributed to the increased viral load. However, it is also possible that this may be attributed to many other potential factors such as SNS effects on other cell types or the changes in the innervation of the vascular system following loss of 6-OHDA treatment. Coupled with the vascular changes associated with LCMV CL13 infection [466], altered innervation of vessels can result in dysregulation of normal vessel pressure and integrity which could lead to a change in how the virus is disseminated throughout the body. Additionally, whether or not the role of nerves supplying the spleen is limited to the control of blood flow to the spleen and how they might modulate other splenic functions that could affect immune responses remain unclear. While 6-OHDA depletion of splenic nerves is effective, it has to be acknowledged that the depletion not only depletes the fibres within the spleen but also those associated with blood vessels [449]. Thus, the depletion of fibres in other systems as a result of 6-OHDA treatment may contribute to the altered responses to LCMV in these experiments. A more refined method could involve

surgical denervation of the celiac ganglia for a more targeted effect of SNS depletion in the spleen though that comes with some technical complexities.

The overall lack of SNS influence on T cell responses in LCMV infection may also be attributed to the observation that TH⁺ fibres in the spleen are lost over the natural course of infection, occurring as early as 3 days post-infection. This begs the question of how much of an impact the SNS can have on the splenic cells during the course of the immune response to LCMV. It should be noted that some lymphocytes have been found to express TH, producing endogenous catecholamines and thus may contribute to signalling pathways of the SNS [467-469]. Together with the previous results from this chapter, this adds further weight to the thought that SNS signals may have a greater influence on T cell priming rather than directly affecting effector T cells themselves. The loss of TH⁺ fibres is consistent with decreased levels of NA in the spleen. The affect appears to be localised and LCMV had no effect on levels of NA in the plasma. Plasma levels of NA may have remained relatively constant due to compensatory mechanisms by the adrenal gland. Future work would involve measuring levels of NA in other organs that are innervated by the SNS and also infected over the course of LCMV infection to observe if the same decrease is observed, allowing for determination of whether or not this decrease in NA occurs globally in the body. Both nerve loss and decreased NA levels following LCMV infection recovered as time progressed. This mimics the recovery seen following 6-OHDA depletion where nerve terminals were able to sprout and regenerate from undamaged axons [470]. However, the results in this chapter could not ascertain if the loss in TH⁺ fibres following LCMV infection was due to a downregulation of TH or if the terminals were lost due to cell death. While β III-tubulin is a widely used marker of cells of neuronal origin [471, 472], it was not possible to determine if the levels of β III-tubulin associated with nerve fibres were altered following LCMV infection as other cells in the spleen

also expressed β III-tubulin. A study on human tissue found expression of β III-tubulin by stromal cells and endothelial cells [473] which may be what was observed in the spleen sections considering other work in the lab have shown co-localisation of these β III-tubulin expressing cells with the endothelial marker, CD31. As such, it was difficult to distinguish between these other cells and nerve fibres that lost TH⁺ expression. Additionally, an alternative to determine if the loss of TH⁺ fibres in the spleen is due to downregulation or nerve damage would have been to stain for other markers that can be used to distinguish sympathetic nerve terminals such as NPY [65, 66] though our attempts at doing so in the spleen were not successful in these experiments.

Various components of the immune response to LCMV were investigated in this chapter to identify a possible mechanism for the nerve loss accompanying LCMV infection. There is a large recruitment in NK cells following LCMV infection though they are not essential for virus clearance [453]. Interestingly, depletion of NK cells decreased the loss in nerve fibres whereas depletion of CD4 or CD8 T cells did not. Additionally, this same phenomenon was observed in IFN γ ^{-/-} mice but not IFNAR mice. NK cells and CD8 T cells are the main producers of IFN γ . IFN γ has been suggested to be detrimental to nerve growth and survival [474, 475]. As such, these results imply a role for IFN γ in potentially contributing to the nerve loss though it is not entirely responsible for it. As IFN γ plays a crucial role in the clearance of LCMV infection and the generation of an effective CD8 T cell response [436], the loss of IFN γ could result in increased viral replication through the poor control of infection and killing of stromal cells resulting in damage to splenic architecture thus contributing to the loss of innervation in an indirect way.

Depletion of CD8 T cells as a producer of IFN γ may not have been effective in preventing the loss of splenic TH⁺ fibres as NK cells would have already produced large

amounts of IFN γ early in the immune response and so have already contributed to the nerve loss. Further work could involve investigating alternative cytokines such as TNF α that may couple with the actions of IFN γ to result in the loss of splenic TH $^+$ fibres.

Whether the decrease in *Th* gene expression in the CG following LCMV infection is a result of the nerve changes in the spleen or if the nerves are lost because of the decrease in TH levels in the CG remains unanswered. Retrograde or anterograde tracing of these fibres would provide more information on the relationship between LCMV infection and the sympathetic nerves of the spleen. Some pilot tracing experiments were carried out but there were various technical difficulties including solubility of the nerve tracers in the small volume to be injected in mice, making it difficult to reliably inject the dye for tracing. Based on the genes sampled in these experiments, gene expression in the CG following infection is conducive to nerve growth and regeneration with increased expression of neurotrophins including nerve growth factor (NGF), neurotrophin factor 3 (NT3), and brain derived nerve growth factor (BDNF). This is consistent with previous findings where NGF and NT3 were important for peripheral nerve regeneration [476]. This gene expression profile could be an attempt to restore the nerve terminals in the spleen in response to the loss of splenic TH $^+$ nerve fibres. There is also the possibility that the downregulation of *Th* gene expression in the CG can result in a switch to a parasympathetic phenotype through the upregulation of *Chat*. The upregulation of *Chat* has been documented in T cells responding to LCMV infection [477] suggesting there may be a preference for parasympathetic signals in the response to LCMV. However, this increase is not observed in the results here which may be due to the dilution of T cell mRNA in the spleen with mRNA from other cell types. Further validation would be required to ascertain if there is a change in the CG to preference parasympathetic fibres.

Alternatively, future experiments could instead try to ascertain if there is potential increase in sensory innervation of the spleen to accommodate for the loss of sympathetic innervation.

Other infections of the spleen were tested for their ability to produce the nerve changes observed during the course of LCMV infection and determine if this is a general phenomenon of splenic infections. No changes were observed in the case of *Listeria monocytogenes* or *Plasmodium berghei* ANKA. One point to note is that the immune responses elicited by listeria and malaria infections are different to viral infections. These infections result in the generation of immune responses of different magnitude and distinct inflammatory cytokine production. For example, while *Listeria* infection results in a strong IFN γ response similar to LCMV [478], IL-6 production has also been found to be a key player for the control of the infection [479]. Interestingly, when secreted, IL-6 has also been found to support nerve growth in the absence of NGF [480]. This may be a possible mechanism by which nerve growth might be supported despite the inhibitory growth conditions in the presence of IFN γ . Another possibility is if LCMV also directly infects splenic nerves. The receptor utilised by LCMV, α -dystroglycan, is also expressed on neuronal cells [481]. Visualising this through co-staining infected spleen sections for LCMV particles and a general nerve marker might be possible though an optimisation of time frame and infectious dose for this would be required. Additionally, whether this effect on splenic nerve fibres is limited to LCMV infection only or applicable to other viral infection of the spleen has not been determined.

Pharmacological β -adrenoceptor stimulation with isoprotenerol treatment had minimal effect on the immune response to LCMV Armstrong. In contrast, stimulation of the SNS during LCMV CL13 infection resulted in improved IFN γ production. It is plausible that effects of SNS signalling on the immune system differ depending on the infection, given that

SNS signals appear to be beneficial in HSV infection as previously mentioned [188]. The increase of β -adrenergic signalling may thus be beneficial in compensating for the loss of SNS signalling in the spleen following LCMV infection resulting in better cytokine production. However, this small increase in IFN γ production did not translate to better viral control. In fact, viral load was increased with isoprotenerol treatment similar to that with 6-OHDA treatment. This is paradoxical as 6-OHDA treatment and isoprotenerol treatment should have opposing effects. Again, there is a possibility that the altered viral titres may be attributed to changes in the vasculature. In rats, 6-OHDA treatment or persistent stimulation of β -adrenoceptors result in a drop in the mean arterial pressure [482, 483]. Repeated treatment of mice with isoprotenerol can also result in initial decreases in blood pressure followed by an increase with consistent treatment [484]. Interestingly, treatment of LCMV-infected mice with vasoconstrictors resulted in increased viral titres [477]. It may be that the regulated maintenance of blood pressure is important in the control of LCMV viral load.

Additionally, isoprotenerol treatment also did not affect the number of other populations of immune cells with the exception of B cells. B cells have been documented to have more adrenoceptor expression than T cells [160] and thus may be more susceptible to the effects of adrenoceptor stimulation. The treatment regime in these experiments could be improved by utilising osmotic minipumps to deliver controlled doses of isoprotenerol over time rather than a daily single bolus injection. This might better reflect the continual stimulation through the SNS following the release of endogenous catecholamines. Contrary to previous studies where DCs were documented to produce IL-10 following LCMV CL13 infection [432], there was only a very small proportion of DCs that produced detectable amounts of IL-10 in both groups. This may be due to the early measurement of the IL-10 production at 7 days post-infection. Previous studies have only measured the IL-10 levels 9

days post-infection for DCs while a study by Ejrnaes *et al.* found that peak IL-10 production by T cells were 20 days post-infection [485]. Further validation of IL-10 expression by the different cells following isoprotenerol treatment is needed.

Overall, the results of this chapter indicate that the SNS control of immune responses to viral infections, particularly in response to LCMV, may be more intricate than expected and that there may be many contributing mechanisms in maintaining the sufficient amount of SNS signalling for normal responses. The relationship between the nervous and immune system is not one directional, but rather each side is able to influence the other.

Chapter 4 – T cell responses in **the immune privileged cornea**

4.1 Introduction

The environments of many organs in the body are carefully regulated to maintain normal functions and keep the individual alive with some more tightly regulated than others. One of these regulatory properties is immune privilege, which is the removal of foreign antigens in the absence of systemic inflammation to reduce the amount of damage that may occur as a result of the immune response. The cornea is thought to be an immune privileged site to preserve the function of sight in an individual [304, 305]. Despite the cornea microenvironment being generally immunosuppressive [296], there are possibilities of immune cells entering the cornea following injury or infection [347]. Herpes simplex virus (HSV) infection of the cornea is a leading cause of viral blindness as a result of a condition known as herpes stromal keratitis (HSK) [356]. Mouse models of HSK have revealed the role for both innate and adaptive immune cells such as neutrophils and T cells in causing damage to the cornea as they attempt to clear the virus, contributing to HSK. However, various aspects of the immune response are still not well understood. One particular question is whether or not T cells are able to establish a population of resident memory T (T_{RM}) cells following the resolution of corneal HSV infections. T_{RM} cells have been documented to persist at sites of previous infection or injury and provide early rapid responses following secondary encounters of the similar pathogen [238, 239].

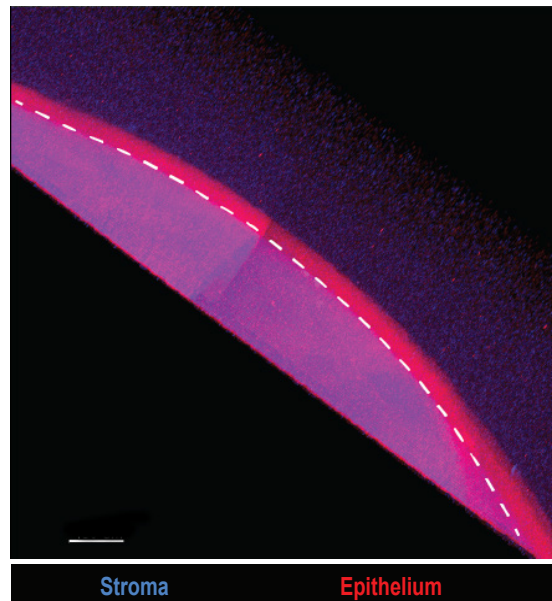
This chapter aims to use microscopy and flow cytometry to establish a model for studying T cell memory, in particular $CD8^+$ T cell memory, following infection with HSV-KOS. The possibility of using other methods to induce infiltration of cells into the cornea is also explored. Finally, this chapter will investigate the role of antigen and the signalling molecule $TGF\beta$ in establishing a population of memory T cells in the cornea.

4.2 Results

4.2.1 *Imaging the cornea*

Two-photon imaging of tissues facilitates analysis of immune cell responses at the site of imaging. Visualising the structure of the cornea is also important in understanding the distribution of immune cells that enter the cornea as a result of immune responses. To do this, B6 mice with epithelial cells that express the tdTomato fluorescent proteins on the cell membrane, highlighting the epithelial layer of the cornea, were prepared for two photon imaging of the cornea. A three-dimensional view of the cornea showing not only the curvature of the cornea but also the basal membrane and stromal layer can be produced (Movie S1). The demarcation of the upper epithelial layer and collagen-rich stromal layer of the cornea visualised using second harmonics generation (SHG) is depicted by the white dashed lines in the image (Fig. 4.1a). Most models of cornea HSV infection use the RE strain of HSV as a large portion of studies focused on understanding the mechanisms behind HSK. It is possible for an infection to occur without the sequelae of HSK if using the KOS strain in B6 mice [381]. To confirm this, corneas of mice were lightly scratched using a 30G needle to remove the epithelial layer and infected with HSV-KOS, an attenuated strain of HSV, expressing GFP. 2 days post infection, corneas were collected, and growth of the virus was observed using confocal microscopy. Patches of the virus (green) can be observed in multiple areas of the cornea suggesting growth of the virus (Fig.4.1b). These results show that microscopy is an effective method of studying the corneal layers and the growth of HSV-KOS in the cornea.

a)



b)

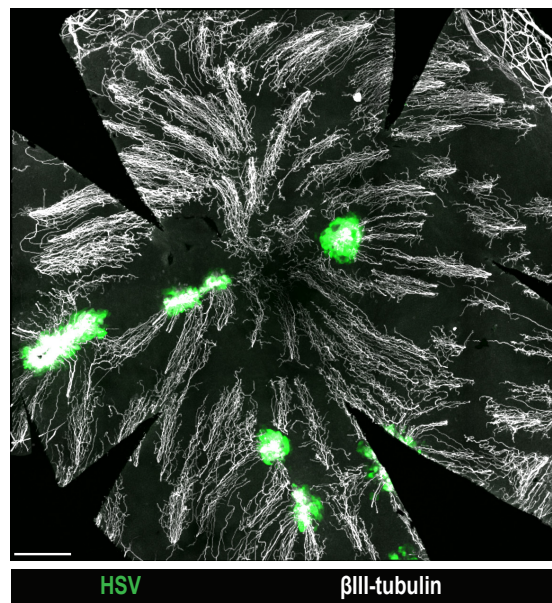


Figure 4.1 Imaging the cornea **a)** Snapshot of 2-photon movie depicting the different layers of a mouse cornea. White dashed lines demarcate the epithelial and stromal layers. See also Movie S1. **b)** Mice were infected with 10^6 pfu HSV-GFP in the cornea and corneas harvested, fixed and prepared for whole mount immunofluorescence. Growth of the virus is depicted in green. **(a)** Image representative of 1 independent experiment with $n=3$ mice. Scale bar, $100\mu\text{m}$ **(b)** Images are representative of 2 independent experiments with $n=3-4$ mice per group per experiment. Scale bar, $300\mu\text{m}$

4.2.2 Corneal infiltration by T cells following HSV-KOS infection

To study T cell response to corneal HSV infection, corneas of mice seeded with gBT-I and gDT-II cells were scratched and infected with HSV-KOS and the T cell response in the draining LN, spleen, and cornea was investigated at various time points (Fig 4.2a). Numbers of gBT-I and gDT-II cells in the draining LN peak at 6 days post infection and progressively retracts from 10 days post infection onwards with numbers 28 days post infection remaining higher than the originally detected numbers (Fig 4.2b and Fig 4.2c). Elevated numbers of gBT-I and gDT-II cells can also be found in the spleen in a similar trend though the peak occurs 7 days post infection instead (Fig 4.2b and Fig 4.2d). Recovery of corneal cells was determined after digestion of the corneal tissues either with collagenase, dispase or liberase. Collagenase and dispase digestion of the cornea resulted in the recovery of relatively equal amounts of gBT-I and gDT-II cells with a substantial amount detected 7 days post infection and the highest amount at 10 days post infection (Fig 4.2b and Fig 4.2e). Very low numbers of T cells were found in the cornea 28 days post infection. Similarly, the digestion with Liberase showed the same infiltration kinetics though lower numbers of gDT-II cells were recovered (Fig 4.2f).

