UNIVERSITY OF BIRMINGHAM

Exploring adaptive – like biology in human and murine γδ T cells

By

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To mama and baba,

for everything

The effect of the COVID-19 pandemic on the research project

During the third year of this project the COVID-19 pandemic happened. This lead the university, as most of the country, to shut down for a prolonged period during a national lockdown. At the University of Birmingham all research had to be stopped from March 2020 to July 2020, until the appropriate precautions were put in place. During this period access to all laboratories and the animal facilities was denied, causing a substantial loss of lab time.

Unfortunately, during the lockdown, most mouse colonies were culled to reduce the numbers of unused mice in the animal facility. Some colonies used in this thesis, such as the Great Smart mice, were severely depleted and took a long time to become viable again, even after the reopening of the university. This also contributed to a delay in research, difficulty procuring the required mice and generating required data.

Abstract

Human Vδ1 γδ T cells in the blood have been observed to have an adaptive biology but many questions about them are difficult to explore in humans. Therefore mouse models may be useful to introduce. In this thesis I aim to investigate the presence and characteristics of murine adaptive-like γδ T cells, in both steady state mice and following infections.

In healthy mice we identified naïve (CD62L- CD44+), central memory-like (cm-like) (CD62L+ CD44+) and effector (CD62L- CD44+) γδ T cells, all of which were present in lymphoid tissue, whilst only effector cells were present in the gut. We characterised the populations through their marker expression, migratory capacity and development and found their characteristics often aligning with a naïve and effector phenotype. Interestingly, the TCR repertoire of all three populations was very polyclonal with few expansions.

In acute and chronic infection models (*L. monocytogenes* and *S. typhimurium*), a polyclonal subset of effector and cm-like γδ T cells release IFNγ or IL-17 within 24 hours in an innate-like response. The naïve population however did not respond in this immediate fashion.

To accompany the murine results, I investigated the human Vδ1 γδ subset in lymphoid tissue and detected greater numbers of naïve cells in the tonsil than blood. The tonsil also had cm-like and effector populations, corresponding with murine subsets. The phenotypic characterisation of the populations aligned with murine cells, however human effector and cm-like cells showed clonal expansions in their TCR repertoire.

This thesis includes a thorough characterisation and comparison of adaptive-like populations in both mice and humans. This will help in shaping our understanding of the immunological role of these cells and guide future research in the field.

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Abbreviations

-/-	Knock out
α4β7	Integrin receptor $\alpha_4\beta_7$
APC	Antigen presenting cells
BFA	Brefeldin A
BMSU	Biomedical Services Unit, University of Birmingham
BTN	Butyrophilin
BTNL	Butyrophilin-like
CCL	Chemokine (C-C motif) ligand
CCR	Chemokine (C-C motif) receptor
CD	Cluster of differentiation
CD62L	L-selectin
CDR	Complementary determining region
CMV	Cytomegalovirus
CXCR	C-X-C chemokine receptor
DCs	Dendritic cells
DETC	Dendritic epidermal T cells
DNA	Deoxyribonucleic acid
EBV	Epstein Barr virus
EDTA	Ethylenediaminetetraacetic acid
Eomes	Eomesodermin
EPCR	Endothelial protein C receptor
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
Foxp3	Forkhead box protein P3
GF	Germ-free
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HBSS	Hanks' Balanced Salt Solution
HMBPP	(E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate
ICAM1	Intercellular Adhesion Molecule 1
ID3	DNA-binding protein inhibitor ID-3
IEL	Intraepithelial lymphocytes
Ig	Immunoglobulin
IL-	Interleukin
ILN	Inguinal lymph node
IPP	Isopentenyl pyrophosphate
KLRG1	Killer cell lectin like receptor G1
LB	Lysogeny broth
LFA-1	Lymphocyte function-associated antigen 1
LPL	Lamina propria lymphocytes
MACS	Magnetic activated cell sorting
MAdCAM-1	Mucosal vascular addressin cell adhesion molecule 1
MCMV	Murine cytomegalovirus
MCP-1	Monocyte chemoattractant protein-1
MHC	Major histocompatibility complex
MHCI	Major histocompatibility complex class I

MHCII	Major histocompatibility complex class II
MICA	MHC class I polypeptide–related sequence A
MICB	MHC class I polypeptide–related sequence B
mg	Milligram
ml	Millilitre
µl	Microlitre
µm	Micrometer
MLN	Mesenteric lymph node
NK cells	Natural killer cells
NKG2	Killer cell lectin-like receptor subfamily C, member 1
NLRP	Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain containing
P-Ag	Phosphoantigen
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pLN	Peripheral lymph node
PLZF	Promyelocytic leukemia zinc finger
PMA	Phorbol 12-myristate 13-acetate
pMHC	Peptide - Major histocompatibility complex
PRR	Pattern recognition receptor
RAG	Recombination-activating gene
RNA	Ribonucleic acid
RANTES	Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted
RoRyt	RAR-related orphan receptor gamma
SCID	Severe combined immunodeficiency
SLO	Secondary lymphoid organs
SPF	Specific pathogen free
T-bet	T-box transcription factor TBX21
TCM	Central memory T cells
TCR	T cell receptor
TEM	Effector memory T cell
TEMRA	CD45RA+ effector memory T cell
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
Vδ	Variable delta chain
Vy	Variable gamma chain
WT	Wild type

Chapter 1 – Introduction

1.1 The innate immune system

1.1.1 Mechanisms of action in the innate immune system

Barrier surfaces in the body act as a first defence to prevent entry of pathogens and the establishment of infection. If the barrier is breached a wide plethora of immune cells respond to the incoming threat. Cells of the innate immune system recognise incoming pathogens in a broad manner through the recognition of self vs non-self molecules. Many of these non-self molecules are conserved across multiple pathogens and have pathogen associated molecular patterns (PAMPs) on their surface. These patterns are recognised by pattern recognition receptors (PRRs) on the immune cells (Male, 2013).

Recognition of PAMPs via PRRs triggers a phagocytic and inflammatory innate immune response (Coico & Sunshine, 2015). Phagocytic cells such as macrophages patrol the body and are prominent in sites that are frequently exposed to pathogens, for example, the gut and lungs (Male, 2013). Upon triggering of their PRRs, they attempt to phagocytose the pathogen by engulfing it and destroying it. Alongside their phagocytic response, an inflammatory response is triggered in macrophages and they undergo changes in gene expression to produce interleukins and cytokines. This then recruits and activates other phagocytic cells such as neutrophils and cells of the adaptive immune system such as B cells and T cells (Coico & Sunshine, 2015; Male, 2013).

Alongside phagocytic cells, other innate cells such as eosinophils and basophils respond through their release of cytotoxic granules into the cellular environment. The granules contain enzymes and reactive oxygen species that kill the targeted cells (Coico & Sunshine, 2015; Rich et al., 2008).

NK cells are also classed as part of the innate immune response. They have cytotoxic effector functions that can be directed to the targeted killing both of foreign or damaged host cells (Trinchieri, 1989). The activation of NK cells is dependent on the triggering of their activatory receptors which recognise stressed self-ligands and antigen from pathogens (Lanier, 2005; Sivori et al., 2004; Vivier et al., 2004, 2008). NK cells can also detect target cells coated by antibodies through the Fc receptor CD16 (Vivier et al., 2008).

1.1.2 Hallmarks of innate immunity

The key role of the innate immune response is to provide immediate protection against incoming pathogens. It protects the body in the early stages whilst the slower adaptive immune response is being generated. Alongside this immediacy in response is its inability to distinguish between various pathogens and its protection is inherently broad. It produces an invariant and consistent reaction when encountering both external pathogens and other non-self antigens. Due to its immediate and invariant nature, the innate immune response does not generally generate an antigen specific memory response and will respond to recurrent exposure to the same antigen in the same method (Janeway et al., 2001).

1.2 The adaptive immune system

1.2.1 Mechanisms of activation in the adaptive immune system

The adaptive immune response is generated by lymphocytes that include B cells and T cells. B cells are activated via their B cell antigen receptor which is a surface immunoglobulin. When the receptor senses antigen it triggers signalling into the cell and activates it. Once activated B cells proliferate and become antibody-producing plasma cells (Male, 2013).

Immature dendritic cells (DCs) are phagocytic cells that are resident in most tissues and phagocytose incoming pathogens. Upon ingestion of pathogens, DCs become activated, mature and present peptides derived from the pathogen on their surface through a system called antigen presentation (Alberts et al., 2002). DCs are “professional” APCs, while other immune cells such as B cells and macrophages can act as non-professional APCs and present antigens to adaptive lymphocytes (Barker et al., 2002; Nakayama et al., 2011).

Antigen presenting cells (APCs) travel through the lymphatic system to local lymph nodes to present pathogen-derived peptides to naïve recirculating T lymphocytes. Upon recognition of the antigen/peptide by antigen-specific T cells, APCs produce cytokines that drive the proliferation, development and expansion of the antigen-specific population (Alberts et al., 2002). The pathway of T cell activation and expansion will be discussed in greater detail in section 1.4.2 below.

1.2.2 Hallmarks of adaptive immune responses

The adaptive immune response is slower to develop than the innate response when exposed to a new antigen. Whilst it is a more delayed response, it is also highly antigen-specific and provides pathogen targeted clearance and killing.

The adaptive response also produces antigen specific memory cells that are long lasting and respond rapidly upon re-exposure to the original antigen. Memory cells can respond within hours compared to days in a novel adaptive immune response, allowing continuous protection against repeated infections (Janeway et al., 2001).

1.3 T cells development and trafficking

1.3.1 T cell subsets and development

T cells are formed from bone marrow precursors that migrate to the thymus to undergo T cell receptor (TCR) rearrangement and maturation before release into the peripheral circulation. Migration of the precursor cells to their target area of the thymus is tightly controlled by chemokine gradients (Liu et al., 2006; Nitta et al., 2009; Petrie & Zúñiga-Pflücker, 2007). Once within the thymus, T cell precursors interact with thymic epithelial cells to develop into different T cell subsets with specific functions. They also are tolerised against self-antigen that is presented by the thymic epithelial cells (Klein et al., 2014; Kurd & Robey, 2016).

T cells are broadly divided into naïve, effector, memory or regulatory T cells. Regulatory T cells are required for maintaining tolerance to self-antigens. Naïve T cells circulate mostly through the lymphatic system and blood in search of pathogens whereas effector cells travel to the site of infection and act to enhance clearance of the pathogen. Activated effector cells have a short life and most will die whilst a few remain as memory cells to have a secondary recall response if needed (Kumar et al., 2018).

In children, most T cells produced by the thymus are naïve or regulatory cells (Kumar et al., 2018). As humans age their thymic output decreases, hence naïve and regulatory T cell populations decrease. Memory T cells however increase over time following a rise in exposure to diverse pathogens (Thome et al., 2015; Douek et al., 1998; Junge et al., 2007). Thymic output was long thought to decline dramatically following puberty but recent studies suggest thymocytes are seen at regular levels until the age of 40, suggesting thymic output may decrease later in life than originally thought (Thome et al., 2016).

The changes in the proportion of T cell compartments may be due to different immune requirements throughout life. In early life, the body is continuously exposed to new pathogens which require naïve T cells to respond. Regulatory T cells are also important to establish tolerance to commonly found harmless stimuli. However in older age, the focus of the immune response is to maintain homeostasis and the recognition of previously encountered pathogens which accounts for the high proportion of memory T cells.

Memory T cells can be subdivided into various categories, 3 of which include: effector memory (TEM) with low CD62L and CCR7 expression, central memory (TCM) with high CCR7 and CD62L expression and in humans, a terminally differentiated TEM subset that also expresses CD45RA (TEMRA). The expression of CCR7 and CD62L on TCM cells allow them to circulate through lymphoid tissue and blood whilst TEM cells express chemokine receptors and integrins needed for access to peripheral, inflamed tissues (Martin & Badovinac, 2018a; Sallusto et al., 1999a).

TEM cells mainly populate mucosal tissues where they have a tissue resident phenotype and are maintained throughout life. Their levels in the tissue are relatively stable over a lifespan whilst increasing in the blood and lymphatic system throughout life. TCM cells however are stable throughout life in both mucosal tissue and the lymphatic system (Thome et al., 2014a).

1.3.2 T cell receptor rearrangement

The T cell receptor (TCR) on T cells can recognise a wide plethora of antigens. Its production is essential in a well organised T cell lineage developmental pathway with checkpoints at important stages. Thymic precursor cells travel from the bone marrow to the thymus and become double negative (DN; CD4- CD8-) precursor cells. They receive specification to the T cell lineage and begin the rearrangement of their TCR gamma (γ) gene segment followed by

their delta (δ) and beta (β) gene segments (Ciofani & Zúñiga-Pflücker, 2007; Shah & Zúñiga-Pflücker, 2014; Sherwood et al., 2011).

For αβ T cells, cells rearrange their TCR β chain and express it alongside an invariant pre-TCR α chain and CD3 in a pre-TCR complex. Signals from this complex are essential for continued development along the αβ T cell lineage. Cells then transition to a double positive stage (CD4+ CD8+) where a mature α chain replaces the invariant α chain (Starr et al., 2003). Cells undergo positive and negative selection before exiting to the periphery as single positive (CD4+ or CD8+) mature αβ T cells (Singer et al., 2008).

For the production of yδ T cells, the precursors diverge from the αβ T cell lineage at stage DN3 of development in the thymus. DN3 is where T cells express CD25 but are negative for both CD8 and CD4. If successful y and δ TCR chains have rearranged, expression of the transcriptional regulator ID3 is thought to help T cells onto a yδ T cell trajectory (Ciofani et al., 2006; Lauritsen et al., 2009). Signal strength is thought to also play a role in the αβ vs yδ T cell delineation by cells. Stronger signals through the TCR drives DN3 cells to the yδ T cell pathway whilst weaker signals preferentially encourage αβ T cell development (Haks et al., 2005; Hayes et al., 2005).

The yδ TCR is produced by VDJ recombination of the y and δ chains. The y chain rearranges first followed by the δ chain and the TCR β (Livák Ferenc et al., 1999; Sherwood et al., 2011). Most β chains are not fully rearranged in yδ T cells and the β chains that are expressed are not productive as they are out of frame (Joachims et al., 2006). Although there are fewer y and δ gene segments compared to the α and β chains, the yδ TCR has higher diversity than the αβ TCR. The diversity in yδ T cells is enriched by high levels of N-nucleotide additions and the presence of Diversity (D) gene segments in the δ locus which can be read in all reading frames (Legut et al., 2015). This provides the δ chain of the yδ TCR with many possible CDR3

sequences through different combinations of Variable, Diversity and Joining segments (Davis, 2004). A figure showing the structure of the y and δ chains of the TCR can be seen in Figure 1.1 below.

Diversity is also generated through a wide variety of CDR3 lengths. αβ TCR CDR3 have a limited length of 6-12 amino acids as they are selected to recognise peptide – MHC complexes. yδ TCRs are not MHC restricted however and hence have no length restrictions on their CDR3 in the delta chain (Rock et al., 1994). The CDR3 lengths of human Vδ1+ T cells in particular showed great diversity and ranged from 9-28+ amino acids in recent studies (Davey et al., 2017b; Hunter et al., 2018).

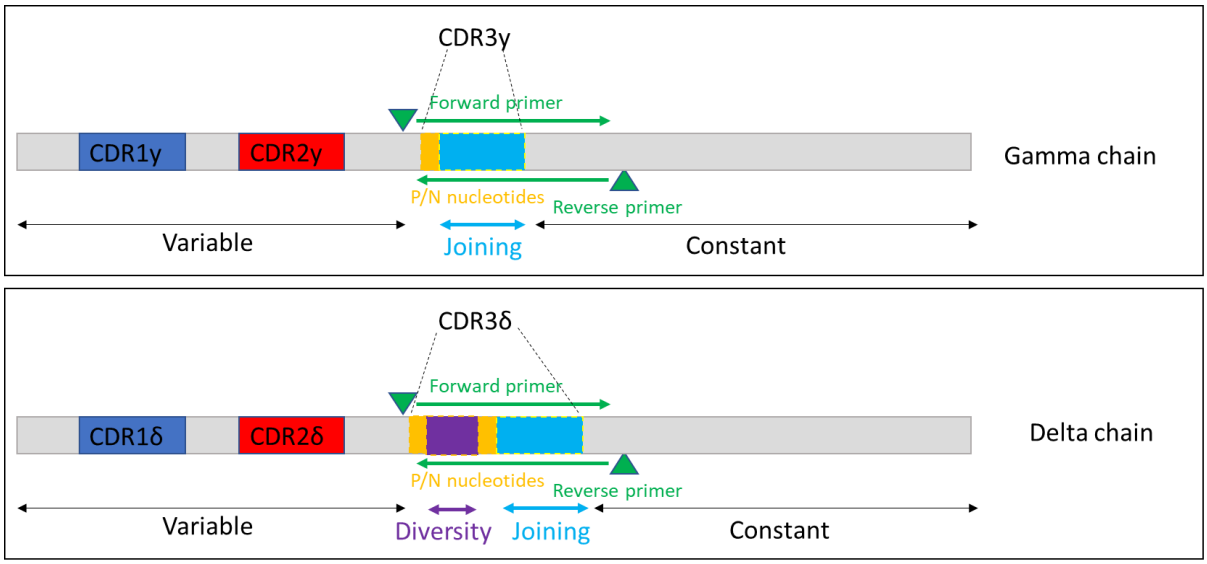


Figure 1.1: A diagram of the γ and δ chains of the γδ T cell receptor. Both gamma and delta chains are formed through VDJ recombination. The gamma chain is comprised of variable, joining and constant regions, with nucleotides added between the variable and joining regions. In the delta chain, variable, diversity, joining and constant regions combine, with nucleotides before and after the diversity region. The addition of P or N nucleotides between the regions increases the diversity in the TCR repertoire. This leads to CDR3 being hypervariable, compared to the germline CDR1 and CDR2. In order to study TCR repertoire in this thesis, the CDR3 region and the sequence directly before and after it were analysed, as seen with the primer locations shown in the diagram.

1.3.3 T cell trafficking in the periphery

Chemokine gradients are essential for the trafficking of all T cells (Stein & Nombela-Arrieta, 2005). T cells egress from the thymus and circulate around secondary lymphoid organs to detect invading pathogens. The high expression of CD62L and CCR7 on naïve T cells and TCM cells allow them to access lymph nodes (Weninger et al., 2001). To enter lymph nodes T cells tether and roll on the endothelium via binding to the CD62L receptor (Gallatin et al., 1983). CCR7 on the T cell surface binds CCL21 on the endothelium which activates LFA-1 on the T cell. LFA-1 binds then to ICAM1 on the endothelium causing the cell to stop and transmigrate into the lymph node (Bao et al., 2010; Masopust & Schenkel, 2013).

Following the encounter of their antigens in the lymph nodes T cells undergo expansion, proliferation and a change to an activated effector phenotype. They then migrate to non-lymphoid sites of injury or inflammation (Lewis et al., 2008; Masopust & Schenkel, 2013). A downregulation of CCR7 on activated T cells allows their egress from secondary lymphoid organs (Pham et al., 2008). Effector T Cells upregulate their expression of tissue homing receptors that allow them entry to the target tissue. These receptors appear to be tissue specific, for example CCR9 and α4β7 that interact with CCL25 and MAdCAM-1 on the gut endothelium (Berlin et al., 1993; Papadakis et al., 2000).

Following clearance of the pathogen, most T cells die with only a small percentage of the cells remaining as memory cells. Some memory cells retain their circulatory ability and can access lymphoid tissue in search of previously encountered antigen (Sallusto et al., 1999). Other memory cells become resident in the previously challenged organs and become long-lived, non-circulating tissue resident memory T cells. This is observed in the mucosal and peripheral tissue of both humans and mice (Thome et al., 2014b; Thome & Farber, 2015).

1.4 T cells in infection

1.4.1 Timeline of T cell responses in infection

In bacterial and viral infection models, activated murine naive T cells expanded by day 6-10 of infection, before contracting and returning to basal levels (Badovinac et al., 2007; Porter & Harty, 2006). In humans it is difficult to study the changes in T cell populations during infection. However studies have been performed in humans directly after vaccination against viral target pathogens (smallpox, yellow fever) and CD8 specific T cells peaked in the blood around 14-21 days after exposure to the pathogen and returned to base line by day 30 post exposure (Akondy et al., 2015; Miller et al., 2008). Memory cells to previously vaccinated pathogens have been shown to persist for decades (Hammarlund et al., 2003).

1.4.2 TCR triggering and expansion

V(D)J recombination produces naïve T cells with diverse TCR sequences that circulate around the body to detect antigen. There are a large number of unique antigen specificities within the naïve T cell population. One study estimated that naïve murine αβ T cells had roughly 2×10^6 clonotypes (Casrouge et al., 2000). Once a TCR clone recognises its cognate antigen it can begin its activation.

In αβ T cells, TCR-peptide (pMHC) interactions are needed alongside co-stimulation and cytokines to become activated (Williams & Bevan, 2007). TCR signalling drives IL-2 secretion that has an autocrine and paracrine effect that causes the cells to proliferate (Pennock et al., 2013). Two well characterised co-stimulatory molecules on αβ T cells are CD28 and CD137 (4-1BB) that bind to their ligands on antigen presenting cells (Dharmadhikari et al., 2016;

Sharpe & Freeman, 2002). A deficiency in either of these co-stimulatory molecules impairs activation but does not curtail it completely. However if both are disrupted then cells show dramatic reduction in their activation. This suggests some redundancy between the co-stimulatory pathways (Shahinian et al., 1993; Tan et al., 1999; Tan et al., 2000; Williams & Bevan, 2007).

As in αβ T cells, the γδ TCR is thought to be involved in the activation of some γδ T cell subsets, alongside concurrent interleukin activation such as IL-15 and IL-12 (García et al., 1997; García et al., 1998; Marlin et al., 2017). NK receptor binding to ligand alongside TCR binding was also shown to increase γδ T cell responsiveness with enhanced cytotoxic function and the release of cytolytic granules upon activation (Rincon-Orozco et al., 2005).

However, in contrast to αβ T cells, TCR binding is not always required for the activation of γδ T cells. The interaction of NKG2D with its ligand induces rapid proliferation in skin murine γδ T cells (Strid et al., 2008). Engagement of NKG2C on the surface of human γδ T cells was also found to increase their cytotoxicity in HIV patients with increased lysis of infected CD4 T cells (Fausther-Bovendo et al., 2008). Engagement of NKp30 also had a similar anti-viral effect as it caused the release of chemokines that suppressed the replication of HIV-infected CD4 T cells (Hudspeth et al., 2012) .

Interestingly, one essential difference between αβ and γδ T cells is that the MHC is not required for activation and antigen recognition. γδ T cells are detected in both MHC I and MHC II knockout mice (Grusby et al., 1993). Nevertheless other studies have shown that γδ T cell clones from humans and mice can recognise MHC-like molecules (Benveniste et al., 2018; Matis et al., 1989). A γδ T cell clone from a Cytomegalovirus infected donor bound Endothelial protein C receptor (EPCR) which has a structure resembling MHC and is upregulated in cells upon viral infection and stress (Willcox et al., 2012).

Following recognition of antigen by a T cell clone, the cell undergoes preferential expansion. This clonal expansion produces further cells that can target the pathogen but can also drive differentiation of the cells (Adams et al., 2020). Long term exposure to the antigen is not necessarily needed, with even short acute exposure times driving proliferation and differentiation (Kaech & Ahmed, 2001). However the length of stimulation correlates with greater expansion and magnitude of the immune response (Prlic et al., 2006).

The size of the proliferation can also be affected by the frequency of the naïve T cell precursor in the circulation, the initial pathogen load and inflammation in the environment the T cell resides in (Akondy et al., 2015; Haring et al., 2006; Obst, 2015). In αβ T cells the strength of the peptide binding to the TCR appears to be directly correlated with the size of the response (Chen et al., 2010; Corse et al., 2011).

1.4.3 T cell transition and phenotypic changes

Following activation T cells increase in size, alter their transcriptional profile and their metabolism (Best et al., 2013; Grumont et al., 2004; Maciver et al., 2013). In αβ T cells, master transcription factors such as T-bet, Foxp3 and RoRyt drive the transition of the cells from naïve to effector or regulatory phenotypes. T-bet drives the differentiation towards a cytotoxic effector phenotype, aided by Eomesodermin (Pearce et al., 2003; Szabo et al., 2002), Foxp3 drives T cells into a regulatory profile (Hori et al., 2003) and RoRyt an IL-17 profile (Ivanov et al., 2006). This is also observed in murine yδ T cells where T-bet is a master transcription factor for IFNy producing cells and RoRyt is needed for IL-17 producing cell development (Barros-Martins et al., 2016). In human yδ T cells, T-bet+ T cells also produce high levels of IFNy (McMurray et

al, in preparation), however the presence of an IL-17 producing human subset in the steady state has limited evidence currently (McKenzie et al., 2018).

It has been shown in αβ T cells that metabolic pathways are altered upon activation and this drives phenotypic changes, with naïve T cells quiescent until activated. Quiescent cells have a catabolic metabolism where they process and break down nutrients such as amino acids and glucose to generate energy for their survival. However following activation cells become anabolic metabolisers and nutrients are used to build complex macromolecules required for proliferation (Fox et al., 2005). For yδ T cells, the effects of activation on metabolism have not been widely researched at present.

Alongside internal changes due to proliferation, T cells also have changes in their surface phenotype and their production of cytokines. For both human cytotoxic αβ and yδ T cells, differentiation into an effector phenotype drives the production of proinflammatory cytokine such as IFNy and TNFα. They also release vesicles containing perforin and granzymes to kill neighbouring cells (Davey et al., 2017b; Pennock et al., 2013).

Alongside an increase in expression of cytotoxic markers, yδ effector T cells also show increased expression of tissue specific homing markers such as CXCR3/6 for the liver and α4β7 and CCR9 for the gut. They also decrease their expression of lymph node homing markers such as CD62L and CCR7 (Ajuebor et al., 2008; Hunter et al., 2018; Mann et al., 2012; Uehara et al., 2002).This allows them to home to inflamed peripheral tissue and begin to target the pathogen.

When transitioning from an effector to a memory state, T cells become quiescent once again. They regain their ability to proliferate in response to interleukin such as IL-7 and IL-15 (Becker et al., 2002; Schluns et al., 2000). They also have antigen independent self-renewal that allows continuous low level homeostatic turnover (Murali-Krishna et al., 1999). Importantly they also

retain the ability to respond rapidly to infection and mount effector responses. Memory cells can respond within hours to a recurrent pathogen, unlike naïve cells which traditionally require days to transition to an effector phenotype (Barber et al., 2003; Bevington et al., 2016).

1.5 Innate-like γδ T cells in mice

1.5.1 The TCR repertoire of innate-like γδ T cells

γδ T cells are the first T cells to develop in the murine thymus. Many γδ T cell subsets in mice are produced in developmental waves perinatally. They leave the thymus and seed peripheral tissues where they undergo homeostatic proliferation in situ. This allows the maintenance of a sizable population throughout the life of the mouse. The vast majority of murine γδ T cells are in peripheral tissues whilst only accounting for around 2% of T cells in the lymphoid organs (Asarnow et al., 1989; Goodman & Lefrancois, 1989; Havran & Allison, 1988, 1990; Ito et al., 1989; Itohara et al., 1990). A compiled summary of the different murine γδ T cell populations that will be discussed in this chapter is listed in Table 1.1 below.

Table 1.1: A summation of murine yδ T cell populations. A summary of the different populations identified in mice and what is known about their biology (development, TCR repertoire, location, activation and response to stimulation).

Subset (mouse)	Development	TCR repertoire	Location	Activation	Response to stimulation
Vy5 Vδ1 (DETC)	In the thymus, during embryogenesis. They require binding to the thymic epithelial cell receptor, Skint-1, for their maturation (Boyden et al, 2008).	Canonical TCR identical across all cells (Asarnow et al, 1988).	Epidermis of the skin (Havran and Allison, 1990).	Via TCR by stress antigens (Giradi et al, 2001, Jameson et al, 2004). Via NKG2D in murine skin carcinogenesis models (Strid et al, 2008).	IFNy, TNFα production (Witherden et al, 2010). Insulin like growth factor– 1 production after injury to uphold barrier integrity (Sharp et al, 2005). Lymphotactin and RANTES to increase immune cell infiltration in inflammation (Boismenu et al, 1996).
Vy6 Vδ1	In the thymus, during embryogenesis (Itohara et al, 1990).	Invariant Vδ1 chain identical to DETC subset. Vy6 mostly invariant, CDR3 in the Vy6 chain is identical to DETC Vy5 but V region differs (Itohara et al, 1990).	Uterus, lung, tongue, MLN (Itohara et al, 1990).	Via TCR (Sheridan et al, 2013). Non-TCR activation: Unknown	IL-17 production (Sheridan et al, 2013, Khairallah et al, 2021).
Vy4 Vδ5	In the thymus, during embryogenesis. Can also be made after birth but in very small numbers (Kashani et al, 2015).	Semi-invariant Vy4 chain. A germline Vδ5 chain, without any junctional diversity (Kashani et al, 2015).	Skin, Lung, liver, LNs, spleen (Kashani et al, 2015).	TCR activation: Unknown IL-1β, IL-23 can drive proliferation through Toll like receptors (Ribot et al, 2010).	IL-17 production (Jensen et al, 2008, Ribot et al, 2009).

Vγ1 Vδ6.3	Produced in the thymus. Detected 2 weeks after birth. Binding of the TCR to a ligand in the thymus programmes their effector phenotype (Kreslavsky et al, 2009).	Little junctional diversity and reduced variable region usage (Wei et al, 2015).	Liver, spleen (Azuara et al, 1997, Gerber et al, 1999, Kreslavsky et al, 2009).	Via TCR (Azuara et al, 1997) Non-TCR activation: Unknown	IL-4, IFNγ, GM-CSF in response to infection (Azuara et al, 1997, Mamedov et al, 2018).
Vγ1 (multiple δ chains)	Produced after birth throughout life (Carding & Egan, 2002; Jensen & Chien, 2009)	Diverse, junctional diversity, different gamma and delta chain pairings (Carding & Egan, 2002; Jensen & Chien, 2009)	Spleen, LNs, MLN, blood (Carding & Egan, 2002; Jensen & Chien, 2009)	Unknown	Unknown
Vγ4 (multiple δ chains)	Produced after birth throughout life (Carding & Egan, 2002; Jensen & Chien, 2009)	Diverse, junctional diversity, different gamma and delta chain pairings (Carding & Egan, 2002; Jensen & Chien, 2009)	Spleen, LNs, blood (Carding & Egan, 2002; Jensen & Chien, 2009)	Unknown	Unknown
Vγ7 (multiple δ chains)	Can develop within thymus or extrathymically. Expands and becomes dominant in the gut by 2-3 weeks post birth. They require the expression of a butyrophilin-like molecule, Btnl1, on gut enterocytes for their development (Di Marco Barros et al, 2016).	High junctional diversity, varied gamma and delta chain pairings (Goodman & Lefrancois, 1989, Takagaki et al, 1989).	Small intestine (Takagaki et al, 1989).	Unknown	Antimicrobial factors against bacteria (Ismail et al, 2011). Keratinocyte growth factors at site of injury (Chen et al, 2002)

The waves of yδ T cell development in the neonatal murine thymus are dependent on the Gamma chain usage of their TCR (Carding & Egan, 2002; Heilig & Tonegawa, 1986). Each Gamma chain is linked to a developmental timeframe and a specific target tissue. The Vy5 chain is expressed in the first wave and seeds the epidermis (Havran & Allison, 1990) whilst Vy6 T cells are in the second wave and some seed the uterus and lungs (Itohara et al., 1990). Vy7 T cells seed the intestinal epithelium and reside as progenitors until 3 weeks of age where they become a mature compartment (Di Marco Barros et al., 2016; Goodman & Lefrancois, 1989; Takagaki et al., 1989).

Many of these subsets that seed tissues early in life have restricted TCR repertoires. This could suggest an innate-like biology with recognition of a limited range of antigens that may be upregulated by multiple infectious stimuli or stress. The Vy5 T cell skin seeding subset, termed DETCs, all express an identical, monomorphic TCR sequence. Their TCR has an invariant germline Vy5 chain and Vδ1 chain. The Vy6 T cell subset also uses the same germline Vδ1 sequence. Its TCR repertoire appears monomorphic with no junctional diversity and short CDR3 sequences (Asarnow et al., 1988; Carding & Egan, 2002; Havran et al., 1989; Hayes et al., 1996; Itohara et al., 1990; O'Brien et al., 2009).

Interestingly the Vy7 T cell subset in the gut has greater diversity. The Vy7 chain can pair with a range of δ chains, different joining segments can be used and non-germline encoded nontemplated (N)-nucleotides are added by the enzyme terminal deoxynucleotide transferase (TdT) which is not expressed in the embryonic thymus (Asarnow et al., 1989; Takagaki et al., 1989). There has also been reports of an NKT-like yδ T cell subset with a semi invariant Vy1 chain and restricted Vδ6 junctions in the liver and spleen (Azuara et al., 1997; Gerber et al., 1999; Kreslavsky et al., 2009). These cells often have little junctional diversity or N region diversity and reduced Variable gene combinations (Wei et al., 2015).

1.5.2 The role of innate-like γδ T cell subsets in anti-microbial defence and tissue repair

Many of these tissue resident subsets respond in an innate-like fashion to stimuli. They are primed effector cells and are believed to be present at barrier sites to form an innate-like immediate response to pathogens before a traditional adaptive immune response begins (Hayday, 2000).

Skin Vγ5 T cells respond in a rapid manner without the need for traditional clonal expansion or proliferation. Their triggering can be via their TCR or can be TCR independent and through NKG2D on their surface (Girardi et al., 2001; Jameson et al., 2004; Strid et al., 2008). They produce inflammatory cytokines such as IFNγ, IL-2 and TNFα in a cytotoxic response when stimulated (Witherden et al., 2010). They can also produce IL-17A in infection which is important in protecting against *Staphylococcus aureus* (Cho et al., 2010). They also produce chemokines such as lymphotactin and RANTES to increase αβ T cell and macrophage presence at the site of inflammation (Boismenu et al., 1996).

Vγ6 γδ T cells also have rapid effector responses upon activation. In infection they can produce IFNγ or IL-17 which is required for pathogen clearance in *E. coli* infection. Mice deficient in the Vγ6 γδ T cell subset had much higher susceptibility to viral infection also (Nishimura et al., 2004; Kensuke Shibata et al., 2007). The NKT-like Vγ1Vδ6 T cell subset also shows rapid responses to pathogens and produces IL-4 and IFNγ upon activation (Azuara et al., 1997). In the gut, Vγ7 T cells block the ability of bacteria to enter the bloodstream by enhancing tight junction complexes (Dalton et al., 2006). They also produce antimicrobial factors when bacteria penetrate the epithelium and can activate local γδ T cell subsets through microbial derived antigens or stress molecules (Ismail et al., 2011).

Alongside their anti-microbial effector function, many of these subsets also play a role in tissue homeostasis. In cases of skin injury, Vy5 T cells produce insulin like growth factor-1 to drive survival of surrounding keratinocytes and to uphold barrier integrity (Sharp et al., 2005). They can also produce IL-17A which promotes the production of host defence molecules by keratinocytes that aid healing (Macleod et al., 2013). In the gut, yδ T cells regulate epithelial cell turnover and the differentiation of intestinal epithelial cells. They also limit intestinal damage through the release of keratinocyte growth factor at the site of injury (Boismenu & Havran, 1994; Chen et al., 2002; Komano et al., 1995; Roberts et al., 1996).

1.5.3 Pre-programmed functional imprinting in innate-like yδ T cell subsets

A hallmark of innate-like yδ T cells in mice is their rapid effector responses to both viral and bacterial pathogens. Many of these yδ T cell subsets leave the thymus primed and with their effector function pre-programmed. In the NKT-like Vy1Vδ6 T cell subset, expression of the transcription factor PLZF in the thymus programs them to rapidly produce cytokines such as IFNy and retain an effector phenotype. It appears that the binding of this particular Vy1Vδ6 TCR by ligand in the thymus is important for switching PLZF on in these cells. So the imprinting of their function seems dependent on their expression of this particular TCR (Kreslavsky et al., 2009).

Skin Vy5 yδ T cells also require a thymic stromal determinant named Skint-1 that is key for their maturation, positive selection and to imprint their function. It is thought to directly bind to the TCR however this has not been directly demonstrated. In mice lacking Skint-1, a yδ T cell population was present in the skin, however it did not express the canonical Vy5Vδ1 DETC TCR (Boyden et al., 2008; Lewis et al., 2006; Turchinovich & Hayday, 2011).

The expression of the marker CD27 has also be linked to the functional imprinting of yδ T cells in the thymus. yδ T cells from mice lacking CD27 were deficient at IFNy production but produced high levels of IL-17. It is suggested that both the binding of the TCR to ligand, and the expression of CD27 in the thymus may drive differentiation into an IFNy producing subset. CD27 induces the expression of the lymphotoxin-β receptor and upregulates genes linked to IFNy production. This functional divide appears to persist in the periphery with CD27+ yδ T cells producing IFNy in infection whilst CD27- yδ T cells produce IL-17. Their functional responses are stable throughout life and do not show the plasticity present in αβ T cells (Jensen et al., 2008b; Ribot et al., 2009a).

Interestingly unlike αβ T cells, not all yδ T cells require the thymus to fully mature. Intestinal yδ T cells in the IEL compartment can develop extrathymically in athymic mice however at lower numbers than WT mice (Bandeira et al., 1991; Antonio Bandeira et al., 1990a). These immature gut bound yδ T cells express high amounts of CCR9 and α4β7 when leaving the thymus (Guy-Grand et al., 2013; Wurbel et al., 2001). A butyrophilin-like molecule, Btnl1 was found to be expressed on murine enterocytes in the gut and to shape the Vy7+ yδ T cells by selectively driving their maturation and proliferation in a TCR-dependent mechanism (Di Marco Barros et al., 2016). Btnl1 forms a co-complex with another butyrophilin-like molecule, Btnl6, with Btnl6 directly binding the Vy7 TCR (Vantourout et al., 2018; Willcox et al., 2019).

The maturation effects driven by Skint-1 for Vy5 yδ T cells and Btnl1/6 with Vy7 yδ T cells suggests the presence of selecting ligands for different yδ T cell subsets. Both Skint-1 and Btnl1/6 can drive the maturation of yδ T cell populations independently of environmental stimuli such as microbial antigens which indicates they are selecting elements just as MHC class I for CD8 T cells (Di Marco Barros et al., 2016; Hayday, 2019). It has been suggested that different subsets in mice home to different tissues as there may be host derived ligands that are specific

for those tissues and select for those populations in that tissue (Janeway et al., 1988). Skint 1 and Btnl1/6 are good candidates for these host derived ligands.

1.5.4 Lymphoid stress surveillance

Alongside their responses to bacterial and viral stimuli, innate-like γδ T cell subsets can also respond rapidly to stress molecules expressed on cancerous or inflamed cells (Constant et al., 1994; Girardi et al., 2001; Strid et al., 2008). Skin Vγ5 γδ T cells have dendrites that are constantly scanning keratinocytes and langerhans cells and become activated by sensing stress and inflammation (Girardi et al., 2001; Jameson et al., 2002; Jameson & Havran, 2007). The Vγ6 γδ T cells can also respond to stress, dysregulated cells and bacteria-free inflammation (Mukasa et al., 1999). The ability of innate-like γδ T cells to respond rapidly to stress signals suggests they are may act as a form of primary, immediate response to changes in homeostasis. This paradigm is known as lymphoid stress surveillance.

Stress caused both by pathogens but also non-microbial stress increases the expression of ligands that bind TCRs, NK receptors and PRRs on γδ T cells. Following engagement of these receptors, γδ T cells respond in an immediate fashion without the requirement for clonal expansion or differentiation and so they persist in an ‘activated yet resting’ state in tissue (Hayday, 2009; Martin et al., 2009; Strid et al., 2008; Sutton et al., 2009).

An innate-like hallmark of these cells is their ability to become activated without TCR stimulation. It has been hypothesised that the ability of these cells to receive antigen stimulation via the TCR during development may cause the TCR to become hyporesponsive in mature cells in the periphery. This would preferentially establish the cells to respond more effectively to NK receptor or PRR ligands and to survey tissue for stress molecules (Wencker et al., 2013).

Whilst an immediate response by innate-like γδ T cells may not clear the pathogen or return homeostasis fully, it may limit the spread of the infection or malignant cells throughout the body (Hayday, 2009; Pennington et al., 2005). Vγ5 T cells become activated after NKG2D on their surface binds NKG2D ligands (NGG2DL) on target cells. They then activate nearby Langerhans cells that lack NKG2D expression and drive the recruitment of other immune cells to the inflamed tissue (Strid et al., 2008). This suggests that γδ T cells may sense changes to the epithelium, activate nearby antigen presenting cells and drive an influx of other lymphocytes. This contrasts with the traditional αβ T cell form of activation where the antigen presenting cell recognises the antigen and then presents it to the T cells (Hayday, 2009).

In summary, many γδ T cell subsets in mice show innate-like features, as detailed in the schematic below (Figure 1.2). They are produced early in life and seed barrier tissues. They often have semi-invariant TCR repertoires with germline encoded sequences. Once within the tissue they are resident with a “activated yet resting” primed phenotype. Their effector function is often pre-programmed within the thymus and does not show plasticity. They can sense both microbial and host derived stress molecules and respond rapidly to activation by lysing infected or maligned cells, producing key anti-microbial cytokines and driving the infiltration of other immune cells.

Alongside their protective function, they also uphold barrier integrity and maintain homeostasis in tissues. Overall, their immediate responses, their lack of functional diversity, their semi-invariant TCR repertoire and their ability to become activated without TCR stimulation suggests an innate-like biology.

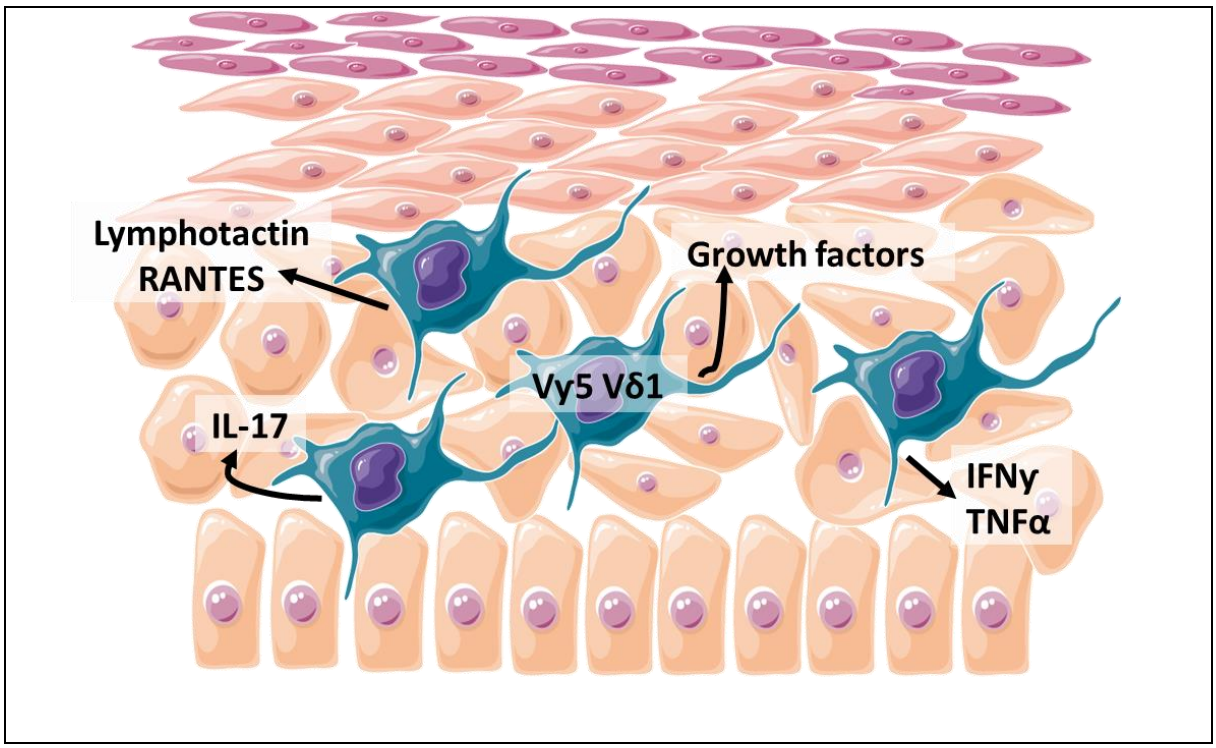


Figure 1.2: The biology of an epidermal innate-like γδ T cell subset in mice. Many murine tissue resident γδ T cell subsets have an innate-like biology with an activated phenotype. They patrol tissues for stress molecules, pathogens and inflammatory stimuli. This is exemplified in epidermal Vγ5Vδ1 T cells with dendrites that scan keratinocytes in the skin. They express an identical, monomorphic TCR with invariant Vγ5 and germline Vδ1 chains. They can be activated via their TCR or via NK receptors on their surface. Upon activation they respond immediately with the production of inflammatory cytokines such as IFN-γ and TNFα and in infection a subset can produce IL-17 that aids pathogen clearance. They also produce chemoattractant factors such as RANTES to aid the recruitment of other immune cells. In tissue injury they release growth factors to uphold barrier integrity and aid wound healing.

1.6 Adaptive-like biology in murine γδ T cells

Alongside innate-like γδ T cell subsets, other γδ T cells in mice egress from the thymus throughout life with a naïve phenotype and circulate around the lymphoid tissue (Zeng et al., 2012). These naïve γδ T cells tend to express the Vγ1 or Vγ4 chains with higher junctional diversity and more complex CDR3 sequences in their TCRs compared to the innate-like γδ T cell subsets (Carding & Egan, 2002; Jensen & Chien, 2009).

Multiple infection models have shown a memory response in murine γδ T cells with improved bacteria clearance and rapid re-expansion upon rechallenge. Dermal Vγ4 IL-17 producing T cells expanded in draining LNs following the application of imiquimod cream to the skin. The cells had an effector phenotype (CD44+ CD62L-) and migrated to the area of imiquimod-induced inflammation where they produced IL-17. Upon rechallenge these cells had a larger response but this seemed to cause enhanced inflammation (Hartwig et al., 2015; Ramírez-Valle et al., 2015).

However, it is disputed whether this response is adaptive, despite the improved secondary response. Dermal Vγ4 T cells are produced in a fixed development window in the neonatal thymus (O'Brien & Born, 2015), which is indicative of an innate-like biology. The inflammation in this experimental protocol was induced by imiquimod which drives innate signalling in the immune system. In mice it binds TLR7 on macrophages and DCs and generates inflammatory cytokines (Hemmi et al., 2002). It can also activate the inflammasome through the NLRP3 pathway, upregulating cytokines such as IL-1β and IL-18 (Kanneganti et al., 2006). The TLR-binding stimulus and the invariant repertoire of responding cells is indicative of an innate-like response.

During oral *Listeria monocytogenes* infection, a Vy6Vδ1 T cells population in the mesenteric lymph node expanded and produced both IFNy and IL-17 with an effector phenotype (CD44+ CD27-). Within a week post infection, this Vy6Vδ1 T cell population appears in the circulation and in the gut. It remained within both the mesenteric lymph node and the gut without any restimulation and appeared long lived. Upon rechallenge the cells expanded at a more rapid rate suggesting some memory response (Romagnoli et al., 2016; Sheridan et al, 2013).

Staphylococcus aureus infection in mice caused peritonitis and a rapid IL-17 response in Vy1 and Vy4 T cells in the draining lymph node. A long lived Vy6 T cell population also responded, expanded and became established in the peritoneum and lymph nodes. Upon rechallenge the Vy6 T cell population expanded more rapidly with an effector phenotype (CD44+ CD27-) and higher production of anti-microbial cytokines (Murphy et al., 2014). This suggests that there may be an immediate innate-like response in the Vy1 and Vy4 T cell populations and a memory recall response upon re-infection in the Vy6 T cell subset.

For both of these infections it is the Vy6 T cell subset that is suggested to have a memory response. However as mentioned previously the Vy6Vδ1 populations are thought to have a semi-invariant repertoire (Asarnow et al., 1988; Itohara et al., 1990) which undermines the concept of an adaptive response. Recently however, it was observed that within the Vy6 responders to *L. monocytogenes* there were cells with semi-invariant, public TCR sequences, but there was also a small population of responders that had a pathogen-specific, clonal TCR repertoire (Khairallah et al., 2021). Therefore there may be multiple subpopulations of Vy6 that respond differently and remain to be fully characterised.

Following infection with *Bordetella pertussis* Vy4 yδ T cells in the lungs rapidly produced IL-17 in an innate-like response. However, two weeks following infection a CD44+ Vy4 population trafficked into the lungs and became resident within the tissue. They proliferated locally

following a secondary challenge and showed improved bacterial clearance. This again suggests that there may be multiple subsets during infection that show different immune responses with the innate-like immediate Vγ4 IL-17 production in early infection and then a memory like Vγ4 population that enter the lungs later in infection (Misiak et al., 2017).

In *Plasmodium chadaudi* infection Vγ1Vδ6 T cells were activated and expanded after the resolution of acute parasitemia which had been dominated by the αβ T cell response. γδ T cells were shown to be essential to stop recrudescence of parasitemia suggesting a long lived memory response. The authors argued that the Vγ1Vδ6 T cells clonally expanded in response to the infection and had an adaptive biology (Mamedov et al., 2018). However the response was in fact polyclonal but shared the V region (TRAV15N-1) in the delta chain. Also the Vγ1 Vδ6 chain pairing was previously linked to an NKT-Like population which has little junctional diversity and reduced variable gene combinations (Wei et al., 2015), suggesting an innate-like biology.

The question remains if many of these responses to bacteria and parasites seen in mice show an adaptive memory response. It is often an innate-like invariant subset that is responding to the infecting pathogen. In the *P. Chaubadi* study, the Vγ1Vδ6 cells had a oligoclonal expansion with a common sequence in the delta chain which is very frequently present and rearranged during development. The lack of a single pathogen specific clonal response is more suggestive of an innate-like expansion of multiple commonly occurring clones (Hayday, 2019; Mamedov et al., 2018). These responses may be quasi-adaptive due to their lack of TCR diversity but they retain hallmarks of an adaptive response such as improved recall responses and the ability to provide protection when given to a new host.

γδ T cells were also thought to have an adaptive-like response to viral pathogens such as murine cytomegalovirus (MCMV). They were essential for the control of MCMV viral load in

the liver, lungs and spleen and for the reduction of organ damage. Vy1 and Vy4 T cells expanded with CDR3 clonotypes that were not observed at high numbers in healthy mice. There was an increase in yδ T cells that had an effector like phenotype (CD44+ CD62L- NKG2D+ CD27-) that persisted for at least a month post infection suggesting a long lived change in phenotype. The expansion of long lived effector yδ T cells with shared CDR3 clonotypes was suggestive of a potential long lived effector memory response. The adoptive transfer of these yδ T cells from a previously CMV-exposed donor mouse into a CD3 knockout mouse gave protection against CMV suggesting a recall ability (Khairallah et al., 2015; Sell et al., 2015).

A potential adaptive-like response was also described against the common B cell antigen, the algae protein-phycoerythrin. Antigen specific binding and recognition by naïve (CD44- CD62L+) yδ T cell clones lead to the activation and maturation of the cells into a effector phenotype (CD44+ CD62L-) that produced high levels of IL-17 upon stimulation (Zeng et al., 2012). The requirement for antigen specific binding and the accompanying transition from a naïve to an effector phenotype has hallmarks of an adaptive biology. However there was little clonal expansion accompanying the activation of the cells which is not indicative of a traditional adaptive response.

In summary, hallmarks of an adaptive response are a naïve T cell population with an initial diverse TCR repertoire, a transition from a naïve to an effector phenotype following activation and long lived recall responses (Willcox et al., 2020). Many of the murine infection models previously discussed lack some of these features. It is innate-like yδ T cell subsets responding with an invariant TCR repertoire and no indication of an antigen specific response. However many of these subsets show memory-like recall upon challenge. It may be that their ability to show adaptive-like characteristics is context dependent and is reliant on certain triggers or inflammatory environments. Identifying cells as fully adaptive or innate can be difficult also due

to the transient phenotype of many cells and the differing perceptions of what adaptive and innate biology can include.

It remains to be fully explored whether fully adaptive murine γδ T cell responses are possible within infection. CMV is a promising model due to a potential clonal expansion and phenotypic change in activated γδ T cells following infection. The long lived and stable nature of the phenotypic change may also indicate an adaptive-like biology. Future work will be required to determine whether murine γδ T cells can fall within innate-like and adaptive-like subsets. This duality in γδ T cell biology would mimic what is currently suggested in humans as discussed in the following sections.

1.7 Development and tissue localisation of human γδ T cells

Human γδ T cells develop very early in life with γδ TCR genes rearranging in the thymus at week 8 of pregnancy (McVay & Carding, 1999). By adulthood, they comprise on average 4% of total T cells in the blood with similar levels in lymphoid tissue (Chien et al., 2014; Groh et al., 1989; Parker et al., 1990). However, their numbers are much higher in peripheral tissues such as the colon where they comprise on average 39% of the intraepithelial lymphocyte compartment (Mayassi & Jabri, 2018; Ullrich et al., 1990). A summary of human γδ T cell populations that will be discussed in this chapter are listed in Table 1.2 below.

Table 1.2: An outline of human yδ T cell populations. A summary of the different populations discovered in humans and what is known about their biology (development, TCR repertoire, location, activation and response to stimulation).

Subset (human)	Development	TCR repertoire	Location	Activation	Response to infection
Vy9 Vδ2	First wave: produced by the thymus before birth. Second wave: produced after birth by the thymus (Davey et al., 2018; Papadopoulou et al., 2020)	First wave: canonical, invariant. Second wave: Higher diversity (n-nucleotides added) in the delta chain compared to the first wave (Davey et al., 2018; Papadopoulou et al., 2020)	Blood (Parker et al, 1990).	Activation can be through both TCR or TCR independent mechanisms eg: NK receptors or IL-12/18 (Bukowski et al, 1995, Rincon-Orozco et al, 2005). Recognition of phosphoantigens is dependent on the binding of Vy9 chain to a butyrophilin molecule (Karunakaran et al, 2020, Rigau et al, 2020).	Proliferation and persistent expansion (Poccia et al, 2006). Can kill infected cells with granulysin, perforin, granzymes, IFNy, TNFα (Dieli et al, 2001, Qin et al, 2012, Tikhonov et al, 2006, Rincon-Orozco et al, 2005). Can recruit other immune cells through CCL3, CCL4, CCL5 and MCP-1 release (Ditirro et al., 1998; Qin et al., 2011).
Vy9-Vδ2	Detected in neonatal cord blood and persists in adulthood but at a lower frequency (Davey et al, 2018).	Diverse, private TCR sequences in both chains (Davey et al, 2018).	Blood, liver (Davey et al, 2018).	Blood subset activated via TCR, induces a transition from a naïve to an effector phenotype. Blood subset unresponsive to TCR-independent stimulation *in vitro*. Unknown if TCR is required for activation in tissue (Davey et al, 2018).	Can expand in CMV and transition to an effector phenotype, expressing granzymes (Davey et al, 2018, Kaminski et al, 2020).

Vδ2- (mostly Vδ1)	Detected in neonatal cord blood and persists in adulthood (Davey et al, 2017).	Private, Diverse TCR sequences in both chains (Davey et al, 2017).	Blood (Dechanet et al, 1999).	TCR dependent. Can not be activated by IL-12/IL-18 alone (Davey et al, 2017).	Transition from a naïve to an effector phenotype. Produce granzymes and perforin (Davey et al, 2017). Persistent expansions in CMV (Dechanet et al, 1999) and Malaria (van Borstel et al, 2021).
Vδ2- (mostly Vδ1)	Unknown	Private, clonal repertoire CD45RA+ population: Shares clones with effector blood Vd1. Hypothesised that have migrated from the blood after activation CD45RA- population: Liver specific clones, hypothesised to be tissue resident (Hunter et al, 2018).	Liver (Hunter et al, 2018).	CD45RA+ population: Require TCR stimulation. CD45RA- population: Can be stimulated by IL-12/IL-18 or TCR. (Hunter et al, 2018).	CD45RA+ population: Produce granzymes and TNFa CD45RA- population: Produces IFNy and TNFa (Hunter et al, 2018).
Vδ2- (mostly Vy4+ Vδ1)	Hypothesis of thymic γδ T cells exposed to IL-2/IL-15 which programmes cells for gut homing (Deusch et al, 1991, Mikulak et al, 2019). Vy4 chain binds butyrophilin-like molecule, BTNL3. Required for survival (Willcox et al, 2019, Melandri et al, 2018, Mayassi et al, 2019).	Germline Vy4 chain with diverse Vδ1 chain (Melandri et al., 2018; Willcox et al., 2019)	Gut (Deusch et al, 1991)	Unknown	Release antimicrobial compounds (Konijnenburg et al, 2017). Some (NKp46+ cells) express Granzymes and IFNy following activation (Mikulak et al, 2019).

In humans, γδ T cells are delineated by Delta chain usage to allow ease of study. Vδ2 T cells that also express the Vγ9 chain are the first T cells to emerge in the foetus and dominate the γδ T cell repertoire in early pregnancy. They become the most prominent γδ T cell subset in adult blood. Their *de novo* production by the thymus decreases after birth but they can expand extrathymically in childhood (Davey et al., 2018; Dimova et al., 2015; Parker et al., 1990). They are also able to reconstitute following stem cell transplantation in adults (Ravens et al., 2017), suggesting that they can be produced if required by adult thymi and do not have a fixed developmental window.

It was previously believed that Vγ9Vδ2 T cells were fetal-derived with a TCR repertoire that was determined before birth (Dimova et al., 2015; Parker et al., 1990). Yet recent research has shown that their TCR repertoire in cord blood and adult blood is different. This suggests some novel production of cells after birth to allow for new TCR sequences to emerge (Davey et al., 2017c; Davey et al., 2018). It is now believed that fetal derived Vγ9Vδ2 T cells are invariant, with pre-programmed functions and expand for early life. However, adult Vγ9Vδ2 T cells are generated by the thymus after birth and are expanded in the periphery following antigen reactivity (Tieppo et al., 2020; Papadopoulou et al., 2020).

The Vδ2- subset of γδ T cells is dominated by cells using the Vδ1+ chain. These cells increase in number in the later stages of pregnancy and are the most prevalent γδ T cell subset produced by paediatric thymi (Ribot et al., 2014). They can also be reconstituted by the adult thymus following transplantation suggesting they also do not have a fixed developmental window (Ravens et al., 2017). They form the minority of γδ T cells in the blood but are the majority in many solid tissues throughout the body such as the liver, gut and skin (Holtmeier et al., 2001; Hunter et al., 2018; Mikulak et al., 2019).

1.8 Innate-like yδ T cells in humans

1.8.1 Innate biology in Vy9Vδ2 T cells

Vy9Vδ2 T cells dominate the yδ T cell population in the blood from childhood. The exposure of the cells to microbial antigens after birth drives their expansion and they remain in an expanded state (Morita et al., 2007; Papadopoulou et al., 2020; Parker et al., 1990; Ravens et al., 2020). They transition from a naïve CD27+ CD45RA+ phenotype in early life to a effector CD45RO+ subset in early life with increased cytotoxic markers (Davey et al., 2018; Parker et al., 1990).

Expanded Vy9Vδ2 T cells in the blood have a semi-invariant TCR repertoire. The majority of Vy9 chains have sequences with restricted CDR3s. These simple sequences are formed by recombination of Vy9 with the JyP gene with the addition of few N-nucleotides. Many of the Vy9 sequences are also public (generated in most individuals) and the same Vy9 sequence can be paired with multiple Vδ2 chains, suggesting they are rearranged independently. Some of these sequences are detected in fetal Vy9 TCRs and persist in adults (Davey et al., 2017b; Davey et al., 2018; Dimova et al., 2015). Vδ2 sequences however are more complex. There are clear differences in J region usage in adult and foetal repertoires and high N nucleotide additions in adult Vδ2 chains (Davey et al., 2018; Papadopoulou et al., 2020).

1.8.2 Activation of Vy9Vδ2 T cells

Vy9Vδ2 T cells are activated by non-peptidic pyrophosphate compounds (phosphoantigens: P-Ags) which drive rapid, innate like, cytotoxic responses within minutes (Lang et al., 1995). These compounds can be produced either by pathogens or by stressed host cells. (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) is a phosphoantigen that is known to

activate Vy9Vδ2 T cells and is produced by most bacteria and protozoa (Morita et al., 1995, 2007; Tanaka et al., 1995; W. Wang et al., 2010). Isoprenyl pyrophosphate (IPP) is a host derived phosphoantigen that can also stimulate Vy9Vδ2 T cells and is expressed at higher levels in cancerous or stressed host cells (Gober et al., 2003).

Both cell to cell contact and TCR stimulation are linked to P-Ag recognition without a requirement for traditional antigen presenting molecules such as MHC, CD1 or MICA/MICB molecules (Lang et al., 1995; Morita et al., 1995). The Vg9Vδ2 TCR alone confers P-Ag reactivity to other cell types as its transduction into Jurkat cell lines lacking an TCR, provided the cells with P-Ag sensitivity (Bukowski et al., 1995).

Alongside the TCR, butyrophilin molecules have been shown to be essential in the P-Ag sensing by Vy9Vδ2 T cells. Butyrophilins (BTNs) and Butyrophilin-like molecules (BTNLs) are molecules that are related structurally to the B7 family of costimulatory molecules that include CD80, CD86 and PD-L1. They have extracellular immunoglobulin (Ig) like domains, a transmembrane region and a large cytoplasmic domain containing a B30.2 domain (Rhodes et al., 2016).

The butyrophilin BTN2A1 directly binds to the germline encoded region of the Vy9 chain to allow for P-Ag recognition. Vy9-Vδ2+ T cells cannot respond to P-Ag, reinforcing the importance of the Vy9 chain (Davey et al., 2018; Karunakaran et al., 2020; Rigau et al., 2020). Alongside the BTN2A1-Vy9 interaction, the CDRs in the Vδ2 chain are also important as mutations within them led to loss of activation. So the Vy9Vδ2 TCR is thought to bind to both BTN2A1 in an innate-like fashion using the CDR2 and HV4 regions of the Vy9 chain but also bind an additional, so far unidentified ligand that is recognised via the CDRs in the Vδ2 chain and the CDR3 of the Vy9 chain (Harly et al., 2012; Karunakaran et al., 2020; Rigau et al., 2020).

BTN2A1 also synergises with another butyrophilin, BTN3A1 on the surface of target cells to allow P-Ag driven activation via the TCR (Harly et al., 2012; Karunakaran et al., 2020; Riãno et al., 2014). BTN3A1 binds P-Ag directly via its cytoplasmic B30.2 domain which induces a confirmational change (Nguyen et al., 2017; Salim et al., 2017; Sandstrom et al., 2014). BTN3A1 can also be upregulated on the surface of infected cells. In Malaria, infected red blood cells express BTN3A1 and BTN2A1. They interact with the Vy9Vδ2 TCR to form cell to cell contact that then activates the cytotoxic effector functions of Vy9Vδ2 T cells that kills the infected cell (Junqueira et al., 2021).

1.8.3 Phenotype and effector response of Vy9Vδ2 T cells

Vy9Vδ2 T cells expand following infections by a myriad of pathogens, most likely due to the increased levels of P-Ags. Pathogens can produce HMBPP through their metabolism and intracellular bacterial infections can drive IPP production by infected host cells (Kabelitz et al., 1990; Morita et al., 2007). Vy9Vδ2 T cells proliferate rapidly in the blood of bacterial infections such as Bacterial Meningitis, parasitic infections such as Malaria, Toxoplasmosis and Leishmaniasis and viral infections such as Epstein Barr virus (De Paoli et al., 1990; Perera et al., 1994; Raziuddin et al., 1994; Russo et al., 1993; Scalise et al., 1992). These expansions persist over time and do not rapidly decrease following antigen clearance unlike traditional αβ T cells (Fabrizio Poccia et al., 2006; Shen et al., 2002).

The presence of expanded Vy9Vδ2 T cells in the blood is thought to be protective. In patients with *Mycobacterium Tuberculosis*, a decrease of Vy9Vδ2 T cells in the blood corresponded with a worsening of the disease (Li et al., 1996). The adoptive transfer of Vy9Vδ2 T cells into severe combined immunodeficiency (SCID) mice allowed the mice to survive both gram-positive (*Staphylococcus aureus*) and gram negative (*Escherichia coli*) bacterial infections, again suggesting a protective function (Wang et al., 2001). In countries with endemic Malaria,

both high numbers of Vy9Vδ2 T cells and their cytotoxic activity correlated with protection from re-infection. They have been shown to kill *Plasmodium falciparum* infected cells *in vitro* (D'Ombrain et al., 2008; Giulia et al., 2011; Jagannathan et al., 2017).

Following activation, Vy9Vδ2 T cells have a rapid cytolytic response and can kill infected cells directly through a CD16 dependent degranulation pathway or through Fas-FasL binding. They also produce cytokines such as IFNy, TNFα, GM-CSF and molecules such as granulysin, perforin and granzymes to kill infected cells or pathogens directly (Dieli et al., 2001; Junqueira et al., 2021; Mamedov et al., 2018; Qin et al., 2012; Rincon-Orozco et al., 2005; Tikhonov et al., 2006). Their rapid, effector function correlates with their phenotype that is mostly central memory-like (CD27+ CD45RA-) with a smaller subset of an effector memory phenotype (CD27- CD45RA-) (Davey et al., 2018).

Alongside their anti-microbial functions, Vy9Vδ2 T cells can also drive arms of the adaptive immune response. They can act as antigen presenting cells through their expression of MHC and co-stimulatory molecules on their surface. They can take up viral particles and present them to αβ T cells to drive antigen specific responses through the proliferation and differentiation of naïve CD8 T cells. They also can increase cytokine production and target cell killing by memory CD8 T cells (Brandes et al., 2005, 2009; Meuter et al., 2010). They also recruit other immune cells through the production of chemokines MCP-1, CCL3, CCL4 and CCL5 that attract macrophages, NK cells and T cells to the site of inflammation (Ditirro et al., 1998; Qin et al., 2011).

Vy9Vδ2 T cells have also been shown to have an anti-tumour function. Tumour cells are recognised due to their increase production of IPP following their heightened metabolism and energy requirements (Gruenbacher & Thurnher, 2015). *In vitro*, Vy9Vδ2 T cells have potent cytotoxicity to cancerous cell lines (Liu et al., 2005) and have also driven improved outcomes

following injection into SCID mice with renal, pancreatic, nasopharyngeal cancer or melanoma (Kabelitz et al., 2004).

In human clinical trials, expansion of Vy9Vδ2 T cells *in vivo* by IL-2 and Zoledronate within B cell, prostate and late stage breast cancer patients has been shown to be safe and leads to an increase in rates of stable disease and some remission. In patients with this improvement there was a persistent and sustained peripheral Vy9Vδ2 T cell population with an effector memory phenotype (Dieli et al., 2007; Meraviglia et al., 2010; Wilhelm et al., 2003). However, a lack of remission is most patients was disappointing and work is in progress to potentially improve Vy9Vδ2 T cells as an anti-tumour treatment.

One potential avenue is the ex vivo expansion of the cells and adoptively transferring them into patients rather than expanding them *in vivo*. Some studies have expanded and activated the cells and then injected them into the site of the tumour which helped reduce satellite malignant nodules in some patients (Wada et al., 2014). Others have followed up the transfer of the expanded cells with the further IL-2 and Vy9Vδ2 T cell activating drugs such as zoledronate in advanced renal carcinoma and showed some remission (Kobayashi et al., 2011).

In summary, the Vy9Vδ2 subset exhibits many hallmarks of an innate biology. Its semi-invariant TCR repertoire with little clonotypic focusing in adults, immediate cytotoxic responses and the recognition of limited ligands points towards an innate-like, pre-programmed biology. This suggests it may more closely correspond to innate-like lymphocytes in the αβ T cell compartment such as invariant NKT cells, rather than the other adaptive-like yδ T cell subsets.

1.9 The biology of human gut γδ T cells

1.9.1 The phenotype of γδ T cells in the gut

γδ T cells are the most prominent T cell within the gut intraepithelial lymphocyte (IEL) compartment. It is hypothesised that thymic γδ T cells are exposed to IL-2 and IL-15 which upregulates the gut homing chemokine, CCR9 and hence leading to trafficking to the gut (Deusch et al., 1991; Mikulak et al., 2019). γδ T cells are important in microbial defence, maintaining gut barrier integrity and tissue homeostasis (Nielsen et al., 2017). When they are exposed to new pathogens they increase their scanning of epithelial cells to detect microbes, along with their antimicrobial gene expression and metabolism. The epithelial cells themselves are thought to be able to sense the pathogen and alert the γδ T cells (Konijnenburg et al., 2017). γδ T cells have also been shown to be a good prognostic marker for improved outcomes and reduction in metastasis in colorectal cancer as the gut resident cells have cytotoxic anti-tumour activity (Meraviglia et al., 2017; Mikulak et al., 2019).

Most of the γδ T cells in the healthy gut express the Vδ1 chain and also preferentially express the Vγ4 chain (Deusch et al., 1991; Landau et al., 1995; Mikulak et al., 2019). Phenotypically the Vδ1 T cells within the gut can be divided by their expression of NKp46. Most NKp46+ve Vδ1 T cells express the Vγ4 chain whilst NKp46-ve Vδ1 T cells express a variety of Vγ gene segments (Mayassi et al., 2019; Mikulak et al., 2019).

NKp46+ Vδ1 T cells are not found in the blood, skin, liver or lymph nodes of humans or in the gut of WT mice and hence appear to be a human gut-specific subset. They form the majority of Vδ1 T cells in the gut, express granzymes and express high levels of activatory NK receptors (NKRs) and low levels of inhibitory NKRs (Mayassi & Jabri, 2018; Mikulak et al., 2019).

The NKp46- Vδ1 T cell population are the minority of Vδ1 T cells in most donors and they have both low IFNy production and low expression of cytolytic molecules such as CD107a and granzyme B compared to the NKp46+ Vδ1 T cell subset (Mikulak et al., 2019). Both NKp46+ and NKp46- Vδ1 T cells have an effector memory phenotype (CD45RA- CCR7-) and express tissue residency markers such as CD69 and CD103 (Mayassi et al., 2019). The delineation in TCR gamma chain usage and cytotoxic phenotype between the two subsets suggests that they may have distinct Vδ1 T cells progenitors that do not overlap between subsets (Mikulak et al., 2019).

1.9.2 Binding of human γδ T cells to Butyrophilin-like molecules (BTNLs) in the gut

Recently it has been found that the gamma chain expressed by γδ T cells in the gut binds directly to a BTNL which is expressed on enterocytes. This interaction drives the activation and expansion of γδ T cells in both mice (Vγ7 chain bind Btnl1/Btnl6) and humans (Vγ4 chain binds BTNL3/BTNL8). In the murine gut this interaction is also essential for the extrathymic maturation of gut γδ T cells as genetic distribution of *btnl1* causes a large decrease in their number. It remains to be determined whether the interaction between BTNLs & human γδ T cells also has that role. BTNLs may also provide a tonic, sub-threshold signals for γδ T cell survival and may also alert them to changes in the environment (Di Marco Barros et al., 2016a; Melandri et al., 2018; Willcox et al., 2019).

In humans it is a germline encoded region in the Vγ4 chain that binds the BTNL3 molecule directly. This shares a conserved method of binding as seen in the BTN2A1 in the blood as both use only germline encoded regions of the Vγ chain to bind which may suggest a shared biology. Alongside this interaction via a germline region, there is also a section of the delta

chain of the TCR (CDRs1-3) that has a diverse repertoire which may bind to specific antigen (Melandri et al., 2018; Willcox et al., 2019).

The TCR in the human gut is thought to combine innate sensing of endogenous signals via the BTNL-gamma chain interaction and a clonal CDR binding potential for a more adaptive-like response. The CDR3 loops of the Vy4 and the Vδ chain are not required for BTNL3 binding but are needed for binding to the previously discovered antigen EPCR (Willcox et al., 2012).

The biology of a germline, innate-like Vy chain that interacts with BTNs or BTNLs alongside a more diverse Vδ chain is also present in the Vy9Vδ2 T cell subset in the blood. Hence this paradigm of yδ T cells potentially binding two different forms of stimuli/interactions via the same TCR can be detected throughout the body (Hayday, 2019). These two forms of binding are not required to be simultaneous. Vy4 cells have been activated in vitro via BTNL3 or the antigen presenting molecule CD1b independently of one another (Melandri et al., 2018; Reijneveld et al., 2020).

Chronic gut inflammation has been shown to alter BTNL expression and affect the yδ T cell compartment. In celiac disease, BTNL8 expression decreases on enterocytes at sites in the gut that had tissue destruction. This was followed by a loss of the steady state cytolytic Nkp46+ Vy4Vδ1 T cells. Concurrently, an atypical gluten sensitive NKp46- Vδ1 T cells expand to fill the gap. These Vδ1 T cells have varied gamma chains with non-germline sequences and do not recognise BTNLs. They also appear to have less degranulation potential than the Nkp46+ Vδ1 T cell population in healthy gut (Mayassi et al., 2019).

Interestingly, following the removal of gluten from the diet BTNL8 expression returned on enterocytes. However the steady state Nkp46+ Vy4Vδ1 T cells did not return (Mayassi et al., 2019). This is in contrast to αβ T cells which normally revert to steady state populations following the exclusion of gluten, alongside a decrease in gluten specific cells (Jabri & Sollid,

2009). It appears that BTNL8 may be needed for the retention of the steady state Nkp46+ Vy4 Vδ1 T cells. Also there may be a developmental window where these cells can seed the gut and then they cannot be replaced outside of that timeframe.

1.9.3 TCR clonotypic expansion in gut Vδ1 T cells

There is debate if TCR specific clones within the Vδ1 T cell population can expand in the gut following inflammation. In celiac patients, the Vδ1 T cell population was clonal when gluten was in their diet. However, when gluten was not eaten, the TCR diversity increased. Nkp46- Vδ1 T cell clones that expanded following gluten exposure were thought to have a shared CDR3 motif. Hence they suggested that gluten drove the expansion of antigen specific clones via the TCR. However when gluten was removed the clones slowly decreased in size or non-expanded Vδ1 T cells were recruited to the gut (Mayassi et al., 2019).

This finding could not be replicated however in a further study that found that the supposed gluten specific CDR3 motif was not common in celiac patients and that their TCR repertoires were in fact more diverse than healthy donors. They suggested that the discrepancy could be due to differences in sorting and sequencing methodologies but believe their findings are a more accurate reflection as they sequenced a larger number of cells (Eggesbø et al., 2019). Therefore the presence of gut specific clonal expansion in the Vδ1 T cell compartment has yet to be proven conclusively.

In summary, gut γδ T cells preferentially express a Vy4Vδ1 TCR in healthy tissue. These cells have an effector-like phenotype and protect the gut from infection whilst also maintaining barrier integrity. They bind BTNL3 via their Vy4 chain and this is hypothesised to provide homeostatic signals for the cell. In chronic inflammation however, BTNL expression is lost, the Vy4Vδ1 T cells subset disappears and a Vy4-Vδ1 T cells subset appears that is not responsive

to BTNLs. This subset was thought to have a clonally expanded repertoire with a shared CDR3 motif that was hypothesised to be antigen specific, however this finding has not been able to be replicated. Therefore it is unclear currently whether γδ T cells in the gut can expand in an antigen specific, clonal manner.

The full biology of gut Vδ1 T cells remains to be elucidated. Their immediate, effector responses and their binding to BTNL molecules may indicate an innate-like biology. However they display adaptive-like features including a diverse range of TCR chains suggesting they may be able to recognise a myriad of antigens. Future work will shed light on whether these cells have an innate-like phenotype such as the Vγ9Vδ2 blood subset or an adaptive-like biology as observed in Vδ1 T cells in the blood as discussed below.

1.10 Adaptive-like γδ T cells in humans

1.10.1 Adaptive-like biology in blood Vδ1 and Vγ9-Vδ2 T cells

γδ T cells were long thought to be innate-like cells that had pre-programmed functions. Though subsets that display this biology (eg: Vγ9Vδ2 T cells) are well characterised, there have been a number of studies in recent years suggesting diversity in the immune phenotype and that a proportion of γδ T cells such as Vδ2- T cells may show an adaptive-like biology.

Vδ2- T cells (mainly Vδ1 usage) form the minority of γδ T cells in the blood but are enriched in solid tissues. They are known to respond to multiple infections such as CMV and HIV (Farnault et al., 2013; Halary et al., 2005; Hunter et al., 2018; Wesch et al., 1998). The first indication of their adaptive-like biology is the diversity in their TCR repertoire. Cord blood Vδ1 T cells have extremely diverse CDR3γ and CDR3δ chains. The high level of diversity is due to the addition

of many N and palindromic (P) nucleotides during recombination and the usage of multiple D gene segments in the delta chain. With age expansion of some clonotypes from the diverse TCR milieu is commonly observed. Such expanded clonotypes are private and remain stable for a long period of time (at least several years) and likely reflect an individual's exposure and/or re-exposure to certain pathogens throughout life. This contrasts with Vy9Vδ2 T cells, which are dominated by a public, semi-invariant, oligoclonal TCR repertoire (Davey et al., 2017a; Ravens et al., 2017).

Concurrently with the clonal expansion, Vδ1 T cells differentiate from a naïve to an effector phenotype, showcasing another hallmark of adaptive-like biology. Naïve Vδ1 T cells share common markers with naïve CD8 T cells, expressing high levels of CD27, CD45RA, central homing markers such as CD62L and CCR7 and transcription factors like TCF7. Effector Vδ1 T cells are similar to effector memory RA+ CD8 T cells (T_{EMRA}) with increased cytotoxic markers (granzymes, perforin), tissue homing markers (CX3CR1) and a decrease of CD27, CD28, IL-7Rα. They also express lower levels of central homing markers and higher levels of transcription factors like Eomes and T-bet. The majority of Vδ1 cells in the blood fall into a naïve or effector population (Davey et al., 2017a, McMurray et al, preparation).

Interestingly, a subset of blood Vδ2 T cells also has an adaptive-like phenotype. Vy9Vδ2 T cells are the minority within the Vδ2 compartment. They typically have a naïve like phenotype similar to naïve Vδ1 T cells (CD27+ CCR7+ CD28+ IL-7Rα+), a diverse TCR repertoire and an inability to respond to P-Ags. Following viral infection in some individuals this subset can undergo clonal expansion and differentiation to an effector state (CD27- CD45RA+ CX3CR1+ Granzyme B+) with phenotypic changes that mirror CD8 T cells (Davey et al., 2018).

The adaptive nature of Vδ2- T cells (of which many are Vδ1) and Vy9-Vδ2 T cells has been highlighted in response to CMV where they expand in the blood following infection (Davey et

al., 2018; Kaminski et al., 2020; Pitard et al., 2008; Ravens et al., 2017). Following hematopoietic stem cell transplantation, many patients exhibit a resurgence in CMV due to their immune suppression. Alongside this CMV resurgence there is a proliferation of blood Vδ2- T cells that aligns with the resolution of infection. This expansion is not observed in CMV-negative transplant patients even if they had EBV so it appears CMV specific (Knight et al., 2010; Lafarge et al., 2001; Scheper et al., 2013).

Expanded blood Vδ1 T cell clones from CMV-infected donors could lyse CMV infected cells *in vitro* and produced high levels of pro-inflammatory cytokines such as TNFα. They also reduced CMV propagation *in vitro* suggesting they can control infection (Devaud et al., 2009; Halary et al., 2005). yδ T cells are generated more rapidly than αβ T cells following Hematopoietic stem cell transplant (Hirokawa et al., 2000) and so they may fill an immunological gap before αβ T cells have recovered.

The CMV driven Vδ2- T cells and Vy9-Vδ2 expansion is thought to be an antigen specific proliferation rather than a polyclonal expansion due to the clonal nature of the expanded cells. Transplant patients without CMV did not have the same degree of clonal focusing in their gamma and delta chains and so the focusing is not an artifact of the transplantation procedure or of homeostatic expansion to fill the 'immunological space' post-transplant. Many of the expanded clones remain as effectors for years suggesting long term recall (Davey et al., 2017a; Davey et al., 2018; Ravens et al., 2017).

CMV- healthy donors also have some clones in the blood Vδ1 T cell repertoire indicating that CMV is not the only pathogen that can drive clonal focusing and the potential for multiple antigens to be recognised (Davey et al., 2017a). Potential *in vivo* clonal expansions in blood Vδ1 T cells can be detected also in Epstein Barr virus infection and in Malaria where they can

mitigate spread of the pathogen and control infection (Farnault et al., 2013; Hviid et al., 2001; Rutishauser et al., 2020).

1.10.2 Adaptive-like biology in liver γδ T cells

Many of the adaptive-like features of Vδ1 and Vγ9-Vδ2 T cells can also be observed in the liver. Both Vδ1 and Vγ9-Vδ2 cells have private expanded clones in healthy and infected donors, with the expanded cells having an effector phenotype. In the matched blood of some donors both subsets of cells have a naïve phenotype with a great diversity in TCR repertoire. Some clones could be detected in both the liver and the blood which suggests that cells may be naïve in the blood until activated, undergo clonal amplification and transition to an effector phenotype, before trafficking to the liver. Transition to the effector state increases their expression of tissue homing markers (eg: CXCR3) and they may be able to enter solid tissue such as the liver (Davey et al., 2018; Hunter et al., 2018). This would mirror a traditional adaptive αβ T cell response with a translocation from the circulation to a peripheral tissue upon activation.

Alongside the Vδ1 T cells in the liver that could have trafficked from the blood, another subset of Vδ1 T cells was observed, which was CD45RA-ve and displayed a tissue resident phenotype. This subset also typically expressed liver-specific, clonotypically expanded TCR sequences that were not detected in blood and could be activated independently of the TCR, via IL-12/IL-18 (Hunter et al., 2018). This suggests there may be two Vδ1 T cell populations in the liver, a population that traffics from the blood after activation and a more tissue resident-like subset that may seed the tissue either early in life or following infection.

In summary, Vδ1 and Vγ9-Vδ2 T cells show an adaptive-like biology in the blood and liver. Their TCR repertoire is diverse early in life which gives scope for clonal expansions, potentially

in response to a multitude of antigens. Following antigen exposure the cells become long lived clones and this is accompanied by a change from a naïve to an effector phenotype. The effector phenotype features expression of tissue homing markers that allow entry into peripheral tissues, which is reinforced by the presence of shared clones between the two tissues. A summary of the working hypothesis in this thesis on the circulation of both Vδ1 and Vy9-Vδ2 T cells is described in Figure 1.3 below.

Whilst studies have begun to explore the adaptive-like biology of human yδ T cells, many questions remain in the field. Which ligands are seen by the cells before expansion? Which further diseases cause an expansion and what differentiates an antigen that drives a clonal response and one that drives a polyclonal response? Do the long-lasting clones retain recall responses following challenge? Are naïve yδ T cells able to access lymphoid tissue at similar rates to effector yδ T cells accessing solid tissue? Many of these answers will help to determine the biology of human yδ T cell subsets and their role in the control of infection or dysregulation in the body.