Expression of the cell surface molecules CD69 and CD103 are typically associated with T_{RM} cells at epithelial sites though CD69 single positive cells that are also considered to be T_{RM} cells can be found in non-epithelial sites [243, 248, 251]. As such, the expression of these markers in corneal T cells were investigated. CD69 expression on memory gBT-I and gDT-II cells in the cornea were relatively equal between the two cell types – approximately 60% (Fig 4.2g). The co-expression of CD69 and CD103 was less prominent with only a small subset of cells being positive for both markers. While non-significant, a larger proportion of gBT-I cells expressed both markers compared to gDT-II cells (Fig 4.2h). Taking these results

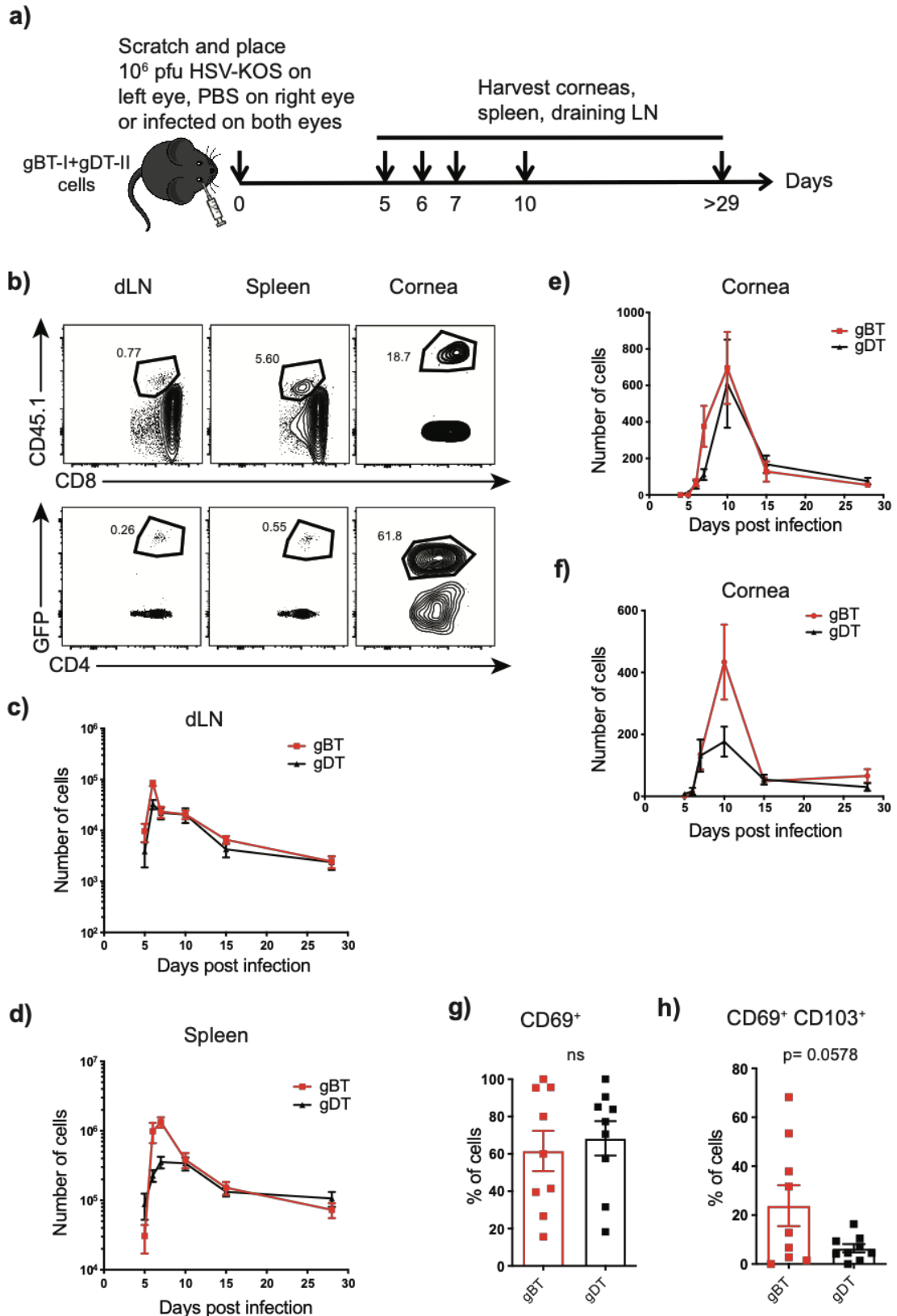
into consideration, T cells are able to enter the cornea following HSV-KOS infection and can be detected through flow cytometry following digestion of the cornea. Liberase digestion will be used in further experiments in this chapter as the yield is comparable. Additionally, given the epithelial component of the cornea, further work on this chapter will focus on CD8 T cells and exploring their capacity to remain resident in the cornea similar to previous studies in the skin [238]. The persistence of these CD8 T cells in the cornea was investigated by enumerating the number of OT-I cells 90 days post HSV-OVA infection (Fig 4.2i). These OT-I cells persist in the spleen at relatively stable numbers (Figure 4.2j). In the cornea, HSV-OVA infections resulted in slightly higher numbers of persisting OT-I cells, though this increase was non-significant compared to the non-infected eye (Figure 4.2k). However, expression of CD69 was only prevalent in the HSV-infected cornea (Figure 4.2l). The proportion of cells co-expressing CD69 and CD103 were also significantly higher in the HSV-infected cornea (Figure 4.2m). Taken together, these results suggest that T cells that infiltrate the cornea following HSV infection are capable of persisting for long periods of time following cessation of immune responses at the site of infection.

Next, the spatial properties of T cell infiltration into the cornea were investigated. Corneas of mice infected with HSV were prepared for confocal microscopy to observe the distribution of T cells in the cornea. Corneal infiltration of gBT-I (green) and gDT-II (red) cells occurred as early as 5 days post infection and could be seen at the edges of the cornea and was relatively unchanged 6 days post infection. Both cell types moved towards the central cornea 7 days post infection and by 10 days post infection a large number of cells could be seen distributed across the whole cornea, with many cells concentrated in the central cornea (Fig 4.3a). gBT-I and gDT-II cells can be observed in both the epithelial and stromal layers of the cornea with that are above the stromal layers exhibiting more varied

morphology (Fig 4.3c, See also movies S2-S5). At memory, few gBT-I cells remained distributed throughout the cornea. gBT-I cells in the cornea at this time appeared to have a distinct morphology, with some having dendrite-like morphology indicative of T_{RM} cells (Fig 4.3b). Unlike in the skin where CD8 T cells were restricted to the epidermis [245], CD8 T cells in the cornea at memory appear to be distributed in both the stromal and epithelial sites (Fig 4.3d, see also Movie S6). To assess if these memory cells come in contact with the circulation, memory corneal HSV-infected mice were injected with fluorescently tagged anti-CD45.1 antibody prior to collection of the corneas and spleen. A population of CD8⁺ cells in the spleen but not the cornea was found to be labelled with the antibody suggesting that the CD8⁺ cells in the cornea do not come into contact with the circulation (Fig 4.3e). Thus, these results indicate that only a small number of cells remain in the cornea despite the large infiltration at the peak of the response. These cells form a heterologous population that appear to be similar to canonical T_{RM} features.

4.2.3 Recruitment of activated T cells into the cornea

It has been shown that activated T cells in the circulation can disseminate and generate T_{RM} cells at peripheral sites following inflammatory events [258]. To investigate if inflammation alone is able to attract and lodge activated T cells in the cornea, mice were injected with *in vitro* activated gBT-I cells and scratched corneas were either infected with HSV-KOS prior to injection or treated with immunomodulatory compounds such as CpG 1668, CpG Combo (modified CpG consisting of both CpG B and CpG P) or lipopolysaccharide (LPS) that are capable of inducing inflammatory responses (Fig 4.4a). Mice seeded with naïve gBT-I cells and infected with HSV-KOS were used for comparison. 8 days post infection, gBT-I cells can be found in the spleen of the mice treated with CpG or LPS, and



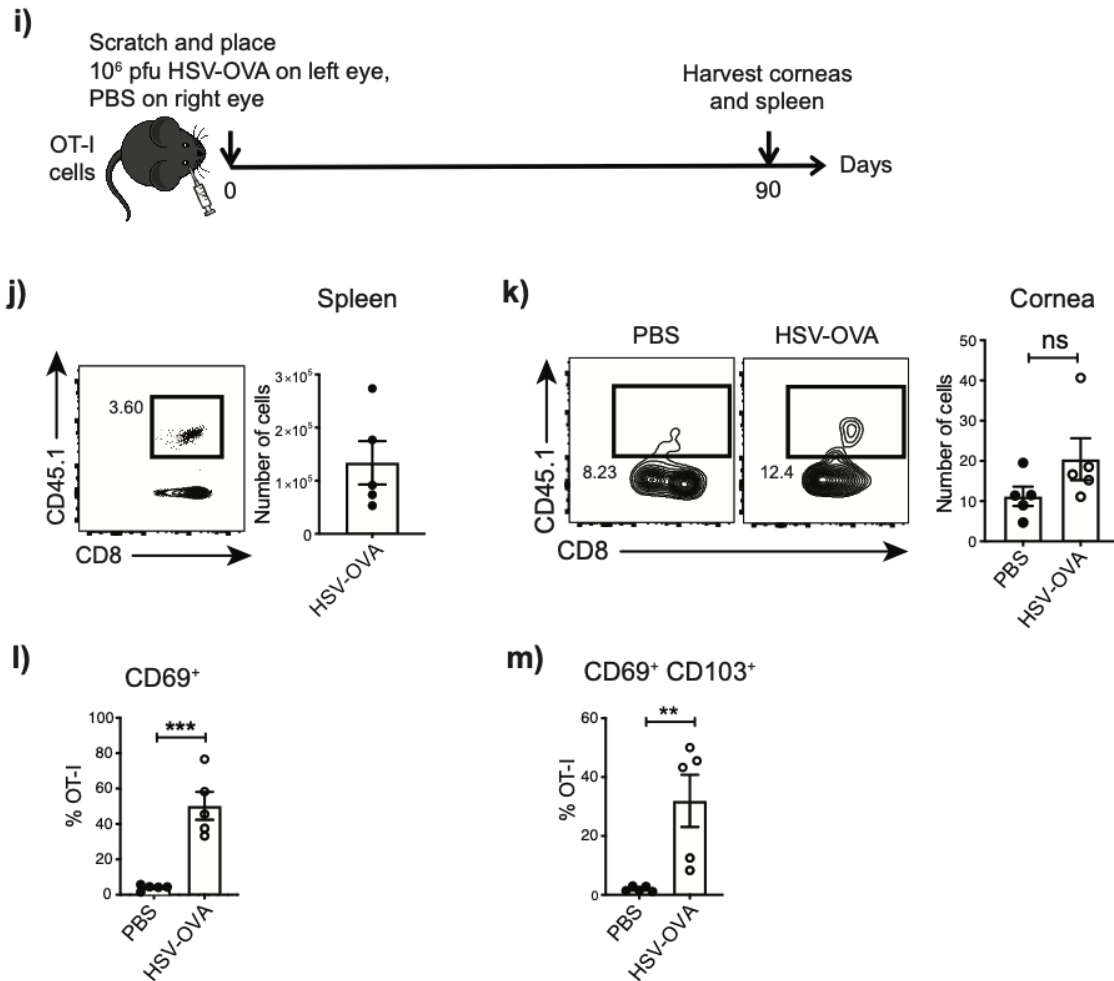


Figure 4.2 Corneal infiltration by T cells following HSV-1 infection

a) Schematic of experimental set up. Corneas of B6 mice seeded with 5×10^4 gBT-I/Ly5.1 and 10^4 gDT-II/uGFP cells were scratched and 10^6 pfu HSV-KOS were placed on the left eye and PBS on the right eye or both eyes were infected with 10^6 pfu HSV-KOS. **b)** Representative FACS plots of gBT-I and gDT-II cells recovered from the draining lymph node, spleen, and cornea of B6 mice 10 days post infection. Cells were gated on live $CD8^+CD4^-$ lymphocytes for identifying gBT-I cells and live $CD8^+CD4^+$ lymphocytes for identifying gDT-II cells. gBT-I and gDT-II cells were enumerated 5, 6, 7, 10, 15, and between 28-32 days post infection from the **c)** draining lymph node and **d)** spleen. **e)** Enumeration of cells from two infected corneas per mouse following collagenase and dispase digestion. **f)** Enumeration of cells from one infected cornea per mouse following liberase digestion. Proportion of gBT-I and gDT-II cells that were **g)** $CD69^+$ and **h)** $CD69^+CD103^+$ were determined. **i)** Corneas of B6 mice injected with 5×10^4 OT-I/Ly5.1 cells were scratched. The right cornea was covered with PBS and the left cornea infected with 10^6 pfu HSV-OVA. Corneas and spleen were harvested 90 days post infection. FACS plot and enumeration of OT-I cells in the **j)** spleen and **k)** cornea. Cells were gated on live $CD8^+MHC-II^-$ lymphocytes. The proportion of OT-I cells that were **l)** $CD69^+$ or **m)** $CD69^+CD103^+$ were determined by flow cytometry. **(b-h)** Data pooled from 2-3 independent experiments. $n = 3-5$ mice per group per experiment. **(j-m)** Data from one experiment. $n = 5$ mice per group. ** $p < 0.01$ *** $p < 0.001$