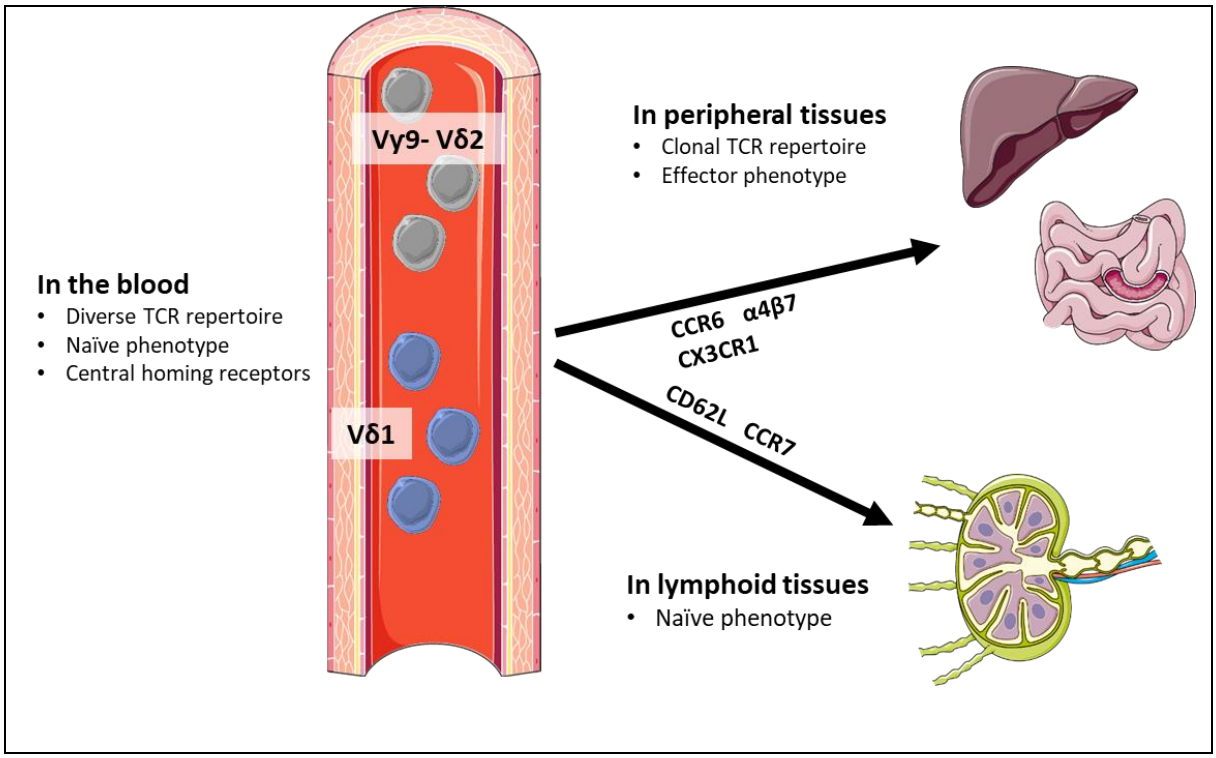


Figure 1.3: A hypothesis of the circulation and biology of human adaptive-like yδ T cell. It has been shown in some donors that subsets of adaptive-like Vδ1 and Vy9-Vδ2 T cells in the blood can have a diverse TCR repertoire and a naïve phenotype. I hypothesise that these cells can access lymphoid tissue through their expression of central homing markers where they would retain a naïve phenotype and search for antigen. Upon activation in the lymphoid tissue or in the blood the cells would transition to an effector phenotype and access the site of inflammation by their expression of peripheral tissue homing markers. This hypothesis is strengthened by the finding that Vδ1 and Vy9-Vδ2 T cells in the liver have an effector phenotype and showed clonal focussing.

1.11 Aims of this investigation

The overarching aim of this investigation was to further explore the adaptive-like biology of human and murine γδ T cells. Understanding this aspect of their function is important as γδ T cells are of therapeutic interest in a plethora of diseases however much of their fundamental biology remains unknown. To explore their potential for adaptive biology, murine models were used to address hypotheses that could not be answered definitively in humans.

The key aims were:

- To identify γδ T cell subsets in healthy murine lymphoid and non-lymphoid tissue and characterise them by their TCR repertoire, surface and intracellular markers and their migratory properties.
- To determine how murine γδ T cells in lymphoid and non-lymphoid tissue respond to both acute and chronic infection by studying their phenotype, cytokine production and the TCR repertoire of responders.
- To complement previous studies on human peripheral blood γδ T cells by establishing the phenotype and TCR repertoire of Vδ1 and Vγ9-Vδ2 T cells in human lymphoid tissue.

Chapter 2 – Materials and Methods

2.1 Media and buffers

Table 2.1: Media and buffers used

Medium	Reagents used
Staining buffer	Dulbecco's PBS (Sigma-Aldrich), 2% Fetal Calf Serum (FCS) (Sigma-Aldrich), 2.5 mM Ethylenediaminetetraacetic acid (EDTA) (Sigma Aldrich)
HBSS + EDTA	Ca and Mg free HBSS (sigma-Aldrich), 2.5 mM EDTA (Sigma-Aldrich)
HBSS + FCS	Ca and Mg free HBSS (Sigma-Aldrich), 2% FCS (Sigma-Aldrich)
Culture media (mouse)	RPMI – 1640 with L-glutamine (Thermofisher), 10% FCS (Sigma-Aldrich) 1% L-glutamine (Sigma-Aldrich), 1% penicillin streptomycin (Sigma-Aldrich).
Culture media (human)	RPMI-1640 (Invitrogen) with 2 mM L-glutamine, 1% sodium pyruvate, 1% penicillin/streptomycin (Invitrogen) and 10% FCS (Sigma-Aldrich).
Gey's solution	10 ml of solution A, 2.5 ml of solution B, 2.5 ml of solution C and 35ml of distilled water. Solution A: 35 g NH_4Cl, 1.85 g KCl, 1.5 g $Na_2HPO_4.12H_2O$, 0.119 g KH_2PO_4, 5.0 g Glucose, 25 g Gelatin, 1.5 mL 1% Phenol red Solution B: 4.2 g $MgCl_2.6H_2O$, 1.4 g $MgSO_4.7H_2O$, 3.4 g $CaCl_2$ Solution C: 22.5 g $NaHCO_5$, distilled H_2O.

2.2 Mice

All studies were carried out in accordance with Home Office regulations and performed under an approved licence. Mice were bred in house or purchased (Harlan or Charles River Laboratories) and all were on a C57BL/6 background. The full list of strains used is available in Table 2.2 below.

Mice were culled through the schedule 1 method of cervical dislocation and were age-matched and sex-matched where possible. All mice used were between 6-12 weeks of age.

Table 2.2: Mouse strains used

Mouse strain	CD45 allotype	Background	Phenotype	Source
WT	CD45.2	C57BL/6	C57BL/6	Biomedical Services Unit (BMSU), Charles River Laboratories
Tg(CAG-tdKaede)15Utr (will be referred to throughout thesis as Kaede)	CD45.2	C57BL/6	Expression of photoconvertible Kaede protein in all cells (Tomura et al., 2008).	BMSU
C.129S4(B6)-Ifngtm3.1Lky/J X B6.129S4-Il17atm1.1Lky/J (will be referred to throughout thesis as Great Smart)	CD45.2	C57BL/6	Dual reporter of IFNyand IL-17 (Price et al., 2012).	BMSU
B6.129S2-Tcratm1Mom/J (will be referred to throughout thesis as TCR α-/-)	CD45.2	C57BL/6	Knockout of αβ T cells	BMSU, Professor Graham Anderson at University of Birmingham
C57BL/6 X B6.SJL-Ptprca Pepcb/BoyJ (will be referred to throughout thesis as C57BL/6 X Boy J)	CD45.1 / CD45.2	C57BL/6	WT	BMSU
***Nr4a3*-Tocky founder line 323** (will be referred to throughout as Nr4a3-TOCKY	CD45.2	C57BL/6	Expression of a temporal photoconvertible protein following Nr4a3 expression. (Bending et al., 2018).	Dr David Bending at University of Birmingham

FVB-Tg(Rag2-EGFP) (will be referred to throughout thesis as RAG2GFP mice)	CD45.2	BALB/c	Reporter for RAG recombinase	Professor Graham Anderson at University of Birmingham
STOCK Il17atm1.1(icre)Stck/J X Gt(ROSA)26Sortm1Hjf (will be referred to throughout thesis as IL-17 - RFP)	CD45.2	C57BL/6	Irreversible RFP reporter for IL-17 expression	BMSU
Germ-free BALB/c	CD45.2	BALB/c	Mice lack a microbiome	Dr Joanne Konkel, University of Manchester

2.3 Infection of mice

Mice were infected with actA-deficient *Listeria monocytogenes-2W1S* (Ertelt et al., 2009) or *Salmonella typhimurium-2W1S* (Mooney et al., 2015) which both express the OVA protein bound to the peptide $2W1S_{52-68}$ (EAWGALANWAVDSA) (Moon et al., 2007). This allowed the detection of an antigen specific immune response by staining with the 2W1S tetramer. Glycerol stock were prepared and stored as described below for both bacterial strains and thawed as required.

2.3.1 Listeria monocytogenes

Bacteria was cultured from a single colony overnight (37 ^{o}C, 250 rpm) in 10 ml LB broth with 20 µg/ml of Chloramphenicol. The following day 1 ml of the culture was placed into 200 ml of LB broth with 20 µg/ml Chloramphenicol and incubated (37 ^{o}C, 250 rpm) until reaching an OD600 of 0.7. The culture was then centrifuged and the pellet resuspended in LB broth with 15% glycerol and frozen in cryovials at - 80 ^{o}C.

To prepare the bacteria for injection into mice, glycerol stocks were thawed before centrifuging them for 12 mins at 4 ^{o}C. The supernatant was removed, the pellet resuspended in 1ml of PBS and centrifuged again. The pellet was then resuspended in sterile PBS and 10^7 bacteria was injected into the mice via intravenous injection in the tail vein.

2.3.2 Salmonella typhimurium

Glycerol stocks were prepared as described for *L. monocytogenes* above (section 2.3.1), however Streptomycin was used as a selecting antibiotic in the place of Chloramphenicol. 1 day prior to infection, mice were administered Streptomycin (20 mg/100 µl sterile PBS) by oral gavage. Glycerol stocks were thawed and washed twice in sterile PBS as described above (section 2.3.1) and resuspended in sterile PBS. The bacteria was then administered via oral gavage at a concentration of 10^9 bacteria per mouse.

2.4 Adoptive transfer

In an attempt to track the response of naïve (CD62L+ CD44-) yδ T cells following infectionIused an adoptive transfer method. Splenocytes from 5 donor TCR α-/- mice (CD45.2) were analysed by fluorescent activated cell sorting and the naïve population sorted. 20,000 naïve cells were injected intravenously into each host BoyJ x C57BL/6 mouse (CD45.1+/CD45.2+).

24 hours post cell transfer the host mice were infected with *L. monocytogenes-2W1S* and culled at different time points post infection (24 hours, 7 days) to determine the presence of donor naïve cells and their potential phenotypic change or expansion during an infection.

2.5 Photoconversion of Kaede mice

Kaede mice express a fluorescent protein in all cells which irreversibly converts from green to red following exposure to UV light (Tomura et al, 2008).

Kaede mice were anaesthetised with gaseous Isoflurane and their flank shaved to access the inguinal lymph node. The lymph nodes were then exposed to UV light through the skin for 5 minutes. The mice were administered a warmed saline solution and monitored during their recovery. At the required timepoint, the mice were culled and the photoconverted lymph node was extracted, alongside contralateral lymph nodes and the spleen.

2.6 Processing of murine tissue

2.6.1 Spleen

Spleen tissue was processed by filtering and crushing it through a 70 µm filter. The cells were centrifuged, the supernatant removed and the pellet resuspended in 5 ml of Gey's Red Blood cell lysis buffer and left on ice for 5 minutes. Lysis was stopped by adding 10 ml of RPMI and the sample was centrifuged again. The supernatant was then removed and the pellet resuspended in an appropriate amount of staining buffer to allow further analysis.

2.6.2 Thymus

Thymi were placed in RPMI and crushed between two glass microscope slides. The slides were then washed in RPMI before the solution was transferred to a falcon tube via a 70 µm filter. A further 5 ml of RPMI was added and the tubes were centrifuged before resuspended in an appropriate amount of staining buffer.

2.6.3 Lymph nodes

Lymph nodes were placed in 3 ml RPMI. Using a dissection microscope, excess fat was removed and the lymph nodes were teased apart. 7.5 µl of Collagenase dispase (100 mg/ml, Sigma-Aldrich) and 7.5 µl of DNase (10 mg/ml, Roche) were added and the cells placed in an incubator for 20 minutes (37 ^{o}C, 5% CO_2). 60 µl of EDTA (0.5 M, Sigma-Aldrich) was added to stop the digestion process and the cells were strained through a 70 µm filter and centrifuged. The pellet was then resuspended in an appropriate amount of staining buffer.

2.6.4 Colon

The tissue was washed in HBSS with FCS immediately after harvesting. All fat, faeces and peyer's patches were removed. The colon was filtered through a mesh and collected in HBSS with EDTA and incubated for 15 mins (37 ^{o}C, 250 rpm). EDTA was used to strip the lining from the colon and therefore to extract the intraepithelial lymphocytes (IELs).

Following incubation the supernatant was collected to harvest the IELs and the remaining tissue was washed in HBSS and re-filtered through a mesh. The incubation was then repeated with fresh HBSS with EDTA to complete the stripping of the epithelium and the supernatant was collected to gather the remainder of the IELs. To conclude the IEL collection, the supernatant from both HBSS with EDTA incubations was filtered through 100 µm and 70 µm filters. The filtered cells were then washed twice in staining buffer before being resuspended at the volume required for further work.

Following the two EDTA incubations, the remaining tissue was then washed twice in HBSS, placed in an Collagenase enzyme and incubated (37 ^{o}C, 250 rpm) for 45 minutes. The Collagenase enzyme mix contained per colon: 10 ml culture media, 8.5 mg Collagenase V (Sigma), 12.5 mg Collagenase D (Roche), 10 mg Dispase (Gibco) and 0.3 mg DNase (Roche).

This allowed the breakdown of the remaining tissue and the freeing of the lamina propria lymphocytes. The digested tissue was then passed through 100 µm and 70 µm filters, washed twice in staining buffer and then resuspended in staining buffer for further work.

2.6.5 Liver

The liver was filtered through a 70 µm strainer and placed in RPMI media. It was then centrifuged, the supernatant discarded and the pellet resuspended in RPMI. The liver was then layered over an Optiprep gradient (Sigma-Aldrich) (1.33 ml Optiprep and 3.67 ml PBS/sample) and centrifuged at 1000 g for 25 mins without brakes as they would disrupt the layering. The lymphocyte layer was collected from the gradient, washed with staining buffer and resuspending in an appropriate amount of staining buffer.

2.7 Processing of human tissue

2.7.1 Harvesting of human PBMCs

All human tissue used were collected with ethical approval from the NRES Committee West Midlands ethical board (REC reference 14/WM/1254) and informed consent from donors. Human blood samples were collected intravenously, centrifuged down and resuspended in culture media. The cells were then layered upon Lymphoprep (STEMCELL technologies) and centrifuged at 1200 g for 30 mins. The lymphocyte layer was then collected, washed in PBS and stained for analysis or cryopreserved as required.

2.7.2 Freezing and thawing of human samples

Cells were cryopreserved at a concentration of $1x10^6$/ml. They were resuspended in freezing media (FCS with 10% DMSO), frozen gradually in an isopropanol chamber at -80^{o}C and then kept in liquid nitrogen for long term storage. When thawing cells, vials were rapidly thawed at 37 ^{o}C and then washed in culture media to remove DMSO. The cell pellet was then resuspended in working media for further analysis.

2.8 Magnetic activated cell separation (MACS)

MACS separation was used to deplete cells that expressed a chosen marker. Cells were incubated with antibodies of the markers of choice (30 minutes, 4 ^{o}C, 1:15 dilution). Following two washes in staining buffer, cells were stained with Microbeads (Miltenyi Biotech) (15 minutes, 4 ^{o}C, 1:5 dilution). Following two subsequent washes in staining buffer, the cells were resuspended in 1 ml of Staining buffer and ran through an LD column (Miltenyi Biotech) for depletion whilst the columns were placed in a Magnetic MACS cell separator (Miltenyi Biotech). The elution was collected for further experimental use and analysis whilst the unwanted cells remained in the LD columns.

2.9 Flow cytometry

For murine tissue analysis, cells were stained for 20 minutes at 4 ^{o}C with an APC eFluor 780 Viability dye (eBioscience, 1:1000 in PBS). Following two washes in staining buffer, surface markers were stained at 4 ^{o}C for 30 minutes. All antibodies used and their chosen

concentrations are shown in Table 2.3 below. Following surface marker staining the cells were washed in staining buffer and then filtered through a 100 µm filter into FACS tubes to be used on the flow cytometer.

For intracellular staining of murine tissue, cells were stained for surface markers and fixed with the BD fixation kit for 1 hour at 4 ^{o}C. They were then washed twice with the Foxp3 Permeabilization Buffer (ebioscience) and stained with intracellular antibodies of interest overnight at room temperature. The following morning the cells were washed twice in the Foxp3 buffer and then resuspended in staining buffer for analysis.

In infection models, 2W1S MHC II tetramer (NIH tetramer facility) staining was performed for 1 hour at room temperature to detect antigen specific T cells. Control MHC II tetramers were used alongside to determine the specificity of the 2W1S tetramer binding.

For human PBMCs and tonsil, cells were stained with Zombie Aqua viability kit (Biolegend, 1:350 in PBS) at room temperature for 10 minutes. They were then washed with staining buffer and stained for surface markers for 30 minutes at 4 ^{o}C. Intracellular staining was performed by fixing the cells for 30 mins at 4 ^{o}C (Foxp3 staining kit, eBioscience) and then staining for intracellular antibodies at 4 ^{o}C for 30 mins. All antibodies used and their chosen concentrations are shown Table 2.4 below.

Spherotech Accucount blank particles (Spherotech) were added to all samples to allow quantification of absolute cell numbers. Samples were analysed using the LSR Fortessa X-20 (BD Biosciences) and data was analysed using the FlowJo software (FlowJo LLC).

Table 2.3: Murine Antibodies used

Antigen	Clone	Conjugate	Manufacturer	Concentration
CD3	17A2	Brilliant UV 395	Biolegend	1:200
		FITC	eBioscience	1:200
		Alexa Fluor 700	eBioscience	1:200
		Brilliant violet 650	Biolegend	1:200
	145-2C11	PE-Cyanine7	eBioscience	1:200
		PE	eBioscience	1:200
CD4	RMA-5	Brilliant violet 510	Biolegend	1:200
		Brilliant violet 786	Biolegend	1:200
CD8	53-6.7	Brilliant violet 650	Biolegend	1:200
CD27	LG.3A10	Brilliant violet 510	Biolegend	1:200
CD44	IM7	Brilliant violet 786	eBioscience	1:200
		PE	Biolegend	1:100
CD45.1	30-F11	Brilliant violet 786	Biolegend	1:200
CD45.2	104	PE	eBioscience	1:200
CD62L	MEL-14	PE	Biolegend	1:200
		APC	Biolegend	1:200
		FITC	Biolegend	1:100
CD69	H1.2F3	Brilliant violet 650	BD Biosciences	1:200
B220	RA3-6B2	APC	eBioscience	1:200
TCR αβ	H57-597	Alexa Fluor 700	Biolegend	1:100
TCR αβ		Brilliant UV 737	Biolegend	1:100

TCR yδ	GL3	APC Brilliant violet 711 PerCP-Cyanine5.5	Biolegend BDBiosciences Biolegend	1:200 1:200 1:200
Vγ1.1	2.11	Brilliant violet 421	BDBiosciences	1:200
Vγ4	UC3-10A6	PE-Cyanine7 FITC	eBioscience Biolegend	1:200 1:100
Ly6C	AL-21	PE-Cyanine7	BDBiosciences	1:200
δ6.3	8F4H7B7	Brilliant violet 650	BDBiosciences	1:200
CCR6	29-2L17	Brilliant violet 605	eBioscience	1:100
KLRG1	2F1	Brilliant violet 785	BDBiosciences	1:200
NK1.1	PK136	Brilliant violet 650	BDBiosciences	1:100
NKp46	29A1.4	Brilliant violet 605	Biolegend	1:100
NKG2A/C/E	20d5	PerCP-eFluor 710	Invitrogen	1:100
NKG2D	CX5	Brilliant Violet 421	Biolegend	1:100
Eomes	Dan11mag	PE	eBioscience	1:100
T-bet	4B10	PE PE-Cyanine 7	Biolegend eBioscience	1:75 1:50
IFNγ	XMG1.2	PE-Cyanine7 PE Alexa Fluor 700	eBioscience Biolegend Biolegend	1:100 1:100 1:100

Table 2.4: Human Antibodies used

Antigen	Clone	Conjugate	Manufacturer	Concentration
CD3	UCHT-1	Brilliant violet 421	Biolegend	1:200
		Brilliant violet 786	Biolegend	1:200
		Brilliant violet 605	Biolegend	1:200
CD8	SK1	Brilliant violet 650	Biolegend	1:100
Vγ9	IMMU360	PE-Cyanine 5	Beckman Coulter	1:200
Vδ1	REA173	FITC	Miltenyi Biotec	1:200
		Pe-Vio770	Miltenyi Biotec	1:200
Vδ2	B6	PE	Biolegend	1:200
	123R3	APC	Miltenyi Biotec	1:200
TCR αβ	IP26	PE	Biolegend	1:200
CD27	M-T271	PE-Dazzle 594	Biolegend	1:200
CD45RA	HI100	Pe-Cyanine 7	Biolegend	1:200
	HI100	Brilliant violet 711	Biolegend	1:200
CD45RO	UCHL1	Brilliant violet 605	BD Bioscience	1:200
CXCR3	FAB160P	PE	R&D systems	1:100
CCR7	G043H7	Alexa Fluor 647	Biolegend	1:100
IL-7Rα	A7R34	Brilliant violet 605	Biolegend	1:100
Granzyme B	GB11	Alexa Fluor 647	Biolegend	1:100
Perforin	B-D48	Brilliant violet 421	Biolegend	1:100
T-bet	4B10	PE	Biolegend	1:100
Eomes	IC6166R	Alexa Fluor 647	R&D systems	1:100
IFNγ	4S.B3	APC	Biolegend	1:100
TNFα	MAb11	Brilliant violet 605	BD biosciences	1:100

2.10 Fluorescence activated cell sorting (FACS)

γδ T cell populations of interest were isolated to a high purity from mice using FACS. Following processing of the spleen and lymph nodes as described above (2.6.1, 2.6.3), the tissues were depleted of B cells and macrophages via MACS depletion. Cells were stained with APC conjugated antibodies for B220 and CD11c for 30 minutes. They were then incubated with anti-APC microbeads(Miltenyi Biotec) for 15 minutes at 4 ^{o}C. LD columns (Miltenyi Biotec) were placed in a MACS separation magnet and cells (with the microbeads) were passed through them. The cells with B220 and CD11c expression had bound microbeads and so were adherent to the sides of the column, whilst the flowthrough was used for FACS. This depletion of other cell types helped reduce the amount of time needed to sort for the required γδ T cells.

FACS was also used to sort human γδ T cells from both PBMCs and tonsil. Cells from neither human tissue underwent any form of depletion before FACS. PBMC samples were sorted fresh whilst tonsil samples were thawed and cultured overnight in culture media with IL-2 (10 ng/ml) and IL-15 (75 ng/ml) to allow the recovery of RNA.

For both murine and human samples, staining of the markers of interest was performed as mentioned above (section 2.9) and then single cells were sorted at 4^{o}C into individual wells of a 96 well plate with the BD FACSAria sorter (BD Biosciences).

2.11 Cell culture

2.11.1 Murine cell culture

Cells were stimulated with PMA (50 ng/ml) and Ionomycin (1.5 µM) in culture media for 4 hours (37 ^{o}C, 5% CO_2). 1 hour into the incubation, BFA (10 µg/ml, BD Biosciences) was added to reduce the loss of the intracellular signals of interest.

For anti-CD3 stimulations, anti-CD3 antibody (10 µl/ml, Biolegend) was aliquoted into each well of a 24 well plate. The plate was then sealed and incubated overnight at 4 ^{o}C. The following day the antibody solution was removed and the wells were washed thrice with sterile PBS to remove any unbound antibody. Cell suspensions from murine tissues were aliquoted into each well at a concentration of 2 x 10^6 cells/ml. The plate was then incubated (37^{o}C, 5% CO_2) for 4 hours with BFA administered 1 hour into the incubation.

In experiments assessing the production of cytokines following infection of WT mice, cell suspensions were cultured with BFA for 3 hours to allow an accumulation of signal within the cells.

2.11.2 Human cell culture

For stimulation assays, Human PBMCs and tonsil cell suspensions were activated by PMA and Ionomycin stimulation. Cells were incubated with PMA (25 ng/ml) and Ionomycin (1 µg/ml) for a total of 6 hours (37^{o}C, 5% CO_2), with Golgi Stop (1 µl/ml, BD Biosciences) added 1 hour into the incubation. Cells were incubated at a concentration of 1 x 10^6 cells/ml.

2.12 Molecular biology

2.12.1 cDNA synthesis

Single cells were sorted from human or murine tissue as described above (section 2.10) into a 96 well plate. Each well contained 2 µl of Reverse Transcriptase enzyme and Buffer with from the SensiFAST cDNA synthesis kit (Bioline) and 0.1% Triton X-100. Immediately after sorting the plates were centrifuges and then incubated at 25 ^{o}C for 10 minutes to allow lysis of the cells in the Triton X-100 and release of mRNA into the buffer, 42 ^{o}C for 60 minutes for the reverse transcription reaction and finally 85 ^{o}C for 5 minutes to inactivate the RT enzyme.

2.12.2 Nested PCR

cDNA was processed using a nested PCR protocol consisting of 2 PCR rounds to ensure amplification of rare TCR cDNAs. Round 1 used external primers (Table 2.5) and Taq polymerase to amplify the sequence of interest upstream of Round 2 primers. Round 2 then used internal primers (Table 2.6) on the product of Round 1 to amplify the sequence of interest more specifically.

Both PCR reactions were run at 95 ^{o}C for 2 mins and then (95 ^{o}C for 30 seconds, 60 ^{o}C for 30 seconds, 72 ^{o}C for 1 minute) for 40 cycles before a final 5 minutes at 72 ^{o}C.

Table 2.5: External Primers for nested PCR

Primer	Species	Sequence (5' to 3')
Vγ1-3 external	Mouse	CAATCAACRACCCTTAGRAGGGAAG
Vγ4 external	Mouse	ATGTCCTTGCAACCCCTACCCAT
Cγ1-3 external	Mouse	GGAAAGAACTTTTCAAGGASACAAAG

Cγ4 external	Mouse	CCCTTATGACTTCAGGAAAGAACTTT
Vδ1 external	Mouse	GGAGGAGATGAGAAAAGTAAGGAC
Vδ2 external	Mouse	CCAAGAAGCATACAAGCAGTATAATG
Vδ4 external	Mouse	GCAATTCTACTGATGGTGGAAAGAG
Vδ5 external	Mouse	CCCATGATGCAGATTTTGTTCAAGG
Vδ6 external	Mouse	TAGTGGAGAGATGGTTTTSCTTATT
Vδ7 external	Mouse	GGAAGMCTCGTCAGCCTGTTGT
Vδ8 external	Mouse	CAGTGTCCGATAAAAAGGAAGATGGA
Vδ9 external	Mouse	AGAGCCTCAAGGGACAAAGAGAAA
Vδ10 external	Mouse	CTTGGCTTCAGGAACAAAGGAGAA
Vδ11 external	Mouse	CTCTTACAAAAAGGAAAATGCAACAG
Vδ12 external	Mouse	GGTCTACAACAAAATACAACCAATAG
Cδ external	Mouse	CCACAATCTTCTTGGATGATCTGAG
Vγ1-8 external	Human	CTGGTACCTACACCAGGAGGGGAAGG
Vγ9 external	Human	AGAGAGACCTGGTGAAGTCATACA
Cγ external	Human	CTGACGATACATCTGTGTTCTTTG
Vδ1 external	Human	CAAGCCCAGTCATCAGTATCC
Cδ external	Human	GCAGGATCAAACTCTGTTATCTTC

Table 2.6: Internal primers for nested PCR

Primer	**Species**	**Sequence (5' to 3')**
Vγ1-3 internal	Mouse	ACAARAAAATTGAAGCAAGTAAAGATTTT
Vγ4 internal	Mouse	CTTAGATAAGGAGTACAAGAAAATGGA
Cγ1-3 internal	Mouse	ACAAAGGTATGTCCCAGTCTTATGGA

Cγ4 internal	Mouse	GGAGACAAAGGTAGGTCCCAGC
Vδ1 internal	Mouse	CGCTAAGCTGGATAAGAAAATGCAG
Vδ2 internal	Mouse	CTCTGTGAACTTCCAGAAAGCAGC
Vδ4 internal	Mouse	CCTCAAAGGGAAAATTAACATTTCAAA
Vδ5 internal	Mouse	CGATTTTCTGTGAAGCACAGCAAG
Vδ6 internal	Mouse	CTAYTCTGTAGTCTTCCAGAAATCA
Vδ7 internal	Mouse	GTMCAATCCTTCTGGGACAAAGCA
Vδ8 internal	Mouse	GATTCACAATCTTCTTCAATAAAAGGGA
Vδ9 internal	Mouse	GGAAGCAGCAGAGGKTTTGAAGC
Vδ10 internal	Mouse	GGGAGGCTAAAGTCAGCATTTGAT
Vδ11 internal	Mouse	GGTCATTATTCTCTGAACTTTCAGAAG
Vδ12 internal	Mouse	GGCTATTGCCTCTGACAGAAAGT
Cδ internal	Mouse	GTCACCTCTTTAGGGTAGAAATCTT
Vγ1-8 internal	Human	TGTGTTGGAATCAGGAVTCAG
Vγ9 internal	Human	GGTGGATAGGATACCTGAAACG
Cγ internal	Human	AATCGTGTTGCTCTTCTTTTCTT
Vδ1 internal	Human	CAACTTCCCAGCAAAGAGATG
Cδ internal	Human	TCCTTCACCAGACAAGCGAC

2.12.3 Sequencing

Products of the nested PCR were added to an ExoSAP-IT enzyme mix (Thermofisher) to clean up the PCR products and remove excess primers and nucleotides. 1 µl of ExoSAP-IT was added per well and the plate was incubated at 37 ^{o}C for 15 minutes and then at 80 ^{o}C for a further 15 minutes to inactivate the enzyme.

The samples were prepared for sequencing using the BigDye Terminator v3.1 sequencing kit (Thermofisher) and the sequencing primer of interest (see Table 2.7 below). The plates were then run at 96^{o}C for 1 min followed by (96 ^{o}C for 10 seconds, 50 ^{o}C for 5 seconds and 60 ^{o}C for 4 minutes) for 25 cycles. The samples were then sequenced by the sequencing facility at the University of Birmingham. Sequencing data was visualised and analysed using the Sequencher software, 5.4.6 (Gene Codes Corporation, Ann Arbor, MI, USA).

Table 2.7: Primers for sequencing

Primer	**Species**	**Sequence (5' to 3')**
Cδ internal	Mouse	GTCACCTCTTTAGGGTAGAAATCTT
Vγ1-3 internal	Mouse	ACAARAAAATTGAAGCAAGTAAAGATTTT
Vγ4 internal	Mouse	CTTAGATAAGGAGTACAAGAAAATGGA
Vδ1 internal	Human	CAACTTCCCAGCAAAGAGATG
Cγ internal	Human	AATCGTGTTGCTCTTCTTTTCTT

2.13 Statistics

Data were analysed using Graph Pad Prism Version 8.0 (GraphPad Software LLC). Data were tested for normality using the Shapiro-Wilk test. For normally distributed data, if two populations were assessed, the paired t-test or two sample t-test were used as required. If more than two populations were analysed then a one-way ANOVA or two-way ANOVA was used dependent on the number of factors measured. For non-parametric data, the Wilcoxon or Mann-Whitney test were used as needed for two samples. If more than two samples were studied then the Kruskal-Wallis test was used. Significance was determined at $p \leq 0.05$. On

graphs, parametric data had a line drawn at the mean, non-parametric data had a line drawn at the median.

Chapter 3 – Phenotypic assessment of murine γδ T cell populations in healthy mice.

3.1 Introduction

Several γδ T cell subsets in mice are of an invariant, innate-like nature and are produced in neonates. They seed peripheral, barrier tissues early in life and proliferate *in situ* throughout adulthood. Their TCR repertoires are semi-invariant with restricted CDR3 regions and little complexity (Asarnow et al., 1988, 1989; Havran et al., 1989; Havran & Allison, 1990). These subsets are tissue specific with expression of a particular gamma chain associated with particular tissues. For example, Vγ5Vδ1 T cells are the first to develop in the mouse thymus at embryonic day 15-17, express an invariant TCR, home to the epidermis, and become dendritic epidermal T cells (DETCs) (Havran & Allison, 1988; Heyborne et al., 1993)

Alongside their limited TCR repertoire, these innate-like γδ T cell subsets in mice have a primed, effector phenotype where they express CD44 and cytotoxic granules. They respond rapidly to cytokines or danger associated molecular patterns (DAMPs) and produce IFNγ or IL-17 at barrier sites (Khairallah et al., 2018; Martin et al., 2009; Sutton et al., 2009). During development their ability to produce IFNγ or IL-17 upon activation is preprogrammed in the thymus. It was suggested that γδ T cells that did not encounter a selecting ligand in the thymus became IL-17 producers, whilst cells that encountered a selecting ligand became IFNγ producers. The selecting ligand for the DETC population was characterised as the thymic epithelial cell molecule, Skint-1. Following engagement of Skint-1 by thymic γδ T cells the cells differentiate to produce IFNγ and develop reduced RoRγt levels and IL-17 producing capacity (Jensen et al., 2008; Turchinovich & Hayday, 2011). It is not thought that the other subset produced during gestation, the IL-17 producing Vγ6Vδ1 subset, encounters ligand in the thymus (Jensen et al., 2008).

However not all murine γδ T cells have an innate biology. Other subsets have a less pre-programmed biology with a naïve phenotype, more complex CDR3 sequences and higher

junctional diversity compared to the innate, pre-programmed subsets (Carding & Egan, 2002; Jensen & Chien, 2009; Zeng et al., 2012).

In humans an adaptive-like biology somewhat analogous to that of αβ T cell responses has been proposed in blood Vδ1 T cells. In cord blood, Vδ1 T cells have a diverse, private TCR repertoire and a naïve T cell phenotype. Whilst in many adult blood samples clonal amplifications within the population are observed in the Vδ1 compartment. These clonal expansions are long lived and correspond with a transition to an effector memory phenotype. Expanded effector blood Vδ1 T cell clones upregulate peripheral homing markers, suggesting they may enter non-lymphoid, peripheral tissues in inflammatory settings (Davey et al., 2017a; Ravens et al., 2017).

Many questions regarding adaptive-like γδ T cell biology remain to be answered, many of which are challenging to address in humans. For example, it is unclear if and how these cells circulate around lymphoid tissue, how they respond *in vivo* to infectious stimuli, their requirement for TCR involvement in their activation, and how phenotypic differences relate to tissue distribution across the body.

Hence, to address some of these questions, murine models were a useful tool as they allowed experimentation *in vivo* and study of responses to infection in real time. However the presence of an adaptive-like γδ T cell subset in mice remains controversial and further work is needed to characterise this potential population.

3.2 Project aims in this chapter

Murine γδ T cell biology has previously focused mainly on innate-like γδ T cells at barrier sites. In this chapter I wished to explore potential adaptive-like γδ T cell subsets in mice and to characterise their biology within the mouse in steady state conditions.

Our aims were:

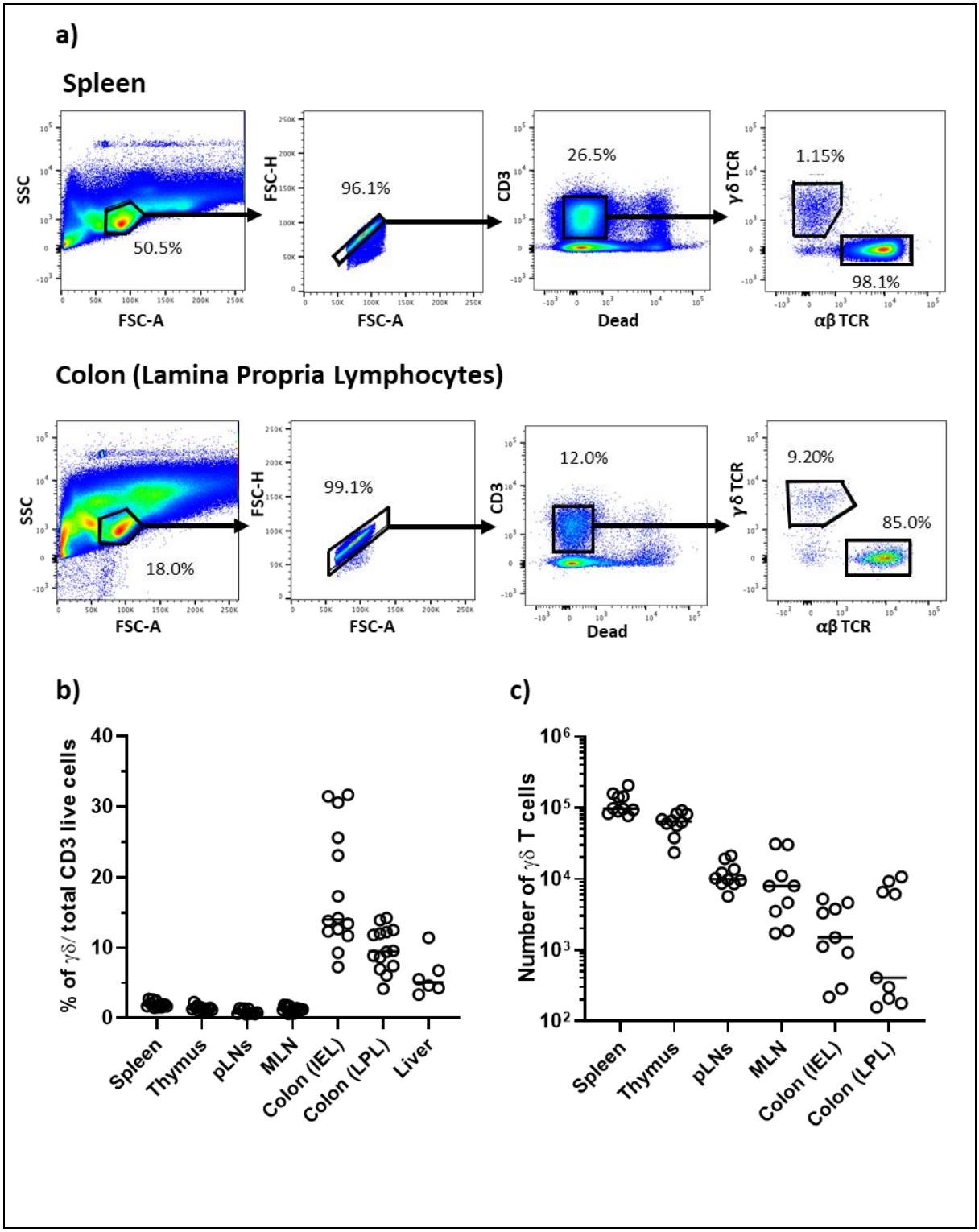
- To systematically assess in multiple tissues how phenotype correlates with localisation in lymphoid and peripheral, non-lymphoid murine γδ T cells.
- To test whether γδ T cells can migrate through lymphoid system as predicted from human results and assess if migration is preferentially associated with a naïve-like phenotype.
- To examine the early development of murine γδ T cell subsets and their lifespan in lymphoid tissues.

3.3 Results

3.3.1 The proportion of γδ T cells in steady state murine tissue

To begin to study the different γδ T cell populations in the mouse, I wished to establish appropriate identification of them in both peripheral and lymphoid tissue in the steady state. γδ T cells are thought to form a high proportion of the T cell compartment at barrier sites and to be less prominent in lymphoid tissue (Havran & Allison, 1990; Itohara et al., 1990) and this was recapitulated here.