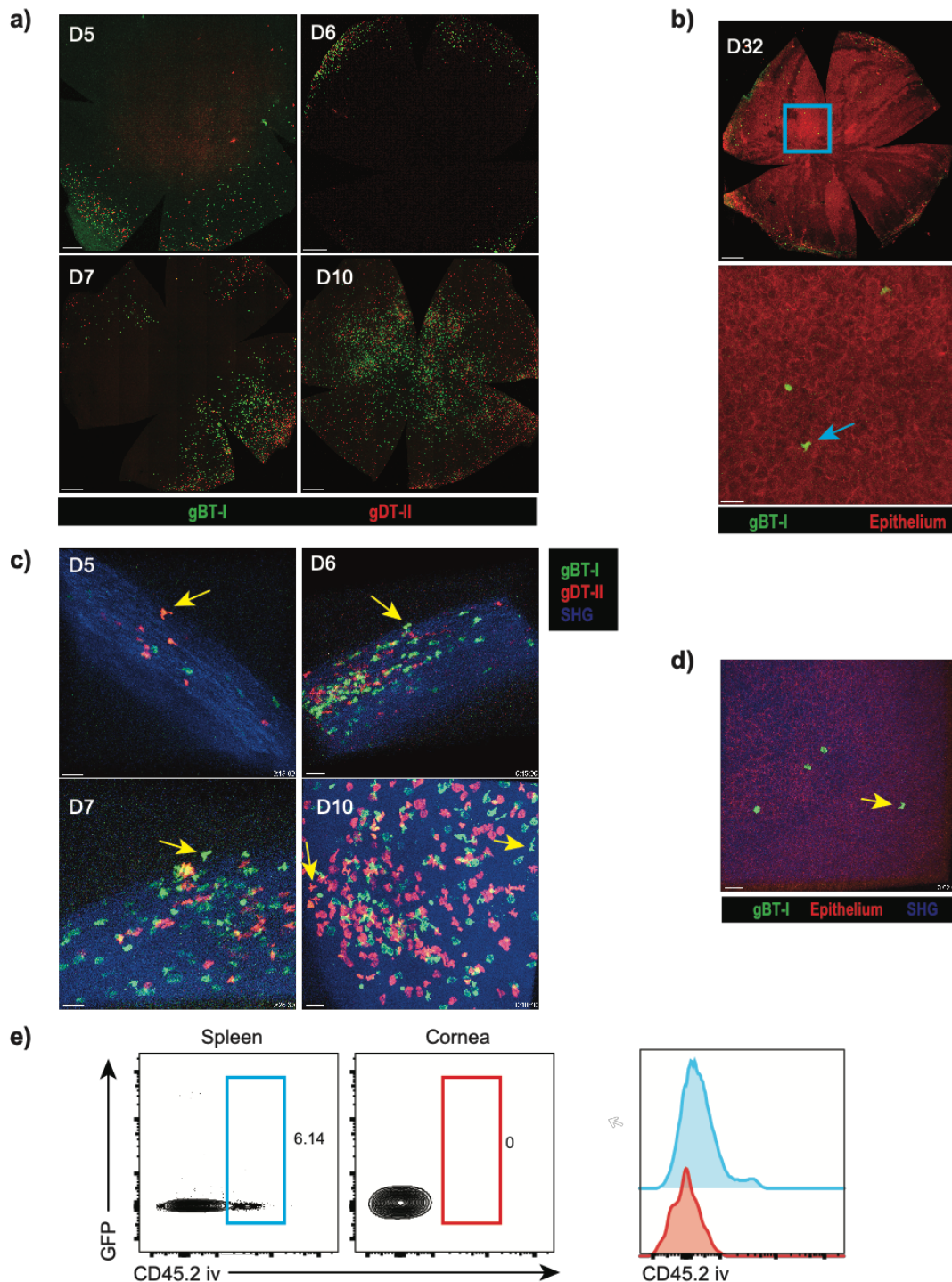


Figure 4.3 Progressive infiltration by T cells following HSV-1 corneal infection

a) Whole mount images of corneas 5, 6, 7, and 10 days post HSV-KOS infection. gBT-I (green) and gDT-II (red) cells can be seen moving into the central cornea. **b)** Whole mount and close up (blue box) image of the cornea 32 days post HSV infection with gBT-I (green) cells. Blue arrow shows gBT-I cell with T_{RM} -like morphology (See also Movie S5). **c)** Maximum intensity projection of a frame of 2-photon imaging of HSV infected corneas 5, 6, 7, and 10 days post infection. gBT-I and gDT-II cells with T_{RM} -like morphology (yellow arrows) can be seen above the stromal layer depicted in blue through second harmonics generation (See also movies S2-S5). **d)** Maximum intensity projection of a frame of 2-photon imaging of HSV infected corneas 32 days post infection. T_{RM} -like gBT-I cells (yellow arrow) can be seen in the epithelial layer represented in red. (See also movie S6) **(e)** FACS plots and histogram of cells from spleen and cornea of mice 28 days post HSV infection. Mice were injected with labelled anti-CD45.2 antibody and euthanised after 5 minutes. Gated on live CD8⁺ cells. **(a-d)** Images are representative of 2 independent experiments with n= 1-4 mice per group per experiment. Scale bar **(a)** 200µm or 300µm **(b)** 400µm or 30µm for close up **(c)** 40µm **(e)** Data representative of 2 independent experiments. n=3-5 mice per experiment.

those infected with HSV with greater numbers in groups injected with activated gBT-I cells (Fig 4.4b). Both CpG compounds and LPS did not appear to draw more cells into the cornea than corneal damage and PBS alone. HSV infection and transfer of activated gBT-I cells was slightly better at recruiting cells into the cornea. However, infection of mice containing naïve gBT-I cells resulted in recruitment of the highest number of cells into the cornea (Fig 4.4c). Activated gBT-I cells were able to persist at high numbers in the spleen at memory time points while the naïve gBT-I group generated fewer splenic effector T cells (Fig 4.4d). In the cornea, gBT-I numbers in all groups were low though both HSV-infected groups with naïve or activated gBT-I cells had more cells compared to the control cornea (Fig 4.4e). Of the gBT-I cells found in the cornea at memory, CD69 expression was also the highest in the HSV groups followed by LPS, and the CpG and control groups were the lowest (Fig 4.4f). The expression of CD69 was also varied and heterogeneous within each group. CD69⁺ CD103⁺ gBT-I CD8⁺ T cells only formed in significant numbers after HSV infection and not with the immunomodulatory compound treatment (Fig 4.4g). However, this co-expression was also only at relatively low levels and did not form a homogenous population. These results indicate that inflammation caused by CpG or LPS is insufficient to induce T_{RM} formation in the cornea and that antigen may be required for the formation of corneal T_{RM}.

4.2.4 Non-specific recruitment of T cells into the cornea

To further explore the role of antigen in recruitment of cells into the cornea, mice seeded with OT-I/uGFP and gBT-I/Ly5.1 cells were infected with HSV-OVA in the left eye, a HSV-KOS strain modified to also express SIINFEKL from the ovalbumin protein, and HSV-KOS in the right eye (Fig 4.5a). This allows for a scenario where one site of infection has the OVA antigen whereas the other does not. If antigen is important, recruitment and persistence of

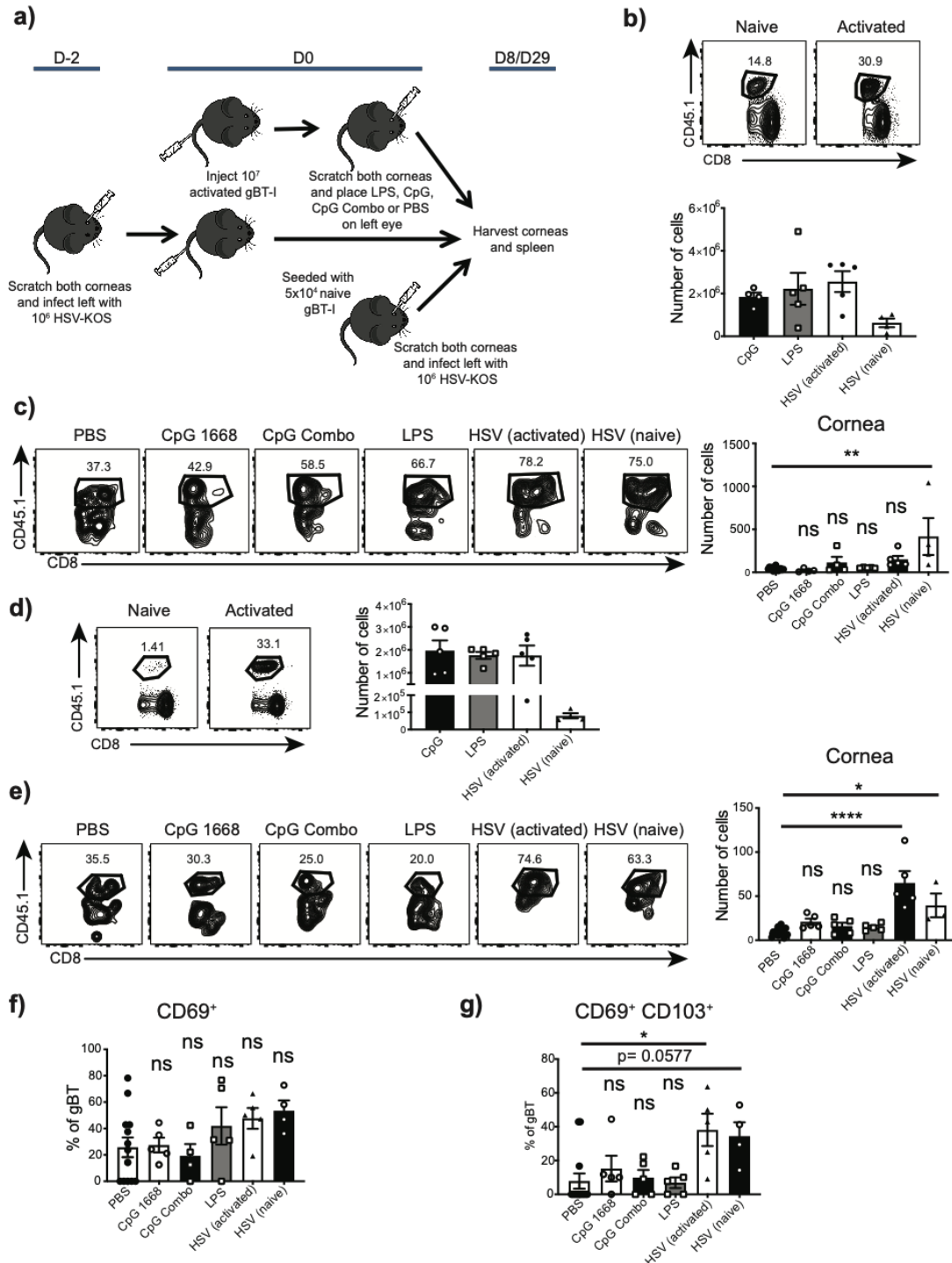


Figure 4.4 Recruitment of activated T cells into the cornea

a) Schematic of experimental set up. B6 mice were either seeded with 5×10^4 gBT-I/Ly5.1 and infected with 10^6 pfu HSV-KOS on the left eye, injected iv with 10^7 in vitro activated gBT-I/Ly5.1 cells then corneas scratched and covered with either 50 μ g LPS, 50 μ g CpG1668, 50 μ g CpG Combo, or PBS, or infected on the left eye with 10^7 pfu HSV-KOS and two days later injected with 10^7 in vitro activated gBT-I/Ly5.1. Cornea and spleen of these mice were collected 8 days or >29 days post infection or treatment. FACS plot and enumeration of gBT-I cells in the **b)** spleen and **c)** cornea 8 days post infection or treatment. Cells were gated on live CD8⁺CD4⁺ lymphocytes. FACS plot and enumeration of gBT-I cells in the **d)** spleen and **e)** cornea 29-32 days post infection or treatment. Cells were gated on live CD8⁺CD4⁺ lymphocytes. The proportion of gBT-I cells that were **f)** CD69⁺ or **g)** CD69⁺CD103⁺ were determined by flow cytometry. Data representative of 2 independent experiments. n= 3-5 mice per group per experiment. * p<0.05 ** p<0.01 **** p<0.0001

OT-I cells should be more prevalent in the eye that was infected with HSV-OVA while gBT-I cell numbers should be relatively consistent between the two.

gBT-I cells appeared to respond more robustly compared to the OT-I cells as higher numbers were detected in the spleen 8 days post infection (Fig 4.5b). This was also the case in the cornea with higher numbers of gBT-I cells recovered compared to OT-I cells, but this difference is not significant. Recruitment of gBT-I cells to corneas infected with HSV-KOS or HSV-OVA were comparable as was the case with the OT-I cells (Fig 4.5c). Thus, T cell recruitment to the cornea was not improved by the availability of local antigen. At the memory time point, gBT-I cells and OT-I cells in the spleen persisted at similar numbers (Fig 4.5f). However, OT-I cells did not persist in corneas infected with HSV-KOS infected cornea and the few cells present in these mice at memory lacked expression of CD69 and CD103. (Fig 4.5g). Conversely, gBT-I and OT-I cells both persisted in corneas infected with HSV-OVA and expressed CD69, with half of the cells co-expressing CD103 (Fig 4.5h and i). Similar to that observed at day 8 post infection, memory OT-I cells expressed higher levels of CXCR6 compared to gBT-I cells, both in the spleen and the cornea (Fig 4.5j and k). Taken together, these results suggest that antigen specifically may not be required for the recruitment of cells into the cornea but rather may play a role in the formation or persistence of these cells instead.

4.2.5 The role of antigen in corneal memory formation

While antigen did not appear to be required for the recruitment of cells into the cornea, there may be a role for antigen requirement in the formation and subsequent persistence of T_{RM} cells in the cornea. Repeated antigen exposure has been thought to contribute to better T_{RM} accumulation and retention [271]. To assess if this might be the

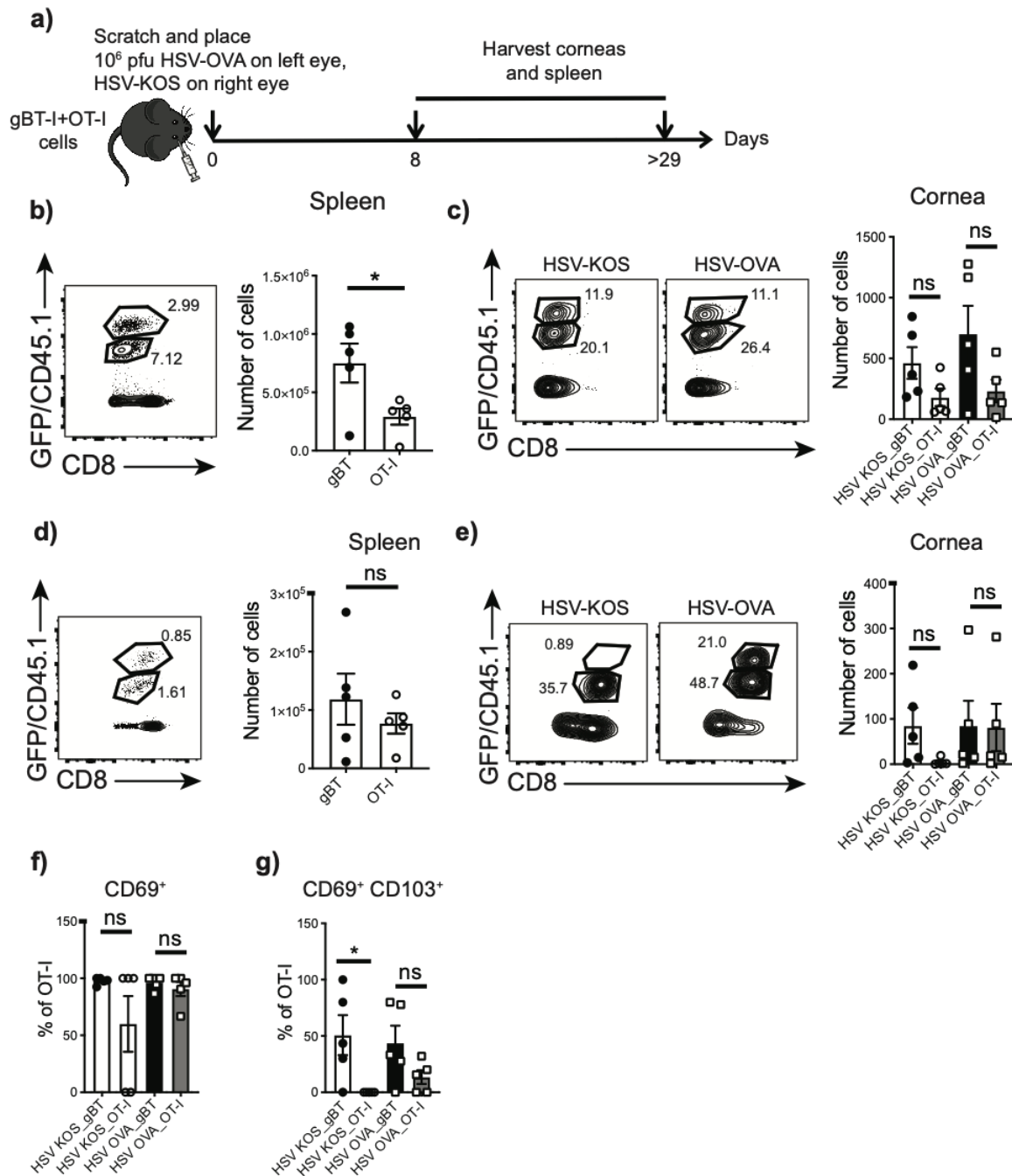


Figure 4.5 Non-specific recruitment of T cells into the cornea

a) Schematic of experimental set up. Corneas of B6 mice seeded with 5×10^4 naive gBT-I/Ly5.1 and 5×10^4 naive OT-I/uGFP cells were scratched and 10^6 pfu HSV-OVA were placed on the left eye and 10^6 HSV-KOS on the right eye. FACS plot and enumeration of gBT-I and OT-I cells in the **b)** spleen and **c)** cornea 8 days post infection or treatment. Cells were gated on live CD8⁺CD4⁺ lymphocytes. FACS plot and enumeration of gBT-I and OT-I cells in the **d)** spleen and **e)** cornea 29 days post infection. Cells were gated on live CD8⁺CD4⁺ lymphocytes. The proportion of gBT-I and OT-I cells that were **f)** CD69⁺ or **g)** CD69⁺CD103⁺ were determined by flow cytometry. Data representative of 2 independent experiments. $n = 4-5$ mice per group per experiment.

* $p < 0.05$

case for the cornea, B6 mice that were infected with HSV-OVA in both corneas and injected with activated OT-I cells were given topical treatments of SIINFEKL peptide on the left cornea and vehicle (10% DMSO) on the right cornea (Fig 4.6a). A large number of the activated cells were found in the spleen following peptide treatment (Fig 4.6b). Treatment with the peptide increased corneal OT-I cell numbers 14 days post infection (Fig 4.6c). However, peptide treatment also resulted in lower proportions of CD69⁺ OT-I cells (Fig 4.6d). Accordingly, CD69 and CD103 co-expression was also affected in the cornea though this difference was not significant (Fig 4.6e). 29 days post infection, the OT-I cells remained in the spleen (Fig 4.6f), while numbers in the cornea were equivalent in both the non-treated and peptide treated corneas (Fig 4.6g). Corneal OT-I T cells expressed slightly less CD69 (Fig 4.6h), although an equivalent number of cells co-expressed CD69 and CD103 (Fig 4.6i). Thus, additional treatment with antigen did not boost corneal CD8 T cell number or increase the formation of memory T cells in the cornea.

4.2.6 Investigating the role for TGFβ for corneal memory T cell formation

Transforming growth factor β (TGFβ) has been demonstrated to be important for the formation of T_{RM} cells at various epithelial sites through regulation of adhesion molecules such as CD103 [242, 275]. However, T_{RM} have also been documented to form in the tissues such as the gut and SLO independent of TGFβ signalling [286, 486]. TGFβ is found in the corneal epithelium and extracellular matrix as part of the anti-inflammatory micro-environment [312, 487] and so may also be important in the formation of memory T cells in the cornea. Following corneal injury, TGFβII in particular is upregulated and secreted into the stroma [313, 488]. Cells from OT-I/Ly5.1^{+/+}/TGFβR-II KO (TGFβRKO cells) mice were co-transferred with OT-I/Ly5.1^{+/-} cells (WT cells) into B6 mice prior to corneal infection with HSV-

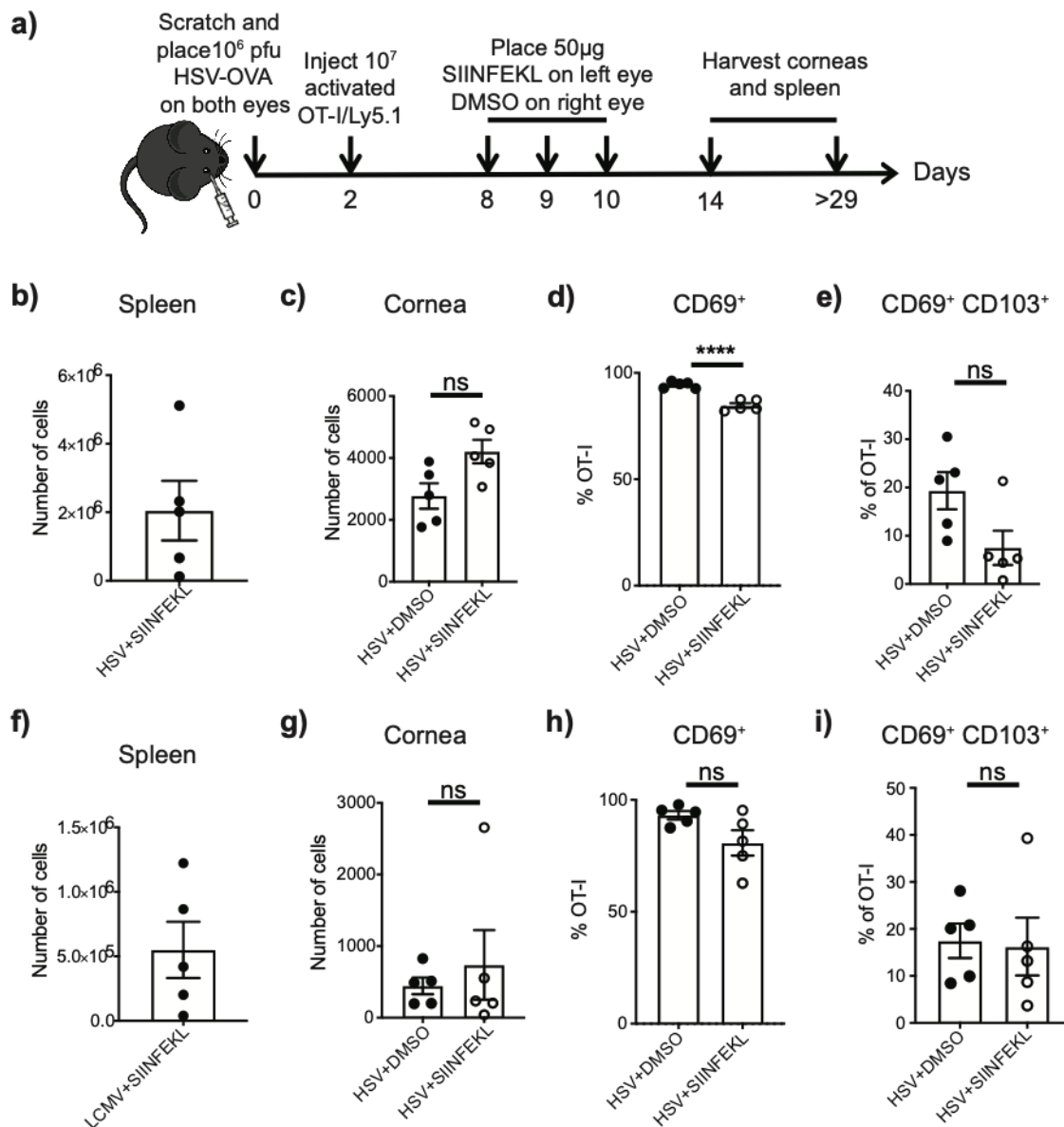


Figure 4.6 The role of antigen in corneal memory formation

a) Schematic of experimental set up. Corneas of B6 mice were scratched and 10^6 pfu HSV-OVA were placed on the left eye. Two days post infection mice were injected with 10^7 *in vitro* activated OT-I/Ly5.1. 50 μ g of SIINFEKL peptide was placed on the left eye and 10% DMSO on the right eye 8, 9, and 10 days post infection. OT-I cells in the **b)** spleen and **c)** cornea 14 days post infection were enumerated. The proportion of OT-I cells that were **d)** CD69⁺ or **e)** CD69⁺CD103⁺ were also determined by flow cytometry. The number of OT-I cells in the **f)** spleen and **g)** cornea 28 days post infection was quantified. The proportion of gBT-I and OT-I cells that were **h)** CD69⁺ or **i)** CD69⁺CD103⁺ were determined by flow cytometry. Data representative of 2 independent experiments. n= 4-5 mice per group per experiment. **** $p < 0.001$

OVA to investigate if formation of corneal memory T cells, specifically T_{RM} cells, would be impacted (Fig 4.7a). Both the WT and TGFβRKO cells were found at relatively equal numbers in the spleen 8 days post infection (Fig 4.7b) suggesting no inherent deficiency in response to the infection. Both TGFβRKO cells and WT cells entered the infected cornea in similar numbers (Fig 4.7c). Both WT and TGFβRKO cells also expressed CD69 in similar proportions (Fig 4.7d). However, the TGFβRKO cells lacked expression of CD103 (Fig 4.7e).

Both WT and KO T cell persisted in the spleen at memory (Fig 4.7f) and were found in the cornea (Fig 4.7g). There were fewer TGFβRKO cells compared to WT cells but this was not significant. Both WT and TGFβRKO expressed CD69 (Fig 4.7h) but the TGFβRKO cells that persisted in the cornea did not express CD103 (Fig 4.7i). This reinforces the role for TGFβ signalling in the expression of CD103. These results illustrate that TGFβ may be required for the establishment of T_{RM} cells in the cornea though the lack of TGFβ signalling does not completely abolish the presence of memory CD8 T cells in the cornea.

4.2.7 T cell infiltration of the cornea using other models of infection

Given the low number of memory cells in the cornea following HSV infection, I wanted to assess if other models of infection might recruit T cells into the cornea that are able to better persist at the memory phase. LCMV Armstrong is capable of infecting stromal cells and have been documented to cause eye pathology following intraocular infection [489]. To determine if LCMV is capable of establishing corneal infection, corneas of mice injected intravenously with P14/Ly5.1 cells (CD8 T cells with T cell receptors specific for LCMV) were scratched and infected with LCMV (Fig 4.8a). Control eyes were scratched, and PBS applied. Priming and activation of the P14 cells from the corneal infection was apparent as demonstrated by the

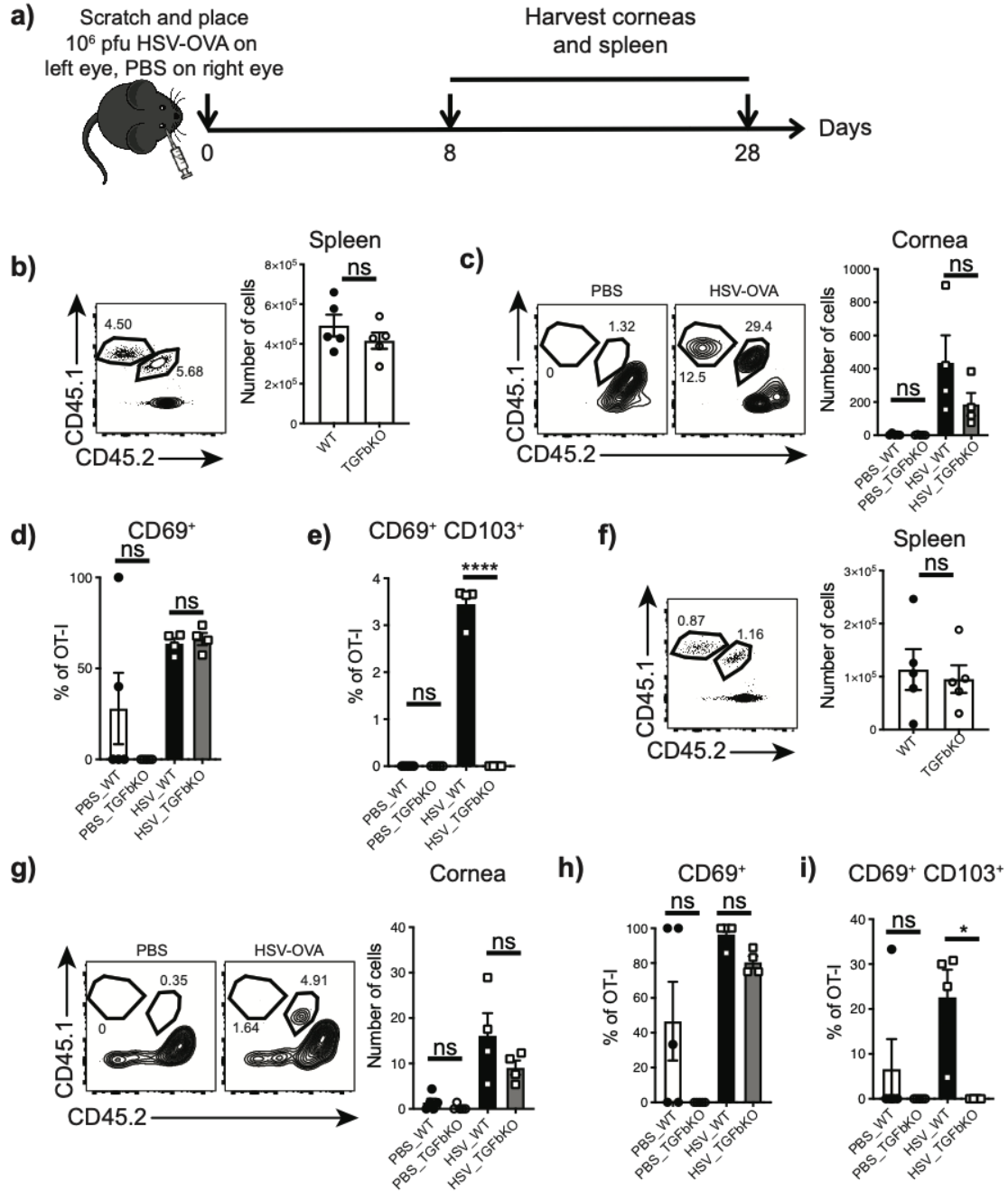


Figure 4.7 Investigating the role of TGF β for corneal memory T cell formation

a) Schematic of experimental set up. B6 mice were seeded with 5×10^4 OT-1/Ly5.1^{+/+} (WT) and 5×10^4 OT-1/Ly5.1^{+/+}/TGF β RII^{-/-} (TGFbKO) and infected with 10^6 pfu HSV-KOS on the left eye. Cornea and spleen of these mice were collected 8 days or 28 days post infection or treatment. FACS plot and enumeration of gBT-I cells in the **b)** spleen and **c)** cornea 8 days post infection or treatment. Cells were gated on live CD8⁺CD4⁻ lymphocytes. The proportion of OT-I WT and OT-I TGFbKO cells that were **d)** CD69⁺ or **e)** CD69⁺CD103⁺ were determined by flow cytometry. FACS plot and enumeration of gBT-I cells in the **f)** spleen and **g)** cornea 28 days post infection or treatment. Cells were gated on live CD8⁺CD4⁻ lymphocytes. The proportion of OT-I WT and OT-I TGFbKO cells that were **h)** CD69⁺ or **i)** CD69⁺CD103⁺ were determined by flow cytometry. Data representative of 2 independent experiments. n= 4-5 mice per group per experiment. * $p < 0.05$