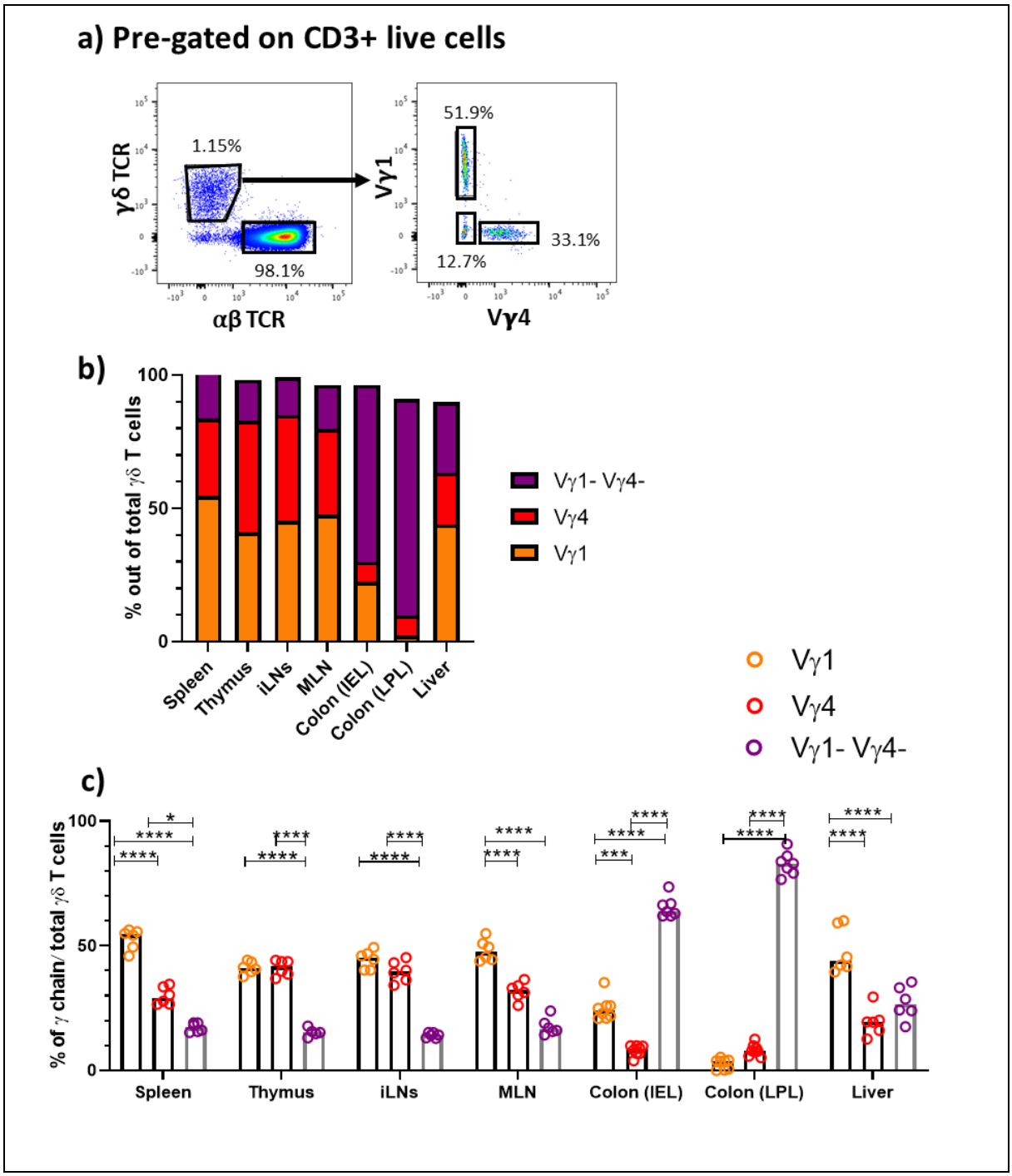
Whilst αβ T cells formed the vast majority (>95%) of live CD3+ T cells in the lymphoid tissue, there were consistent and stable populations of γδ T cells which ranged from 0.5-3% of total live CD3+ T cells in the spleen, thymus and lymph nodes (Figure 3.1b). There was a high absolute number of γδ T cells in these lymphoid tissues (Figure 3.1c), reflecting the large number of live CD3+ T cells in these tissues. In peripheral tissue such as the colon and the liver there was a higher percentage of γδ T cells, ranging between 3-15% of the total live CD3+ T cells (Figure 3.1b), as the αβ T cell population was smaller in these sites.


a)
Spleen
SSC
FSC-A
50.5%
FSC-H
FSC-A
96.1%
CD3
Dead
26.5%
γδ TCR
αβ TCR
1.15%
98.1%
Colon (Lamina Propria Lymphocytes)
SSC
FSC-A
18.0%
FSC-H
FSC-A
99.1%
CD3
Dead
12.0%
γδ TCR
αβ TCR
9.20%
85.0%
b)
% of γδ/ total CD3 live cells
Spleen
Thymus
pLNs
MLN
Colon (IEL)
Colon (LPL)
Liver
c)
Number of γδ T cells
Spleen
Thymus
pLNs
MLN
Colon (IEL)
Colon (LPL)

3.3.2 TCR chain usage in γδ T cells in steady state murine tissues

As described in Chapter 1, neonatal murine γδ T cells develop in waves with each being dominated by a distinct gamma chain usage and seeding set peripheral tissues (Carding & Egan, 2002). I wished to further characterise the populations detected at steady state by determining their Vγ chain usage. In the gut, the vast majority of γδ T cells are Vγ7+ that are seeded early in life (Di Marco Barros et al., 2016; Itohara et al., 1990). In our WT mice a significantly large majority of γδ T cells in the gut were Vγ1- Vγ4- and are likely this population (Figure 3.2b,c). Unfortunately there is currently no commercial antibody available for the Vγ7 chain and therefore could not be confirmed. Lower proportions of Vγ1- Vγ4- T cells were also located in the liver, spleen and LNs (Figure 3.2b,c). The Vγ1- Vγ4- liver population is most likely Vγ6 T cells as they have been found previously in this organ (Kodaira et al., 1996).

Unlike the neonatal tissue-specific subsets, systemic γδ T cells are dominated by the Vγ1 and Vγ4 chains (Carding & Egan, 2002; Jensen & Chien, 2009). Following gating on γδ T cells in lymphoid tissue, sizable Vγ1 and Vγ4 populations were present (Figure 3.2b,c) Therefore, Vγ1 and Vγ4 γδ T cells dominate lymphoid tissue, whilst the Vγ1- Vγ4- cells are present in higher numbers in the colon and also visible in the liver.


a) Pre-gated on CD3+ live cells
γδ TCR
αβ TCR
1.15%
98.1%
Vγ1
Vγ4
51.9%
33.1%
12.7%
b)
% out of total γδ T cells
100
50
0
Vγ1- Vγ4-
Vγ4
Vγ1
Spleen
Thymus
iLNs
MLN
Colon (IEL)
Colon (LPL)
Liver
Vγ1
Vγ4
Vγ1- Vγ4-
c)
% of γ chain/ total γδ T cells
100
50
0
*
****
***
Spleen
Thymus
iLNs
MLN
Colon (IEL)
Colon (LPL)
Liver

3.3.3 The classification of potential naïve, central memory-like and effector γδ T cell subsets in steady state murine tissue

A wide range of markers have been proposed to separate γδ T cell subpopulations in mice. To elucidate potential adaptive subsets I looked at two markers that are used to demarcate naïve, effector and central memory αβ T cells; CD44 and CD62L (Budd et al., 1987; Sallusto et al., 1999; Swain, 1994). CD62L is essential for migration of naïve and central memory T cells into secondary lymphoid organs whilst CD44 allows activated cells to adhere to the endothelial walls of the vasculature and enter inflammatory sites (Butcher & Picker, 1996; DeGrendele et al., 1996, 1997; Gunn et al., 1999; Waters et al., 2003).

Central memory αβ T cells are commonly classified as CD62L+ CD44+, naïve αβ T cells as CD62L+ CD44- and effector αβ T cells as CD44+ CD62L- (Golubovskaya & Wu, 2016). These distinct populations of CD44 and CD62L expression are present in splenic murine αβ T cells and importantly γδ T cells (Figure 3.3a,c,d,e) whilst the colon is dominated by effector CD62L- CD44+ cells in both αβ and γδ T cells (Figure 3.3b,c).

In summary, in lymphoid tissue there are larger populations of CD62L+ CD44- naïve γδ T cells and CD62L+ CD44- central memory like γδ T cells and fewer CD62L- CD44+ effector γδ T cells. Conversely, in peripheral tissue there are large populations of CD62L- CD44+ effector γδ T cells and few CD62L+ CD44- naïve γδ T cells and CD62L+ CD44+ central memory-like γδ T cells. This nomenclature for the three populations (naïve, effector and cm-like γδ T cells) will be used throughout this thesis.

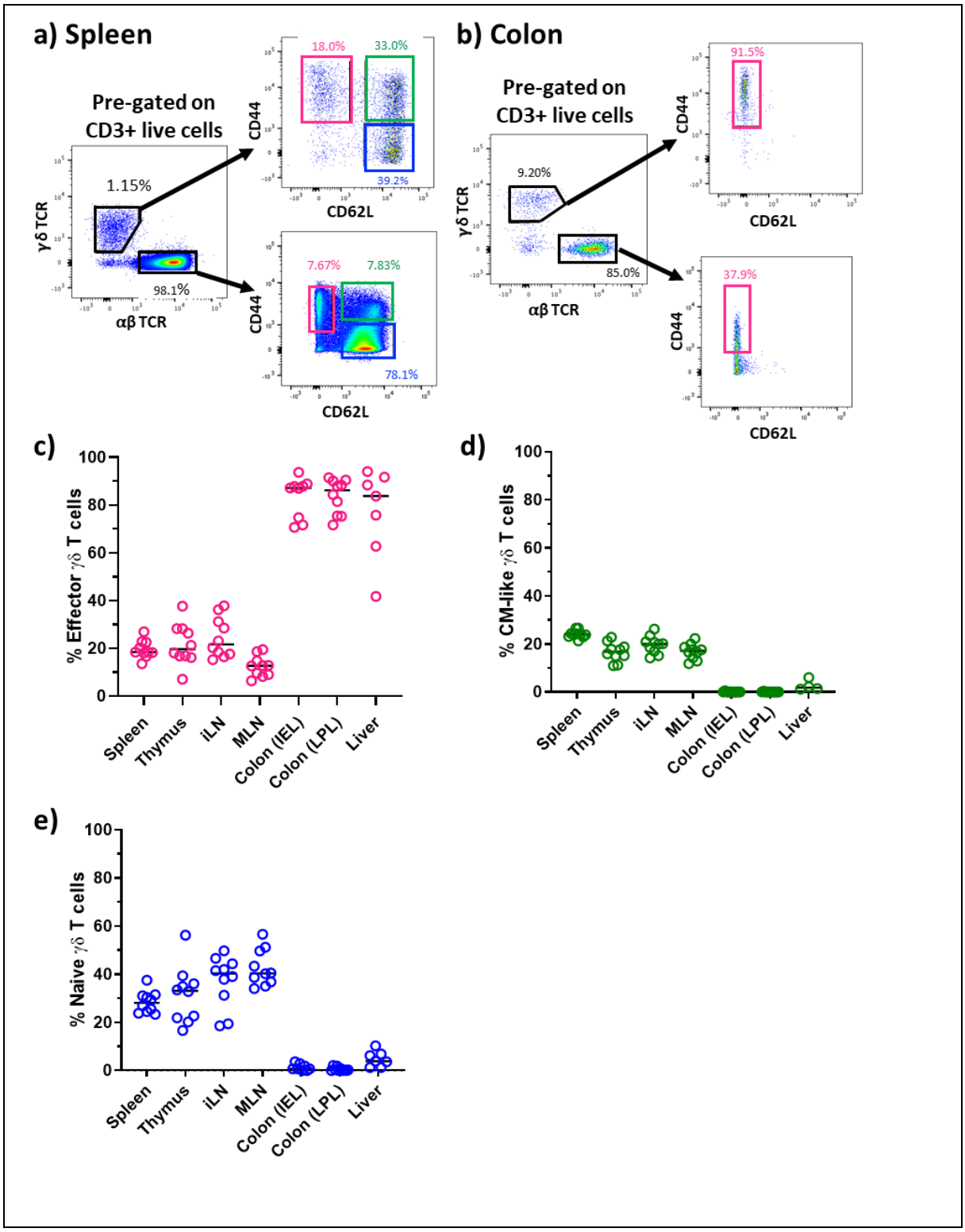

a) Spleen
Pre-gated on CD3+ live cells
1.15%
98.1%
γδ TCR
αβ TCR
18.0%
33.0%
39.2%
CD44
CD62L
7.67%
7.83%
78.1%
b) Colon
9.20%
85.0%
91.5%
37.9%
c)
% Effector γδ T cells
d)
% CM-like γδ T cells
e)
% Naive γδ T cells
Spleen
Thymus
iLN
MLN
Colon (IEL)
Colon (LPL)
Liver

Figure 3.3: The proportion of naïve (CD62L+ CD44-), central memory-like (Cm-like) (CD62L+ CD44+) and effector (CD62L- CD44+) γδ T cells in lymphoid and peripheral tissues. Tissue was harvested from the spleen, thymus, inguinal lymph nodes (ILN), mesenteric lymph nodes (MLN), colon and liver of WT mice as described in the methods and analysed via flow cytometry. Each data point represents an individual mouse and data were pooled from a minimum of 3 experiments.

a) Representative flow cytometry gating strategy for the naïve, central memory and effector αβ T cell and γδ T cell subsets in the spleen.

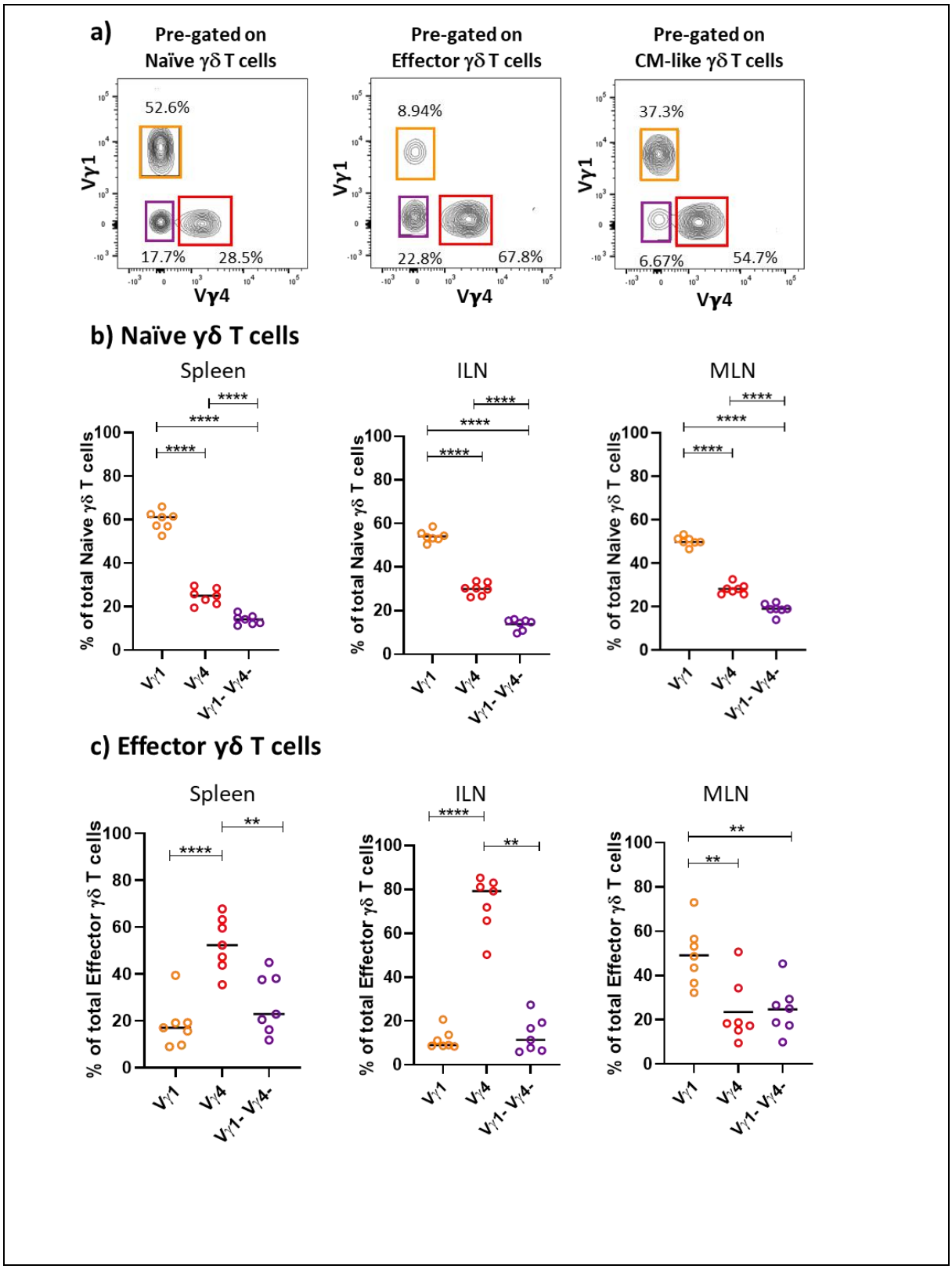
b) Representative flow cytometry gating strategy for effector αβ T cells and γδ T cell subsets in the colon.

c) The percentage of effector γδ T cells in the total γδ T cell population of the spleen (n=10), thymus (n=10), ILN (n=10), MLN (n=10), colonic IELs (n=19), colonic LPLs (n=10) and liver (n=7).

d) The percentage of cm-like γδ T cells in the total γδ T cell population of the spleen (n=10), thymus (n=10), ILN (n=10), MLN (n=10), colonic IELs (n=10), colonic LPLs (n=10) and liver (n=4).

e) The percentage of naive γδ T cells in the total γδ T cell population of the spleen (n=10), thymus (n=10), ILN (n=10), MLN (n=10), colonic IELs (n=8), colonic LPLs (n=10) and liver (n=6).

Having identified the 3 populations in the murine γδ T cell compartment, I wished to determine their Vγ chain usage. The naïve γδ T cell population in the spleen, MLN and ILN were dominated by the Vγ1 chain with a smaller population of Vγ4+ T cells (Figure 3.4b). This contrasts with the effector γδ T cell population that has prominent Vγ4 chain usage in the spleen and ILN (Figure 3.4c). The central memory γδ T cell population had high levels of Vγ1 and Vγ4 chain usage in all tissues (Figure 3.4d). As predicted, the Vγ1- Vγ4- population was not represented in high proportions in any of the naïve, effector or cm-like subsets in the spleen, ILN or MLN (Figure 3.4b,c,d) as this population is not commonly found in lymphoid tissue.


a)
Pre-gated on Naïve γδ T cells
Pre-gated on Effector γδ T cells
Pre-gated on CM-like γδ T cells
52.6%
17.7%
28.5%
8.94%
22.8%
67.8%
37.3%
6.67%
54.7%
Vγ1
Vγ4
b) Naïve γδ T cells
Spleen
ILN
MLN
% of total Naive γδ T cells
Vγ1
Vγ4
Vγ1- Vγ4-
c) Effector γδ T cells
Spleen
ILN
MLN
% of total Effector γδ T cells

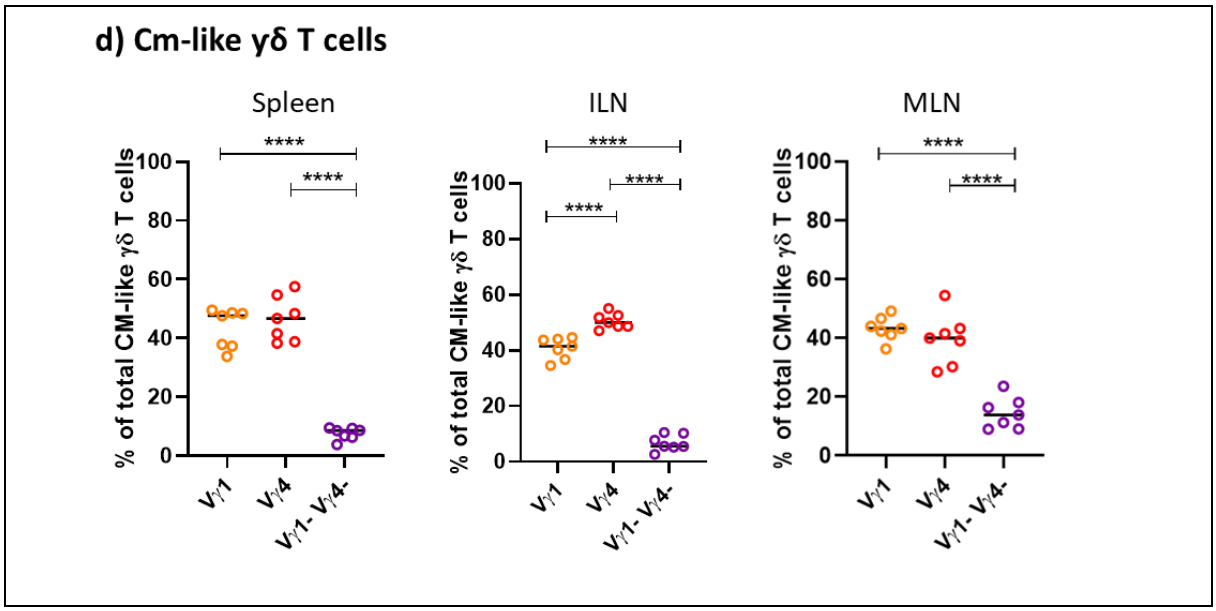


Figure 3.4: Vy Chain usage in the naïve, effector and cm-like yδ T cell populations of steady state mice. Tissue was harvested from the spleen, MLN and ILN of WT mice as described in methods and analysed via flow cytometry. Each data point represents an individual mouse and data were pooled from a minimum of 3 experiments. Data were analysed via a one way ANOVA with Tukey's post hoc test or a Kruskal Wallis with Dunn's post hoc test * p0.05 **p0.01 *** p0.001 **** p0.0001
a) Gating strategy for y chains of the naïve, effector and cm-like populations in the spleen.
b) The percentage of Vy1 (n=7), Vy4 (n=7) and Vy1-Vy4- (n=7) naïve T cells in the spleen, ILN and MLN.
c) The percentage of Vy1 (n=7), Vy4 (n=7) and Vy1-Vy4- (n=7) effector T cells in the spleen, ILN and MLN.
d) The percentage of Vy1 (n=7), Vy4 (n=7) and Vy1-Vy4- (n=7) cm-like T cells in the spleen, ILN and MLN.

Some of the cells within the Vγ4+ effector population may be IL-17 producing innate-like cells that express an invariant Vγ4Vδ5 TCR (Kashani et al., 2015). These innate-like γδ T cells are pre-programmed in the neonatal thymus to produce set cytokines and respond immediately to infectious stimuli and frequently to express the Vγ4 or the Vγ6 chain (Jensen et al., 2008b; Lockhart et al., 2006; McKenzie et al., 2018; Narayan et al., 2012; Ribot et al., 2009a). Using IL-17 fate mapped mice I determined that lymphoid γδ T cells that had previously or were currently producing IL-17 were found only in the effector population (Figure 3.5b,c) and were dominated by Vγ4+ and Vγ1- Vγ4- (presumably Vγ6+) subsets (Figure 3.5d). Therefore it is clear that neither the naïve or cm-like population had any history of IL-17 production, in contrast to the effector population. Hence the effector population is hypothesised to contain both an adaptive effector compartment that shares similarities with αβ T cell biology and an innate-like compartment.

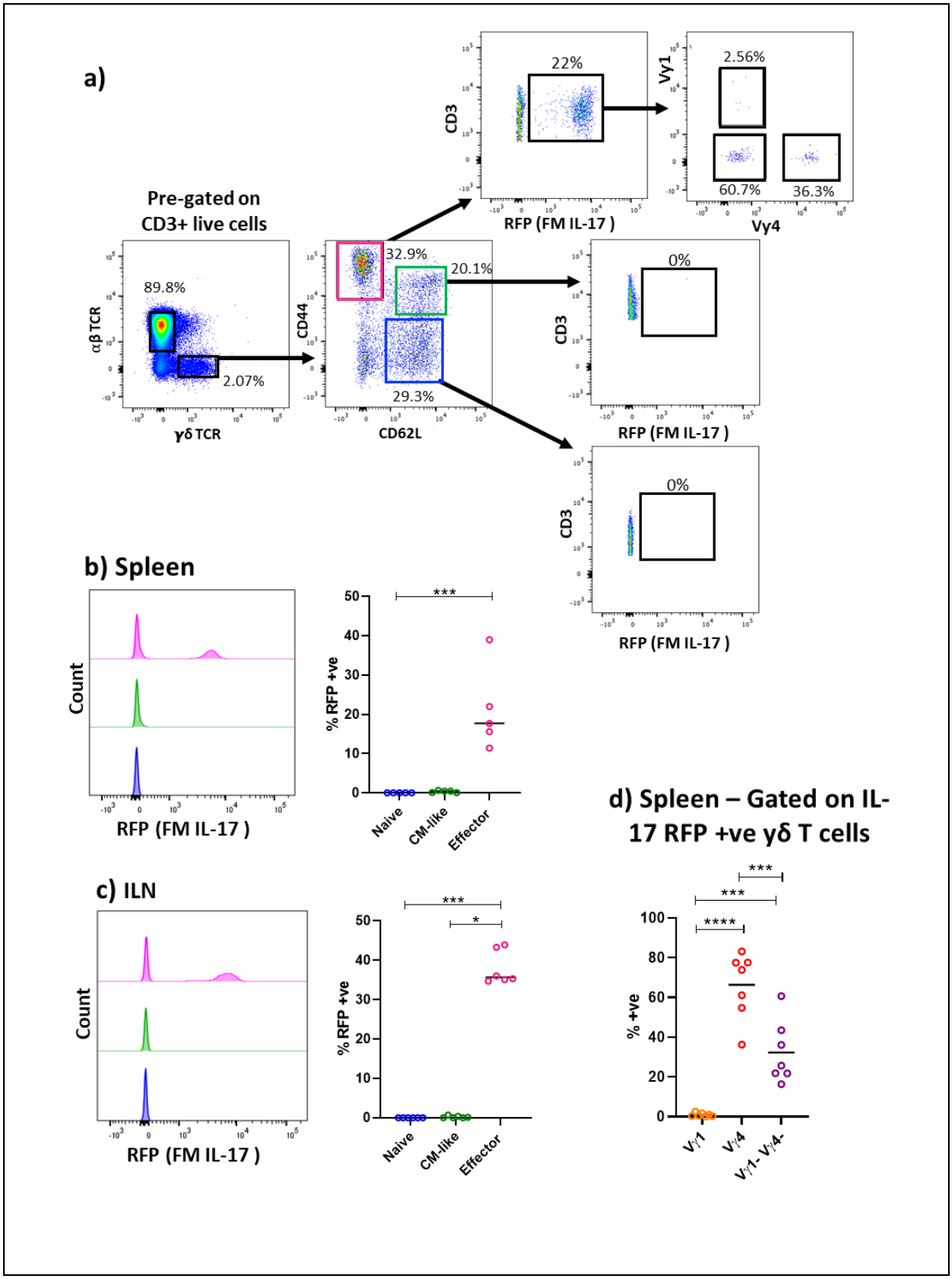

a)
Pre-gated on
CD3+ live cells
89.8%
2.07%
αβ TCR
γδ TCR
32.9%
20.1%
29.3%
CD44
CD62L
22%
CD3
RFP (FM IL-17)
2.56%
60.7%
36.3%
Vγ1
Vγ4
0%
0%
b) Spleen
Count
RFP (FM IL-17)
% RFP +ve
Naive
CM-like
Effector
c) ILN
d) Spleen – Gated on IL-17 RFP +ve γδ T cells
% +ve
Vγ1
Vγ4
Vγ1- Vγ4-

Figure 3.5: The naïve yδ T cell compartment is not pre-programmed to express IL-17 in the thymus. Tissue was harvested from the spleen and inguinal lymph nodes (ILN) of IL-17-RFP fate mapped mice as described in methods and analysed via flow cytometry. Each data point represents an individual mouse and data were pooled from a minimum of 2 experiments. Data were analysed via one way ANOVA with Tukey's post hoc test or Kruskal Wallis with Dunn's post hoc test * p≤0.05 **p≤0.01 *** p≤0.001 **** p≤0.0001

a) Representative flow cytometry gating strategy for RFP+ cells in the naïve, cm-like and effector yδ T cells in the spleen

b) The percentage of RFP+ cells in the naïve, cm-like and effector yδ T cell populations in the spleen

c) The percentage of RFP+ cells in the naïve, cm-like and effector yδ T cell populations in the ILN

d) The percentage of splenic Vy1+, Vy4+ and Vy1- Vy4- yδ T cells within the RFP+ compartment

3.3.4 Phenotypic marker expression of naive, effector and cm-like γδ T cell subsets in steady state mice

To further characterise the naïve, effector and cm-like γδ T cell populations, I wished to explore their surface marker and transcription factor expression. In murine γδ T cell biology, CD27 has traditionally been used as a marker to distinguish IFNγ (CD27+) or IL-17 producing (CD27-) γδ T cells (Ribot et al., 2009, 2010). However, it has also recently been used as an important surface marker to delineate adaptive naïve and effector γδ T cells. Human and murine naïve γδ T cells express high levels of CD27 whilst effector γδ T cells have low levels of expression (Davey et al., 2017b; Ugur et al., 2018). This is recapitulated in data from Figure 3.6 where the percentage of CD27+ cells was high in both naïve and cm-like γδ T cell populations in the spleen and ILN, whilst low in effector γδ T cells (Figure 3.6b). Its low CD27 expression is thought to be linked to a fully differentiated state with a reduced proliferative capacity (Larbi & Fulop, 2014).

CD69 was also included as it is a tissue residency marker in most lymphocytes and can also be induced following activation (Cibrián & Sánchez-Madrid, 2017; Mackay et al., 2012). In humans it is found on liver CD27- effector Vδ1 T cells (Hunter et al., 2018) which aligns with the murine data here with the lymphoid effector γδ T cells having a greater number of CD69+ γδ T cells compared to the naïve γδ T cell subset (Figure 3.6c).

Ly6C and CD44 have been used to delineate effector and naïve like γδ T cell populations in mice. $Ly6C^+$ $CD44^+$ γδ T cells share similarities with αβ memory cells and $Ly6C^-$ $CD44^-$ γδ T cells with naïve αβ T cells (Lombes et al., 2015). Ly6C expression was most represented in the cm-like population in all secondary lymphoid organs (Figure 3.6d) and may represent the $Ly6C^+$ $CD44^+$ found in the previously referenced paper.

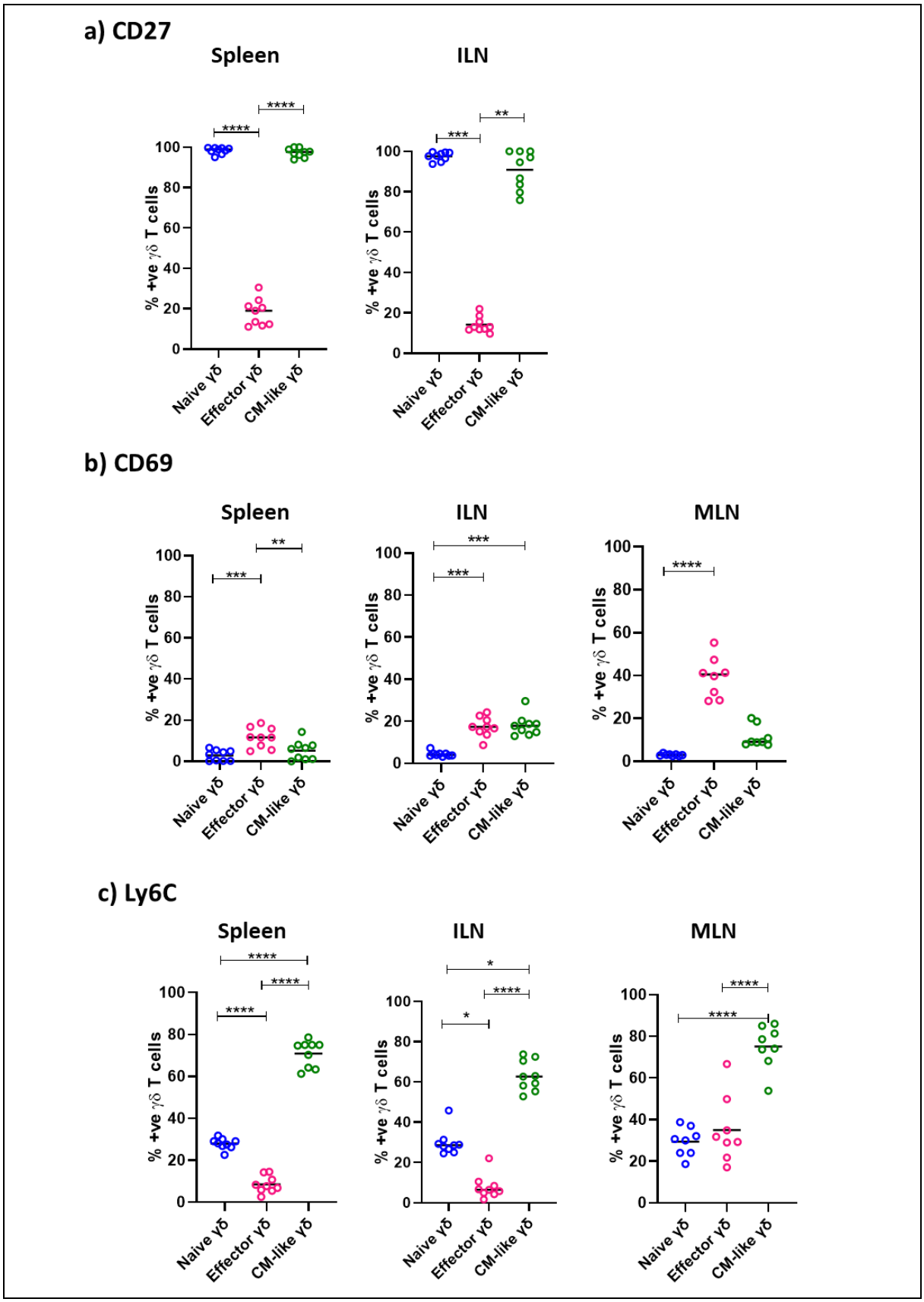

a) CD27
Spleen
ILN
% +ve γδ T cells
Naive γδ
Effector γδ
CM-like γδ
b) CD69
Spleen
ILN
MLN
c) Ly6C
Spleen
ILN
MLN

Figure 3.6: T cell surface marker expression in naïve, central memory-like and effector γδ T cells. Tissue was harvested from the spleen, inguinal lymph nodes (iLN) and mesenteric lymph nodes (MLN) of WT mice as described in the methods and analysed via flow cytometry. Each data point represents an individual mouse and data were pooled from a minimum of 3 experiments. Data were analysed via one way ANOVA with Tukey's post hoc test or Kruskal Wallis with Dunn's post hoc test * p0.05 **p0.01 *** p0.001 **** p0.0001
a) The percentage of CD27+ γδ T cells in the naïve, effector and cm-like compartments of the spleen and ILN.
b) The percentage of CD69+ γδ T cells in the naïve, effector and cm-like compartments of the spleen, ILN and MLN.
c) The percentage of Ly6c+ γδ T cells in the naïve, effector and cm-like compartments of the spleen, ILN and MLN.

Having observed difference in surface marker expression between the subsets, their expression of transcription factors was studied. T-bet and Eomesodermin were chosen as they are transcription factors expressed in differentiated effector/memory T cells and drive effector programming. They are involved in IFNy production and T-bet has also been implicated in the translocation of cells to inflamed tissue (Groom & Luster, 2011; Kallies & Good-Jacobson, 2017; Pearce et al., 2003; Sullivan et al., 2003; Szabo et al., 2002). T-bet expression can also drive IFNy production in yδ T cells in both humans and mice (Barros-Martins et al., 2016; Ribot et al., 2014) with higher expression of both T-bet and Eomes in effector-like CD27- yδ T cells (Rodríguez-Gómez et al., 2019). Lymphoid effector yδ T cells had a higher percentage of T-bet+ cells and Eomes+ cells than the naïve population (Figure 3.7b,c,d). Interestingly the cm-like yδ T cell subset had the highest levels of both transcription factors in the spleen, MLN and ILN (Figure 3.7b,c,d).

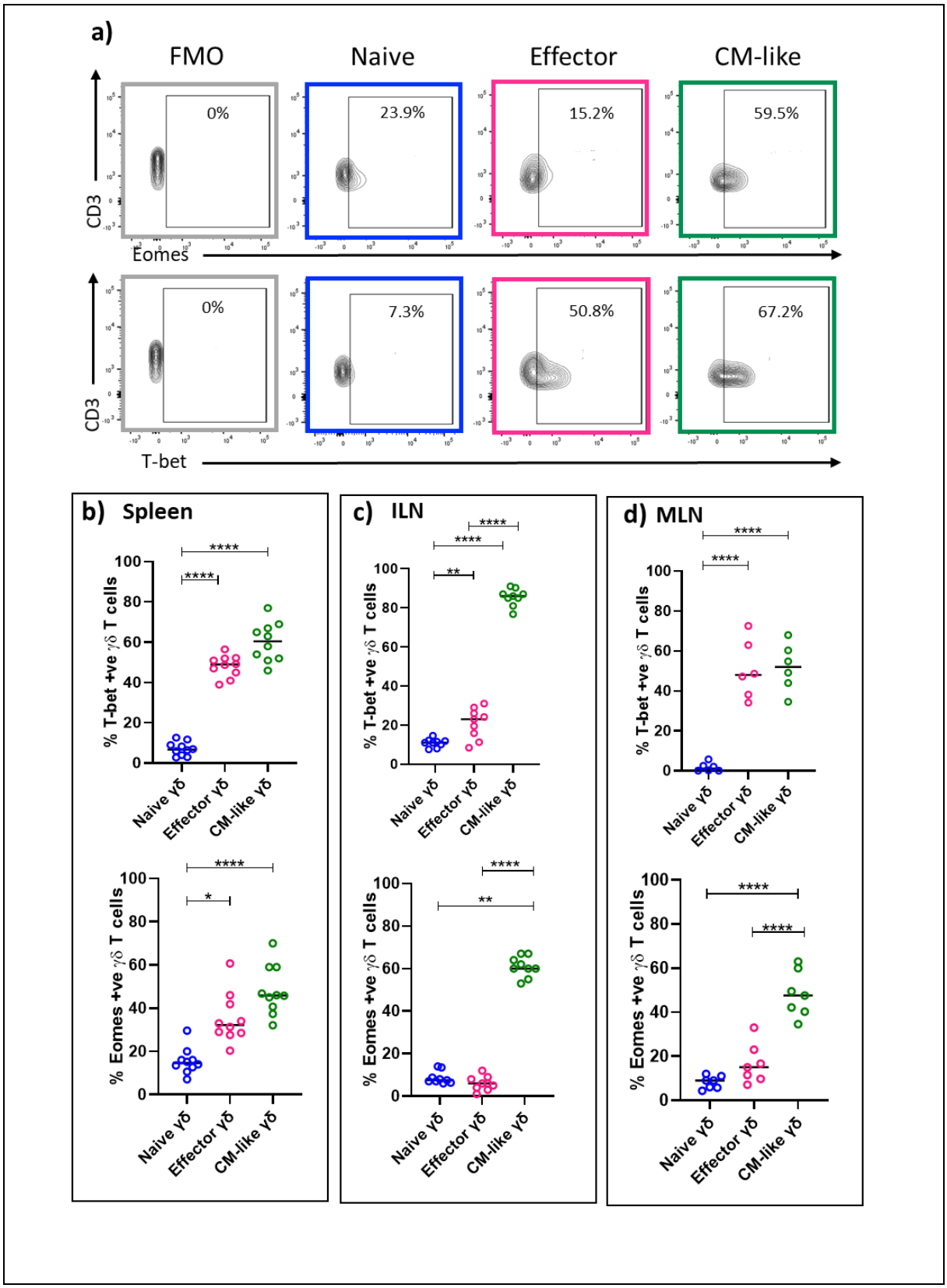

a)
FMO
Naive
Effector
CM-like
0%
23.9%
15.2%
59.5%
CD3
Eomes
0%
7.3%
50.8%
67.2%
CD3
T-bet
b) Spleen
c) ILN
d) MLN
% T-bet +ve γδ T cells
% Eomes +ve γδ T cells
Naive γδ
Effector γδ
CM-like γδ

Figure 3.7: Transcription factor expression in naïve, central memory-like and effector γδ T cells. Tissue was harvested from the spleen, inguinal lymph nodes (iLN) and mesenteric lymph nodes (MLN) of WT mice as described in methods and analysed via flow cytometry. Each data point represents an individual mouse and data were pooled from a minimum of 2 experiments. Data were analysed via one way ANOVA with Tukey's post hoc test or Kruskal Wallis with Dunn's post hoc test * p0.05 **p0.01 *** p0.001 **** p0.0001

a) Representative Eomes and T-bet staining in naïve, effector and cm-like γδ T cell populations in the spleen

b) Transcription factor expression in the spleen. The percentage of T-bet+ γδ T cells within the naïve (n=10), effector (n=10) and cm-like (n=10) populations. The percentage of Eomes+ γδ T cells within the naïve (n=10), effector (n=10) and cm-like (n=10) populations.

c) Transcription factor expression in the ILN. The percentage of T-bet+ γδ T cells within the naïve (n=9), effector (n=9) and cm-like (n=9) populations. The percentage of Eomes+ γδ T cells within the naïve (n=9), effector (n=9) and cm-like (n=9) populations.

d) Transcription factor expression in the MLN. The percentage of T-bet+ γδ T cells within the naïve (n=6), effector (n=6) and cm-like (n=6) populations. The percentage of Eomes+ γδ T cells within the naïve (n=7), effector (n=7) and cm-like (n=7) populations.

3.3.5 NK receptor expression on naïve, effector and cm-like γδ T cells in lymphatic tissue

Alongside their activation via the TCR, it has been shown that some subsets of murine and human γδ T cells can respond to stimulation through NK receptors (NKRs) alone (Nitahara et al., 2006; Rincon-Orozco et al., 2005). It would be expected that innate-like γδ T cell subsets within the effector compartment may be able to be activated by this route. Also in potential adaptive-like cells within the three populations, NKRs may act to influence a TCR driven response. I wished to explore the expression of both activatory and inhibitory NKRs on the naïve, effector and cm-like subsets.

The NKRs were all expressed at significantly higher levels in lymphoid effector and cm-like γδ T cell populations compared to the naïve γδ T cell subset which lacked them (Figure 3.8a,b,c). Their high and retained expression in cm-like γδ T cells is unexpected as it indicates these cells may be able to respond independently to the TCR or that they may require co-activation with both signals. Their expression in the effector subset recapitulates previous findings in αβ T cells as NK receptors are commonly expressed on cytotoxic cells. They may act as an extra modulator of activatory or inhibitory stimuli bound by the TCR. NKG2D is an activation receptor on γδ T cells that allow the γδ T cells to respond to stressed or infected nearby cells (Das et al., 2001; Wensveen et al., 2018). NK1.1 is also expressed on CD44+ effector γδ T cells in mice (Haas et al., 2009) and NKG2A is highly expressed on both innate-like Vγ9Vδ2 T cells and on adaptive effector Vδ1 cells in humans (McMurray et al, in preparation) and is thought to potentially regulate the TCR activation threshold (Boullier et al., 1998).

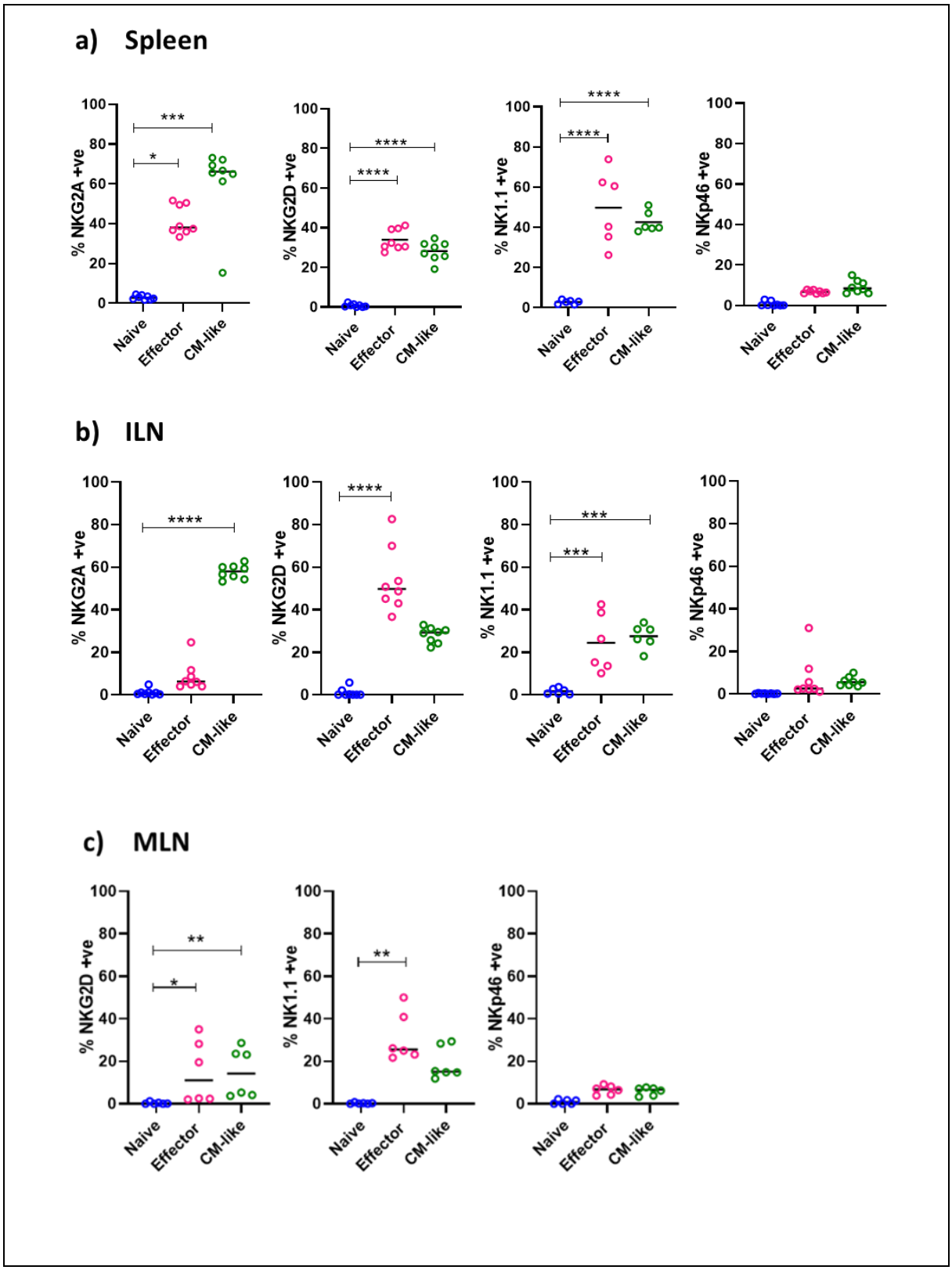

a) Spleen
% NKG2A +ve
% NKG2D +ve
% NK1.1 +ve
% NKp46 +ve
Naive
Effector
CM-like
b) ILN
% NKG2A +ve
% NKG2D +ve
% NK1.1 +ve
% NKp46 +ve
Naive
Effector
CM-like
c) MLN
% NKG2D +ve
% NK1.1 +ve
% NKp46 +ve
Naive
Effector
CM-like

Figure 3.8: NK receptor expression in naïve, central memory-like and effector yδ T cells. Tissue was harvested from the spleen, mesenteric lymph nodes (MLN) and inguinal lymph nodes (ILN) of WT mice as described in methods and analysed via flow cytometry. Each data point represents an individual mouse and data were pooled from a minimum of 3 experiments. Data were analysed via one way ANOVA with Tukey's post hoc test or Kruskal Wallis with Dunn's post hoc test * p0.05 **p0.01 *** p0.001 **** p0.0001

a) The percentage of naïve, effector and cm-like yδ T cells that express NKG2A (n=8), NKG2D (n=8) or NK1.1 (n=6) and NKp46 (n=8) in the spleen.

b) The percentage of naïve, effector and cm-like yδ T cells that express NKG2A (n=8), NKG2D (n=8) or NK1.1 (n=6) and NKp46 (n=8) in the ILN.

c) The percentage of naïve, effector and cm-like yδ T cells that express NKG2D (n=6) or NK1.1 (n=6) and NKp46 (n=6) in the MLN.

3.3.6 Single cell TCR sequencing of naïve and effector γδ T cell populations in steady state lymphoid tissue

The TCR repertoire can be used to elucidate the biology of cell subsets. A diverse TCR repertoire with private sequences suggests an ability to respond to a wide range of antigens through a clonal specific interaction, whilst an expansion of a private TCR clone indicated past antigenic exposure. In human blood Vδ1 T cells, cells with a naïve phenotype have a private, diverse repertoire whilst in some donors, cells with an effector phenotype have a greater number of large clonal expansions that persist for at least two years (Davey et al., 2017b; Ravens et al., 2017). This suggests an antigen-specific clonal expansion that coincides with the transition of cells from a naïve to an effector phenotype. In contrast, the Vγ9Vδ2 p-Ag reactive subset expresses a repertoire of simple, often shared, Vγ9 rearrangements, lacking in the great diversity seen in the Vγ rearrangements of Vδ1 T cells (Davey et al., 2017; Davey et al., 2018). In this case, all Vγ9 rearrangements are thought to recognise the epitope on BTN2A1 and other molecules involved in pAg recognition, and hence this subset is not subject to specific clonal expansion due to antigen exposure (Karunakaran et al., 2020).

To determine whether murine effector γδ T cells were the result of (presumably antigen-driven) clonal expansion as in human Vδ1 T cells, the variable CDR3 region was sequenced from γδ T cells isolated from the spleen and MLN. γδ T cells were harvested from WT mice and single cell sorted based on their expression of CD62L and CD44. In an effort to increase our chances of sequencing adaptive-like γδ T cells, all cells sorted were also either Vγ1+ or Vγ4+ as these gamma chains had previously been linked to quasi-adaptive murine responses (Mamedov et al., 2018; Sell et al., 2015; Zeng et al., 2012).

The Vδ chain usage is relatively consistent between naïve and effector MLN γδ T cells, with DV5 and DV6 prominent in both subsets (Figure 3.9a). This similarity between the subsets

was also noted in the CDR3 sequence repertoire in both gamma and delta chains, with no distinct expansions in either (Figure 3.9b,c). In the effector splenic population however, there was an increase detected in DV5 chain usage in the effector population and a recurring sequence in both gamma and delta chains (Figure 3.9). Unfortunately the expanded sequence in both chains were not sequenced in pairs and therefore it was not possible to ascertain if it was the same TCR expressing both sequences. The recurring sequences in splenic effector yδ T cells (gamma chain CSYGLYSSGFHKVF) and (Delta chain CASGYIGGIRATDKLVF) (Figure 3.9b,c) are sequences that are close to germline and so it is unclear whether it is a true clone or merely a commonly expressed sequence due to its frequent rearrangement.

In both tissues, the majority of sequences were very simple, with few rearrangements or nucleotide additions (Appendix 1). The gamma sequences in particular were not complex with the same sequences observed between subsets and tissues such as CAVWRSGTSWVKIF and CAVWIKGTSWVKIF present in naïve and effector splenic yδ T cells and CAVWRSGTSWVKIF and CASGYIGGIRATDKLVF expressed by both splenic effector and MLN naïve yδ T cells.

Therefore in steady state effector yδ T cells I was unable to detect high proportions of clonal expansions. Perhaps in the absence of infection, some naïve yδ T cells mature into effector T cells. The sequences of both Vy and Vδ chains are mostly simple, germline rearrangements suggesting a lack of antigen-specific recognition.

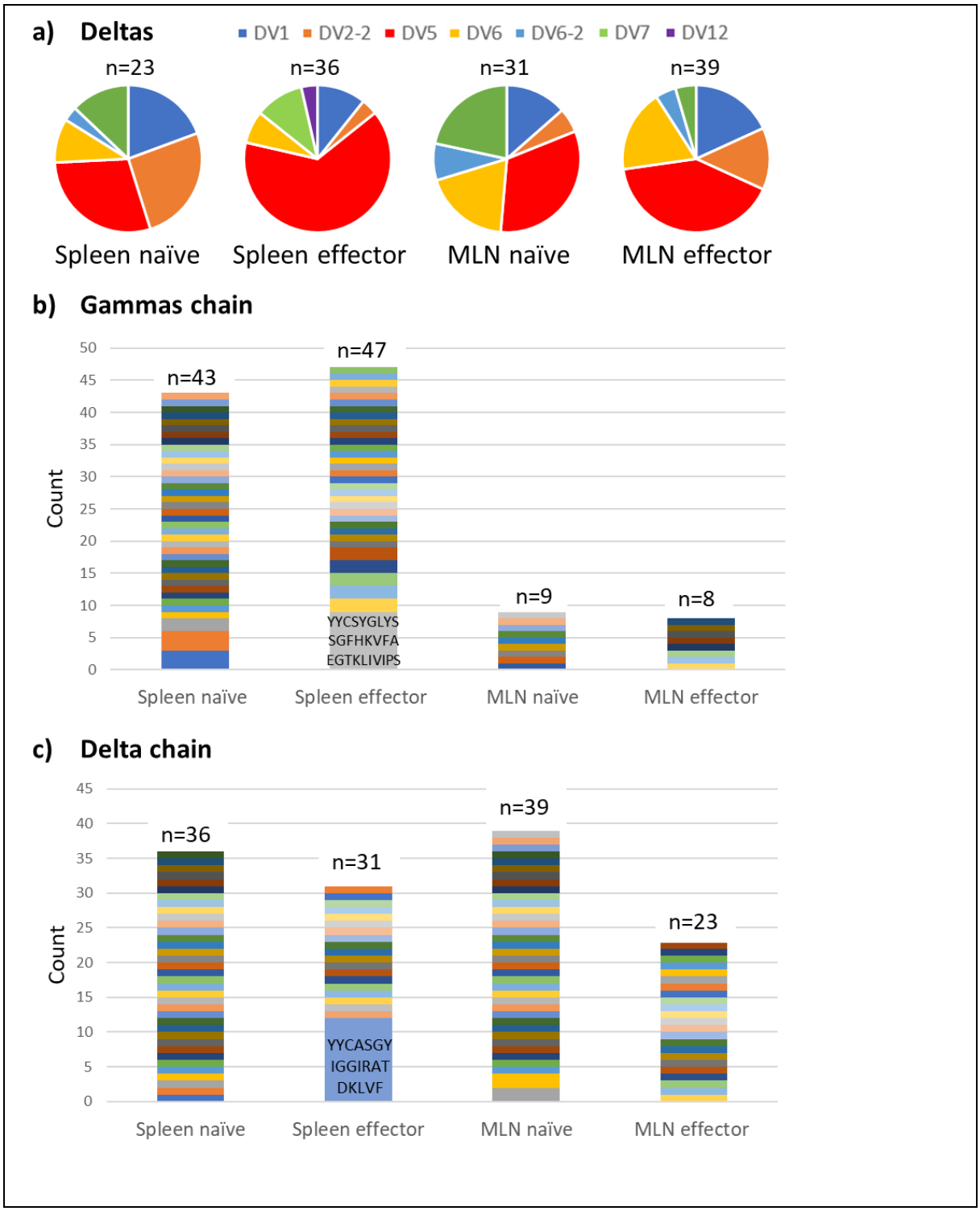

a) Deltas
DV1 DV2-2 DV5 DV6 DV6-2 DV7 DV12
n=23
n=36
n=31
n=39
Spleen naïve
Spleen effector
MLN naïve
MLN effector
b) Gammas chain
Count
n=43
n=47
n=9
n=8
YYCSYGLYS
SGFHKVFA
EGTKLIVIPS
Spleen naïve
Spleen effector
MLN naïve
MLN effector
c) Delta chain
Count
n=36
n=31
n=39
n=23
YYCASGY
IGGIRAT
DKLVF
Spleen naïve
Spleen effector
MLN naïve
MLN effector

Figure 3.9: Single cell sequencing of the variable CDR3 regions of naïve and effector splenic and mesenteric yδ T cells. Cells were harvested from the spleen and mesenteric lymph nodes of WT mice. They were then delineated and single cell FACS sorted through their expression of CD44 and CD62L into naïve (CD62L+ CD44-) and effector (CD62L-CD44+) yδ T cell populations. The variable region of the CDR3 from each cell was sequenced and analysed using Sequencher software.

a) The proportion of delta chain usage in splenic and mesenteric naïve and effector yδ T populations.

b) The composition of the gamma chain repertoire in splenic and mesenteric naïve and effector yδ T populations. Each coloured bar represents an individual sequence. The same colour across categories on the x axis does not correspond to the same sequence.

c) The composition of the delta chain repertoire in splenic and mesenteric naïve and effector yδ T populations. Each coloured bar represents an individual sequence. The same colour across categories on the x axis does not correspond to the same sequence.

3.3.7 The naïve γδ T cell subset has higher RAG-recombinase expression than effector and cm-like γδ T cells

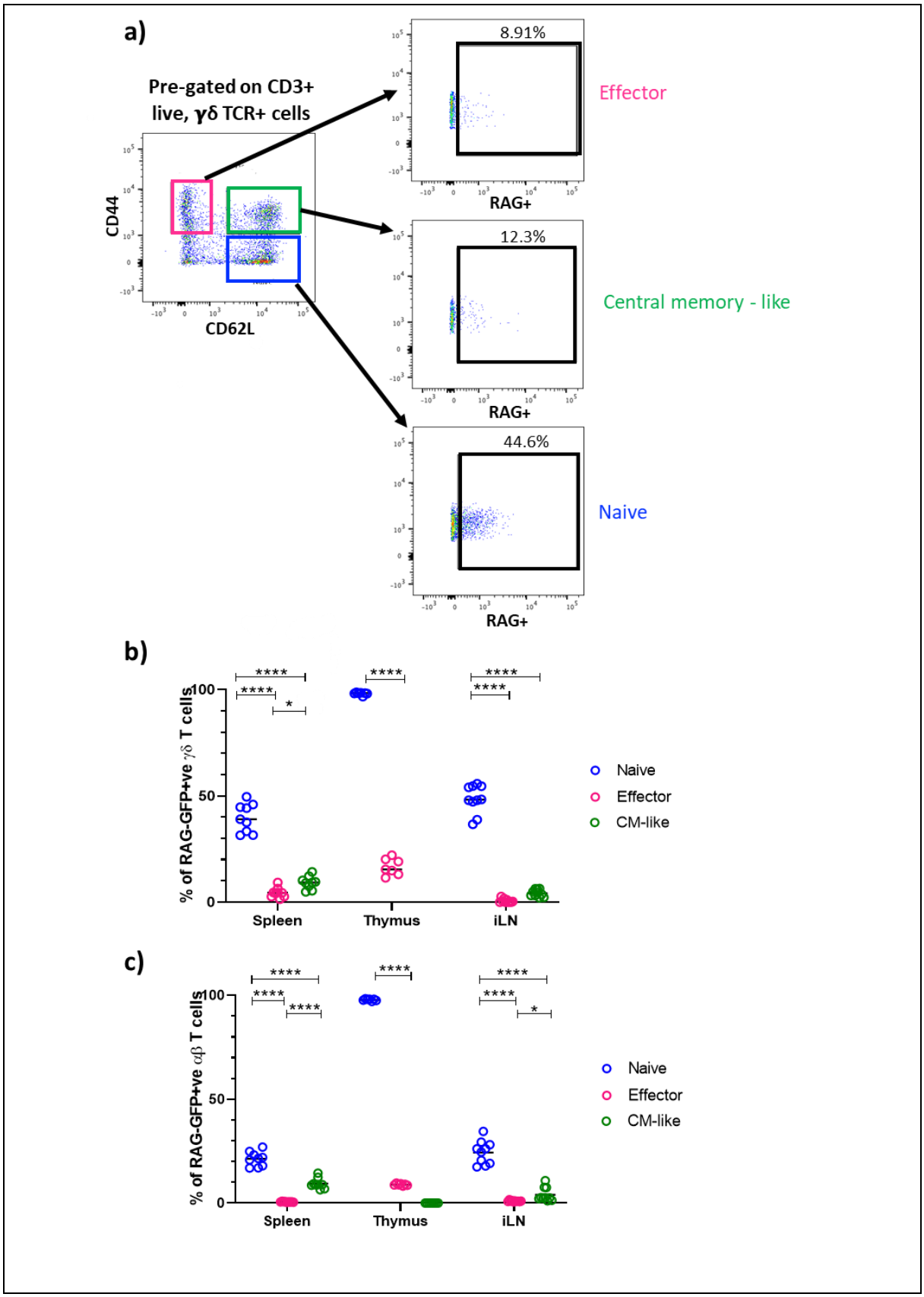
As the naïve γδ T cell population had phenotypic hallmarks of a naïve adaptive subset I wanted to further identify the lifespan of these cells and their potential function in tissues. The peripheral T cell pool, dominated by αβ T cells, is relatively stable throughout life with constant turnover and egress of T cells from the thymus (Tough & Sprent, 1994). I wished to determine if the naïve γδ T cell subset shared this characteristic.

One way of inferring the age of a T cell is through RAG recombinase expression. RAG recombinase is a protein required in VDJ recombination of the T cell receptor (TCR) and a RAG-GFP reporter mouse was developed to study T cell development (Matthews & Oettinger, 2009; Yu et al., 1999). RAG recombinase expression is tightly controlled and temporally limited. The GFP protein under the control of the RAG recombinase promoter decays over 2-3 weeks following expression. Therefore the level of GFP correlates with the age of the cell post VDJ rearrangement (Boursalian et al., 2004; Houston et al., 2008).

In the spleen, thymus and ILN, the naïve γδ T cells had a significantly higher percentage of GFP+ cells than effector and cm-like cells (Figure 3.10b). This suggests that a higher proportion of naïve cells were recently produced with a continuous turnover, compared to the older effector and cm-like populations. It could also however indicate that the effector and cm-like subsets proliferate more rapidly after their egress from the thymus and lose their GFP signal at a quicker rate than the naive population. Interestingly, there was a higher percentage of GFP+ cells in the naïve γδ compartment compared to naïve αβ T cells (Figure 3.10b,c), suggesting there is a more rapid turnover in the naïve γδ T cell compartment.

As expected, nearly all naïve γδ T cells in the thymus expressed RAG, consistent with their recent thymic development (Figure 3.10b). Up to 50% of naïve γδ T cells in the spleen and lLN rearranged their TCR and egressed the thymus within 2-3 weeks prior to the experiment (Figure 3.10b). Murine naïve T cell populations are sustained by continuous thymic output, even in aged mice (den Braber et al., 2012). The high percentage of GFP+ cells were noted in both naïve Vγ1 and Vγ4 subsets indicating that Vγ chain usage does not correlate with a variation in cell turnover (Figure 3.10d,e).

In summary, the naïve γδ T cell compartment has very rapid turnover and continuous output from the thymus throughout life. It remains to be seen however if they transition to an effector phenotype with age or apoptose if not engaged.


a)
Pre-gated on CD3+
live, γδ TCR+ cells
CD44
CD62L
8.91%
RAG+
Effector
12.3%
RAG+
Central memory - like
44.6%
RAG+
Naive
b)
% of RAG-GFP+ve γδ T cells
Spleen
Thymus
iLN
Naive
Effector
CM-like
c)
% of RAG-GFP+ve αβ T cells
Spleen
Thymus
iLN
Naive
Effector
CM-like

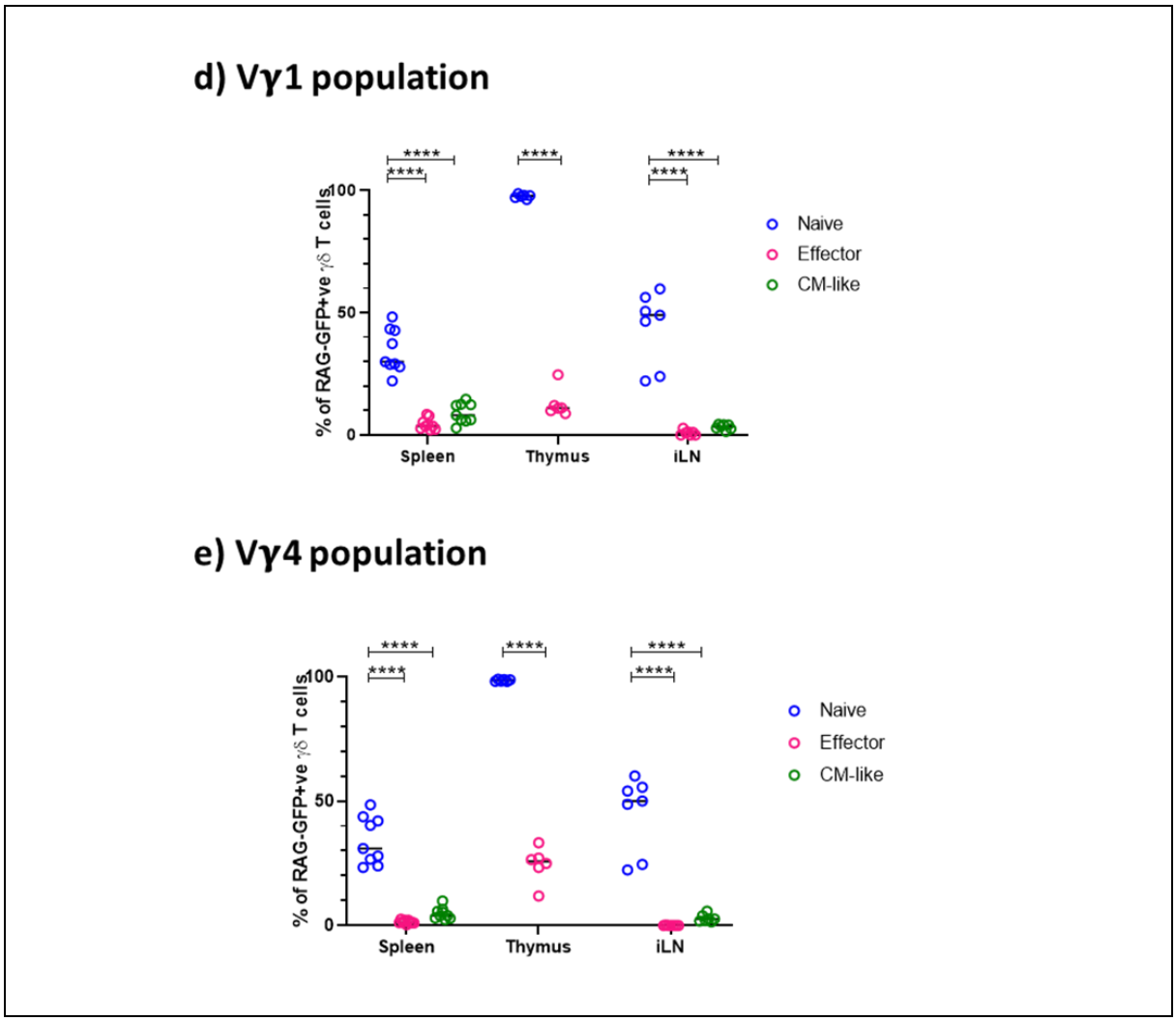


Figure 3.10: Naïve γδ T cells have higher RAG expression than effector and cm-like γδ T cells. Tissue was harvested from the spleen, inguinal lymph nodes (ILN) and thymus of RAG-GFP mice as described in the methods and analysed via flow cytometry. Each data point represents an individual mouse and data were pooled from a minimum of 3 experiments. Data analysed via two way ANOVA with Tukey's post hoc test. * p0.05 **p0.01 *** p0.001 **** p0.0001

a) Gating strategy for the RAG-GFP+ signal in splenic naïve, effector and cm-like γδ T cells

b) The percentage of RAG-GFP+ naive, effector and cm-like γδ T cells in the spleen (n=9), thymus (n=7) and ILN (n=10).

c) The percentage of RAG-GFP+ naive, effector and cm-like αβ T cells in the spleen (n=9), thymus (n=9) and ILN (n=10).

d) The percentage of RAG+ cells in the Vγ1 compartment of the spleen (n=9), thymus (n=6) and ILN (n=7).

e) The percentage of RAG+ cells in the Vγ4 compartment of the spleen (n=9), thymus (n=6) and ILN (n=7).

3.3.8 Naïve γδ T cells recirculate in the lymphoid system

Naïve adaptive αβ T cells circulate around the lymphoid system to allow TCR clones to detect cognate antigen in lymph node. However, it is unclear whether γδ T cells also conform to this paradigm. The migration of naïve, effector and cm-like γδ T cells was explored *in vivo* via Kaede mice. Cells in this model express a fluorescent protein that irreversibly photoconverts from green to red following exposure to UV light (Tomura et al., 2008). This allows labelling of tissues *in vivo* and tracking the circulation of the cells that were present in the photoconverted tissue at the time of labelling to other areas of the mouse. A similar model has previously shown that αβ T cells that were irreversibly photoconverted to red in one lymph node could then be found in a contralateral lymph node and spleen (Ugur et al., 2018).

The entire inguinal lymph node was photoconverted with a UV light as previously described and validated (Dutton et al., 2019; Marriott et al., 2017) and the tissues harvested from the mice 24 hours later, to allow time for potential migratory behaviour from the photoconverted tissue. Photoconverted naïve γδ T cells and cm-like γδ T cells could be detected in the spleen and a contralateral lymph node 24 hours later at significantly higher levels than effector γδ T cells (Figure 3.11b,c,d,e). There were also significantly higher numbers of non-photoconverted naïve and cm-like γδ T cells in the photoconverted lymph node, suggesting these cells had entered the lymph node within the last 24 hours after the labelling had taken place (Figure 3.11f,g).

This data provides direct evidence that naïve and cm-like γδ T cells are able to traffic to other secondary lymphoid organs from a lymph node. However, the effector γδ T cells appear to circulate in the lymphoid system at a lesser degree. The circulation of naïve and cm-like γδ T cells throughout lymphoid tissue is a key marker of a potential adaptive-like biology.

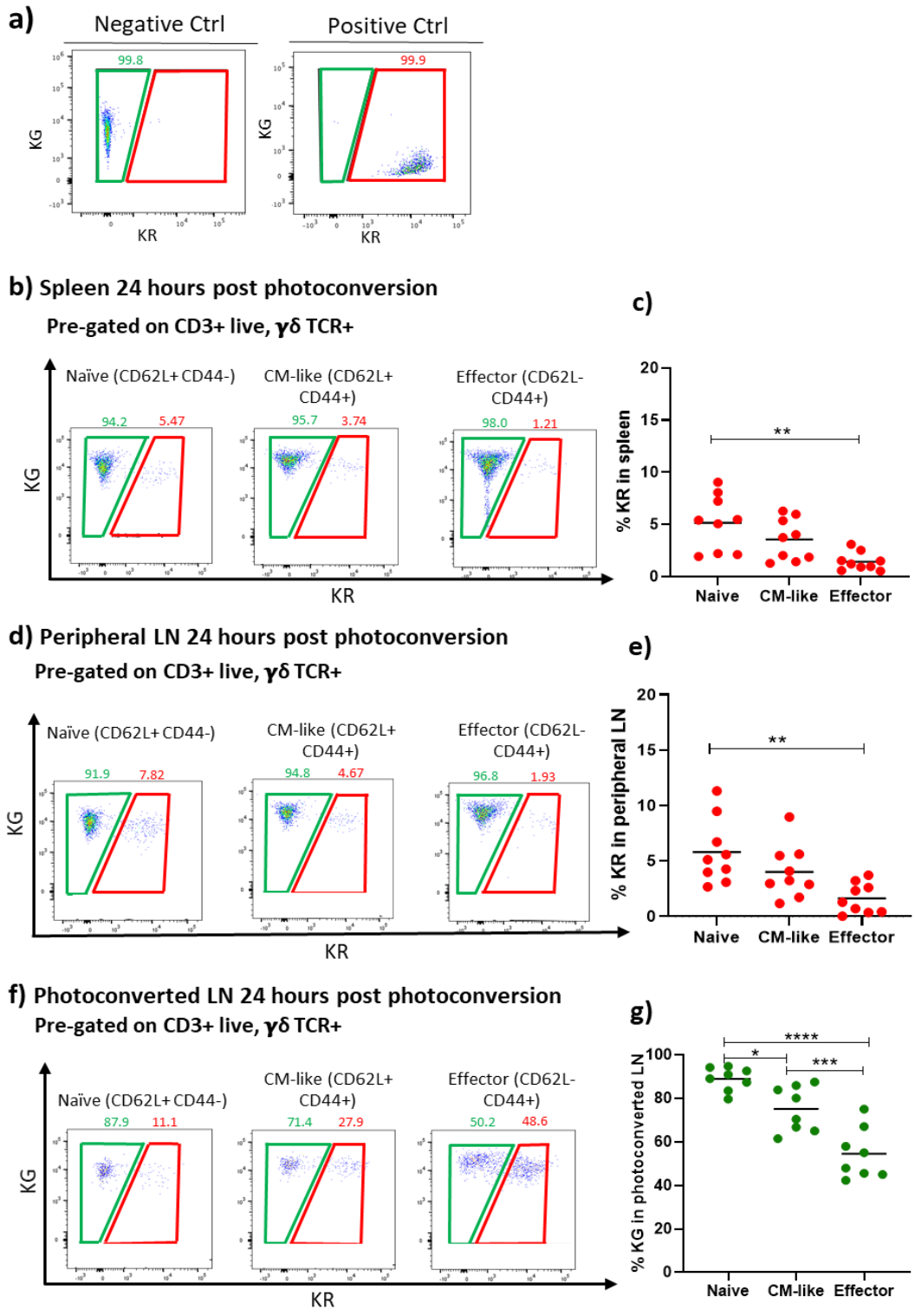

a)
Negative Ctrl
Positive Ctrl
99.8
99.9
KG
KR
b) Spleen 24 hours post photoconversion
Pre-gated on CD3+ live, γδ TCR+
Naïve (CD62L+ CD44-)
CM-like (CD62L+ CD44+)
Effector (CD62L- CD44+)
94.2
5.47
95.7
3.74
98.0
1.21
c)
% KR in spleen
Naive
CM-like
Effector
d) Peripheral LN 24 hours post photoconversion
Pre-gated on CD3+ live, γδ TCR+
91.9
7.82
94.8
4.67
96.8
1.93
e)
% KR in peripheral LN
f) Photoconverted LN 24 hours post photoconversion
Pre-gated on CD3+ live, γδ TCR+
87.9
11.1
71.4
27.9
50.2
48.6
g)
% KG in photoconverted LN

Figure 3.11: Naïve γδ T cells circulate around lymphoid tissue at a higher proportion than effector γδ T cells. Inguinal lymph nodes were photoconverted in Kaede mice as described in the methods. 24 hours after photoconversion the mice were sacrificed, the labelled lymph nodes, unlabelled lymph nodes and spleen were harvested and analysed with flow cytometry. In the spleen and unlabelled lymph nodes red cells represent cells migrated from the labelled lymph node. In the labelled lymph node green cells represent cells that have entered hat particular lymph node in the last 24 hours. Each data point represents an individual mouse and data were pooled from a minimum of 3 experiments. Data were analysed via one way ANOVA with Tukey's post hoc test or Kruskal Wallis with Dunn's post hoc test * p0.05 **p0.01 *** p0.001 **** p0.0001

a) An unlabelled Kaede lymph node with green cells (negative control) and a labelled lymph node showing photoconversion of all cells to red (positive control)

b) Representative FACS plots of unlabelled Kaede green (KG) and labelled Kaede red (KR) cells within naïve, effector and cm-like γδ subsets in the spleen 24 hours post photoconversion.

c) The percentage of naïve, effector and cm-like γδ labelled Kaede red (KR) cells in the spleen 24 hours post photoconversion.

d) Representative FACS plots of unlabelled Kaede green (KG) and labelled Kaede red (KR) cells within naïve, effector and cm-like γδ subsets in a peripheral unphotoconverted lymph node 24 hours post photoconversion.

e) The percentage of naïve, effector and cm-like γδ labelled Kaede red (KR) cells in a peripheral lymph node.

f) Representative FACS plots of unlabelled Kaede green (KG) and labelled Kaede red (KR) cells within naïve, effector and cm-like γδ subsets in a peripheral photoconverted lymph node 24 hours post photoconversion.

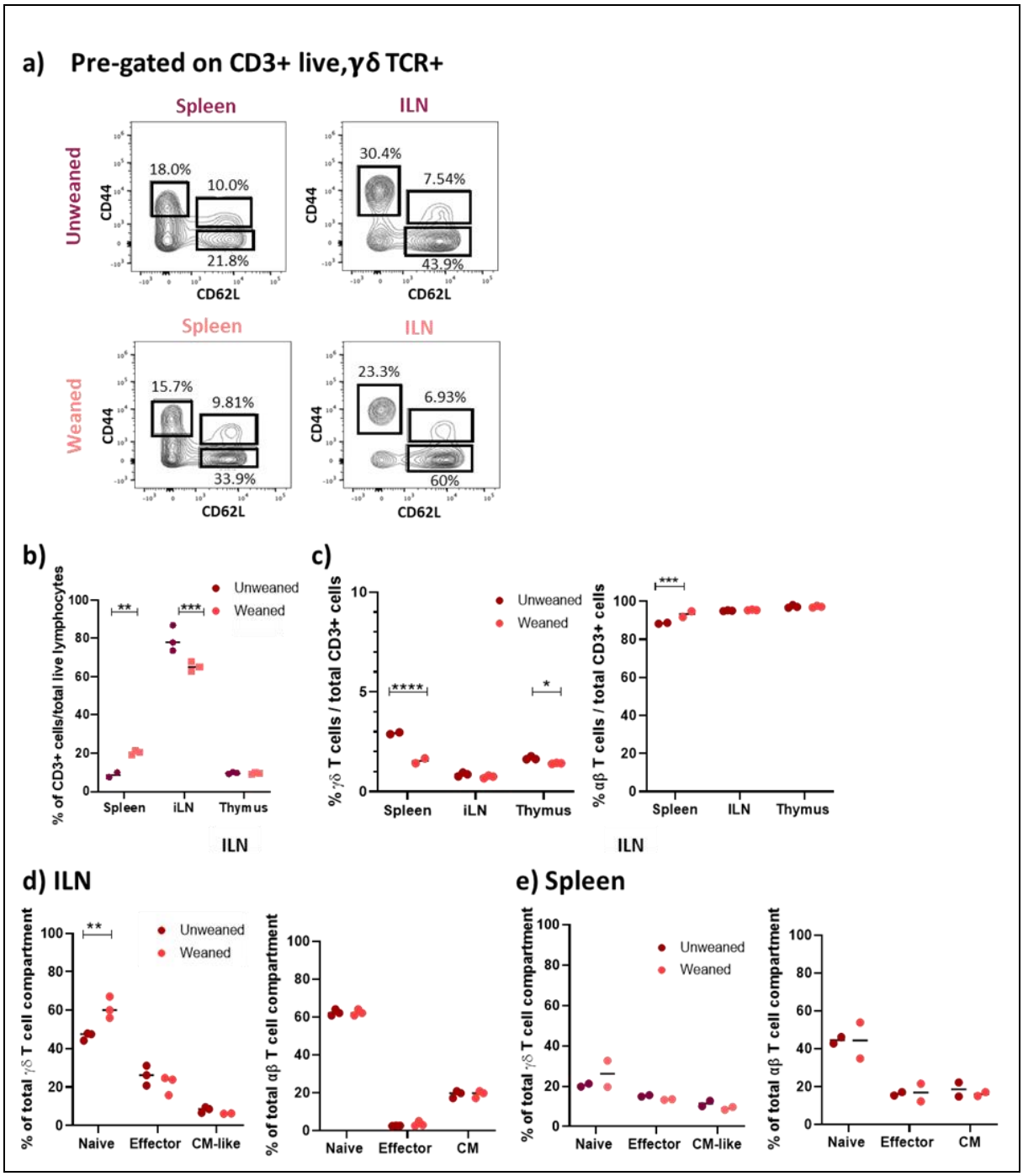
g) The percentage of naïve, effector and cm-like γδ unlabelled Kaede green (KG) cells in the photoconverted lymph node.

3.3.9 The effects of the microbiota and weaning on naïve, effector and cm-like γδ T cells

Having defined the phenotype, migration and composition of lymphoid γδ T cell populations, I wished to study how effector and cm-like γδ T cells populations were established in the absence of infection. I speculated if the microbiota was important in driving the presence of these populations in early life following antigen recognition by naïve γδ T cells.

We began by investigating the populations in the lymphoid tissue of mice before and after weaning onto solid foods, as an immune response to new pathogens during weaning is linked to the induction of some αβ T cell populations (Al Nabhani et al., 2019). I compared γδ T cells in the spleens of mice pre (2 weeks old) and post weaning (6 weeks or older). The percentage of total live CD3+ T cells increased in the spleen following weaning, however there was a decrease in the percentage of γδ T cells (Figure 3.12b), suggesting that the increase in live CD3+ T cells after weaning may be αβ T cell driven. In the ILN the percentage of total live CD3+ T cells decreased after weaning but γδ T cell populations did not change in size, reflecting that this is also αβ T cell driven (Figure 3.12b).

The overall trend of γδ T cell populations in lymphoid tissues was that the size of the populations were not altered following weaning (Figure 3.12c,d). However the gamma chain usage within each population changed significantly. In both the spleen and ILN, Vγ1 usage increased in the naïve γδ T cell compartment whilst Vγ1- Vγ4- usage decreased (Figure 3.12e,f). In the effector compartment, Vγ4 chain usage increased in both tissues whilst the Vγ1-Vγ4- usage decreased (Figure 3.12e,f). This suggests that after weaning there is an increase in the production and release of Vγ1 and Vγ4 cells into the lymphoid system with an accompanying decrease in other γ chains.


a) Pre-gated on CD3+ live,γδ TCR+
Spleen
ILN
Unweaned
Weaned
CD44
CD62L
18.0%
10.0%
21.8%
30.4%
7.54%
43.9%
15.7%
9.81%
33.9%
23.3%
6.93%
60%
b)
% of CD3+ cells/total live lymphocytes
Unweaned
Weaned
Spleen
iLN
Thymus
c)
% γδ T cells / total CD3+ cells
% αβ T cells / total CD3+ cells
ILN
d) ILN
e) Spleen
% of total γδ T cell compartment
% of total αβ T cell compartment
Naive
Effector
CM-like
CM

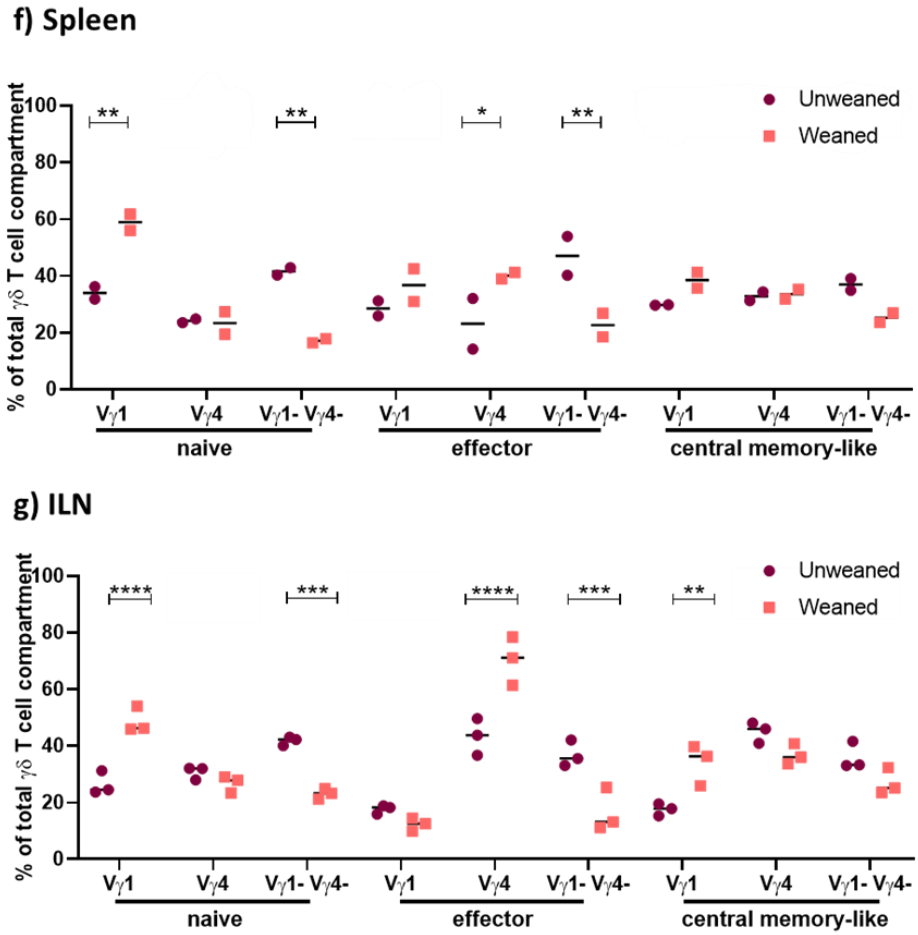


Figure 3.12: The effect of weaning on the naïve, effector and cm-like yδ T cell compartments. Tissue was harvested from unweaned 2 week old mice and weaned adult mice and analysed via flow cytometry. Each data point represents an individual mouse. A repeat of this experiment was not possible due to COVID-19. Data analysed via two way ANOVA with Tukey's post hoc test. * p0.05 **p0.01 *** p0.001 **** p0.0001

a) Representative flow plots of naïve, effector and cm-like yδ T cell compartments in the spleen and ILN of unweaned and weaned mice.

b) The percentage of live CD3+ T cells in the spleen (n=2), ILN (n=3) and thymus (n=3) of unweaned and weaned mice.

c) The percentage of yδ T cells and αβ T cells in the spleen (n=2), ILN (n=3) and thymus (n=3) of unweaned and weaned mice.

d) The percentage of naïve (n=3), effector (n=3) and cm-like (n=3) yδ T cells and αβ T cells in the ILN of unweaned and weaned mice.

e) The percentage of naïve (n=3), effector (n=3) and cm-like (n=3) yδ T cells and αβ T cells in the spleen of unweaned and weaned mice.

f) The percentage of Vy1 (n=2), Vy4 (n=2) and Vy1- Vy4- (n=2) cells within the naïve, effector and cm-like yδ T cell compartments in the spleen.

g) The percentage of Vy1 (n=3), Vy4 (n=3) and Vy1- Vy4- (n=3) cells within the naïve, effector and cm-like yδ T cell compartments in the ILN.