large expansion of P14 cells in the spleen 8 days post infection (Fig 4.8b). However, only a relatively low number of cells were found in the cornea. Cells were found in both infected and control corneas at similar levels (Fig 4.8c). At memory, P14 cells persisted in the spleen (Fig 4.8d), and in both control and infected corneas (Fig 4.8e). Only a low proportion of these P14 cells expressed CD69 (Fig 4.8f), while the majority of these cells lacked expression of CD103 (Fig 4.8g).

Next, a model of vaccinia infection was compared to the HSV infection model. Vaccinia virus has also been documented to cause corneal infection and pathology similar to that of HSV [490]. A modified strain of vaccinia virus expressing the SIINFEKL portion of ovalbumin (VV-OVA) was used to infect B6 mice seeded with OT-I/GFP. HSV-OVA was used as a comparison (Fig 4.8h). The OT-I cells did not expand in VV-OVA infected mice suggesting that the infection did not sufficiently prime the OT-I T cells (Fig 4.8i). This is reflected in the cornea where OT-I cells recovered from the VV-OVA infected cornea was comparable to that of the PBS control cornea (Fig 4.8j). Infiltration of OT-I cells in the HSV-infected cornea was as in previous experiments. There was an overall increase of endogenous CD8 T cell infiltration into VV-OVA infected corneas that was comparable to HSV-OVA which indicates that some immune response was elicited by the infection (Fig 4.8k). Taken together, it would appear that neither the LCMV nor vaccinia models tested in this thesis appeared to be effective models for the formation of corneal T_{RM} cells.

4.2.8 Improved infiltration of cornea by circulating memory T cells

Despite corneal memory CD8 T cells found being heterologous populations, it is possible that they are still capable of generating rapid responses similar to those of conventional T_{RM} cells. To assess if these cells are able to increase recruitment of cells from

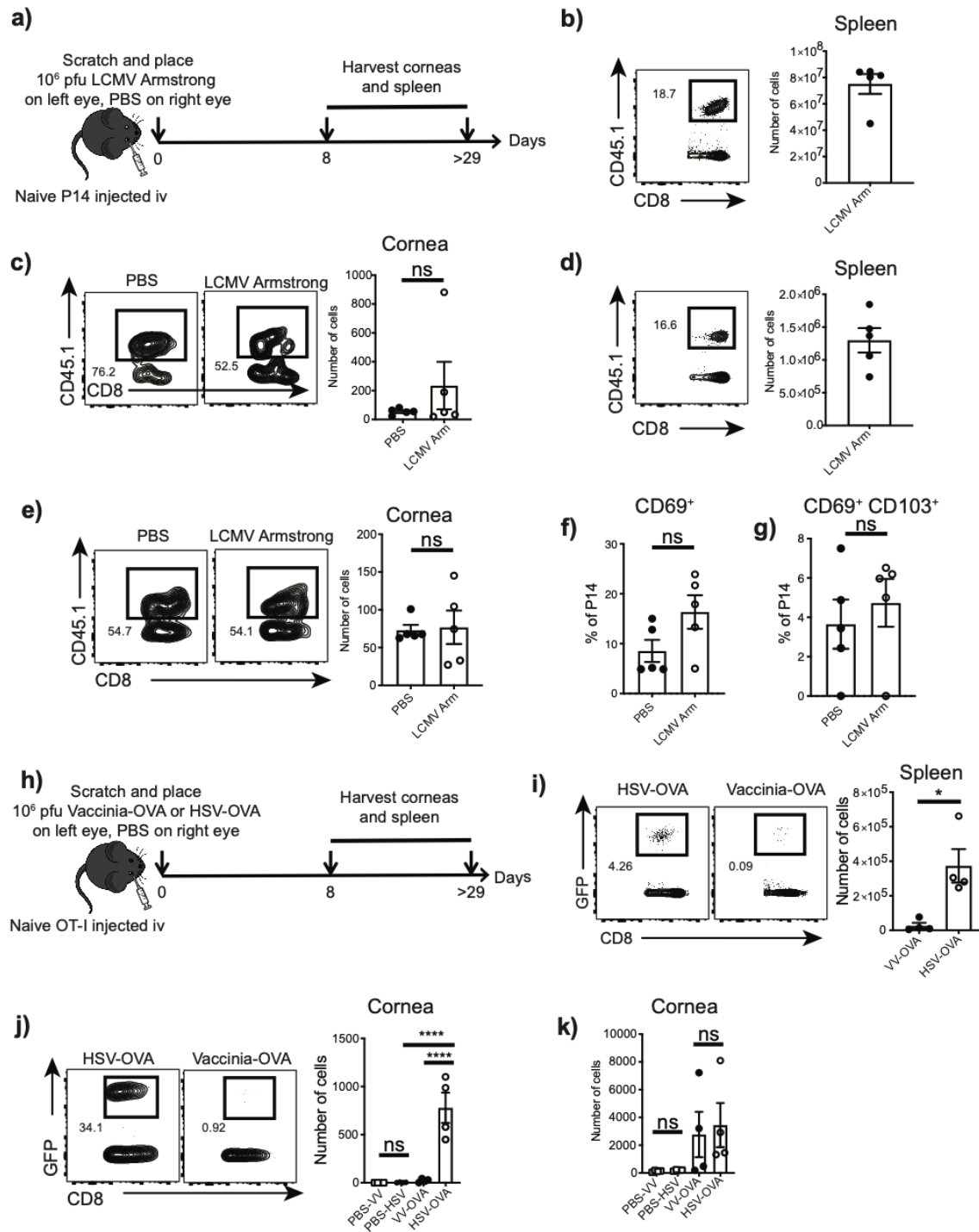


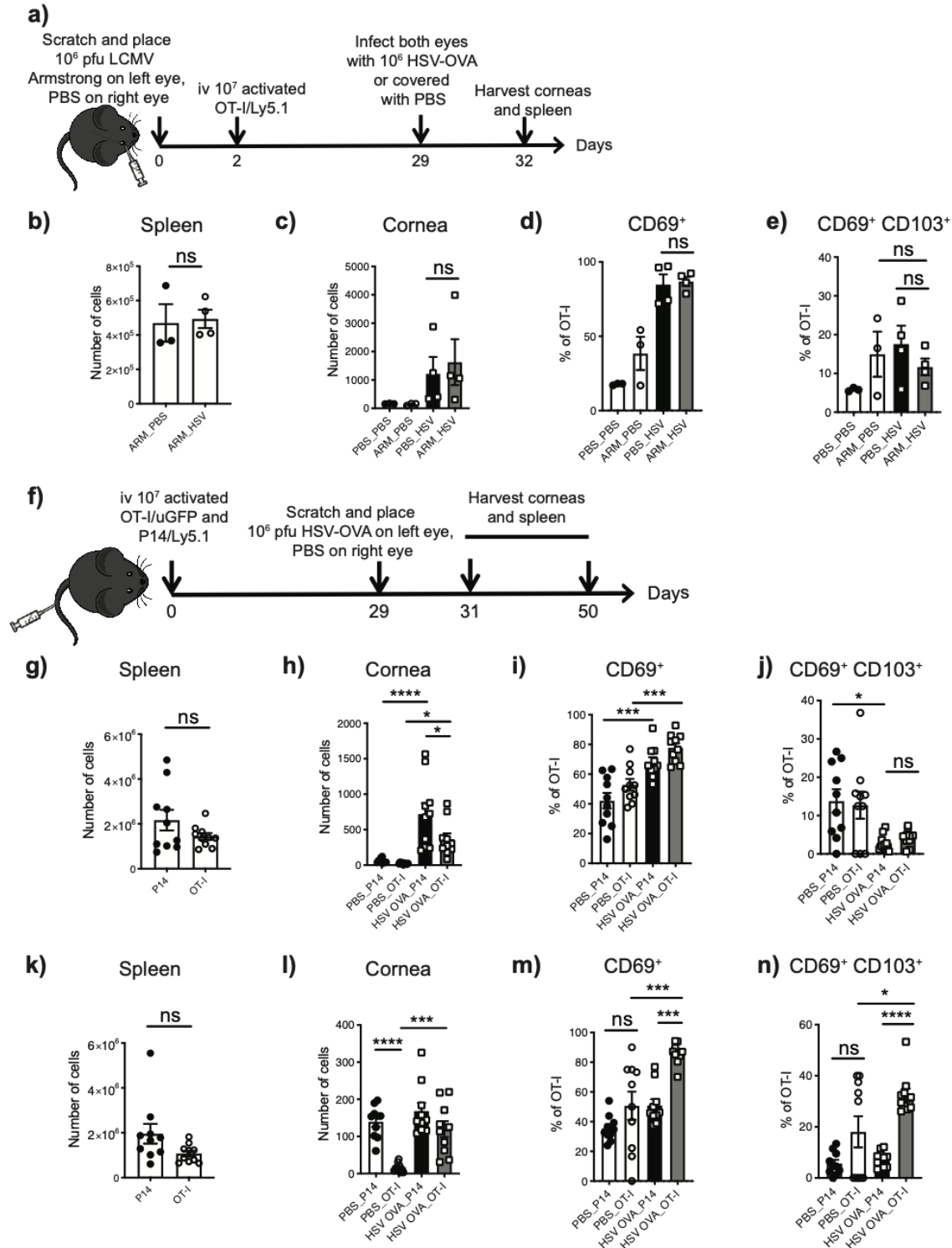
Figure 4.8 T cell infiltration of the cornea using other models of infection

a) Schematic of experimental set up. Corneas of B6 mice seeded with 5×10^4 naive P14/Ly5.1 were scratched and 10^6 pfu LCMV Armstrong were placed on the left eye. FACS plot and enumeration of P14 cells in the **b)** spleen and **c)** cornea 8 days post infection or treatment. Cells were gated on live CD8⁺CD4⁺ lymphocytes. FACS plot and enumeration of P14 cells in the **d)** spleen and **e)** cornea 29 days post infection or treatment. Cells were gated on live CD8⁺CD4⁺ lymphocytes. The proportion of gBT-I and OT-I cells that were **f)** CD69⁺ or **g)** CD69⁺CD103⁺ were determined by flow cytometry. **h)** Schematic of experimental set up. Corneas of B6 mice seeded with 5×10^4 naive OT-I/uGFP were scratched and 10^6 pfu Vaccinia-OVA or HSV-OVA were placed on the left eye. FACS plot and enumeration of OT-I cells in the **i)** spleen and **j)** cornea 8 days post infection or treatment. Cells were gated on live CD8⁺CD4⁺ lymphocytes. **k)** Enumeration of the endogenous response of CD8⁺ T cells in the cornea. Data from one experiment. n= 4-5 mice per group per experiment. * $p < 0.05$ **** $p < 0.0001$

the circulation, I attempted to generate corneal memory CD8 T cells using LCMV Armstrong to create an inflammatory micro-environment in the cornea prior to iv injection of *in vitro* activated OT-I/Ly5.1 cells. 29 days post infection with LCMV, one group of mice had both corneas scratched again and infected with HSV-OVA while a separate group had both corneas scratched and covered with PBS only (Fig 4.9a). The number of OT-I cells in the spleen 3 days post HSV infection were relatively equal between the HSV-infected and non-infected groups (Fig 4.9b). Prior LCMV infection had no effect on the recruitment of OT-I cells as demonstrated by the large infiltration of OT-I cells following HSV-OVA infection in both LCMV infected and non-infected groups. Only a low number of OT-I cells were detected in the cornea of mice infected with LCMV but not infected with HSV (Fig 4.9c). Almost all OT-I cells in the HSV-infected corneas with or without LCMV infection expressed CD69 while a slightly higher proportion of OT-I cells (not significant) expressed CD69 in the LCMV infected but not HSV-infected cornea compared to the cornea that was uninfected (Fig 4.9d). There was a proportion of CD103 co-expression with CD69 by OT-I cells in the cornea though this was not influenced by the different combination of infections (Fig 4.9e). Overall, prior infection with LCMV failed to enhance responses to HSV-OVA though the overall infiltration response by circulating memory T cells appear to be more rapid than in a naïve setting.

Next, I wanted to assess if the improved infiltration of the cornea by memory T cells at the acute time point was also independent of the presence of antigen at the cornea (See 4.2.4). B6 mice were injected iv with *in vitro* activated OT-I/uGFP and P14/Ly5.1 cells and left for 28 days to form memory cells. Corneas of these mice were then scratched and either infected with HSV-OVA or covered with PBS only and the immune response 3 days post infection was assessed (Fig 4.9f). Both OT-I and P14 cells were found at relatively equal numbers in the spleen (Fig 4.9g). A higher number of T cells were found in the corneas

infected with HSV compared to the control. Interestingly, both P14 and OT-I memory cells were able to enter the cornea at high numbers, providing further evidence that antigen is not required for the recruitment of cells into the cornea (Fig 4.9h). Similar proportions of both OT-I and P14 cells were expressing CD69 in the HSV-infected cornea while a lower proportion were positive for CD69 in the control cornea (Fig 4.9i). CD103 and CD69 co-expression was not observed in the infected corneas with only a very small proportion being positive for both markers. This co-expression was seen in a larger proportion of the small number of P14 and OT-I cells found in the control cornea (Fig 4.9j). The resulting memory cells from the HSV challenge was determined after 21 days post infection to assess if antigen can play a role in the persistence of these memory cells in the cornea. Numbers of P14 and OT-I cells in the spleen show a slightly higher number of P14 cells though this difference is not significant (Figure 4.9k). In the cornea, a modest amount of P14 and OT-I cells were found in the infected cornea at similar numbers. Notably, there also appeared to be a population of P14 cells, but not OT-I cells, in the control cornea (Fig 4.9l). OT-I cells in the infected cornea had the highest proportion of CD69 expression – more than that of the P14 cells in the same cornea which was comparable to the CD69 expression of cells in the control cornea (Fig 4.9m). OT-I cells in the infected cornea also showed the highest proportion of cells co-expressing CD103 and CD69. While some OT-I cells in the control cornea appeared to express both markers as well, the numbers of these cells make them negligible (Fig 4.9n). There was close to no co-expression in the P14 cells in both corneas. These results show that memory T cells are able to enter the cornea at an earlier time point without the need for antigen, similar to naïve cells, but the expression of canonical T_{RM} marker CD103 in the cornea following this infiltration is impaired.