Following the discovery that naïve, effector and cm-like γδ T cell populations were not altered significantly by weaning, I wished to determine if a more dramatic perturbation in the microbiota may have an effect. Hence I compared γδ T cell populations in age-matched and strain-matched germ free (GF) and SPF mice. It was previously suggested that GF mice show reduced αβ T cells in the SLOs and gut (Mazmanian et al., 2005; Umesaki et al., 1993), however in our samples the percentage of γδ T cells and αβ T cells did not differ in the spleen, thymus, ILN and MLN between GF and SPF mice (Figure 3.13b,c).

The naïve, effector and cm-like γδ T cell subsets were also consistent between SPF and GF mice in all tissues (Figure 3.14b,c,d,e). Surprisingly therefore there was a substantial effector γδ T cell population even without the presence of a microbiota, suggesting their presence in young SPF mice is not driven by responses to the microbiome. Overall, these results from weaning and GF mice suggest that the microbiota does not play a large role in the generation and proportion of γδ T cell compartments in secondary lymphoid organs.

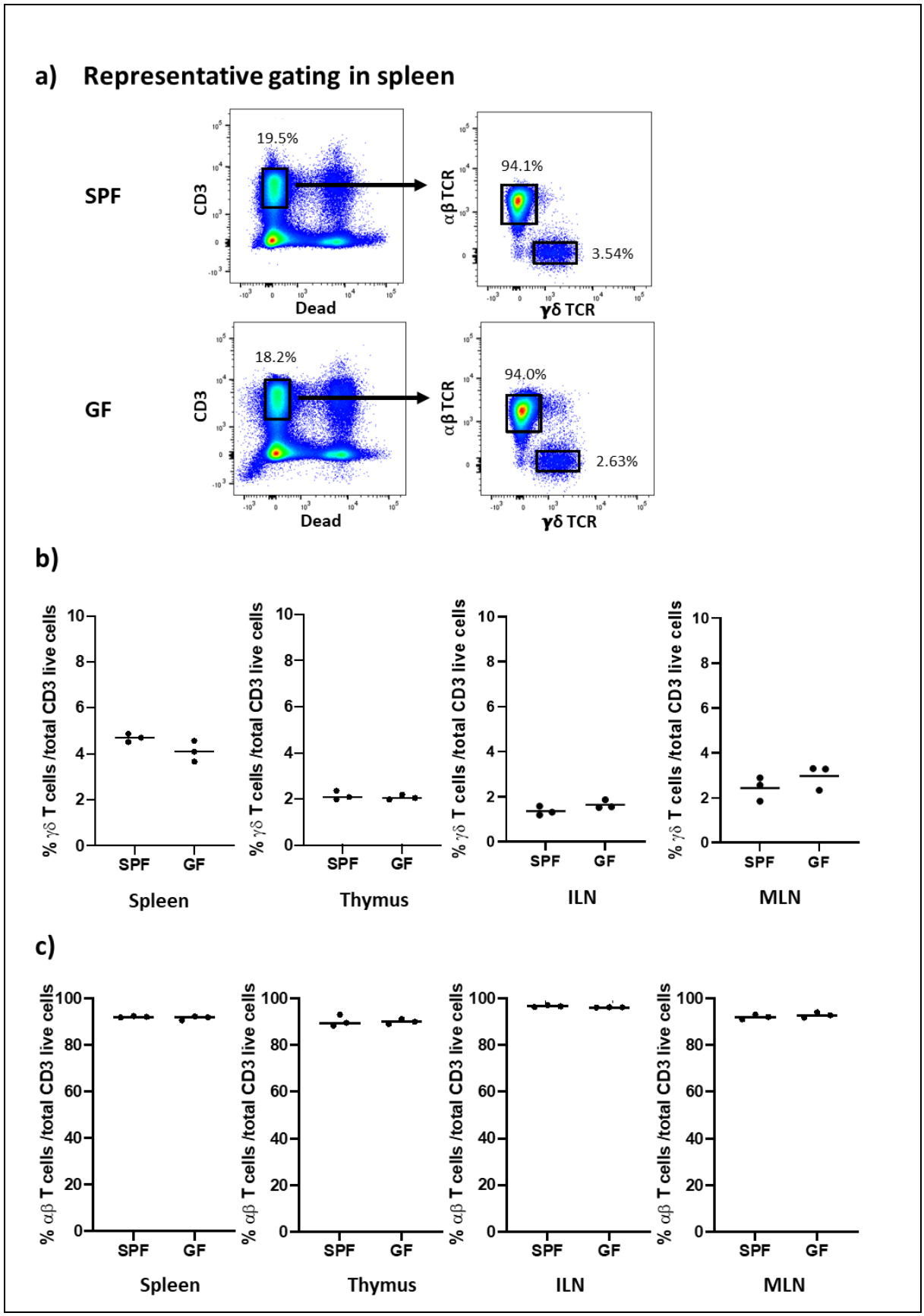
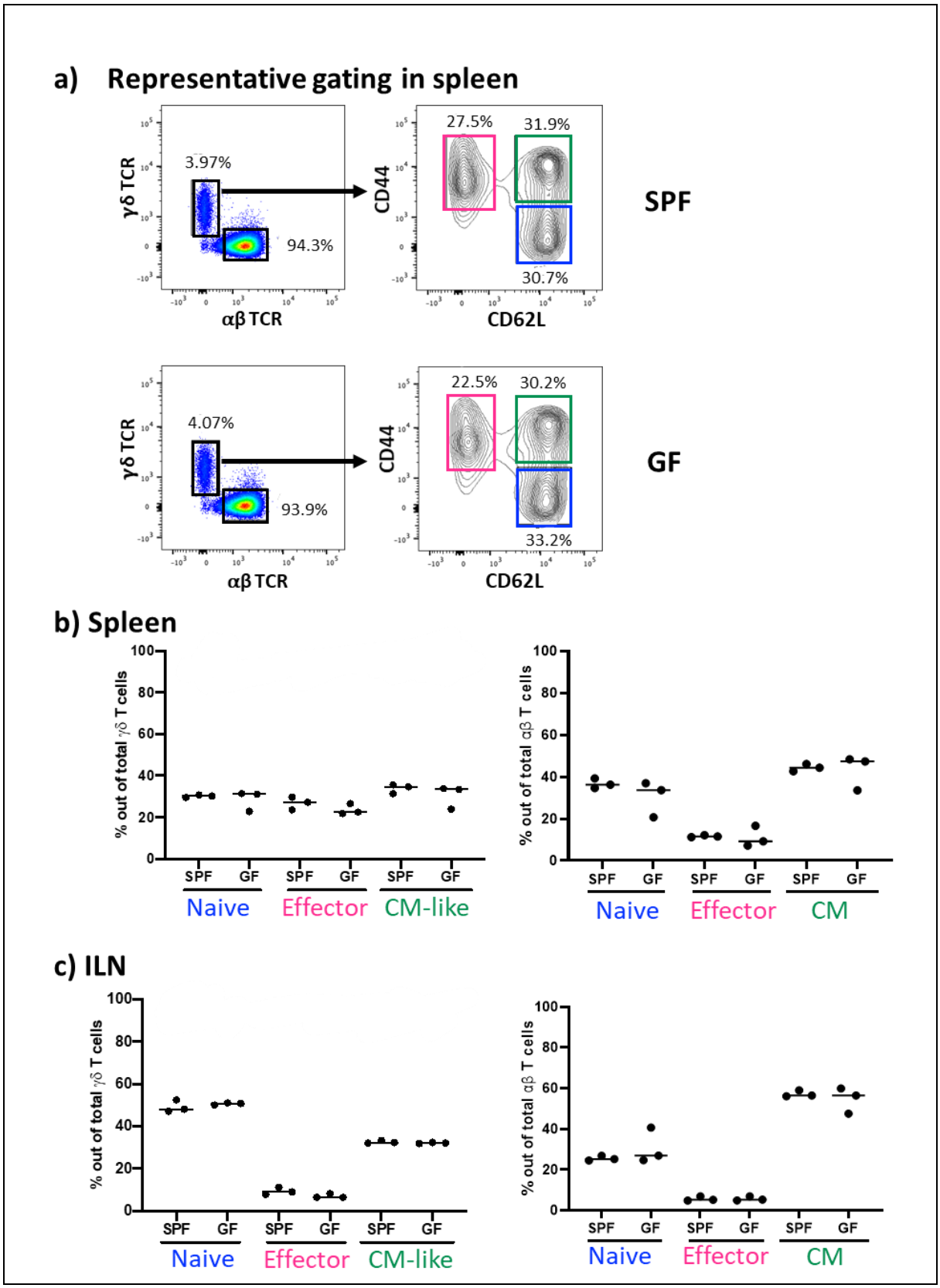

a) Representative gating in spleen
SPF
GF
CD3
Dead
19.5%
18.2%
αβ TCR
γδ TCR
94.1%
3.54%
94.0%
2.63%
b)
% γδ T cells /total CD3 live cells
SPF
GF
Spleen
Thymus
ILN
MLN
c)
% αβ T cells /total CD3 live cells

Figure 3.13: The proportion of yδ and αβ T cells are not significantly different between germ free and specific pathogen free mice. Tissue was harvested from GF and SPF mice and analysed with flow cytometry. Bars on graphs represent the median and each data point represents an individual mouse. A repeat of this experiment was not possible due to COVID-19. Data were analysed via an unpaired t-test or a mann whitney test. * p0.05 **p0.01 *** p0.001 **** p0.0001

a) Gating strategy for yδ and αβ T cells in the spleen of GF or SPF mice

b) The percentage of yδ T cells in the spleen, thymus, ILN and MLN of GF (n=3) and SPF (n=3) mice

c) The percentage of αβ T cells in the spleen, thymus, ILN and MLN of GF (n=3) and SPF (n=3) mice


a) Representative gating in spleen
γδ TCR
αβ TCR
3.97%
94.3%
CD44
CD62L
27.5%
31.9%
30.7%
SPF
4.07%
93.9%
22.5%
30.2%
33.2%
GF
b) Spleen
% out of total γδ T cells
% out of total αβ T cells
SPF
GF
Naive
Effector
CM-like
CM
c) ILN

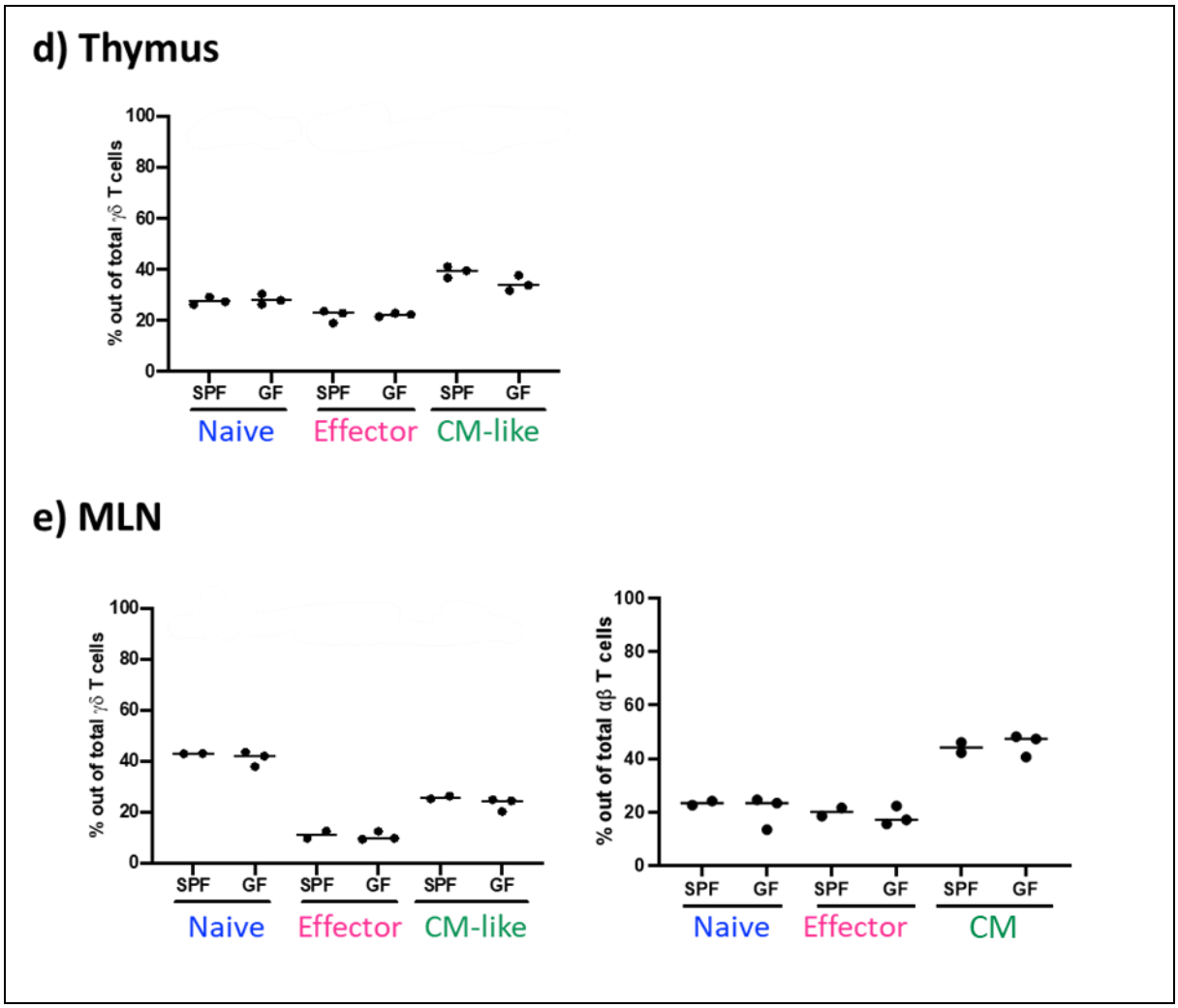


Figure 3.14: The proportion of naïve, cm-like and effector yδ T cells are not significantly different between germ free and specific pathogen free mice. Tissue was harvested from GF and SPF mice and analysed with flow cytometry. Each data point represents an individual mouse and data were analysed via two way ANOVA with Tukey's post hoc. * p0.05 **p0.01 *** p0.001 **** p0.0001. A repeat of this experiment was not possible due to COVID-19.
a) Gating strategy for naïve, effector and cm-like yδ T cells in the spleen of GF or SPF mice
b) The percentage of naïve, effector and cm-like yδ T cells in the spleen of GF (n=3) and SPF (n=3) mice. The percentage of naïve, effector and cm αβ T cells in the spleen of GF (n=3) and SPF (n=3) mice.
c) The percentage of naïve, effector and cm-like yδ T cells in the ILN of GF (n=3) and SPF (n=3) mice. The percentage of naïve, effector and cm αβ T cells in the ILN of GF (n=3) and SPF (n=3) mice.
d) The percentage of naïve, effector and cm-like yδ T cells in the thymus of GF (n=3) and SPF (n=3) mice.
e) The percentage of naïve, effector and cm-like yδ T cells in the MLN of GF (n=3) and SPF (n=3) mice. The percentage of naïve, effector and cm αβ T cells in the spleen of GF (n=3) and SPF (n=3) mice.

3.4 Discussion

In this chapter I phenotypically characterised murine γδ T cells to investigate adaptive-like populations. Hallmarks of an adaptive biology in αβ T cells are naïve T cell populations that are produced throughout life by the thymus and recirculate around lymphoid tissue in search of antigen. Upon recognition of cognate antigen and TCR driven activation, the cells clonally expand and transition from a naïve to an effector T cell phenotype. The effector T cells can access peripheral tissues to target the area of infection/inflammation. Following the resolution of the infection, long lived memory cells with expanded clones persist to protect against future infection. Using both WT and transgenic murine models I was able to study both phenotypic and functional features of murine γδ T cells and determine any adaptive-like features and answer questions that could not be studied in humans.

I have shown that naïve (CD62L+ CD44-) and cm-like (CD62L+ CD44+) γδ T cell populations are present in lymphoid tissue whilst not in peripheral tissue such as the gut. I also identify an effector (CD62L- CD44+) γδ T cell population that is present in both lymphoid and peripheral tissue. The naïve γδ T cell population has characteristic expression of surface markers also detected on naïve αβ T cells whilst lacking NK receptors and transcription factors associated with an effector state. This population also appears to be produced continuously by the thymus and circulates around the lymphoid tissue. These characteristics are hallmarks of a traditional adaptive αβ T cell subset and suggests that this γδ T cell population may have an adaptive-like biology in the mouse and have key similarities with naïve αβ T cells.

3.4.1 The presence of naïve, effector and cm-like phenotypic γδ T cells in murine tissue.

One feature of an adaptive biology is the existence of naïve, effector and memory T cell subsets. Adaptive T cells can transition over time from one subset to another in an antigen driven response. Naïve T cells clonally expand following activation, transition to an effector phenotype and then contract following pathogen clearance. The majority of effector T cells die whilst a small proportion transition to a memory phenotype. The presence of γδ T cell populations that share similarities with αβ T cell naïve, effector and memory populations, suggests they may share an adaptive reaction.

As mentioned above naïve and cm-like γδ T cell populations were only found in lymphoid tissue (Figure 3.3) where they had high levels of Vγ1 and Vγ4 chain usage (Figure 3.4). These gamma chains are of interest in an adaptive paradigm as they have previously been implicated in quasi-adaptive immune responses to murine CMV, Malaria and the model antigen Phycoerythrin (Khairallah et al., 2015; Mamedov et al., 2018; Sell et al., 2015; Zeng et al., 2012).

The effector γδ T cell population was present in both lymphoid and peripheral tissue and is dominated by Vγ4 and Vγ1- Vγ4- populations (Figures 3.3 and 3.4). This suggests that many of these cells are innate-like IL-17 γδ T cells which frequently express an Vγ6 or Vγ4 chain, or in the gut, an invariant Vγ7 population (Haas et al., 2012; Itohara et al., 1990). This was further confirmed when a subset of effector γδ T cells that have historic IL-17 production in adult IL-17-fate mapped mice were also mostly Vγ4 cells (Figure 3.5). The lack of a substantial innate-like IL-17 effector population in the MLN (McKenzie et al., 2018) may explain reduced Vγ4 effector γδ T cells in the MLN compared to the ILN or spleen (Figure 3.4).

Further phenotyping of the three populations identified their surface and intracellular expression of adaptive markers. The naïve subset expresses CD62L which suggests that they circulate around lymphoid tissue, potentially in the search of antigen. They also have low expression of NKRs and transcription factors linked to effector cells. CD27 is a marker used alongside CD45RA to identify naïve and effector CD8 and Vδ1 T cell populations in human PBMCs with naïve cells expressing both markers (Davey et al., 2017b; Tomiyama et al., 2002). This pattern was also detected in mice with a significantly high percentage of naïve and cm-like yδ T cells expressing CD27 compared to the effector subset in SLOs (Figure 3.6).

CD27 low expression on effector T cells may be due to their full differentiation and a decrease in replicative capacity. The loss of it on the surface indicates decreased telomerase activity and could suggest the progression towards proliferative senescence. However it is not definitively a marker of exhaustion or loss of function as it is now thought that stopping proliferation is a method to reduce molecular damage and to retain functional response (Henson et al., 2009; Larbi & Fulop, 2014; Reinke et al.,2013).

CD27 expression has also been shown to increase on T cells as they develop into memory populations from effector cells. It is hypothesised that it can be used as a marker to delineate effector populations that will apoptose and those that will develop into memory cells (Dolfi et al., 2008; Martin & Badovinac, 2018). Hence it is not surprising that our cm-like yδ T cell subset also has high CD27 levels. CD27 has also been used in mice to delineate the IFNy producing and IL-17 producing innate-like yδ T cells (Ribot et al., 2009, 2010). Interestingly the naïve population in our data also had high CD27 levels compared the effector population. However this marker cannot be used to fully delineate the two populations, given the potential multiple sub-populations within the effector gate.

The activatory marker CD69 was most prominently expressed in the effector γδ T cell population compared to the other subsets, though the percentage of positive cells was not large (Figure 3.6). CD69 is commonly found on lymphocytes in inflammation or following activation (Cibrián & Sánchez-Madrid, 2017). However it was expressed at low levels on most γδ T cell populations in the SLO (Figure 3.6). Interestingly, unlike other lymphoid tissue, the MLN had a substantial percentage of CD69+ effector γδ T cells. It may be that these cells have drained from the gut as gut γδ T cells regularly have high levels of CD69 expression (Do et al., 2017). CD69 is also a marker of tissue residency as it interferes with the sphingosine-1-phosphate receptor which is essential for tissue egress (Mackay et al., 2015). Hence CD69 expression on effector γδ T cells may also represent tissue residency in the MLN and the lack of it on naïve and cm-like cells could suggest recirculation potential.

Ly6C is a differentiation marker which increases as cells transition to the central memory compartment. It appears to be required for migration of the cells to lymph nodes and blocking it on CD8 TCM cells recapitulates the blocking of CD62L (Bamezai Anil, 2004; DeLong et al., 2018; Hänninen et al., 2011). It is not surprising therefore that it is most highly expressed on the cm-like γδ T cell subset (Figure 3.6). Ly6C+ CD44+ CD27+ γδ T cells have previously been described in murine lymph nodes and appear to share characteristics with the cm-like γδ T cell population characterised in this thesis. It was found that this Ly6C+ CD44+ subset had high expression of CD122, Fas ligand, CD137 and OX40 (Lombes et al., 2015). It would have been interesting to stain the cm-like γδ T cell population for these markers and determine if they are indeed the same population.

Ly6C also delineates between naïve and memory like γδ T cells with Ly6C- CD27+ γδ T cells having a naïve phenotype and Ly6C+ CD27+ γδ T cells being more cytotoxic. This was shown both through surface marker expression and single cell sequencing with the gene signature of each subset sharing similarities with naïve T cells and CD8 T cells, NK cells and mature human

γδ T cells respectively (Wiesheu et al., 2020). This is recapitulated in this thesis with the naïve γδ T cell population having low Ly6C expression and high CD27 expression (Figure 3.6). The small proportion of naïve γδ T cells that do express Ly6C could be undergoing homeostatic proliferation where for a short period of time they express memory markers such as Ly6C and CD44 before returning back to their naïve state (Goldrath et al., 2000).

Lymphoid naïve γδ T cells also no NKRs on their surface whilst effector and cm-like cells expressing NKG2D, NKG2A and NK1.1 (Figure 3.8). Their expression on effector γδ T cells is consistent with previous work as NK1.1 and NKG2D were shown to be on murine CD44+ γδ T cells (Haas et al., 2009; Strid et al., 2011). NKG2D is an activatory receptor expressed at low levels on naïve T cells and upregulated upon activation. Its ligands are expressed at higher levels on stressed or infected cells which allows recognition of non-self or loss of homeostasis (Raulet, 2003). NKG2A is an inhibitory receptor which can prevent terminally differentiated effector cells from releasing cytotoxic molecules, including Vy9 Vδ2 T cells (Angelini et al., 2011). In infection human Vδ2- T cells were mostly NKG2D positive but had reduced NKG2A expression (Couzi et al., 2009). In barrier sites such as the gut with a constant requirement for cytotoxic functions many Vδ2- cells also had low NKG2A expression (Mikulak et al., 2019) suggesting that it has an inhibitory effect when expressed.

Interestingly NKp46 expression was low in all three compartments in the spleen and ILN (Figure 3.8) so does not seem to be a delineating factor across the subsets in the SLOs of mice. The human blood Vδ1 compartment was mostly NKp46- but NKp46 expression could be induced by TCR engagement and cytokine stimulation and in the human gut many resident, cytotoxic Vδ1 T cells are NKp46+ (Correia et al., 2011; Mikulak et al., 2019). It appears however that NKp46 is not expressed in murine γδ T cells in the steady state, even within the effector subset.

Transcription factor staining recapitulated the patterns noted on surface marker expression. Effector and cm-like cells yδ T cells expressed high levels of T-bet and Eomes, transcription factors associated with an effector phenotype, in the spleen and MLN (Figure 3.7). These transcription factors are expressed in effector αβ T cells whilst not present in naïve αβ T cells (Pearce et al., 2003; Szabo et al., 2000). TCR stimulation of yδ T cell thymocytes increases T-bet expression, a decrease in RoRyt and drives a Th1 phenotype. In human yδ T cells, both T-bet and Eomes are markers of the effector Vδ1 compartment in human blood (McMurray et al, in preparation) and this is recapitulated in the murine effector subset.

Interestingly cm-like yδ T cells had the highest percentage of T-bet and Eomes expressing cells (Figure 3.7) which contrasts with CD8 TCM cells which express them at low levels (McLane et al., 2013). However, this study was in humans and so may differ from murine T cells. In mice, effector CD8 T cells that lack Eomes do not become TCM cells as they are not proficient at long term survival and hence there are few Eomes negative cells in the TCM pool (Banerjee et al., 2010; Li et al., 2013). This would suggest that Eomes is required for the transition to the TCM compartment and explains its high expression on cm-like yδ T cells.

One interesting angle would have been to explore RoRyt expression in lymphoid yδ T cells. RoRyt is an essential transcription factor for IL-17 expressing cells and is produced in a mutually exclusive manner to T-bet. It is expressed at high levels on IL-17+ yδ T cells and a lack of RoRyt reduced this population in both lymphoid tissue and mucosal tissue (Ivanov et al., 2006; Lochner et al., 2008; Shibata et al., 2011). It is likely that it would have been expressed at high levels in the effector subset due to the likely presence of IL-17 responders within that population.

In summary, a feature of an adaptive response is the presence of a naïve T cell population that transitions to effector cytotoxic cells. I identified three populations of yδ T cells that share

many characteristic surface and intracellular marker expression with αβ T cell subsets. To further explore the populations I explored the ability of the subsets to circulate around lymphoid tissue.

3.4.2 The ability of naïve γδ T cells to recirculate around lymphoid tissue

A key feature of adaptive biology is the ability of naïve T cells to circulate throughout lymphoid organs to increase their chance of interacting with cognate antigen and becoming activated. For murine γδ T cells, all of the naïve, effector and cm-like subsets were present in the spleen and lymph nodes, whilst only the effector subset can access the gut and liver (Figure 3.3). The presence of effector γδ T cells in lymphoid tissue is surprising given their lack of CD62L expression. They may lose expression of CD62L and become effector T cells upon entering lymphoid tissue as in αβ T cells. Some effector γδ T cells have previously been shown to be able to move between tissues such as the skin and the ILN in steady state conditions. However they enter the tissue at slower rates than CD62L+ populations such as central memory T cells (Gray et al., 2011; Jiang et al., 2017; McKenzie et al., 2018; Nakamizo et al., 2015).

The expression of CD62L on both naïve and cm-like γδ T cells indicate they have the ability to circulate between lymphoid tissues. Previous work has shown that CD62L+ CD44- γδ T cells in murine lymph nodes have a higher expression of lymphoid migratory genes compared to the effector CD62L- CD44+ subset (Ugur et al., 2018). Using the fluorescent Kaede mouse, developed by (Tomura et al., 2008), I was able to photoconvert a lymph node and track the cells from that lymph node to other tissues in the body. I assessed the migration of these cells and showed that naïve and cm-like γδ T cells could circulate through the LNs and the spleen. I also determined that within a LN, the majority of naïve and cm-like cells were replaced within 24 hours (Figure 3.11). Therefore there is continuous ingress and egress throughout lymphoid tissue in both of these subsets.

We also noted that a higher percentage of effector γδ T cells remained in a lymph node for 24 hours compared to the other subsets (Figure 3.11). Therefore they showed a reduced ingress into lymphoid tissue and a longer retention time within the tissue. Some may become resident following activation in the tissue. However, the 24 hour time point in our study is too short a period to determine whether it is indeed a tissue resident population or whether they simply migrate at a slower rate than the naïve and cm-like subsets. The fluorescent signal in the Kaede model is only retained for 7 days and hence it is difficult to examine the populations for a long period of time. A recent study however analysed the displacement of CD62L- CD44+ effector γδ T cells in a longer lasting fluorescent mouse model. They analysed the population over a period of 28 days and found 40% of γδ effector cells remained within the LN of interest (Ugur et al., 2018).

Alongside this however, there was also a clear population of effector γδ T cells that left the photoconverted LN within 24 hours (Figure 3.11), suggesting there may be effector subpopulations divided by their speed of migration. Further phenotyping of these sub populations within the effector γδ T cell compartment would have been interesting. Future work is also needed to determine whether migrating effector γδ T cells travel to peripheral sites if homing to these tissues is instructed in the LN.

In conjunction with investigating the migratory properties of the three populations, their production in the thymus was also studied. Consistent production of T cells by the thymus is essential to maintain TCR diversity in the periphery (Yager et al., 2008). In murine αβ T cells, naïve cells are continuously produced and released by the thymus throughout the life of the mouse and circulate in the periphery before dying or becoming activated (den Braber et al., 2012). To elucidate the age of γδ T cell subsets, RAG recombinase reporter mice were used. RAG recombinase is an enzyme required for VDJ recombination and its expression in this model is detected by a GFP reporter protein that decays over 2 weeks. Therefore the strength

of the RAG recombinase signal correlates with the time since VDJ recombination and production of the cells.

The naïve γδ T cell population had a significantly higher percentage of GFP+ cells in the spleen, thymus and ILNs compared to effector and cm-like subsets (Figure 3.10). Therefore a significantly higher population of the naïve compartment where continuously produced by the thymus and recapitulated previous data on naïve αβ T cells. In adult mice the naïve T cell pool is continuously replenished through thymic output, in contrast to adult humans where the peripheral naïve T cell pool is maintained by proliferation in the periphery (den Braber et al., 2012).

The lower GFP+ populations of effector and cm-like cells indicates they have been in the periphery for longer. This could be through their transition from naïve to an effector/cm-like phenotype, however this is not definitive. γδ T cells in the thymus were found to take up BRDU rapidly before release into the periphery, followed by differentiation into a mature naïve CD62L+ CD44- phenotype and then dying, gaining an effector phenotype or becoming long lived mature naïve cells with a very slow turnover (Tough & Sprent, 1998). It may also be that during their transition to an effector/memory phenotype their turnover increases and this dilutes their GFP+ signal. Staining the cells with a proliferation marker such as Ki67 could help answer this angle.

Intriguingly naïve γδ T cells had a higher percentage of GFP+ cells than naïve αβ T cells in the lymph nodes and spleen (Figure 3.10). This is consistent with previous data showing that the γδ compartment has a higher turnover than αβ T cells (Tough & Sprent, 1998). Unlike γδ T cells, after development mature αβ T cells egress from the thymus slowly over 1-2 weeks (Egerton et al., 1990). The lower levels of GFP+ αβ T cells in the periphery could be due to them being retained in the thymus for longer, allowing their GFP signal to begin to degrade

before they reach other lymphoid tissue. This argument is also supported by the comparable levels of GFP+ γδ T cells and αβ T cells in the thymus, suggesting that γδ T cells do not express inherently higher levels.

The low RAG expression in the effector γδ T cells may be affected by the presence of innate-like effector γδ T cells in the population. Many of these cells only produced only during embryogenesis and the perinatal period (Kashani et al., 2015; Shibata et al., 2008) and hence have not recently egressed from the thymus. The effector population in the thymus with RAG expression may represent Vγ4 and Vγ1 innate-like cells that are produced throughout life with more varied TCR sequences and pairings (Carding & Egan, 2002).

The cm-like γδ T cell compartment also had a significantly lower percentage of GFP+ cells compared to the naïve γδ T cell subset in SLOs (Figure 3.10). This is as expected as central memory T cells are long lived cells that retain their ability to circulate around the lymphoid system and are generated following an antigen driven immune response (Sallusto et al., 1999; Wherry et al., 2003). They also were not present as a clear population in the thymus (Figure 3.10) which also suggests they are produced in the periphery.

Unexpectedly cm-like γδ T cells expressed a higher levels of RAG compared to the effector subset. This is surprising as memory cells are thought to originate from effector cells and are produced at a later time frame following T cell activation (Pennock et al., 2013) and therefore would have lower RAG expression than the effector subset. Interestingly however recent modelling and mouse model work has suggested that in healthy mice some naïve αβ T cells differentiate into a central memory population without a need for antigen stimulation (Gossel et al., 2017). Therefore some cm-like cells may transition from naïve T cells and undergo less proliferation than effector cells.

Hence, the naïve γδ T cell population shows further characteristics of an adaptive biology with the continuous production by the thymus throughout life. This was also accompanied by their ability to circulate around lymphoid tissue, could be for the detection of antigen. The very small populations of RAG+ cm-like and effector γδ T cells suggests they may develop in the periphery and are not produced straight from the thymus.

3.4.3 The generation of effector and cm-like γδ T cell subsets in the mouse

A key feature of an adaptive biology is antigen driven activation of naïve T cells through their TCR and their transition to an effector phenotype. In this thesis, the TCR repertoire of both naïve and effector γδ T cell subsets was explored in the spleen and MLN. In αβ T cell biology, memory and effector subsets display expanded clonotypes with identical sequences and chain pairing which is noted also in adaptive effector Vδ1 cells in human blood and tissue (Davey et al., 2017c; Hunter et al., 2018).

However in the mouse both naïve and effector γδ T cells had a diverse TCR repertoire with little clonal focusing. One CDR3 DV5 sequence did recur in the effector subset in the spleen which was represented in the increase in DV5 chain usage in this population compared to naïve subset (Figure 3.9). There was also a corresponding recurring gamma sequence that had expanded in the spleen. In this experiment it was not possible to determine paired gamma and delta sequences, so it is not possible to say if these were expressed together each time. However, these sequences were identified as also being expressed together by a Vγ4Vδ5 innate like IL-17 producing subset (Kashani et al., 2015), so it is likely these were matched. It would also have been interesting to sequence the TCRs of cm-like γδ T cells to see if they shared similarities to either subset and if they have greater clonal focussing than effector cells.

It could be that cm-like are more adaptive and have expanded clones whilst effectors are more innate-like.

Alongside the lack of clonal expansion in the effector subset, the sequences in both subsets tend to be germline and simple, particularly in the gamma chain (Appendix 1). Some sequences are also present between naïve and effector subsets and across tissues. Hence it is unlikely that each TCR sequence is antigen specific and can recognise only one cognate stimulus. The low numbers of sequences analysed may have limited this analysis as it does not exclude the possibility of some complex TCR sequences that are clonal in the effector subset. It may be that the simple and frequent sequences are very common, whilst more antigen specific, complex sequences are rarer within the populations. Bulk deep sequencing would have recognised rarer sequences and it would have been an interesting avenue to pursue if time had allowed it.

The lack of clear clonal expansions and the use of simple germline sequences suggests some effectors do not expand and transition to their phenotyping after the triggering of their TCR. The presence of naïve TCR sequences unexpanded in the effector TCR repertoire and across tissues is indicative of the expression of simple sequences that require little recombination and are produced frequently due to its reduced complexity. It may also be due to steady state transition of naïve cells to an effector pool. In infection-free mice, αβ T cells transitioned from the naïve to effector and cm compartments at a consistent rate (Gossel et al., 2017).

Alongside the finding that the TCR repertoire does not indicate antigen specific activation of clones in the generation of the effector γδ T cell subset, I noticed that even young healthy SPF mice had sizable populations of effector and cm-like γδ T cells. I wondered if this expansion required signals from commensal bacteria or if their generation was antigen independent. The microbiota has been shown to drive the maturation and function of immune cells. Its

composition is highly variable early in life and sensitive to environmental changes before stabilising. This limited temporal window is hypothesised as the period of time when the immune system can be altered in a durable fashion that lasts throughout life (Gensollen et al., 2016; Palmer et al., 2007; Yatsunenko et al., 2012).

In germ free mice, there are much lower numbers of αβ intraepithelial T lymphocytes in the gut compared to WT controls and antigen activated cells were not able to transition into memory cells and had reduced function (Bachem et al., 2019). Interestingly the αβ T cell population could be rescued later in life by the introduction of commensal bacteria to the gut, however the γδ T cell population never recovered (Umesaki et al., 1993). This links with data proving that the Vγ7 IEL population had a fixed temporal development window early in life (Di Marco Barros et al., 2016). The SLOs of GF mice exhibit structural defects in lymphoid architecture, reduced CD3+ T cells and a lack of expanded T cells (Bauer et al., 1963; Hrncir et al., 2008; Kernbauer et al., 2014).

To explore the effects of the microbiota on our naïve, effector and cm-like γδ T cell populations, I used two experimental methods: studying mice pre and post weaning and using GF mice. Following weaning, mice are exposed to solid food and to a wide range of microbes which drives changes in the microbiota. These changes cause a large immune perturbation which has a set window in time (Al Nabhani et al., 2019). These two methods allowed us to observe what may happen following changes in the microbiota compared to an absence of one.

Following weaning, the population of CD3+ live cells did increase, however this was reflected in the increase in αβ T cells (Figure 3.12). Much of this could have been the CD4 T regulatory population which expands following weaning in the gut (Nabhani et al., 2019), it is unclear whether such increases are present in other tissue. Within the γδ T cell population, the

proportion of naïve, effector and cm-like γδ T cells did not change significantly overall in the SLOs (Figure 3.12).

In GF mice there was also no significant differences in the 3 populations in the SLOs or thymus compared to SPF littermates (Figure 3.14). This suggests that the microbiota is not instrumental to the development and size of the naïve, effector or cm-like γδ T cell populations in the SLOs. This corroborates previous work in the gut which shows that the development and maturation of the Vγ7 tissue resident subset occurs early in life and is independent of microbial stimuli (Di Marco Barros et al., 2016). However this is not conclusive as other innate-like subsets require the microbiota for their development and function, such as IL-17 producing γδ T cells in the liver and lungs (Cheng et al., 2014; Duan et al., 2010; Li et al., 2017). This indicates that whilst some of the IL-17 producing tissue resident innate subsets require the microbiota, the lymphoid populations characterised here are less dependent on it.

Interestingly following weaning naïve γδ T cells increased their Vγ1 usage and decreased in Vγ1- Vγ4- usage and effector γδ T cells had higher Vγ4 chain usage (Figure 3). This may be due to the waves of development for different γδ T cell subsets expressing varied Vγ chains. Systemic Vγ1 and Vγ4 chains are produced consistently through life whilst many Vγ1- Vγ4- subsets such as Vγ6 are produced during embryogenesis (Carding & Egan, 2002). Hence some of the changes in chain usage may be temporally driven instead of a microbiota related phenomenon. Equally however, some of the innate-like subsets are dependent on the presence of set microbial populations such as the Vγ4+ IL-17 producers in the dermis that require *Corynebacterium accolas* for their survival (Ridaura et al., 2018). It may be that some of the bacterial population changes following weaning favour some Vγ4 and Vγ1 innate-like populations and allow their establishment.

One aspect that could have been explored is the effect of diet on the naïve, effector and cm-like γδ T cells in GF mice. Many GF mice are fed food with low levels of LPS due to difficulty in achieving complete decontamination. This has been shown to alter CD3 population size and increase expansion of T cells in the MLN (Hrncir et al., 2008). It may be that the GF mice used in this experiment had actually ingested low levels of LPS and this drove the expansion and development of the immune system independent of the microbiota. This may have reduced the changes visible between SPF and GF mice.

It would have also been interesting to look at the gut of weaned and GF mice alongside their SLOs. However for γδ T cells the microbiota is not necessary for γδ T cells to home to the intestine (Antonio Bandeira et al., 1990) or skin (Naik et al., 2012) but the lack of a microbiota may have an effect on γδ T cells once they reach the tissue. The gut resident Vγ7 population's development is not dependent on the microbiota (Di Marco Barros et al., 2016) but less prominent populations may be microbiota reliant. In GF mice, gut αβ T cells retain a naïve phenotype and do not transition to an effector phenotype in the absence of commensal bacteria (Williams et al., 2006). An innate-like effector γδ T cell population was found to be decreased in the gut of GF mice and the microbiota has also been suggested as being important in the activation of these cells (Duan et al., 2010; Li et al., 2017). It would also have been interesting to test the functional capability of these cells by in vitro stimulation ex vivo. This would have allowed us to compare their health and functionality rather than just their number. Another limitation of this section is the small sample size for both pre/post weaning and GF mice. Unfortunately these experiments were intended to be repeated but the COVID-19 pandemic did not allow us to source the mice before my thesis submission. Therefore to further strength this data and to be able to draw confident conclusions repeat experiments would have to be performed.

3.5 Conclusion

In summary, in this chapter I have characterised phenotypic, migratory and developmental features of candidate populations in an attempt to establish if adaptive-like populations exist in mice. I comprehensively analysed the murine γδ T cells in secondary lymphoid and peripheral tissue. I identified 3 distinct γδ T cell populations in the SLOs of mice: naïve (CD62L+ CD44-), effector (CD62L- CD44+) and cm-like γδ T cells (CD62L+ CD44+) that showed hallmarks of adaptive immunity. I explored the populations through their surface and intracellular protein expression, migratory ability, egress from the thymus and retention in the periphery and their colonisation of the gut. I also investigated their clonal repertoire in both the lymph nodes and spleen, which did not show evidence of clonal expansion in effector compartments.