4.9 Improved infiltration of the cornea by circulating memory T cells

a) Schematic of experimental set up. B6 mice were injected with 10^7 *in vitro* activated OT-I/Ly5.1. Corneas of the mice were scratched and 10^6 pfu LCMV Armstrong were placed on the left eye and PBS on the right. 29 days post infection, corneas were scratched again and either covered with 10^6 pfu of HSV-OVA or PBS. Corneas and spleen were harvest 3 days post HSV infection. OT-I cell numbers in the **b)** spleen and **c)** cornea 3 days post HSV infection were determined. The proportion of OT-I cells that were **d)** CD69⁺ or **e)** CD69⁺CD103⁺ were determined by flow cytometry. **f)** Schematic of experimental set up. B6 mice were injected with 10^7 *in vitro* activated OT-I/uGFP and P14/Ly5.1 to form memory cells. 29 days later, corneas of the mice were scratched and 10^6 pfu HSV-OVA were placed on the left eye and PBS on the right. Number of OT-I cells in the **g)** spleen and **h)** cornea 3 days post infection were quantified. 21 days post infection, OT-I cells in the **k)** spleen and **l)** cornea were also determined. The proportion of these OT-I cells that were **m)** CD69⁺ or **n)** CD69⁺CD103⁺ were also determined. Data pooled from two independent experiments. n= 3-5 mice per group per experiment. * $p < 0.05$ *** $p < 0.001$ **** $p < 0.0001$

4.2.9 Secondary responses to antigen in the cornea

T_{RM} cells are capable of rapidly responding to antigen or secondary infections by producing cytokines, recruiting other immune cells, or dividing *in situ* to increase responses [271, 287, 288]. The ability of corneal memory T cells to respond to antigen was assessed via application of peptide to the cornea. Corneas of B6 mice seeded with gBT-i/Ly5.1 were scratched and infected with HSV-KOS on one eye or PBS applied on the other eye. 21 days post infection, gB peptide was applied to both eyes without scratching to minimise responses to due tissue damaged-induced local inflammation, and the immune response assessed 24 hours later (Fig 4.10a). Memory gBT-I T cells formed in the spleen (Fig 4.10b). Following antigen application to the cornea, recruitment of gBT-I cells was greater in the previously infected cornea (Fig 4.10c). CD69 expression was higher in the HSV-infected cornea (Fig 4.10d). The proportion of gBT-I cells co-expressing CD103 and CD69 following gB treatment was low, possibly due to a large influx of gBT-I cells from the circulation, and unaffected by whether or not there was prior infection (Fig 4.10e).

To examine how TRM cells respond to antigen *in situ*, memory T cells in the cornea were imaged by intravital two-photon microscopy and any immediate responses to local antigen delivery was observed. Corneal memory cells became less motile following administration of gB peptide (red arrow) and this decrease in speed was sustained for at least one-hour post administration (Fig 4.10f, see also Movie S7). Cells also became rounded, losing the dendrite-like morphology following treatment with gB peptide (Fig 4.10g and Fig 4.10h, see also Movie S7). These results show that memory corneal T cells are capable of immediate response to antigen and may be beneficial in increasing protection against secondary encounters of a pathogen.

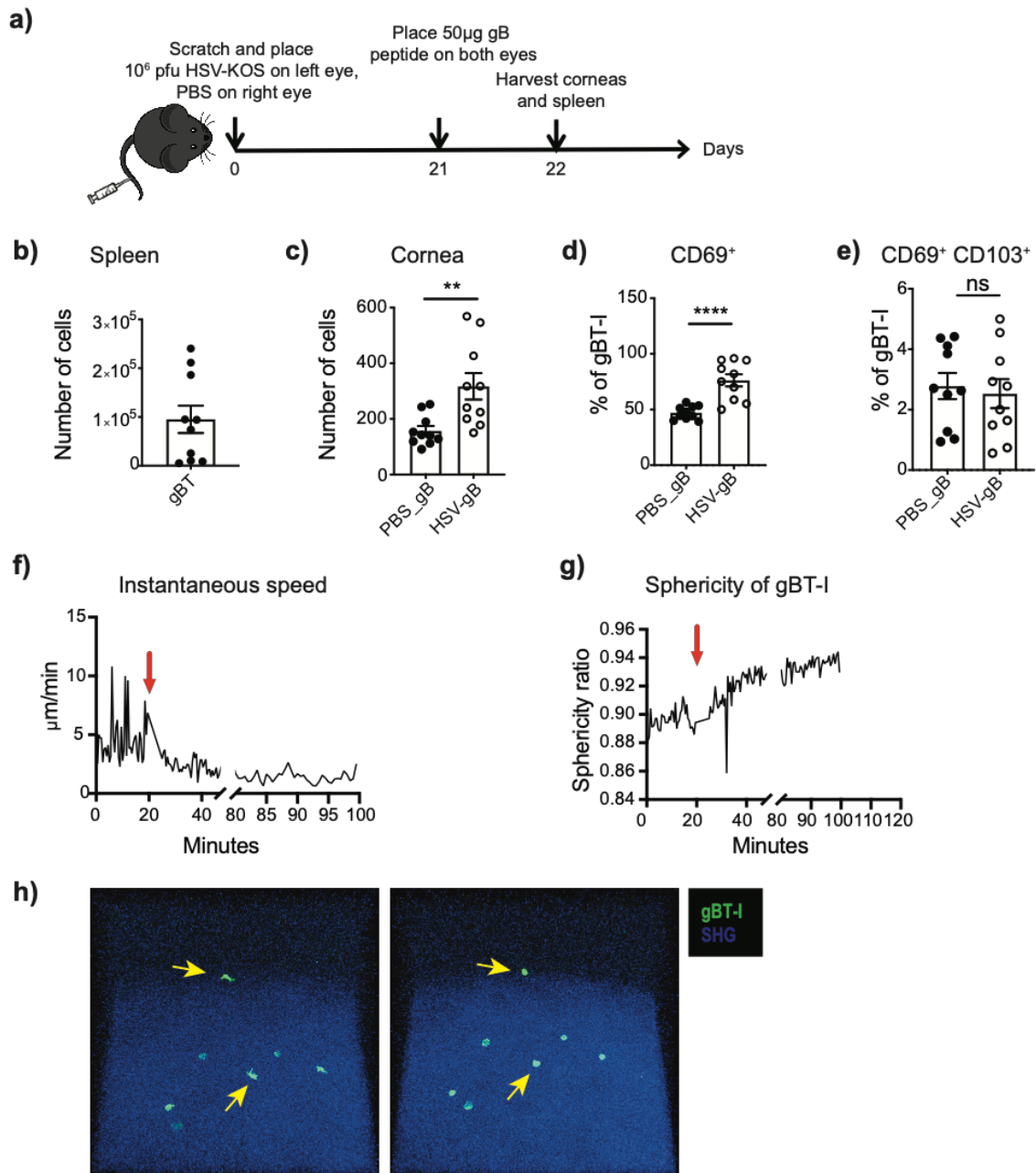


Figure 4.10 Secondary responses to antigen in the cornea

a) Schematic of experimental set up. Corneas of B6 mice seeded with 5×10^4 gBT-I/Ly5.1 were scratched and 10^6 pfu HSV-OVA were placed on the left eye and PBS on the right. 21 days later, the left eye of these mice were treated with $50\mu\text{g}$ of gB peptide and the right eye covered with PBS containing 10% DMSO. The number of gBT-I cells in the **b)** spleen and **c)** cornea 24 hours post treatment were quantified. The proportion of these gBT-I cells that were **d)** CD69⁺ or **e)** CD69⁺CD103⁺ were also determined. **f)** Quantification of the instantaneous speed of gBT-I cells in the cornea following administration of $50\mu\text{g}$ of gB peptide into the eye imaging solution (red arrow). **g)** Measurement of the sphericity of gBT-I cells in the cornea following administration of $50\mu\text{g}$ of gB peptide into the eye imaging solution (red arrow). **(g)** Maximum intensity projection of a frame of 2-photon imaging of HSV infected corneas 32 days post infection before (left) and after (right) stimulation with gB peptide. T_{RM}-like gBT-I cells (yellow arrow) can be seen rounding following treatment. **(a-e)** Data pooled from two independent experiments. $n = 5$ mice per group per experiment. **(f,g)** Data representative two independent experiments. $n = 3-4$ mice per group per experiment. **(f,g)** Image representative of two independent experiments. $n = 3-4$ mice per experiment ** $p < 0.01$ **** $p < 0.0001$ (See also Movie S7)

4.3 Discussion

The cornea is an immune privileged site that modulates immune responses in a manner that prevents excessive damage to the cornea. Much of the work on corneal HSV infections utilise the RE strain of HSV which results in corneal clouding leading to HSK which is contributed to by the action of the infiltrating T cells following a breach of this immune privilege property [360, 491]. This can lead to complications in studying the traits of the immune response that make it so unique, especially in the context of memory cells in the cornea. Here, a sub-clinical model of HSV infection that does not result in HSK was successfully used to investigate the kinetics and formation of corneal memory T cells. Infiltration of T cells was confirmed through flow cytometry and microscopy, providing data on both the number and location of these cells over time. Memory CD8 T cells were found in low numbers though a small population of them express the canonical T_{RM} cell surface markers CD69 and CD103. Additionally, various conditions such as the requirement for antigen or the use of immunomodulatory compounds were tested to identify potential factors that can influence memory cell formation. Finally, the functional capacity of memory T cells in relation to the cornea was also explored.

Given that the cornea is an easily accessible and relatively thin tissue, it is highly suitable for two-photon imaging. Being able to identify the different layers of the cornea allows for identification of which layer the immune cell infiltrates mostly populate. The visible growth of patches of HSV in the cornea complements the study by Rowe *et al.* where they have showed successful growth of HSV-KOS from the corneas in their study [381]. Infiltration of the cornea by T cells in response to the HSV-infection shows similar kinetics to that of epicutaneous HSV-infection with the exception of the peak of expansion in being approximately one day later in the cornea [405]. It was not possible to directly compare the

number of CD4 and CD8 T cells that expanded in response to the corneal HSV infection due to the difference in the initial number of cells injected. However, as the number of cells recovered from the spleen were comparable across the different time points despite the lower initial numbers, it may be that CD4 T cells are able to expand at a greater capacity compared to CD8 T cells, similar to what has been observed in the epicutaneous model [405]. The limbus of the cornea contains vessels that act as an entry site for immune cells [336] thus explaining the infiltration of T cells from the edges of the cornea and progressively moving towards the central area over time. While not all cells at memory expressed both CD69 and CD103, a larger proportion of CD8 T cells co-expressed both markers compared to CD4 T cells which is consistent with expression patterns of T_{RM} cells in the skin [245]. Additionally, CD69⁺CD103⁺ CD8 T cells were found persisting in the cornea 90 days post infection with HSV, providing evidence of long-lived T_{RM} cell formation in the cornea. Given the heterogeneity in the population of T cells in the cornea at memory time points, it is possible that these are indicative of different populations of these cells occupying the different niches available in each corneal layer. Further microscopy experiments utilising labelled antibodies for T_{RM} cell surface markers may be useful in identifying if there is a correlation between these populations and the corneal layers. The lack of *in vivo* labelling of memory CD8 T cells in the cornea may be attributed to the lack of neovascularisation of the central cornea where most cells are found thus leading to no chance of the cells encountering any labelled antibodies in the circulation. While the results here showed that T cells are able to enter the different parts of the cornea after HSV-KOS infection and a small number of them remain in the cornea at the resolution of the immune response, whether these cells are truly resident in the cornea would require further experiments. This may involve parabiosis or corneal implants of corneas containing the

resident-like T cells to observe if those cells return to the circulation over an extended period of time.

The use of immunomodulatory compounds was not effective at recruiting significant numbers of activated T cells into the cornea. The immunomodulatory compounds used – CpG and LPS, act by triggering inflammatory responses initiated by binding with Toll-like receptors (TLR) 4 and 9 [492, 493]. Anterior Chamber-Associated Immune Deviation (ACAID) is a property of the cornea where future potentially damaging responses are suppressed [494]. It is possible that ACAID is able to disrupt the inflammatory signals triggered by CpG and LPS. It may be that stronger inflammatory signals or other signals are required to overcome ACAID such as the establishment of an infection. If this was the case, a combination of immunomodulatory compounds and/or infection may alter the magnitude of the response. In these set of experiments, the ability for activated cells to enter the cornea compared to naïve cells was also assessed. While there were fewer activated cells compared to naïve cells in the cornea 8 days post infection, there were comparable numbers at the memory time point. It may be that a lower number of activated cells were required for the clearance of the virus at the acute time point but the ones that do enter are able to persist like their naïve counterparts.

Additionally, the other models of infection tested in this chapter did not result in the same magnitude of corneal T cell infiltration as HSV infection. It could be possible that in the case of LCMV infection, LCMV was restricted in its growth as dystroglycan, the receptor used by LCMV, is only expressed in the corneal endothelium [495] and so the cells do not populate the stromal and epithelial layers of the cornea. This would also explain the lack of CD103 expression by the CD8 T cells as the cells do not need to enter the epithelial layer of the cornea and CD103 is linked to adhesion to epithelial sites [252]. Moreover, since LCMV

is a systemic infection, that may have caused the infection of the endothelium of the control cornea thus resulting in T cell infiltration into that cornea as well. Further validation using fluorescently tagged LCMV and microscopy would provide a better idea of the restriction of LCMV infection in the cornea.

The vaccinia model used in this chapter was not sufficiently optimised to produce robust results. While there was no OT-I T cell response initiated against the virus, the infiltration of endogenous CD8 T cells in the infected corneas suggests that there may have been some replication of the virus, but the OVA component of the virus was not well expressed. It is also pertinent to note that the infectious dose of 10^6 pfu was used to be consistent with the HSV model but a previous study with vaccinia and the cornea used a higher infectious dose which may be what is required for an infection to occur [490]. Perhaps a different strain of VV expressing other well recognised antigens, such as gB, could be used instead. Future experiments to optimise the vaccinia model would allow for a better comparison of immune responses in the cornea.