Following a well-rounded initial investigation in steady state tissue, I thought the best way to explore this further would be within an infection model to examine if the cells responded in an adaptive-like manner. Hence I examined their responses to both acute and chronic infections and that forms the basis of the following chapter.

Chapter 4 – The response of lymphoid murine γδ T cells in infection.

4.1 Introduction

γδ T cells are important in pathogen clearance and establishing an effective immune response in many infections. Frequently in murine models, it is innate-like γδ T cell subsets that respond, acting as a first line of defence and reducing the spread of infection (Hayday, 2009). In a model of *Staphylococcus aureus* infection, the Vγ5 DETC subset can rapidly produce IL-17 within 8 hours of infection, recruiting other immune cells and clearing infection (Cho et al., 2010). Epithelial Vγ6Vδ1 T cells protect against intravaginal colonisation in *Herpes simplex* infection and against *E. coli* dissemination in the peritoneum through the rapid production of IFNγ (Nishimura et al., 2004; Shibata et al., 2007).

In other murine infections γδ T cells have been suggested to make adaptive-like responses. In *Plasmodium chabaudi* infection, Vγ1Vδ6.3 cells expand late in infection following the resolution of acute parasitaemia. They were essential to stop its recurrence via their expression of macrophage colony stimulating factor and chemokines such as CCL3/5 that recruited other immune cells. The authors argued that the delayed response of the Vγ1Vδ6.3 cells and the specific expansion of this subset within the γδ T cell compartment were indicative of an antigen-specific adaptive response (Mamedov et al., 2018). However TCR repertoire analysis indicated these expansions were oligoclonal and they featured a commonly occurring delta chain sequence. An alternative explanation for the expansion of the Vγ1Vδ6.3 subset is an innate-like expansion of multiple common clones (Hayday, 2019).

CMV has also been proposed to generate an adaptive-like γδ T cell response. γδ T cells are required for the control of CMV load and reduction of viral damage in the liver, lungs and spleen. Following infection both Vγ1 and Vγ4 subsets expand to form long lived, cytotoxic clones with CDR3 gamma clonotypes not detected commonly expanded in healthy mice. Also interestingly, γδ T cells from previously infected donor mice could control viral replication in

naïve recipient mice suggesting a form of memory response (Khairallah et al., 2015; Sell et al., 2015).

In the previous chapter I identified naïve, effector and central memory-like yδ T cell populations that shared phenotypic similarities with αβ T cells. I wished to investigate if they had an adaptive-like response to well characterised bacterial infections models. I studied their response to a rapid, acute infection (attenuated *Listeria monocytogenes*) and a chronic infection model (*Salmonella typhimurium*). *L. monocytogenes* is a gram-positive bacterium that generates rapid responses in mice infected intravenously. The bacteria spreads swiftly to the spleen and liver and becomes engulfed by macrophages (Portnoy et al., 2002). Innate immune cells such as NK cells, granulocytes and monocytes control the infection in the spleen and liver at early stages but T cells are required to clear it (Bhardwaj et al., 1998; Kang et al., 2008). By day 8 post infection the effector αβ T cell response is at its peak, followed by a rapid contraction phase and the clearance of the pathogen (Obar et al., 2011; Pepper et al., 2010a).

Previous work with yδ T cells in *L. monocytogenes* has focused on innate-like murine subsets. In the MLN, an effector, IL-17 and IFNy producing Vy6Vδ1 population expanded following infection via oral administration and was systemic and in the gut within a week. They remained in the MLN and gut following pathogen clearance and upon rechallenge responded at a more rapid rate, indicative of a recall response. Yet the TCR repertoire of the expanded cells was semi-invariant with TCR sequences frequently present in the steady state (Romagnoli et al., 2016; Sheridan et al., 2013; Simonian et al., 2009). Recently however deep sequencing of the Vy6Vδ1 responders to oral *L .monocytogenes* showed a small proportion of non-canonical Vδ1 sequences expanded, which may indicate more of an adaptive-like response (Khairallah et al., 2021). Therefore the response to infection may be multifaceted within each yδ T cell subpopulation.

S. typhimurium is a gram-positive bacterium that infects the host via a food-borne pathway. In humans the bacteria colonise the epithelium of the gut, reside in macrophages and cause localised inflammation. In rare cases the bacteria breaches the mucosal barrier it can also cause bacteraemia (Santos et al., 2009). Wild type *S. typhimurium* does not colonise the gut of healthy mice, hence mice must be pre-treated with an antibiotic such as Streptomycin. This promotes gut inflammation and bacterial colonisation and is more representative of human disease. The antibiotic is thought to clear the gut of commensal bacteria and produce a less hostile environment for the ingested bacteria (Barthel et al., 2003; Que et al., 1986).

Following the use of an antibiotic to drive *S. typhimurium* gut colonisation, the infection becomes systemic in mice. The bacteria breaches the gut epithelial barrier and spreads to the peyer's patches, MLN, liver and spleen by 24 hours post infection. However the majority of the tissue damage remains in the gut (Barthel et al., 2003; Coburn et al., 2005; Collins, 1972; Philip Carter & Collins, 1974). αβ T cells are important in the defence against *S. typhimurium* with bacteria specific effector Th17 and Th1 cells expanding and producing IL-17, T-bet, TNFα and IFNy (Mittrücker et al., 2002; Pham & McSorley, 2015; Salazar-Gonzalez et al., 2006; Srinivasan et al., 2004).

In humans, Vy9Vδ2 blood yδ T cells are expanded in donors infected with *S. typhimurium* and respond to it *in vitro* (Hara et al., 1992). The expansion of this subset is also noted in macaques following vaccination with *S. typhimurium* and activated yδ T cells can be found in the MLN of infected calves (Hedges et al., 2007; Workalemahu et al., 2014). These findings indicate yδ T cells play a role in the response to *S. typhimurium* in many species. However little has been done in mice so their importance in the murine immune response remains unclear.

4.2 Project aims in this chapter

Following the identification and characterisation of naïve, effector and cm-like yδ T cell populations in steady state conditions, I wished to study these subsets in the context of both acute and chronic infection. I hoped to further elucidate their characteristics in both lymphoid and peripheral tissue and to determine whether they exhibited traits of an adaptive immune response.

Our aims were:

- Identify the proportion of naïve, effector and cm-like yδ T cells that respond to *L. monocytogenes* and *S. typhimurium.*
- Study the production of IFNy by effector and cm-like yδ T cells throughout *L. monocytogenes* and *S. typhimurium* infection.
- Explore the role of the TCR in the activation of yδ T cells that respond to *L. monocytogenes* and *S. typhimurium.*
- Characterise the TCR repertoire of yδ T cell responders following *L. monocytogenes* and *S. typhimurium* infection.

4.3 Results

4.3.1 The response of yδ T cells in murine *L. monocytogenes* infection

To investigate the response of naïve, effector and cm-like yδ T cells, mice were infected with attenuated *L. monocytogenes,* which models an acute infection. Following IV administration the bacteria disseminated to the liver and spleen before spreading systemically. It can be cleared within a week due to its attenuated nature (Pepper et al., 2010b; Pepper & Jenkins,

2011; Portnoy et al., 2002). It is attenuated because it is ActA deficient, a bacterial protein required for actin polymerisation and the motility needed to move between cells (Ertelt et al., 2009; Portnoy et al., 2002).

We began by investigating the very early responses to *L. monocytogenes* to determine if γδ T cells showed an immediate innate-like response. Using dual cytokine reporter mice, I was able to monitor changes in cytokine levels without requiring artificial stimulations. At one day post infection a sub-population of both αβ and γδ T cells produce IFNγ+ in the spleen (Figure 4.1a). However, the increase in the percentage of these IFNγ+ cells post infection was only significant in the γδ T cell compartment (Figure 4.1b). Within the IFNγ+ cells, γδ T cells were a significantly higher proportion than the αβ T cells (Figure 4.1c), suggesting that the early IFNγ responses to the bacteria are heavily driven by γδ T Cells au lieu of αβ T cells. This was also seen in IL-17+ cells which only significantly increased in the γδ T cells one day post infection (Figure 4.1d). The immediate responses are in reaction to bacterial burden which peaks in this strain of *L. monocytogenes* between 12 and 36 hours post infection (Porter & Harty, 2006).

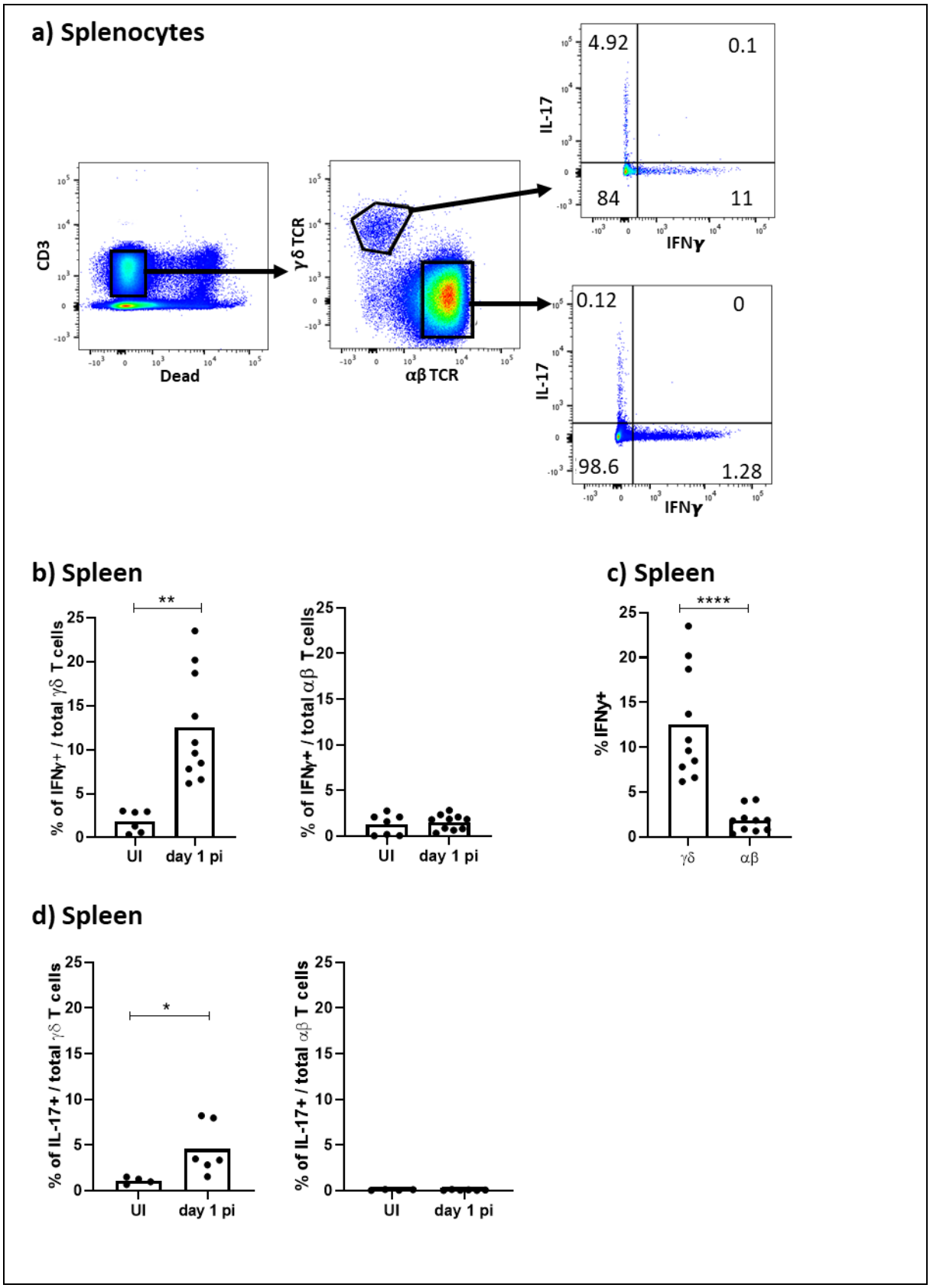

a) Splenocytes
CD3
Dead
γδ TCR
αβ TCR
IL-17
IFNγ
4.92
0.1
84
11
0.12
0
98.6
1.28
b) Spleen
% of IFNγ+ / total γδ T cells
% of IFNγ+ / total αβ T cells
UI
day 1 pi
**
c) Spleen
% IFNγ+
****
γδ
αβ
d) Spleen
% of IL-17+ / total γδ T cells
% of IL-17+ / total αβ T cells
*

Figure 4.1: A proportion of γδ T cells respond within 24 hours to Listeria monocytogenes infection. Great Smart mice (dual IFNy and IL-17 reporters) were infected intravenously with attenuated *L. monocytogenes.* Tissue was then harvested after 24 hours from the spleen as described in the methods and analysed via flow cytometry. Each data point represents an individual mouse and data were pooled from a minimum of 3 experiments. Data was analysed via an unpaired t-test * p0.05 **p0.01 *** p0.001 **** p0.0001

a) Gating strategy for IL-17 or IFNy expressing cells in the γδ or αβ T cell populations.

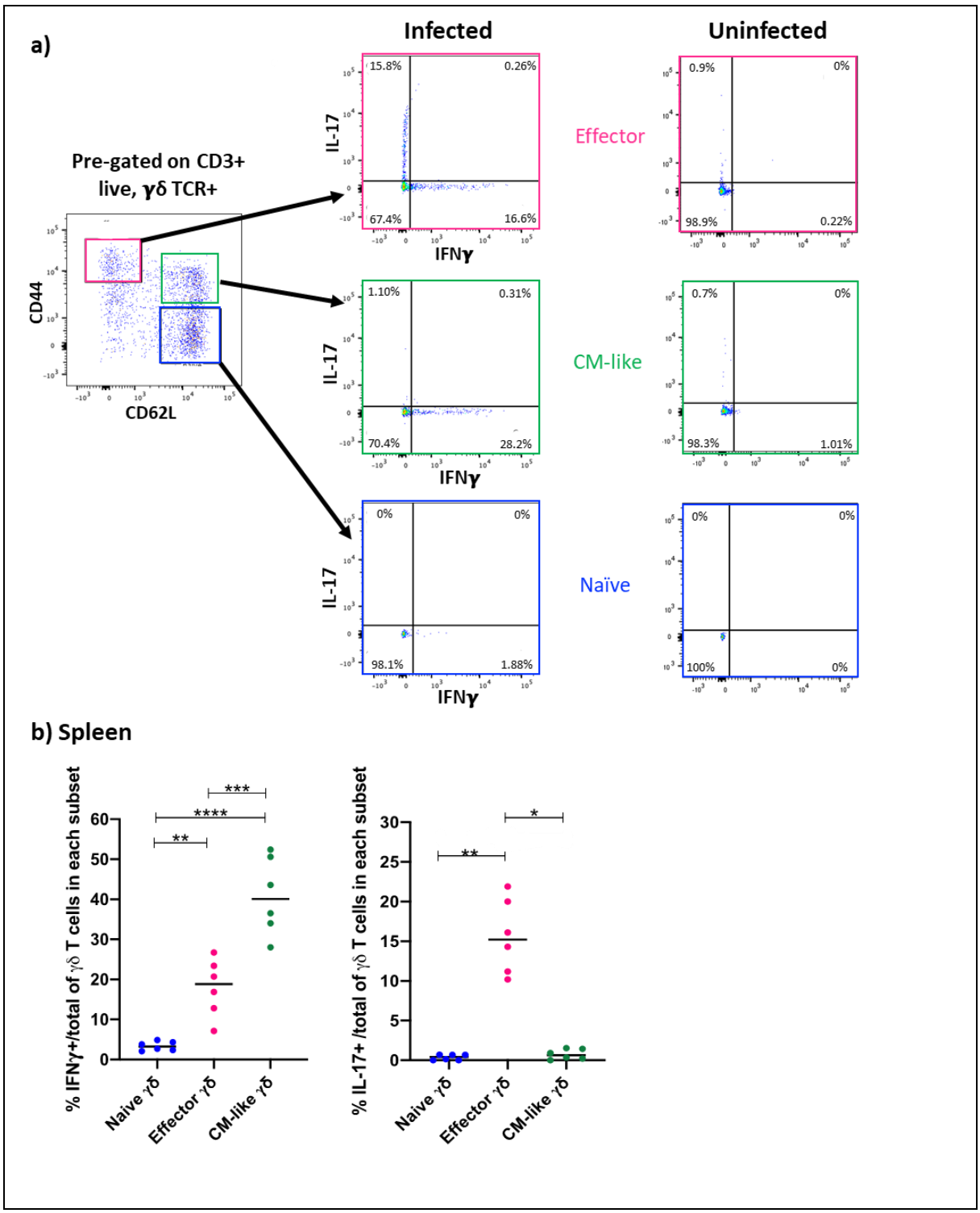
b) The percentage of IFNγ+ γδ T cells in uninfected mice (n=6) and infected mice one day post infection (n=10). The percentage of αβ T cells that express IFNyin uninfected mice (n=7) and infected mice one day post infection (n=10).

c) The percentage of IFNγ+ γδ T cells (n=10) or IFNγ+ αβ T cells (n=10) one day post infection.

d) The percentage of IL-17+ γδ T cells in uninfected mice (n=4) and infected mice one day post infection (n=6). The percentage of αβ T cells that express IL-17 in uninfected mice (n=4) and infected mice one day post infection (n=6).

In the previous chapter I used CD44 and CD62L to delineate 3 populations: naïve, effector and cm-like γδ T cells. I wished to determine which of these subsets of produced the IFNγ observed early in infection. I would expect the naïve population to not respond this rapidly but the effector subset may include rapid responders. As predicted splenic naïve γδ T cells did not respond within 24 hours of infection, suggesting a lack of a cytotoxic phenotype. Effector γδ T cells however produced both IFNγ or IL-17 rapidly following infection whilst the cm-like γδ subset produced only IFNγ (Figure 4.2a). Cm-like γδ T cells formed the significant majority of IFNγ producing cells whilst IL-17 was produced by the effector γδ T cells alone (Figure 4.2b). This recapitulates previous findings that effector, innate-like γδ T cells in the MLN respond with IL-17 production immediately post *L. monocytogenes* infection (Sheridan et al, 2013). The production of IFNγ by cm-like γδ T cells as a defense mechanism also correlates with the αβ T cell response where many lymphoid CD8 responders to *L. monocytogenes* have an effector or CM phenotype (Obar & Lefrançois, 2010; Wherry et al., 2003).

The IFNγ+ γδ T cells at 24 hours post *L. monocytogenes* infection had no significant bias in gamma chain usage (Figure 4.2c). However the vast majority of IL-17+ γδ T cells expressed Vγ1- Vγ4- or Vγ4 chains (Figure 4.2c). Previous work has shown that in *L. monocytogenes* infection the majority of IL-17 producing cells are Vγ4 or Vγ6 cells. The IL-17 drives the initiation of an adaptive immune response and aids pathogen clearance (Hamada et al., 2008). Some Vγ6 cells were found to produce both IFNγ and IL-17 (Sheridan et al, 2013) which may explain why Vγ1- Vγ4- cells seem to constitute a proportion of the IFNγ+ and IL-17+ responders (Figure 4.2c).


a)
Infected
Uninfected
Pre-gated on CD3+
live, γδ TCR+
CD44
CD62L
IL-17
IFNγ
15.8%
0.26%
67.4%
16.6%
0.9%
0%
98.9%
0.22%
Effector
1.10%
0.31%
70.4%
28.2%
0.7%
0%
98.3%
1.01%
CM-like
0%
0%
98.1%
1.88%
0%
0%
100%
0%
Naïve
b) Spleen
% IFNγ+/total of γδ T cells in each subset
% IL-17+ /total of γδ T cells in each subset
Naive γδ
Effector γδ
CM-like γδ
**
****
***
**
*

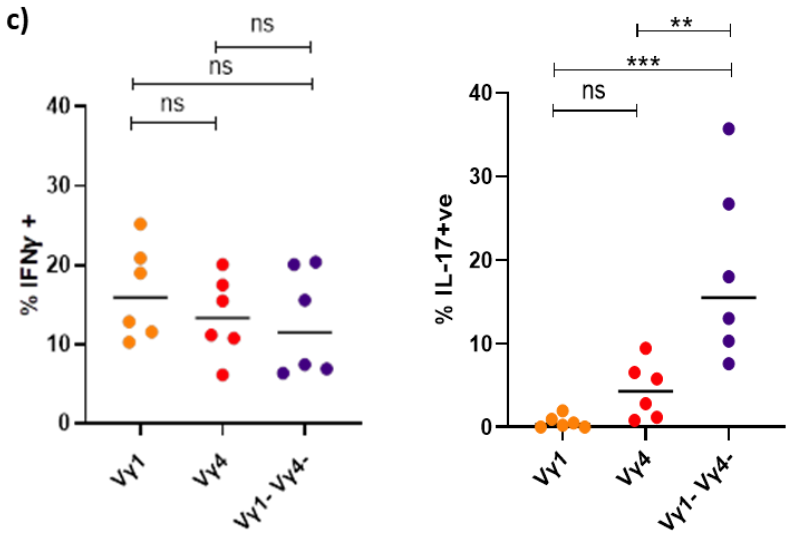


Figure 4.2: Effector and central memory-like γδ T cells respond within 24 hours of *L. monocytogenes* infection. Great Smart mice (dual IFNγ and IL-17 reporters) were infected with attenuated *2W1S-Listeria monocytogenes.* Tissue was then harvested after 24 hours as described in the methods and analysed via flow cytometry. Data pooled from a minimum of 3 experiments and each data point represents an individual mouse. Data were analysed via Kruskal Wallis test with Dunn's post-hoc test or with a one way ANOVA with Tukey's post hoc test * p0.05 **p0.01 *** p0.001 **** p0.0001

a) Gating strategy for IFNγ and IL-17 expression in naïve, effector and cm-like γδ T cells from uninfected and infected mice.

b) The percentage of IFNγ producing γδ T cells in splenic naïve (n=6), effector (n=6) and cm-like (n=6) γδ T cells at 24 hours post infection. The percentage of IL-17 producing γδ T cells in splenic naïve (n=6), effector (n=6) and cm-like (n=6) γδ T cells at 24 hours post infection.

c) The proportion of Vγ1 (n=6), Vγ4 (n=6) and Vγ1- Vγ4- (n=6) cells within the IFNγ+ population. The proportion of Vγ1 (n=6), Vγ4 (n=6) and Vγ1- Vγ4- (n=6) cells within the IL-17+ population.

In summary, following infection with *L. monocytogenes* a proportion of γδ T cells rapidly produce IFNγ or IL-17. The IFNγ+ cells are present in both the effector and cm-like γδ T cell compartments and can express Vγ1, Vγ4 or Vγ1- Vγ4- chains. The IL-17+ responders are in the effector γδ T cell compartment and are dominated by the Vγ1- Vγ4- chain and a small proportion of Vγ4 cells.

After identifying that a subset of effector and cm-like γδ T cells react immediately post infection, I wanted to determine if the γδ T cell compartment and response changed over time, which is consistent with an adaptive response. Following rapid IFNγ production at day 1 post infection, the proportion of IFNγ+ γδ T cells remained high at day 5 post infection before decreasing by day 7 (Figure 4.3a). Unlike the rapid response in γδ T cells, αβ T cells did not produce IFNγ until day 5 post infection (Figure 4.3b). This may suggest a primary adaptive response with antigen specific αβ T cells responding to infection. This correlates with previous findings that identify CD4 *L. monocytogenes* specific T cells expand and peak at day 5 of infection (Malhotra et al., 2020; Pepper et al., 2010).

Throughout all time points the cm-like subset consistently formed the majority of IFNγ+ γδ T cells (Figure 4.3c). Interestingly the percentage of IFNγ+ effector γδ T cells decreased at day 5 before increasing again at day 7 (Figure 4.3c). This corresponded with an expansion of the effector compartment at day 5 (Figure 4.3d). This may indicate that an innate effector subset produced IFNγ early in infection and then decreased by day 5. This could have been accompanied by the expansion and activation of an adaptive-like subset at day 5 which then produces IFNγ by day 7. However further characterisation of the effector population would be required before coming to this conclusion decisively.

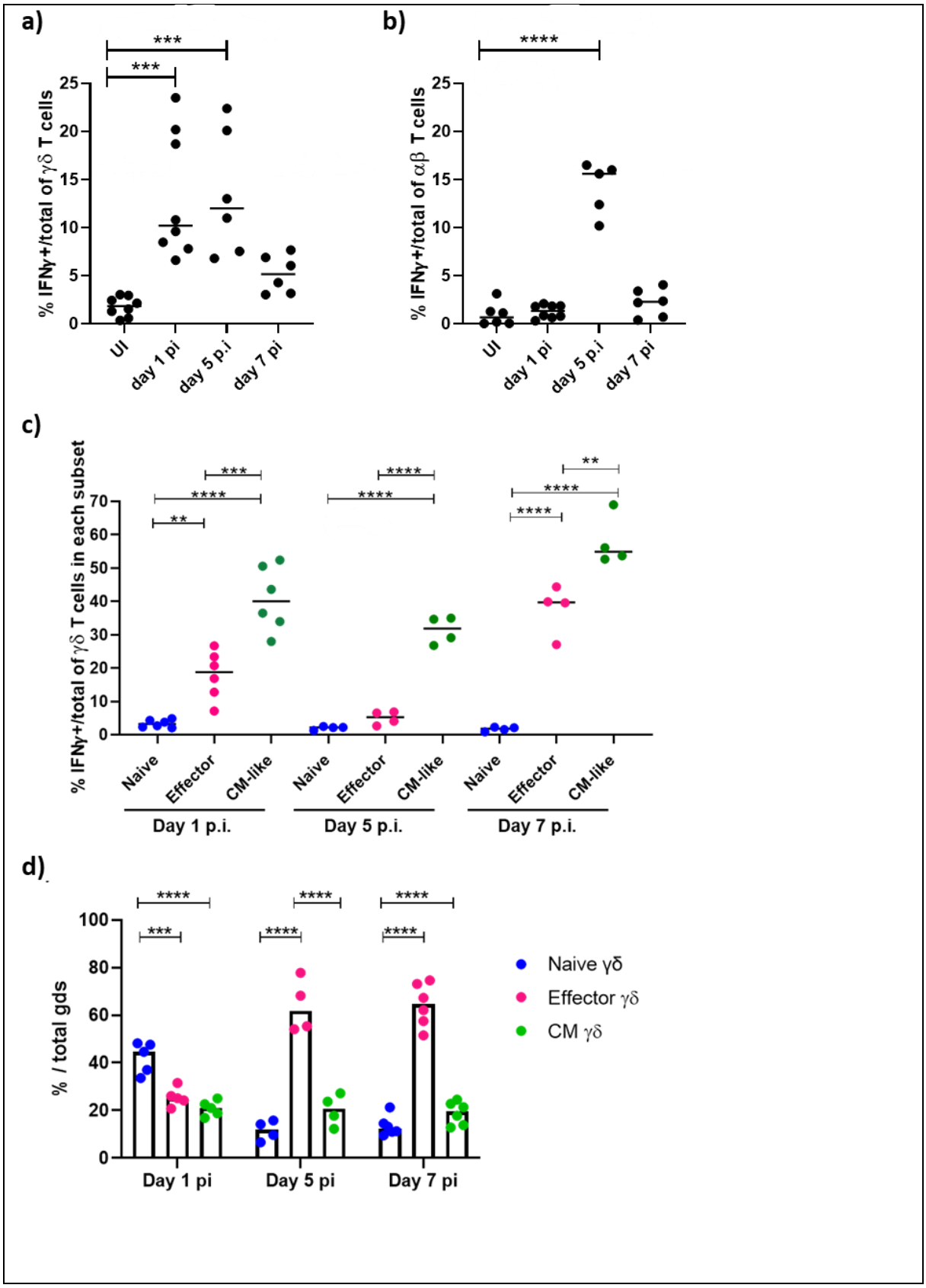

a)
% IFNγ+/total of γδ T cells
UI
day 1 pi
day 5 p.i
day 7 pi
b)
% IFNγ+/total of αβ T cells
c)
% IFNγ+/total of γδ T cells in each subset
Naive
Effector
CM-like
Day 1 p.i.
Day 5 p.i.
Day 7 p.i.
d)
% / total gds
Day 1 pi
Day 5 pi
Day 7 pi
Naive γδ
Effector γδ
CM γδ

Figure 4.3: A timecourse of yδ and αβ T cell responses to *L. monocytogenes* infection. Great Smart mice (dual IFNyand IL-17 reporters) were infected with attenuated *2W1S-Listeria monocytogenes.* Tissue was then harvested at day 1, 5 and 7 post infection as described in the methods and analysed via flow cytometry. Each data point represents an individual mouse and data pooled from a minimum of 3 experiments. Data was analysed via one way ANOVA with Tukey's post hoc or two-way ANOVA with Tukey's post hoc test * p0.05 **p0.01 *** p0.001 **** p0.0001

a) The percentage of IFNγ+ yδ T cells in uninfected mice (n=8) and at day 1 (n=8), day 5 (n=6) and day 7 (n=6) post infection.

b) The percentage of IFNγ+ αβ T cells in uninfected mice (n=6) and at day 1 (n=8), day 5 (n=6) and day 7 (n=6) post infection.

c) The percentage of IFNγ+ cells in the naïve, effector and cm-like yδ T cells subsets throughout infection.

d) The percentage of naïve, effector and cm-like subsets within the yδ T cell compartment throughout infection.

4.3.2 Naïve γδ T cells are confirmed via adoptive transfer to not respond to *L. monocytogenes* within 24 hours of infection

Naïve γδ T cells were predicted (Section 4.3.1) to not respond within 24 hours of infection, however this was difficult to definitively prove. Naïve γδ T cells could have transitioned to an effector population without being detected. Therefore it was important to try and track a naïve population and their response following infection. Hence an adoptive transfer experiment was planned with trackable naïve γδ T cells transferred from a donor (CD45.2 allotype) to a host (CD45.1/CD45.2 allotype) and the host was then infected with *L. monocytogenes.* The naïve T cells were tracked in the host by their lack of CD45.1 expression. The experimental procedure is shown in Figure 4.4a. At day 1 post infection in the host, naïve donor cells did not expand and retained their CD62L+ CD44- phenotype (Figure 4.4b,c). Due to COVID-19 restrictions, the experiment could only be done twice. However the two experiments showed consistent results that suggests that the naïve cells are truly not responsive within 24 hours to *L. monocytogenes* and do not have a preprogrammed effector function in this setting.

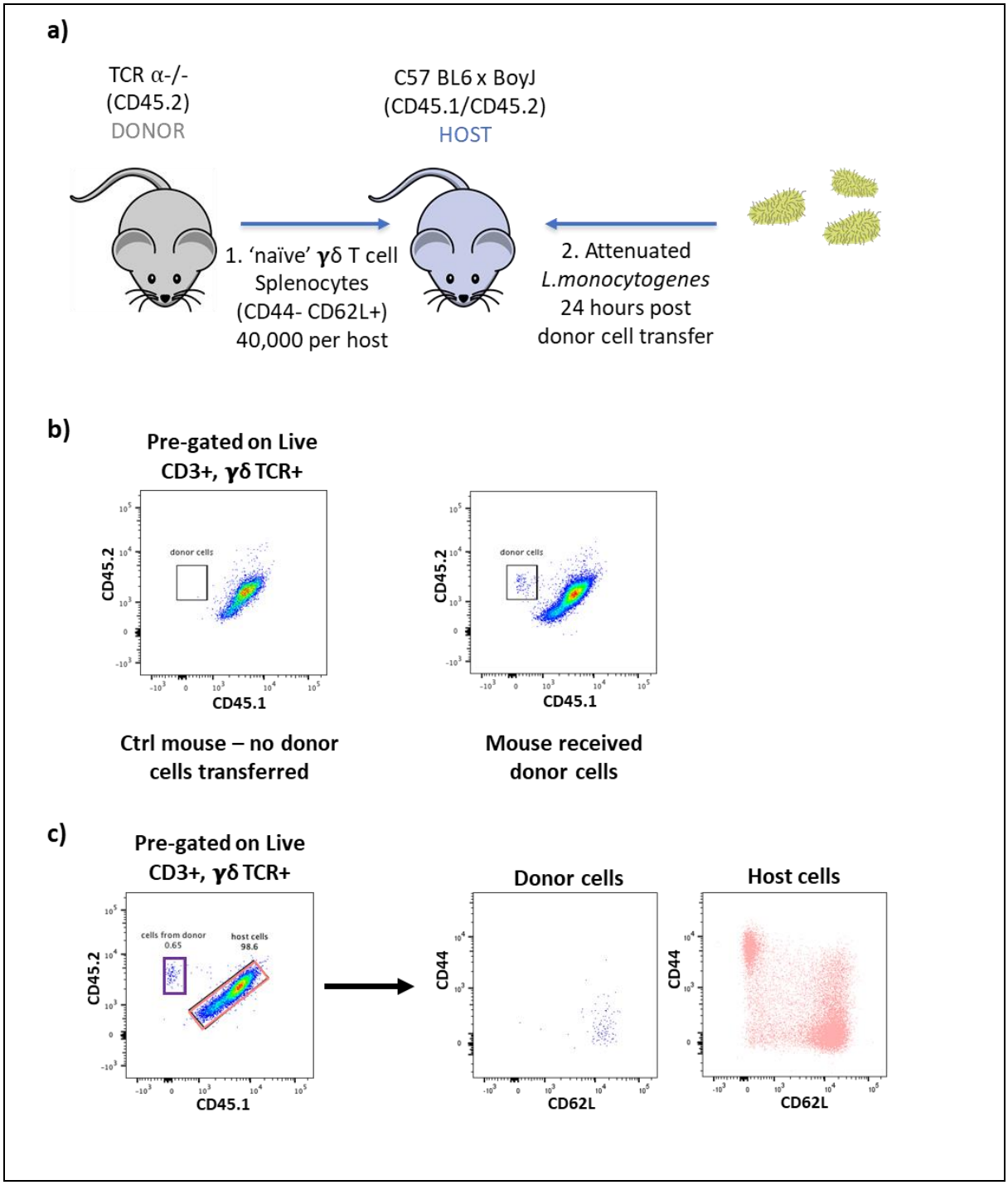

a)
TCR α-/-
(CD45.2)
DONOR
C57 BL6 x BoyJ
(CD45.1/CD45.2)
HOST
1. 'naïve' γδ T cell
Splenocytes
(CD44- CD62L+)
40,000 per host
2. Attenuated
L.monocytogenes
24 hours post
donor cell transfer
b)
Pre-gated on Live
CD3+, γδ TCR+
donor cells
CD45.2
CD45.1
Ctrl mouse – no donor
cells transferred
donor cells
CD45.2
CD45.1
Mouse received
donor cells
c)
Pre-gated on Live
CD3+, γδ TCR+
cells from donor
0.65
host cells
98.6
CD45.2
CD45.1
Donor cells
CD44
CD62L
Host cells
CD44
CD62L

Figure 4.4: Adoptive transfer confirms that naïve γδ T cells do not respond within 24 hours of *L. monocytogenes* infection. Naïve splenocytes were harvested from donor mice (CD45.2 allotype) and injected in host mice (CD45.1/CD45.2). 24 hours post transfer, host mice were infected with *L. monoytogenes.* 24 hours post infection, tissue was harvested from host mice and analysed via flow cytometry. The experiment was performed twice (n=6, 2 mice received host cells and 1 did not in each experiment).

a) Diagram of the experimental protocol

b) Donor cells are present in host mice 2 days post cell transfer

c) The naïve donor population retained their CD44- CD62L+ phenotype 24 hours after host infection with *L. monocytogenes*

4.3.3 Single cell sequencing of the TCR of effector and cm-like IFNγ+ γδ T cells in *L. monocytogenes* infection

Following the identification of IFNγ producing effector and cm-like γδ T cells at 24 hours of infection, I wanted to determine whether the TCR was involved in this response. Clonal expansion in the responding cells would suggest an antigen-driven response via the TCR. The variable region of the CDR3 in both gamma and delta chains of IFNγ+ γδ T cells were sequenced in the spleen of *L. monocytogenes* infected mice.

In the gamma chain repertoire there was only one recurring sequence, which was infrequent and appeared in a 10th of all sequenced gamma chains (Figure 4.5b). This sequence and other gamma chains sequences were simple, germline sequences that are very commonly rearranged during T cell development and have few n-nucleotide additions. All sequences of both gamma and delta chains are included in Appendix 2.

Within the splenic IFNγ+ responders at 24 hours post infection there was a wide variety of delta chain usage, with DV7 and DV5 most prominent (Figure 4.5a). This is similar to the splenic effector compartment in steady state mice with the DV5 chain dominating (Chapter 3). The delta chain repertoire in splenic IFNγ+ responders also showed no recurring sequences (Figure 4.5c) but many sequences were variations of the germline CASGYLYGLLIGGIRATDKLVF sequence. This suggests that it is not a clonal response in the effector and cm-like γδ T cells at 24 hours after infection. Rather it is a polyclonal response with a variety of similar germline TCRs which is more indicative of an innate biology. This is to be expected given the rapid nature of the response.

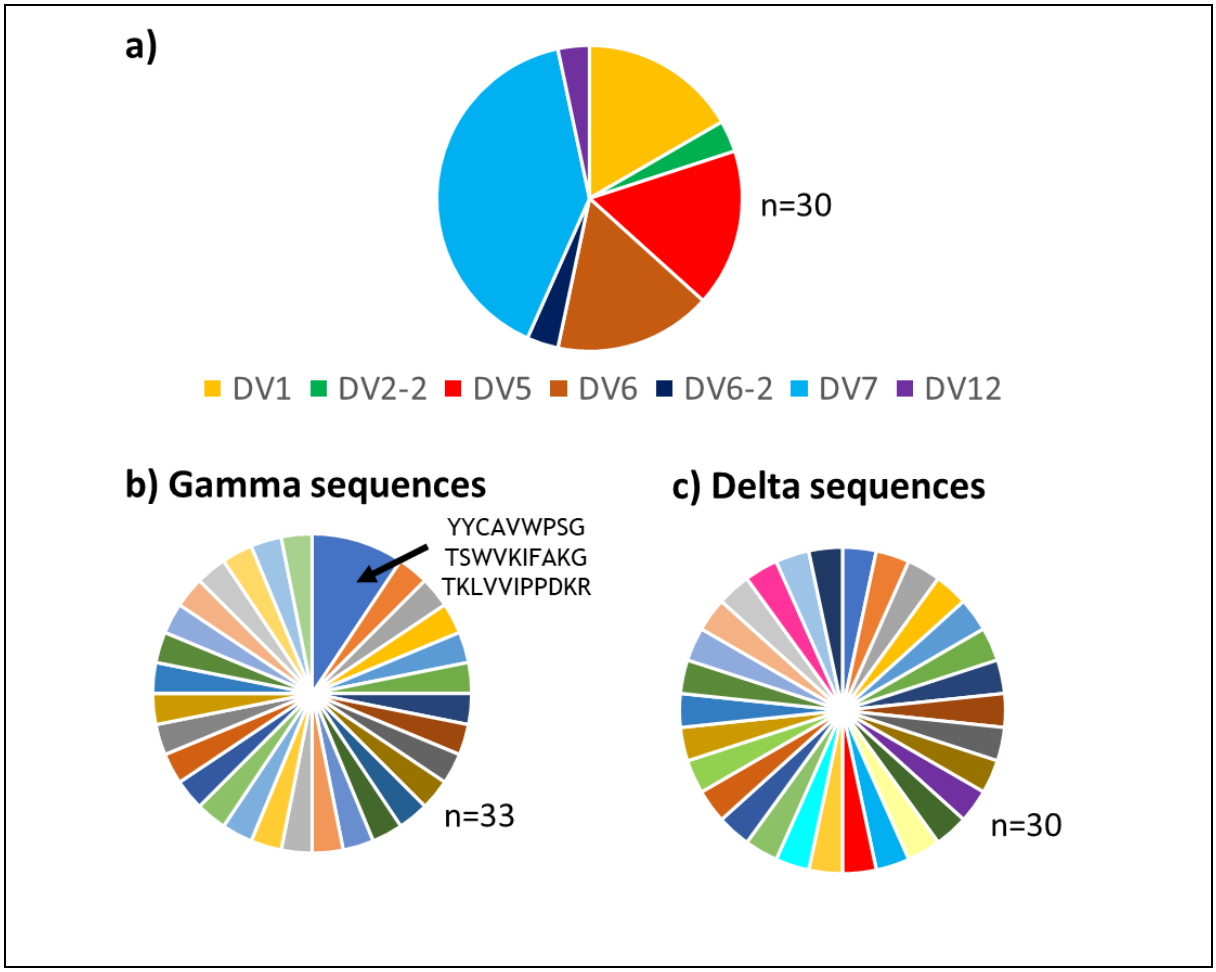

a)
n=30
DV1 DV2-2 DV5 DV6 DV6-2 DV7 DV12
b) Gamma sequences
YYCAVWPSG
TSWVKIFAKG
TKLVVIPPDKR
n=33
c) Delta sequences
n=30

Figure 4.5: Sequencing of the variable region of the CDR3 of effector and cm-like yδ T cells 24 hours post *L. monocytogenes* infection. Great Smart mice (dual IFNy and IL-17 reporters) were infected with attenuated *L. monocytogenes.* The spleen was harvested 24 hours post infection and IFNy+ Vy1 or Vy4 yδ T cells were FACS sorted and the variable region of the CDR3 from each cell was sequenced. Sequences were analysed via the Sequencher software.

a) Delta chain usage in IFNγ+ yδ T cells

b) The clonal repertoire of gamma chain CDR3 sequences in IFNγ+ yδ T cells. Each section of the pie represents an individual sequence.

c) The clonal repertoire of delta chain CDR3 sequences in IFNγ+ yδ T cells. Each section of the pie chart represents an individual sequence.