The role of antigen in corneal T cell responses is unclear and is a topic of interest in this chapter. While antigen may not be required for the initial infiltration of T cells, antigen enhanced the formation of memory cells in the cornea. The infiltration of OT-I cells into infected corneas was intact in the absence of cognate antigen though fewer cells persisted to memory and few formed CD69⁺ CD103⁺ TRM cells. Unlike other epithelial sites that do not require antigen for the establishment of T_{RM} cells [258, 265], corneal memory T cell formation appear to be similar to those of neuronal tissue, which also share some immune privilege properties, such as dorsal root ganglia (DRG) and the brain [264]. While that is the case, it would appear that peptide treatment following recruitment of antigen experienced CD8 T cells into the cornea does not improve the conversion of corneal memory T cells to

express more of the canonical T_{RM} markers. A future consideration would be identifying the type of APCs in the interactions with T_{RM} cells in the cornea in both the establishment and reactivation of these cells. Previous studies have found DCs to be important in reactivating CD8 T_{RM} cells in the skin and female reproductive tract [496, 497]. However, another study has also shown that T_{RM} cells are not only reactivated by haematopoietic cells, but also by non-haematopoietic cells and that activation by either cell type can result in different transcriptional changes [498]. Perhaps this differential gene expression profile can be extended to memory establishment in the cornea given the variety of APCs present in the cornea. DCs are well observed in the corneal epithelium while other APC populations such as macrophages can be found in the stromal compartment [499]. Additionally, corneal epithelial cells have also been found to gain APC functions through upregulation of Ly6C and MHC-II following HSV-1 infection [500]. It may be that the strength of the memory formation and subsequent response is dependent on the interactions between these different types of APCs and the responding T cells. It would also be prudent to consider if antigen presentation in the cornea is not dependent on the resident APCs but rather on migratory DCs that function in a similar manner to those of the skin [405].

Another factor to consider is $TGF\beta$ which has been well documented to not only be involved in T_{RM} cell formation in tissues such as the gut and skin through the regulation of CD103 expression [272, 279] but also implicated to be a broad regulator of residency in multiple tissues [501]. While it was expected that CD103 expression would not be detected in $TGF\beta R$ -KO cells in the cornea, the numbers of these cells at memory compared to wild type appeared unchanged. It may be that the number of T cells in the cornea at memory is not dependent on $TGF\beta$ due to the heterogeneity of the cells. Thus, the loss of the CD103⁺ population would not affect the overall number of T cells in the cornea. Future experiments

involving the overexpression of TGF β signalling to investigate if there will be increased proportions of CD103⁺ cells in the cornea at memory and shift the population to be more homogenous.

How memory cells, both in the circulation and in the cornea, respond to antigen in the cornea is not well known. The relatively large infiltration of memory cells from the circulation shortly after HSV challenge is a result of the canonical rapid response to secondary encounters [234]. This infiltration is indicative of the properties of circulating and effector memory T cells in a standard immune response [227, 234] suggesting that the immune privilege of the cornea does not dampen the secondary response by memory cells in the circulation. Additionally, it would appear that non-responding memory T cells in the circulation are also able to non-specifically infiltrate infected corneas in a similar fashion to their responding counterparts. This recruitment of non-antigen specific memory cells has also been documented in the female reproductive tract [502]. Interestingly, these non-antigen specific cells are able to persist in the cornea following the cessation of the immune response unlike the scenario with non-antigen specific naïve cells. There is likely some cell programming which occurs in memory T cells that allow them to overcome this barrier for persistence in the cornea. Gene analysis and comparison between memory cells in the cornea formed via memory cells as a precursor or naïve cells as a precursor may reveal important factors to answer this question. Peptide application on corneas with memory T cells provides further evidence of rapid immune responses in the cornea through increased infiltration and CD69 expression within 24 hours of treatment. Moreover, physical changes could be observed in the corneal CD8 T cells within minutes of peptide treatment. T_{RM} cells have been documented to produce cytokine *in situ* in other tissues [259-261] and is likely to

do so in the cornea as well. How this affects the corneal microenvironment and its impact on preserving the clarity of the cornea remains to be ascertained.

The results in this chapter provide insight into T cell responses to infection in the cornea, and the formation of resident memory T cell populations though more questions have arisen from these results. The formation of corneal T_{RM} cells are not as prominent as in other tissues and is a largely heterogenous population. However, I have only used relatively mild and acute infections in studying the memory cells in the cornea. Perhaps more severe infections such as the RE strain of HSV or another chronic infection might lead to a different outcome with memory formation. Additionally, whether or not T_{RM} cells contribute to protective immunity or pathology can lead to the development of management strategies for corneal damage and injury. Given the well-known role of immune cells in corneal HSK, it may be that immune cell activity results in more pathology and the poor establishment of T_{RM} cells in the cornea is a mechanism by which excessive damage to the cornea is kept to a minimum.

Chapter 5 – General

discussion

5.1 The major findings of this thesis are:

5.1.1 Chapter 3

Sympathetic signals in the spleen are involved in intricate crosstalk with antiviral responses. Changes in sympathetic signalling can result in downstream effects that can modulate immune responses. Here, the use of chemical sympathectomy with 6-OHDA to deplete peripheral SNS fibres did not affect T cell responses to localised HSV infection at peripheral sites but did marginally modulate effector T cell responses to systemic chronic LCMV infection. Following further investigation of the relationship between LCMV infection and the SNS, it was determined that LCMV infection resulted in the loss of TH⁺ nerve fibres in the spleen and impacted local but not systemic levels of NA. In order to determine the factors responsible for this depletion, various aspects of the immune response to LCMV was investigated. This loss of splenic TH⁺ nerve fibres could be partially attributed to NK cell and IFN γ activity. The loss of splenic TH⁺ fibres was concordant with changes in the celiac ganglia with upregulation of neural growth factor gene expression to counter the loss of splenic fibres. Given the depletion of local endogenous NA in the spleen following LCMV infection, the effects of augmenting downstream sympathetic signalling through treatment with the β -adrenocptor agonist, isoprenaline was investigated. Much like chemical sympathectomy, isoprenaline treatment was also able to modulate effector T cell responses to systemic chronic LCMV infection. Importantly, both chemical sympathectomy and β -adrenoceptor stimulation altered viral control of the chronic LCMV infection. This chapter shows that both the SNS and immune system can have significant effects on the other during the course of LCMV infection, suggesting the possibility that this may extend to other forms of systemic viral infection.

5.1.2 Chapter 4

This chapter explores the role of memory cells in the highly innervated and immune privileged cornea. Here, a model of sub-clinical model of HSV infection was utilised that resulted in successful priming and infiltration of the cornea with T cells without causing excessive corneal pathology. Using confocal and 2-photon microscopy, this chapter shows T cells entering the cornea from the limbus region, progressively move towards to central cornea, and occupy both stromal and epithelial layers of the cornea. Additionally, only infection of the cornea and not application of inflammatory compounds led to non-specific recruitment of CD8 T cells into the cornea. Furthermore, circulating memory CD8 T cells were able to enter the cornea more rapidly than their naïve counterparts. A small pool of memory cells was retained in the cornea after infection. This population was not homogenous with only a small proportion expressing the canonical T_{RM} cell markers CD69 and CD103. To better understand the formation of these cells, the role of local antigen availability was investigated and found to have no effect on the numbers of memory T cells following cessation of acute immune responses though was required for expression of CD103. TGFβ signalling which has been documented to be important in T_{RM} formation also had no effect on the number of corneal memory T cells. Functionally, these corneal memory CD8 T cells were also capable of responding to antigen *in situ* and increased recruitment of responding CD8 T cells from the circulation. Taken together, the results from this chapter suggests that the cornea might not be conducive to memory cell persistence and though the small number of cells that to persist are able to modulate secondary immune responses

5.2.1 Sympathetic signals and antiviral responses

Neuroimmune crosstalk between the SNS and the immune system has been shown to play a role in responses to acute trauma, anti-tumour responses, inflammatory autoimmune responses, and immune responses to foreign pathogens [503-505]. A number of studies have shown SNS signalling to modulate different aspects of the antiviral immune response from influencing cell differentiation to effector functions [185, 186, 188, 506]. The results from this thesis further add to this pool of knowledge by exploring the effects of the SNS on the T cell response to both acute and chronic strains of LCMV, expanding on previous work on localised viral infections. Studies with vesicular stomatitis virus and IAV revealed suppressing effects of the SNS on the cytokine production of responding effector CD8 T cells thus suggesting that SNS signals negatively modulate immune responses to these viruses [185, 186]. However, work with HSV makes this association less clear where the absence of sympathetic signals resulted in the suppression of cytotoxic T cell response (CTL) and memory CTL generation [188]. This ability for the SNS to modulate immune responses is not only restricted to viral infections. Cytokine production by T cells following infection with the bacteria *Listeria monocytogenes* was increased after 6-OHDA treatment [507]. Anti-tumour CD8 T cell responses were also dampened following daily treatment with β -adrenoceptor agonists which led to poorer control of tumours [192]. Here, the overall increase in cytokine production by T cells when LCMV-infected mice were treated with β -adrenoceptor agonists and slight decrease following chemical sympathectomy suggests that sympathetic signals may be important in the immune response to LCMV. Perhaps the increased IFN γ is a result of increased T cell activity despite a dampened inflammatory response leading to reduced viral clearance (See Fig 3.12). This ability for the SNS to either promote or inhibit immune responses is not surprising to allow for flexibility in the desired outcome. An example of this

is central nervous system (CNS) infections by alphaviruses where IFN γ is required for the clearance of the virus but increased levels of IFN γ may lead to damage of the CNS [508]. It may be that in the case with LCMV, the modulation of IFN γ and other cytokine production by the SNS may contribute to the balance of cytokine activity and thus allow for the optimal compromise between excessive tissue damage and clearance of the virus. It could be that different infections can result in different outcomes and thus may affect the amount of downstream sympathetic signalling that occurs in the body. This highlights that the potential for the SNS to modulate the immune response may be dependent on the stimulus that initiated in the immune response. A measure of T cell cytotoxic killing capabilities during models of infection may be useful to ascertain if we are able to manipulate this mechanism to improve T cell activity without excessive inflammation.

Another point of note is the significant effect that changes in SNS signalling has on the control of chronic LCMV viral loads. Both chemical sympathectomy and β -adrenergic receptor agonist treatment resulted in a higher viral load compared to untreated mice. This occurred despite better IFN γ responses in mice treated with agonists and the lack of major effects in other immune cell subsets. Taken together, I was unable to ascertain if the SNS signals acted directly on the immune response through our experiments. It would be pertinent to consider that the SNS modulation of the response to LCMV may involve more complex interactions involving other indirect effects. A potential candidate is the vascular system. LCMV infection results in damage to the vasculature through infection of endothelial cells [466]. Coupling this with the changes in blood pressure due to manipulation of the SNS [482, 483] could potentially result in increased dissemination and replication of the virus. In conclusion, SNS signalling appears to have nuanced effects on LCMV infection and the resulting immune response. Extending on this finding, the relevance of SNS signalling in other chronic systemic

infections may be of interest as well. The studies in this thesis focused on the effector phase of antiviral responses. Future work could consider investigating the effects of SNS signalling on the priming phase of the immune response. Given that SNS signalling appears to modulate the migration of T cells from SLO [166], there may be alterations to the priming or proliferation of these T cells depending on their ability to encounter APCs and the amount of signals they can receive based on the amount of time spent in SLOs. The effect of the SNS on memory formation and secondary responses is also unknown.

Much of the work in this thesis focuses on the effects of β -adrenergic signalling given T cells have the highest expression of β -adrenoceptors. However, it is important to consider the effects of activating other signalling pathways downstream of the SNS, as NA and adrenaline bind both α and β -adrenoceptors on different cell types with different affinities and can result in distinct outcomes. Notably, activation of α -adrenoceptors is typically thought to promote, while β -adrenoceptors can inhibit, innate immune responses [111]. Therefore, an avenue to investigate would be to compare how responses to chronic viral infections might change with stimulation of α -adrenoceptors as there has been evidence that activation of α -adrenoceptors is beneficial in the response to IAV infection [509]. Perhaps the actions of NA binding to both α and β adrenoceptors may result in less pronounced effects of each arm of the SNS on immune responses to viruses. Further work would also need to be done to ascertain the mechanisms by which the SNS modulate immune responses. Perhaps a significant contributing factor of SNS immune crosstalk is dependent on the indirect modulation of T cell function through the effects on other cells or pathways.

β -adrenoceptor signalling pathways have been manipulated to manage pathologies such as those associated with hypertension using medication that are usually lifelong management strategies [510]. This can result in altered downstream signalling in these

signalling pathways which could then lead to changes in the normal immune response to infections over the duration of their life. A greater understanding of these interactions would not only allow for ways to mitigate potentially detrimental changes to immune responses but also lead to novel methods of manipulating immune responses using these modified signalling pathways downstream of sympathetic signals.

5.2.2 Modification of the sympathetic network following infection

The relationship between the immune system and nervous system is bi-directional as signals from the immune system are also capable of affecting the SNS. The maintenance of peripheral nerve fibres may be influenced by the levels of cytokines released by immune cells in inflammation. Muscularis macrophages are capable of utilising the neurons to produce colony stimulating factors (CSF) important for the proliferation of these macrophages by producing bone morphogenic protein 2 (BMP2) which can stimulate neurons [511]. Most notably, IFN γ production, particularly during the course of viral infection, has been shown to be detrimental to nerve survival and growth *in vitro* [474, 475]. Following simian immunodeficiency virus (SIV) infection, not only increased IFN γ but also decreased levels of neurotrophic factors such as nerve growth factor (NGF) and neurotrophin-3 (NTF3) correlated with reduced innervation of LNs [452]. NGF is a molecule essential for growth and survival of peripheral nerves [512, 513] while NTF3 supports the outgrowth of sympathetic fibres [514, 515]. Together, infection with SIV resulted in a microenvironment that appeared to be detrimental to nerve growth and survival. Moreover, in models of autoimmunity, IFN γ also inhibited the remyelination of damaged nerves [516]. As demonstrated in this thesis, this role of IFN γ in nerve damage is also implicated in LCMV infection. Whether or not the decrease is solely due to immune responses, the infection itself, or a combination of factors needs to be

ascertained. Additionally, the changes in the ganglia following LCMV require further exploration to determine if they occur as a result of signals from the splenic fibres or if there is inflammation and immune cell infiltration in the ganglia itself.

Loss of innervation and subsequent reinnervation has also been observed in corneal infections of HSV-1 and have been attributed to altered nerve growth factor production by cells in the corneal microenvironment [517]. However, in both the cornea and here with LCMV, it remains unclear as to whether these changes are triggered by viral infection or a result of the host response. Splenic stromal cells have been documented to not only produce a variety of nerve growth factors but have also been found to express receptors for these factors [518, 519]. To this effect, stromal cells in the spleen become cells of interest to investigate their capacity to produce a range of factors that may then support innervation of SLOs and promote neuroimmune crosstalk. Furthermore, stromal cell functions could be impacted by direct LCMV infection [418]. Given the documented inhibitory effects of SNS signalling on adaptive immune responses [185, 186, 192], it is possible that the loss of splenic nerves is a mechanism to reduce the amount of sympathetic signalling in the spleen during LCMV infection to allow for an effective immune response. By understanding how inflammation from viral infections could potentially affect peripheral nerves, it may be possible to gain insight into strategies in manipulating neuroinflammatory damage and autoimmunity by targeting the different aspects of the inflammatory pathways.

Another point to note when considering the notion that neuroimmune crosstalk is bi-directional is that subsets of immune cells have been documented to produce catecholamines in both mice [520-523] and humans [524-527]. The production of catecholamines is thought to mimic the processes of sympathetic neurons as expression of TH has been shown in these catecholamine producing immune cells [469, 526, 528]. As previously described,

catecholamine activity has a range of effects on the functioning of immune cells. Further investigation could consider if immune cells are capable of TH expression following persistent chronic infections. This evidence of catecholamine production by immune cells provides an opportunity for immune responses to not only regulate itself via sympathetic signals but also augments SNS signalling through increased catecholamine production which can result in altered downstream responses.

5.3 Persistence of memory T cells in the cornea – good or bad?

The clarity of the cornea is important for vision and so various mechanisms are in place to prevent excessive damage to the cornea, particularly in the case of immune responses. One such instance of this is the infection of the cornea by HSV leading to herpes stromal keratitis (HSK) [357, 358]. HSK is a sequela of the immune response [359, 360]. Indeed, in mice that are lacking T cells, the symptoms associated with HSK do not manifest in mice with HSV-infected corneas [361]. Results from this thesis suggest that one of these mechanisms in preventing corneal damage may be the control of persisting memory T cells in the cornea, potentially to prevent excessive immune responses. Memory T cells are well known for eliciting rapid and robust immune responses following secondary encounter of antigen [215-217]. There is the possibility that this may not be beneficial in the cornea as a strong inflammatory response could result in unwanted damage and that a slower response that eventually removes invading pathogens in the cornea is preferable. Thus, the presence of T_{RM} cells in the cornea may not be as desirable as in other tissues such as the skin and mucosal tissues, which may explain the regulation of T cell numbers in the cornea where recruitment only occurs if there is sufficient local damage and inflammation. It is important to note that other immune privileged sites such as the brain and the ganglia have been documented to

harbour T_{RM} cells [264, 385, 529] indicating that it is not impossible for T_{RM} cells to persist in these sites. Therefore, a question to consider would be if the reason for the low numbers of persisting corneal memory T cells is due to a corneal microenvironment that is not conducive to the survival of immune cells. The cornea is rich with anti-inflammatory cytokines such as TGF β [530] which has been documented to impede DC maturation [314]. While results from this thesis have suggested that TGF β did not have a role in the total number of cells persisting in the cornea, it is possible that there are other cytokines that affect the number of cells that are able to persist following cessation of the immune response. However, the well-defined role of TGF β regulation of CD103 is also apparent in the cornea.

The lack of vascularisation, particularly in the central cornea, suggests that many corneal infiltrating T cells perish rather than leave the tissue to re-enter the circulation as cells may not be able to receive sufficient oxygen or survival signals. However, circulating memory T cells were able to enter the cornea rapidly following infection. The rapid increase in numbers shortly after secondary exposure to antigen suggested that circulating memory T cells can rapidly enter the cornea. Given that inflammatory responses are often dampened in the cornea, it at first appears contradictory that cells are able to enter so rapidly to produce a strong secondary response. Yet, this could allow rapid immune surveillance of the cornea when most required. Whether this requires small populations of cells resident in the cornea to support rapid local immunity will require further investigation.

Corneal infection models are a valuable tool in understanding how immune responses develop and are regulated in the eye. It is of growing interest as to how T_{RM} cells are able to occupy niches in a variety of tissues [531] and the cornea provides yet another addition to the list as a quite unique tissue microenvironment. A better understanding of immune cell interactions in the cornea could lead to potential avenues for control of damaging immune

responses that result in the loss of sight. Moreover, further work on determining the effects of the anti-inflammatory microenvironment of the cornea can be extended to provide insight to better management of inflammatory diseases.

Chapter 6 – References

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Chapter 7 –

Thesis amendments and

examiner comments

Examiner #1			
Examiners comment	Student's response	Change made to thesis (if any)	Page reference
On page 36, it is referred that neutrophils can clear the HSV-1 infection. Those studies have used the anti-Gr1 antibody, which targets both neutrophils and inflammatory monocytes. More recent studies have shown the minimal effect of neutrophils in regulating viral load. Other cell types like inflammatory monocytes and NK cells are relatively more important in clearing HSV-1 infection.	Noted. While the older study cited here did use antibodies that can have confounding effects, the recent study by Hor <i>et al.</i> [367] that demonstrates the dispensable role of neutrophils utilised a skin flank infection model instead of a corneal infection. There may be factors in the microenvironments of these different sites of infection that can affect the functioning of these cells.	Added a sentence to juxtapose the role of neutrophils in the response against HSV-1 in other models of infection.	36
The data presented show that systemic LCMV infection reduces the sympathetic innervation and neuropeptide Y expression in spleen. Were studies carried out to determine if NPY treatment given to LCMV infected mice modulates virus-specific T cell response? After LCMV infection, did the loss of sympathetic innervation persist for longer duration and if there was a compensatory increase of the sensory innervation in the spleen?	Unfortunately, I did not have the opportunity to investigate the role of NPY in T cell responses given the focus on adrenoceptors on immune cells. Interestingly, a study has showed a potential role for NPY in the pathology of influenza infection, attributed to impaired antiviral response and increased pro-inflammatory cytokine production [368]. Perhaps the downregulation of NPY in the instance of LCMV infection is a mechanism in attempting to limit the damage. In regard to the persistence of sympathetic nerve loss, I have only observed the effects up until D28-D32 post infection where the restoration of nerves can begin to be observed. I was unsuccessful in my attempts to detect sensory fibres in my sections though this was likely a technical issue which was not pursued.	Included a sentence "Alternatively, future experiments..." to include the potential for investigating sensory innervation in the spleen following infection.	97

Why did the candidate not carry out the challenge experiments with infectious HSV-1(RE) in the corneas harbouring HSV-1 KOS-specific T_{RM} cells to measure the functional efficacy of these memory T cells in clearing the viral infection?	This was a planned experiment that was not completed due to the lack of time, reagents, and other reasons. There was also concern regarding potential cross-reactive antibody responses affecting infection kinetics and the overall antiviral response. An attempt was made to test for the functional role of T_{RM} cells in the cornea by utilising modified virus that express the immunogenic gB peptide though I was unsuccessful in establishing a working model. Additionally, I considered attempting <i>ex vivo</i> peptide stimulation and intracellular staining to measure cytokine production but that also proved to be challenging with the low number of cells recovered.	N/A	N/A
While describing the importance of antigens in the retention of TRM cells in virus-infected corneas, the candidate can speculate about the possible cell types involved in presenting the antigens to TRM cells in infected corneas. Will that be resident DCs or epithelial cells or macrophages?	This is an interesting question that would be beneficial to explore in future experiments. Given the number of DCs in the cornea epithelium, there is a potential for them to be the first responders as APCs. However, there are a variety of APCs present in the cornea in both the epithelium and the stroma. Corneal epithelial cells may also potentially gain APC-like functions following infection [500]. Further experiments would be needed to identify if any of these subsets are important for T_{RM} cell function or if they might be dependent on migratory DCs.	Addition of section beginning from “A future consideration...” to discuss possible corneal APC and T_{RM} interactions	130

Examiner #2			
Examiners comment	Student's response	Change made to thesis (if any)	Page reference
The role of IFN γ depletion in increasing splenic Th+ fibres in LCMV infected mice may be indirect – i.e. it may not be IFN γ driving the loss of splenic Th+ fibres, rather the lack of IFN γ alters the pathogenesis of the virus (e.g. in terms of viral titres, tropism, pathology etc) and this may account for the moderate phenotype change. What is known about the pathogenesis of LCMV in IFN γ -/- mice? Please include mention of this indirect mechanism in the discussion of this chapter	This is a great point and was alluded to in the discussion though perhaps not as clearly. It is known that IFN γ is important in the clearance of LCMV infection and has a role in orchestrating CD8 T cell responses [436]. The loss of IFN γ likely results in increased pathology, not necessarily immune-mediated damage but potentially damage through poorly controlled viral replication. LCMV is known to infect stromal cells which are important in maintaining splenic architecture and thus may contribute to supporting nerve growth.	Included a section “As IFN γ plays a crucial role” detailing the potential for indirect effects of IFN γ in regulating LCMV pathogenesis and hence potential modification of splenic nerve fibres	96
Fig 3.9 c/d/e: All other graphs have shown individual datapoints overlayed over bar graphs – please adjust these accordingly. Please also perform the appropriate statistics to determine if the expression levels are significantly different from the mock. – could any of these gene alterations be responsible for the specific loss of splenic Th fibres? Did you look at gene expression after Listeria and/or Plasmodium infection where the same phenotype was not observed? This is would make an interesting comparison and give you a feel for what was biologically significant. Please discuss	Noted regarding the graph formats. The data for the gene expression in infected mice is presented relative to the expression of genes in non-infected mice using $2^{\Delta\Delta CT}$. Therefore, a direct statistical test for this would not be possible. With regards to gene expression following Listeria or Plasmodium infection, I did not consider looking at this due to time constraints. However, it would definitely provide some insight to which factor is responsible should there be increased expression in these other models. Additionally, it would also be insightful to consider other ways that the system might compensate for a microenvironment that is detrimental to nerve growth.	Changed graphs in Fig.3.9 as requested Added a section “For example... “for discussion with regards to comparing bacterial/ parasitic infections to viral infections and potential gene expression changes	84 98

Does isoprotenerol have any off-target effects? How did you confirm the efficacy of isoprotenerol treatment?	Isoprotenerol is able to act on all cells that express β -adrenoceptors on their cell surface. As highlighted in the thesis, there is the possibility for isoprotenerol to act on other cells such as those in the circulatory system which can in turn indirectly affect T cells and other immune cells. While not included in this thesis, prior work in the lab treating isolated T cells with isoprotenerol has resulted in increased cAMP activity although an assay to measure this activity following <i>in vivo</i> treatment of mice in our models have not been done. However, the administration of isoprenaline to study the effects of SNS signals have been well utilised and reported in the many studies highlighted in this thesis.	N/A	N/A
Was the presence of HSV in the cornea confirmed by viral plaquing?	I made multiple attempts to confirm the growth of the virus both in the cornea and through tear swabs but was unsuccessful each time. However, the presence of the virus can be observed through microscopy as depicted in Fig. 4.1. It would be unlikely to observe the GFP+ staining if there was no productive replication at the site of infection.	N/A	N/A

Was LCMB tropism in the cornea assessed by IHC to determine if it was epithelial or endothelial tropic?	LCMV tropism was not assessed by IHC though it is expected that the virus is likely to be within the endothelium as it was reported that α -dystroglycan, the receptor utilised by LCMV for entry into a cell, is typically only expressed in the corneal endothelium and not the epithelium [495].	N/A	N/A
Typos in table of contents	Noted and changed.	Removed an incorrect subheading	XIII
The last sentence of the thesis aims appears complete.	Noted and corrected.	Completed the sentence with "...better antiviral responses and protection in the cornea."	43
Double dot on page 92	Noted and corrected.	Removed extra period.	92
Methods please list the correct name for PR8	Noted and changed name in Methods section.	Changed to Influenza A virus (IAV) (strain A/Puerto Rico/8/1934 H1N1)	56
In the statistical analysis please confirm that normalcy tests were performed (and state which ones)	Noted and amended	Included statement about normalcy tests used	64

Additional amendments	
Changes made	Page reference
Included Fig 3.7 which was previously missing from the document but was accessible from the download link in the reference list	81
Correction of ketamine and xylazine dose	56-57

Appendix I – Movie figure legends

[Movie S1 Three-dimensional view of the mouse cornea](#)

<https://www.dropbox.com/s/3c4o62oqxuo2uux/Movie%20S1.mp4?dl=0>

Two-photon imaging of corneas of naïve td-Tomato mice. Epithelial and endothelial cells (red) make up the top and bottom layers of the cornea. The stromal layer can be visualised through second harmonics generation (SHG) (blue).

[Movie S2 T cell infiltration of the cornea 5 days post HSV infection](#)

<https://www.dropbox.com/s/6e6d6cmt6kp5c4p/Movie%20S2.mp4?dl=0>

B6 mice were adoptively transferred with gDT-II (red) and gBT-I (green) cells prior to corneal infection with HSV-KOS and imaged 5 days post infection. Cells are seen deep in the field of view at the edge of the cornea, near the corneoscleral junction. The stromal layer can be visualised through second harmonics generation (SHG) (blue).

[Movie S3 T cell infiltration of the cornea 6 days post HSV infection](#)

<https://www.dropbox.com/s/o3d3u292t8x4w2e/Movie%20S3.mp4?dl=0>

B6 mice were adoptively transferred with gDT-II (red) and gBT-I (green) cells prior to corneal infection with HSV-KOS and imaged 6 days post infection. More cells are visible and still close to the corneoscleral junction. The stromal layer can be visualised through second harmonics generation (SHG) (blue).

[Movie S4 T cell infiltration of the cornea 7 days post HSV infection](#)

<https://www.dropbox.com/s/s5xwv0f4n4smt9r/Movie%20S4.mp4?dl=0>

B6 mice were adoptively transferred with gDT-II (red) and gBT-I (green) cells prior to corneal infection with HSV-KOS and imaged 7 days post infection. Cells are seen further up from the corneoscleral junction, moving closer towards the curvature of the central cornea. The stromal layer can be visualised through second harmonics generation (SHG) (blue). Some cells are above the stromal layer, presumably in the epithelium.

[Movie S5 T cell infiltration of the cornea 10 days post HSV infection](#)

<https://www.dropbox.com/s/dg959ir4dztgz7a/Movie%20S5.mp4?dl=0>

B6 mice were adoptively transferred with gDT-II (red) and gBT-I (green) cells prior to corneal infection with HSV-KOS and imaged 10 days post infection. Cells are close to the central cornea and in larger numbers. The stromal layer can be visualised through second harmonics generation (SHG) (blue). More cells are above the stromal layer, presumably in the epithelium.

[Movie S6 T cell infiltration of the cornea 29 days post HSV infection](#)

<https://www.dropbox.com/s/dx8m1l23syqaxgc/Movie%20S6.mp4?dl=0>

Td-Tomato mice were adoptively transferred with gBT-I (green) cells prior to corneal infection with HSV-KOS and imaged 29 days post infection. The stromal layer can be visualised through second harmonics generation (SHG) (blue) and epithelium indicated by Tomato expression (red). Only a few cells can be seen in the field of view with varying morphology.

[Movie S7 Corneal memory T cell response to gB peptide](#)

<https://www.dropbox.com/s/08bram450qkrmni/Movie%20S7.mp4?dl=0>

B6 mice were adoptively transferred with gBT-I (green) cells prior to corneal infection with HSV-KOS and imaged 29-35 days post infection. The stromal layer can be visualised through second harmonics generation (SHG) (blue). A baseline movie was taken prior to addition of 50µg gB peptide into the imaging solution that covers the eye. Corneal T cells can be seen rounding up and slowing down.