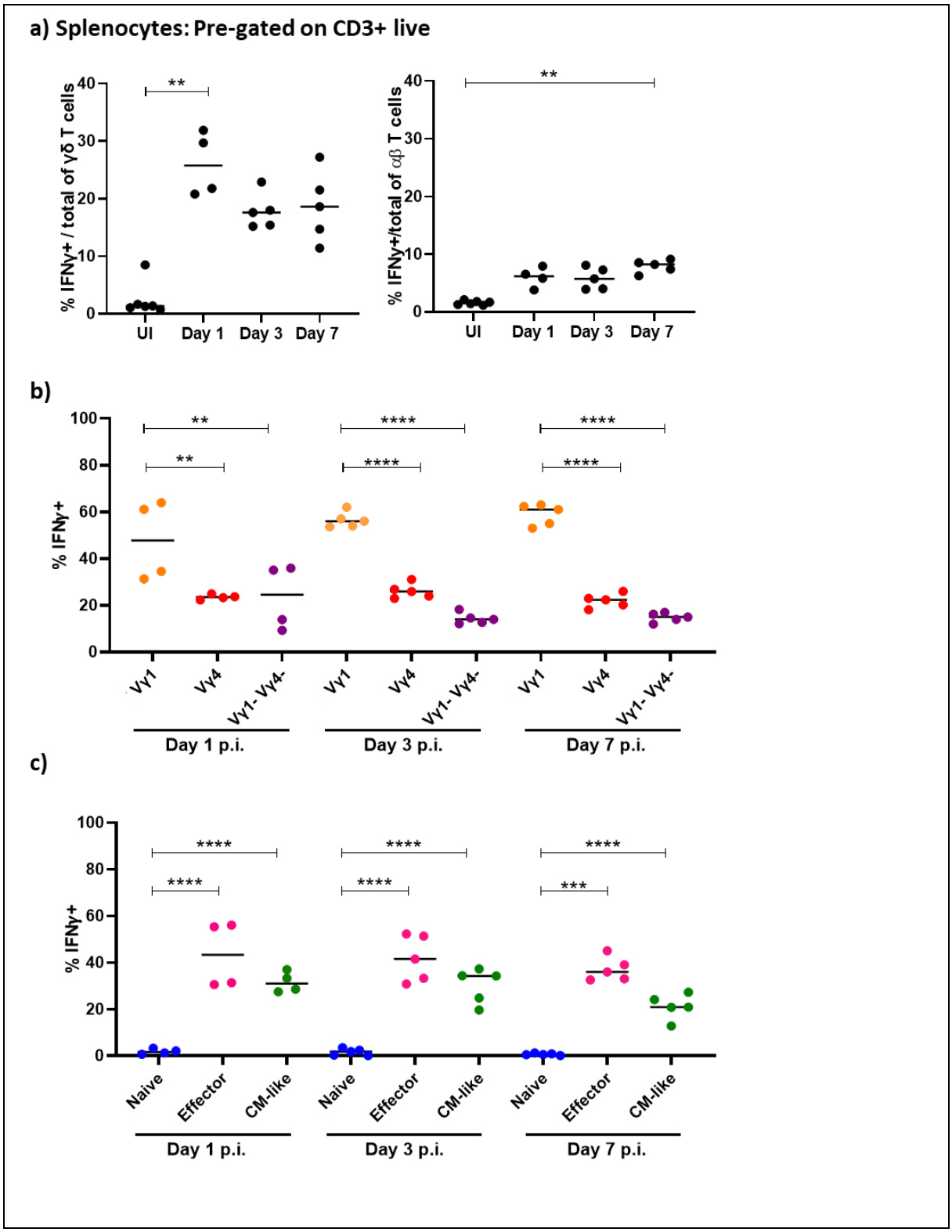
4.3.4 The response of γδ T cells to *S. typhimurium* infection

After studying the γδ T cell response to an acute attenuated *L. monocytogenes* infection, I investigated the response to an unattenuated pathogen that caused a chronic infection. I questioned whether the speed of the clearance of *L. monocytogenes* may have had an impact on the likely effector-dominant γδ T cell response. By studying a chronic model, I could determine if prolonged high bacterial levels could generate a slower γδ T cell adaptive response. IFNγ reporter mice were infected orally with *S. typhimurium*. After ingestion the bacteria crosses the intestinal wall and rapidly spreads throughout the body. At the beginning of infection there are high levels of bacterial replication which is controlled by the host immune system after a few days. There is then a plateau period of multiple weeks with a constant level of high bacterial load which is then cleared eventually by the adaptive immune system (Mäkelä & Hormaeche, 1997; Mittrücker et al., 2002; Mittrucker & Kaufmann, 2000).

As seen in *L. monocytogenes*, by 1 day post infection there is a subset of IFNγ+ γδ T cells in the spleen. However unlike the quick decline in *L. monocytogenes* infection, in *S. typhimurium* this subset persists until the latest timepoint recorded, day 7 post infection, indicating a more chronic infection (Figure 4.6a). Also, αβ T cells a subset of IFNγ+ cells reaches a significantly higher size only at day 7 post infection, suggesting a delayed, adaptive response (Figure 4.6a). Unfortunately the IL-17 response was not tracked in this infection model but it would have been relevant given the importance of Th17 in the containment of infection (Lee et al., 2012).

Throughout the infection the splenic IFNγ+ γδ T cells were dominated by effector and cm-like cells (Figure 4.6c). IFNγ production is essential for typhimurium clearance as mice lacking T-bet, IFNγ receptor and IFNγ cannot resolve infection (Pham & McSorley, 2015). The IFNγ production by effector cells recapitulates the αβ T cell response where many cells have an effector phenotype and produce IFNγ and TNF (Srinivasan et al., 2004). Both effector and cm-

like T cells had significantly high Vy1 chain usage (Figure 4.6b), which would coincide with their role as IFNy producers (Carding & Egan, 2002). Interestingly, at each timepoint there was also a consistent population of Vy1Vδ6.3 cells (Figure 4.6d), which have previously been shown to respond to other pathogens such as Malaria in a oligoclonal fashion and were previously described as effector cells with a semi-invariant TCR repertoire (Azuara et al., 1997, 1998; Mamedov et al., 2018). It may be therefore that a subset of the IFNy+ responders to *L. monocytogenes* have broad pathogen reactivity.


a) Splenocytes: Pre-gated on CD3+ live
% IFNγ+ / total of γδ T cells
% IFNγ+/total of αβ T cells
UI
Day 1
Day 3
Day 7
b)
% IFNγ+
Vγ1
Vγ4
Vγ1- Vγ4-
Day 1 p.i.
Day 3 p.i.
Day 7 p.i.
c)
% IFNγ+
Naive
Effector
CM-like
Day 1 p.i.
Day 3 p.i.
Day 7 p.i.

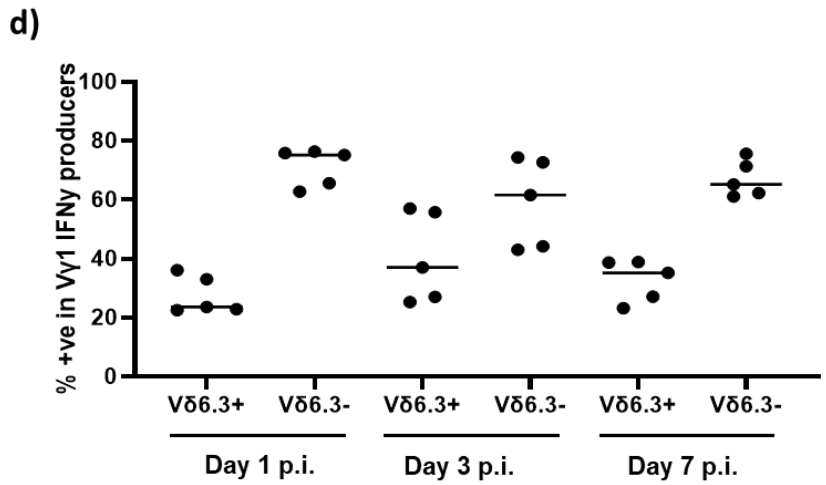


Figure 4.6: The response of γδ T cells throughout *S. typhimurium* infection. Great Smart mice (IFNγ reporters) were infected with *S. typhimurium.* Tissue was then harvested at day 1,3 or 7 post infection as described in the methods and analysed via flow cytometry. Each data point represents an individual mouse and data were pooled from 2 experiments. Data were analysed via one way or two way ANOVA with Tukey's post hoc test * p0.05 **p0.01 *** p0.001 **** p0.0001

a) The percentage of splenic IFNγ+ γδ T cells in uninfected mice (n=6) and at day 1 (n=4), day 3 (n=5) and day 7 (n=5) post infection. Also, the percentage of splenic IFNγ+ αβ T cells in uninfected mice (n=5) and at day 1 (n=4), day 3 (n=5) and day 7 (n=5) post infection.

b) The γ chain usage of splenic IFNγ+ γδ T cells at day 1 (n=4), day 3 (n=5) and day 7 (n=5) days post infection.

c) The percentage of splenic IFNγ+ cells within the naïve, effector and cm-like γδ T cell subsets at day 1,3 and 7 post infection.

d) The Vδ6.3 chain usage in Vγ1 IFNγ+ cells at day 1 (n=5), day 3 (n=5) and day 7 (n=5) post infection.

4.3.5 Sequencing of the TCR repertoire of IFNγ+ γδ T cell responders to *S. typhimurium* infection

After discovering that a subset of effector and cm-like γδ T cells respond to *S. typhimurium* in a similar fashion to *L. monocytogenes*, I wished to explore if the responders had clonal or diverse TCR repertoires. 24 hours post infection, cells were harvested from the MLN, sorted and the variable region of CDR3 in both chains were sequenced to explore their repertoire. The MLN was selected as it is an initial site of infection and is the site of T cell bacterial specific activation (McSorley et al., 2002; J. Moon & McSorley, 2009).

As in *L. monocytogenes* infection the DV5 delta chain is prominent within the IFNγ+ responders (Figure 4.7a). This is similar to MLN effector γδ T cell populations in the steady state MLN (Chapter 3), suggesting that it may be a tissue specific phenomenon rather than infection driven. Within the gamma chain CDR3 repertoire there is a few sequences that occur in 3 cells, however they are again simple rearrangements and their recurrence can merely be due to their frequent rearrangement (Figure 4.7b). In the delta chain repertoire, the common CASGYLYGLLIGGIRATDKLVF sequence occurs frequently (Figure 4.7c). However this sequence was also recurring in the *L. monocytogenes* responders in the spleen, confirming that this is not an pathogen-specific clone. It also suggests that these cells are indiscriminate and respond with the same stimuli to multiple bacterial pathogens and in different tissues. All sequences of both gamma and delta chains are present in Appendix 2.

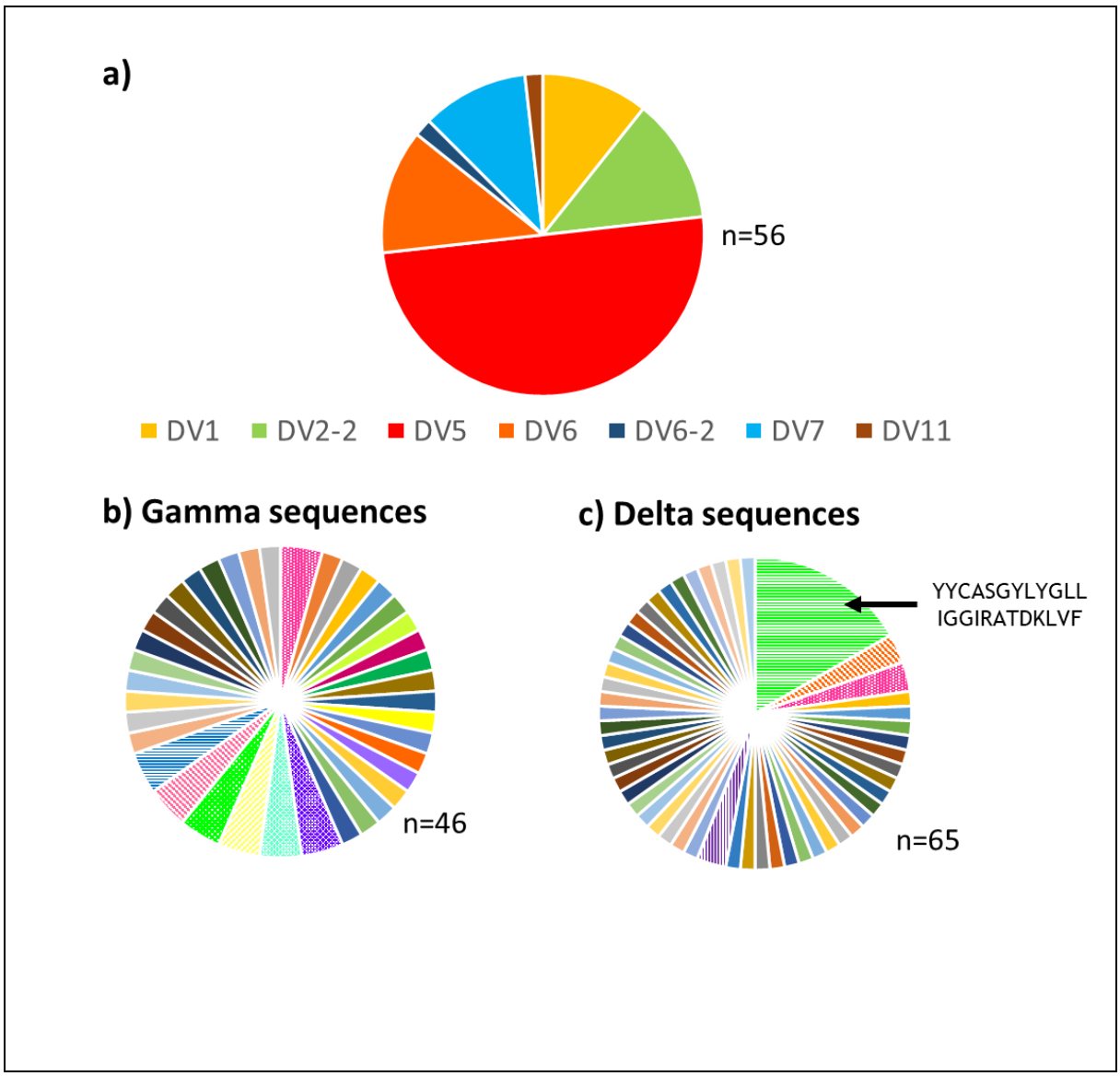

a)
n=56
DV1
DV2-2
DV5
DV6
DV6-2
DV7
DV11
b) Gamma sequences
n=46
c) Delta sequences
YYCASGYLYGLL
IGGIRATDKLVF
n=65

Figure 4.7: Sequencing of the variable region of the CDR3 of effector and cm-like yδ T cells 24 hours post *S. typhimurium* infection. Great Smart mice (dual IFNy and IL-17 reporters) were infected with *S. typhimurium.* The MLN was harvested 24 hours post infection and IFNy+ Vy1+ or Vy4+ yδ T cells were FACS sorted and sequenced on a single cell level. Sequences pooled from three experiments.

a) Delta chain usage in IFNy+ yδ T cells.

b) The composition of gamma chain CDR3 sequences in IFNγ+ yδ T cells. Each section of the pie represents an individual sequence.

c) The clonal repertoire of delta chain CDR3 sequences in IFNγ+ yδ T cells. Each section of the pie chart represents an individual sequence.

4.3.6 Using a TCR activation reporter model to investigate the role of the TCR in γδ T cell responses

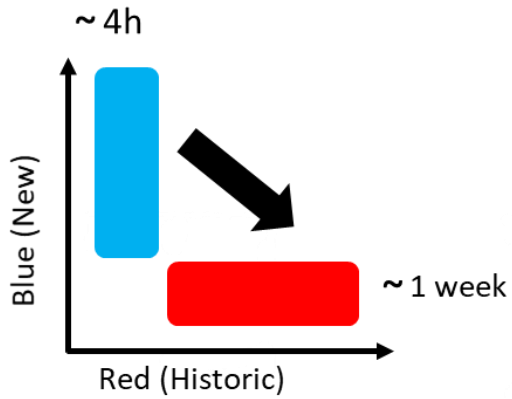
The importance of the TCR in the activation of γδ T cells is a key area of research. Its role remains unclear as only a small number of ligands for both human and murine TCRs have been found and some innate-like subsets in both species can be activated independently of the TCR (Deseke & Prinz, 2020; Girardi et al., 2001; Rincon-Orozco et al., 2005). However other adaptive-like subsets such as human Vδ1 T cells have shown TCR-driven activation and are not able to be activated by cytokines alone. Their expansion following activation is also accompanied with clonal focusing suggesting the focal role of the TCR in antigen specific proliferation (Davey et al., 2017b). I aimed to investigate the role of the TCR in the effector and cm-like responses to both infection models. This was studied through using novel Nr4a3-TOCKY mice to capture further information about TCR triggering.

The Nr4a3-TOCKY model is a TCR activation reporter that allows the temporal tracking of TCR activation *in vivo*. The Nr4a3 protein is fused to a fluorescent timer protein that degrades over time. Immediately following TCR triggering and Nr4a3 upregulation (a nuclear protein downstream of TCR signaling), the timer protein becomes blue and remains so for 4 hours. This is a marker for recent TCR activation. The protein then slowly degrades over 24 hours, becoming red and a marker for historic TCR activation before the signal erodes at one week post activation (Figure 4.8a) (Bending et al., 2018).

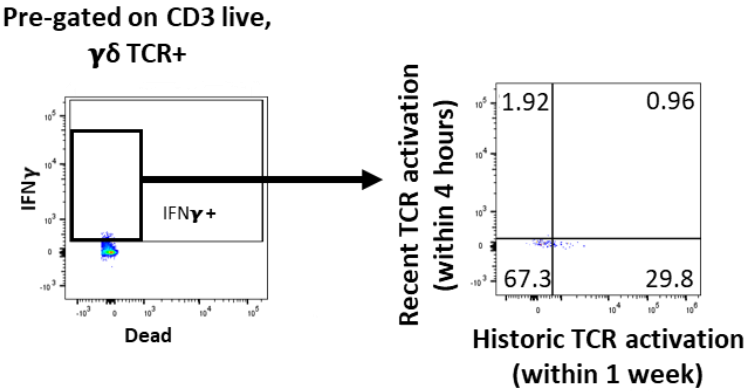
The IFNγ+ γδ T cells at 24 hours post *L. monocytogenes* infection were measured for Nr4a3 signal. As expected in uninfected Nr4a3-TOCKY reporter mice, no IFNγ+ γδ T cells could be observed (Figure 4.8b). Following infection of the mice there was an increase in IFNγ+ γδ T cells at 24 hours post infection. However this population did not appear to have received TCR stimulation within the last week as they had a negative signal for both recent and historic TCR

activation (Figure 4.8c). This suggests that the rapid IFNy releasing response in effector and cm-like yδ T cells may not be TCR-dependent and could depend on other activatory mechanisms such as NKRs which are upregulated on both subsets (Chapter 3).

a) Nr4a3-TOCKY signal



b) Uninfected



c) 1 day post *L. monocytogenes* infection

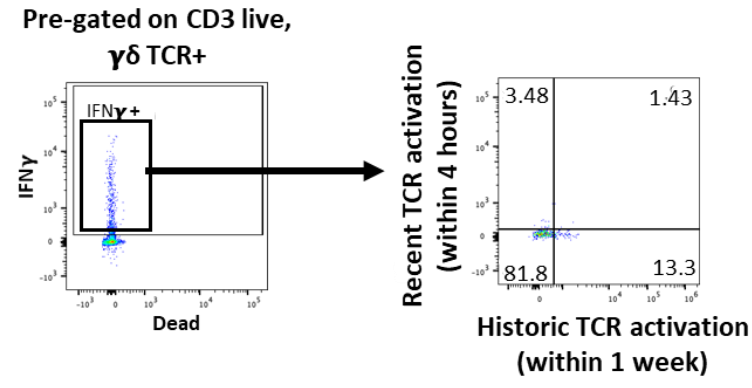


Figure 4.8: A TCR triggering signal could not be detected in IFNyproducing γδ T cells 24 hours post 2W1S-*L. monocytogenes* infection. Nr4a3-TOCKY reporter mice were infected with attenuated 2W1S-*Listeria monocytogenes.* Mice were culled at day 1 post infection. Cells were harvested from the spleen as described in methods and analysed via flow cytometry. Data representative of 2 experiments.

a) Diagram of the Nr4a3-TOCKY protein degradation. Adapted from Bending et al, 2018.

b) IFNγ+ γδ T cells were not present in uninfected mice and did not have a Nr4a3-TOCKY signal.

c) IFNγ+ γδ T cells were present in infected mice and did not have a Nr4a3-TOCKY signal.

Having failed to detect clear evidence for TCR signalling in yδ T cells, I wanted to confirm that TCR signalling in vivo could be accurately detected with the Nr4A3-TOCKY model. I tracked a known *L. monocytogenes* driven αβ T cell expansion using the model. *L. monocytogenes* specific CD4 T cells expand after TCR activation and convert to a CD44+ effector phenotype in lymphoid tissue. I used a 2W1S peptide MHCII tetramer system to identify antigen-specific T cells as previously described (Gajdasik et al., 2020; Pepper et al., 2010b). A bacterial strain of *L. monocytogenes* is used that produces a tetramer of 2W1S-peptide bound to a fluorochrome and the ovalbumin protein. This allows the identification of antigen-specific CD4 T cells that respond to the infection.

As expected the uninfected Nr4A3-reporter mouse did not have a 2W1S-tetramer+ CD4 T cell population (Figure 4.9a). However by day 5 post infection with *2W1S - L. monocytogenes* there was a clear antigen specific tetramer+ subset. Surprisingly this population did not show any TCR activation signal in this Nr4a3-TOCKY model (Figure 4.9b). This finding was recapitulated in a *2W1S-S. typhimurium* model where 2W1S-tetramer+ CD4 T cells could be detected at day 8 post infection in the spleen and the colon, however neither of these CD4 T cell populations had a Nr4a3 signal (Figure 4.10). This surprising result with the lack of a Nr4a3 readout in a recognised TCR driven response is explored in the discussion.

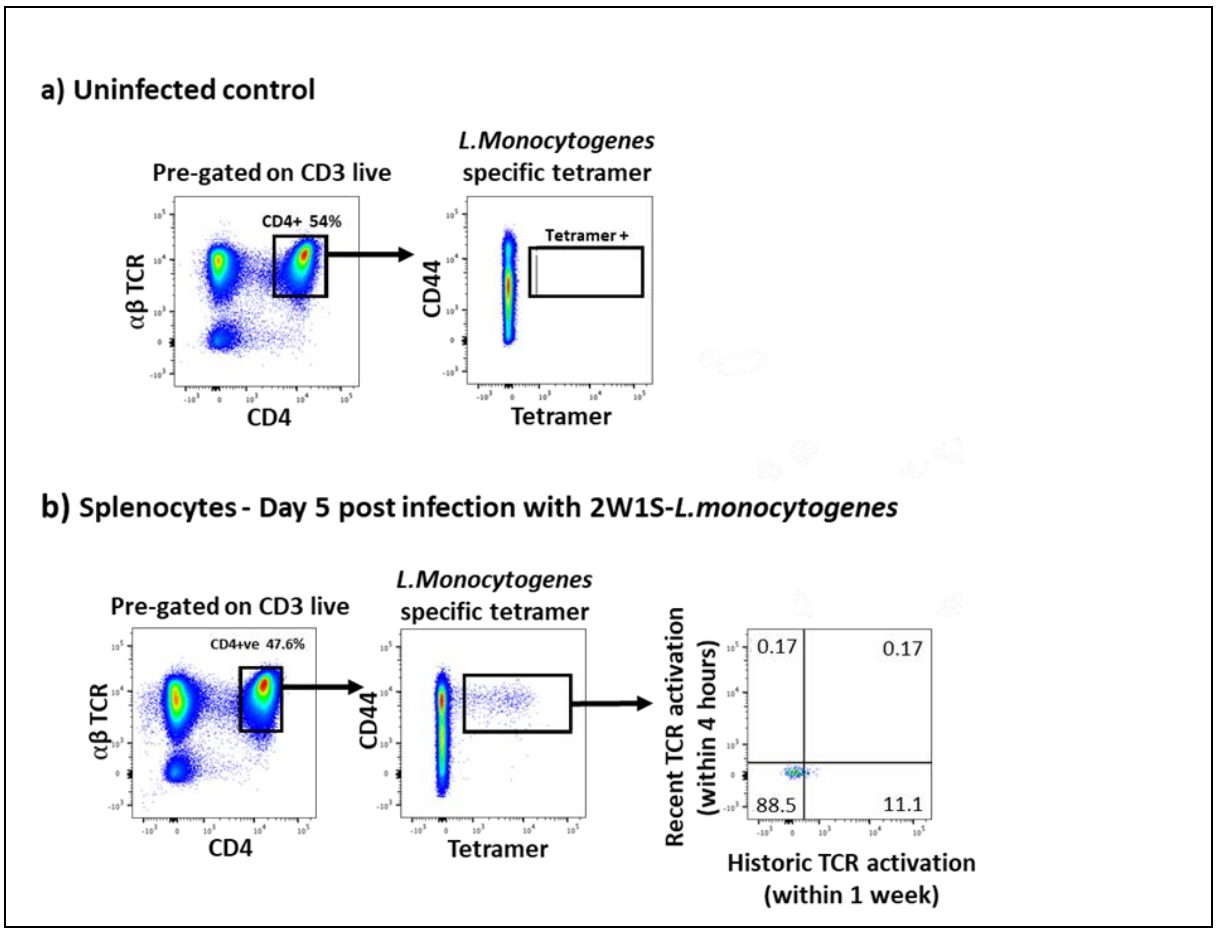
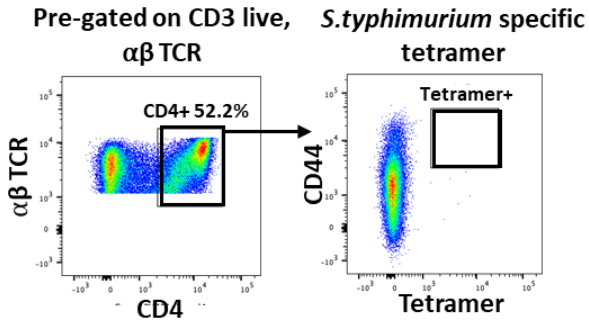

a) Uninfected control
Pre-gated on CD3 live
CD4+ 54%
αβ TCR
CD4
L.Monocytogenes
specific tetramer
Tetramer +
CD44
Tetramer
b) Splenocytes - Day 5 post infection with 2W1S-L.monocytogenes
Pre-gated on CD3 live
CD4+ve 47.6%
αβ TCR
CD4
L.Monocytogenes
specific tetramer
CD44
Tetramer
Recent TCR activation
(within 4 hours)
0.17
0.17
88.5
11.1
Historic TCR activation
(within 1 week)

Figure 4.9: TCR triggering signal could not be detected in 2W1S-L. monocytogenes tetramer positive αβ T cells 5 days post infection. Nr4a3-TOCKY reporter mice were infected with 2W1S-*L. monocytogenes* and culled at day 5 post infection. Cells were harvested from the spleen and stained for antigen specific tetramer in CD4 T cells. Samples were analysed via flow cytometry as described in the methods.

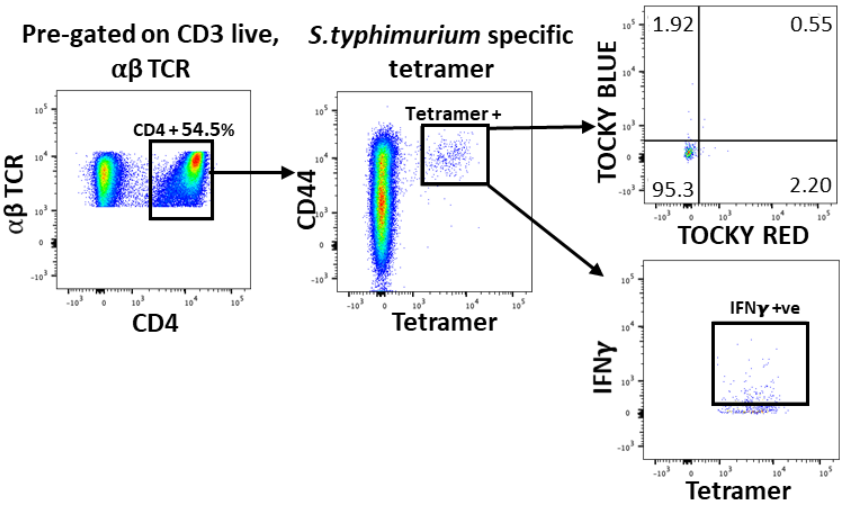
a) The lack of 2W1S- *L. monocytogenes* tetramer specific CD4 T Cells in uninfected mice.

b) The presence of 2W1S- *L. monocytogenes* tetramer specific CD4 T Cells at day 5 post infection which did not have a Nr4a3-TOCKY TCR triggering signal.

a) Uninfected control



b) Splenocytes - Day 8 post infection with 2W1S - *S.typhimurium*



c) Colon - Day 8 post infection with 2W1S - *S.typhimurium*

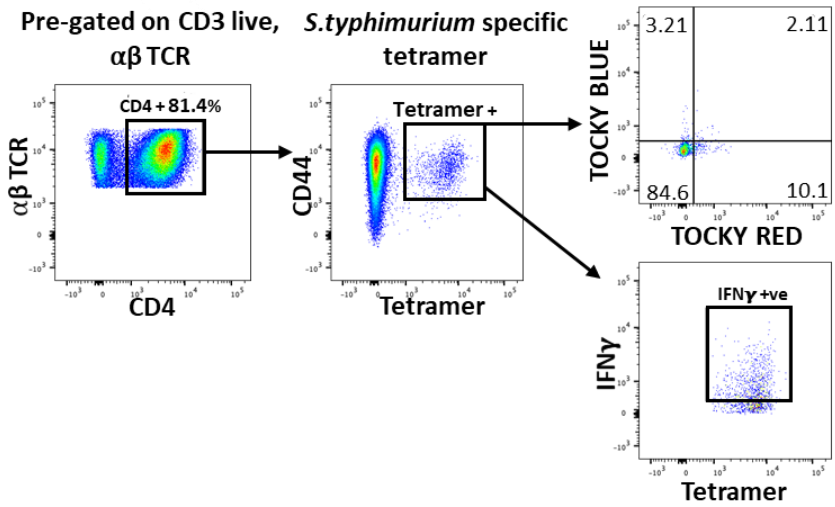


Figure 4.10: TCR triggering signal could not be detected in 2W1S-*S. typhimurium* tetramer positive αβ T cells 8 days post infection. Nr4a3-TOCKY reporter mice were infected with 2W1S-*S. typhimurium* and culled at day 8 post infection. Cells were harvested from the spleen and colon and stained for antigen specific tetramer in CD4 T cells. Samples were analysed via flow cytometry as described in the methods.

a) The lack of 2W1S-*S. typhimurium* tetramer specific CD4 T Cells in uninfected Nr4a3-TOCKY reporter mice

b) Splenic 2W1S-*S. typhimurium* tetramer specific CD4 T Cells were detected at day 8 post infection but they did not have a Nr4a3-TOCKY TCR triggering signal

c) Colonic 2W1S-*S. typhimurium* tetramer specific CD4 T Cells were detected at day 8 post infection but they did not have a Nr4a3-TOCKY TCR triggering signal

4.4 Discussion

Following the identification of lymphoid γδ T cells in the mouse with naïve, effector and cm-like phenotypes, I wished to determine how these cells respond to infectious stimuli. I found that during both an acute *L. monocytogenes* infection and a chronic *S. typhimurium* infection, a subset of effector and cm-like γδ T cells responded within 24 hours. The naïve γδ T cell subset did not respond immediately to either infectious stimuli and retained its phenotype.

4.4.1 The immediate response of effector and cm-like γδ T cells to various infection models

One characteristic of a primary adaptive T cell response is a delayed reaction to the pathogen as a naïve T cell must recognise it, undergo clonal amplification and proliferation and transition to an effector state. This timescale was not clearly exhibited by murine γδ T cells in both infection models studied in this chapter. A subset of effector and cm-like γδ T cells responded with IFNγ or IL-17 production within 24 hours of both infections in a marked difference to the αβ T cell response (Figures 4.1, 4.6). The immediacy of the response suggests the γδ T cell responders may play an innate role in the infection. The immune response to bacterial infections has an early innate response to limit bacterial spread and a later adaptive immune arm that fully clears infection (Andersson et al., 1998).

The γδ T cell responders' production of IFNγ in both models reiterates previous characterisation of the γδ T cell response and the Th1 skewing of the immune response (Matsuzaki et al., 2002; Pepper et al., 2010a). The IFNγ produced very early in infection can drive IFNγ production by NK cells and the development of CD4 Th1 cells alongside causing an decrease in bacterial proliferation and a delay in granuloma formation (Carding & Egan, 2002; Ferrick et al., 1995; Ladel et al., 1996).

The IL-17 production following *L. monocytogenes* also recapitulates previous research describing a IL-17 producing mesenteric yδ T cell population (Romagnoli et al., 2016). In Salmonella infection with the strain *S. enteritidis*, yδ T cells also produced IL-17 following infection (Schulz et al., 2008). Unfortunately the IL-17 response was not measured in *S. typhimurium* experiments which does not allow the comparison of the effector IL-17+ response. It would also have been interesting to have studied the IL-17 response at later timepoints in both infection models.

Interestingly within the subset of responding effector yδ T cells to *L. monocytogenes* infection, some produced IFNy or IL-17 with no dual producers present (Figure 4.1). It has been suggested that innate-like effector yδ T cells are pre-programmed in the thymus to express one of these cytokines following stimulation. CD27 expression can delineate the IFNy and IL-17 producers as it induces expression of genes linked to a Th1 phenotype in the thymus, driving towards an IFNy phenotype (Ribot et al., 2009).

Another study of yδ T cells in *L. monocytogenes* found that mesenteric effector Vy6 T cells can produce both IL-17 and IFNy, however this dual expression is decreased significantly in memory cells 3 months after infection (Sheridan et al., 2013). yδ T cells that are primed in the thymus to produce IL-17 can have the ability to express both IL-17 lineage genes and T-bet and IFNy (Schmolka et al., 2013). It may be that this Vy6Vδ1 population is pre-programmed to express IL-17 but can produce IFNy in inflamed conditions.

Interestingly only around 17% of the yδ T cells responded at 24 hours post *L. monocytogenes* (Figure 4.1). This contrasts with other innate-like reactions such as Vy9Vδ2 cells in humans where the majority of cells respond to the same stimulus (Morita et al., 2007). Both the effector and cm-like subsets may also house less innate-like populations alongside the rapid responders. It is unclear what delineates the responders to *L. monocytogenes* from the

remaining population. It is unlikely to be the TCR as the responders to both infections had diverse repertoires with little clonality or complex recurrent sequences. It may be due to the presence or the strength of expression of activation markers on the cell surface. It could also be due to their ability to sense bacterial specific PAMPs and or stress molecules that are upregulated in inflamed tissue.

In contrast to effector and cm-like subsets, naïve γδ T cells did not expand or produce cytokines (Figure 4.2). This was confirmed by adoptive transfer of donor naïve T cells to a host mouse which was then infected (Figure 4.4). It is likely therefore that the naïve donor γδ T cell population did not expand in the host following infection, was not responsive and retained its CD62L+ CD44- phenotype.

However this experiment in itself is not fully conclusive alone. It may be that the cells were damaged and unable to respond or access lymphoid tissue following the transfer. The population transferred was also a stochastic proportion of the γδ T cells with the naïve phenotype in lymphoid tissue and a T cell with a TCR that can recognise *L. monocytogenes* may have not been transferred. To improve this study the adoptive transfer model should also have been used following *S. typhimurium* infection to determine if the naïve γδ T cell population also did not respond to that stimulus. A higher amount of donor cells could also have been transferred to allow for cell death and to increase the diversity of the TCR repertoire available.

4.4.2 γδ T cell responses at later time points in *L. monocytogenes* and *S. typhimurium* infection

To further elucidate the response of γδ T cell subsets to infection, the IFNγ producers were tracked throughout infection. The longer timepoints highlighted the differences in the infection models. The acute *L. monocytogenes* model showed high IFNγ+ γδ T cell populations at day

1 post infection before decreasing at day 7 (Figure 4.3). Whilst in the chronic *S. typhimurium* model the IFNy+ yδ T cell population was consistent, with a rapid increase at day 1 post infection that persisted at day 7 (Figure 4.6).

This difference in acute vs chronic infection was also observed in the αβ T cell data but in a more delayed adaptive response. The IFNy+ αβ T cells in both models did not respond immediately at day 1 post infection and slowly increased by day 5, persisting in *S. typhimurium* and dropping in *L. monocytogenes* by day 7 (Figures 4.3, 4.6). The delayed response is indicative of an antigen specific activation of a TCR clone and the expansion and proliferation of the cell. It is uncertain whether the naïve yδ T cell population would behave likewise as although it does not respond immediately to infection as described in the previous section, it is unclear if it expands at a later timepoint. To further elucidate its response at later timepoints the population could have been tracked throughout infection following adoptive transfer of the cells to a host. If a clone had responded within the naïve yδ T cell population, it may have been possible to identify expanded donor cells within the host.

Interestingly there are two stages of yδ T cell responses to *L. monocytogenes*. One wave is in early infection and the other after the peak (around 7-10 days). The first wave aids pathogen clearance whilst the second may reduce inflammatory conditions and guide the return to homeostasis. yδ T cells have been shown to produce IL-10 at later stages of infection to decrease inflammation (Carding & Egan, 2002; Hsieh et al., 1996). In our *L. monocytogenes* model, the IFNy+ effector yδ T cells decreased at day 5 post infection, before returning to high levels at day 7 (Figure 4.3). It may be that the effector yδ T cells at each timepoint have a different phenotype and function. The first responders at day 1 may be innate-like cells with preprogrammed functions, whilst the delayed responders may have an anti-inflammatory or memory role. To improve the study, further phenotyping of the population at each timepoint should be performed to detect any differences.

Within the γδ T cell IFNγ response, the cm-like subset dominates in *L. monocytogenes* and the effector population in *S. typhimurium*. Whilst the percentage of IFNγ+ effector γδ T cells decrease at day 5 post *L. monocytogenes*, the total IFNγ+ γδ T cells does not decrease (Figure 4.3). This is likely due to a corresponding expansion in the effector population at day 5, therefore although the proportion of effector γδ T cells that produce IFNγ may have decreased, their number will stay relatively consistent with the early timepoints. The fact that the effector subset's expansion does not correlate with increased IFNγ+ γδ T cells suggests the majority of the expanded effector population are not IFNγ producers. It may be that they are IL-17 producers or are expanding to help with tissue repair by the production of IL-10 (Carding & Egan, 2002; Hsieh et al., 1996).

Unfortunately whilst the IFNγ response was explored throughout both infections, the IL-17 data was not recorded. If repeated the proportion of IL-17+ γδ T cells and the representation amongst effector and cm-like subsets should be noted. It is likely that the effector subset in both diseases is responsible for most of the IL-17 response, continuing the trend noted at day 1 (Figure 4.1). IL-17 is linked to improved bacterial clearance in *S. typhimurium* (Mayuzumi et al., 2010) so its production in both infections and different tissues would have been interesting to track.

Alongside the increased phenotyping and characterisation of the later timepoints of infection, future work could also focus on recall experiments. Studying an improved response or the lack thereof in repeat infections would allow a greater understanding of the immune response enlisted. Repeated infections with *L. monocytogenes* show that mesenteric γδ T cells respond more rapidly and with greater cytokine production (Romagnoli et al., 2016; Sheridan et al., 2013) hence it is likely that some effector and cm-like γδ T cell subset would improve in their response in secondary infections. Recall experiments may also show a greater amount of cm-like γδ T cells responding, if a proportion of this subset was generated from naïve T cells during

the primary response. It remains unclear whether the cm-like subset is comprised of both innate-like immediate responders and adaptive memory T cells and characterizing their response in secondary infections may help to address the question.

Throughout this chapter the populations characterised were in lymphoid tissue, which did not account for subsets in peripheral tissue. Both infections have sizable T cell responses in tissues such as the gut and liver which would be a good comparison to lymphoid populations. yδ T cells in both of these tissues have an effector phenotype and within the gut an innate-like cytotoxic biology (Hunter et al., 2018; Mikulak et al., 2019). They would be an interesting contrast to potential adaptive-like subsets in the spleen and LNs. Future work could phenotype the cytokine response and profile of non-lymphoid responders and determine their response to the infectious stimuli throughout an infection timecourse.

In summary, by tracking the yδ T cell response in both infections I could link the persistence of the response with the chronicity of the pathogen. Both infection models had IFNy driven responses by effector and cm-like yδ T cells from immediately after infection until pathogen clearance. However it was not possible to track the responders to determine if the responders at each time point differed. It could be that whilst a subset of both effector and cm-like yδ T cells respond immediately to infection, there is also a proportion of the cells that are slower to respond and require TCR activation. Further work on the models would need to try and delineate the responders on each day and attempt to track a possible activation in the naïve subset.

4.4.3 The role of the TCR in the γδ T cell response following *L. monocytogenes* and *S. typhimurium* infection

After discovering that immediate anti-bacterial response by some γδ T cells, I wanted to determine definitively that the TCR has no role in the activation of these cells. This was suspected to be true due to the rapid response and the lack of a protracted TCR driven expansion and activation. By sequencing the variable region of the CDR3 in the TCR of IFNγ+ γδ T cell responders and examining their chain usage I could ascertain that there were no indications of an adaptive TCR-driven response.

In both infections the Vγ1 chain dominated the IFNγ+ responders (Figures 4.3, 4.6). This agrees with previous findings showing that Vγ1+ γδ T cells produce IFNγ rapidly upon Listeria and Salmonella infection with many of these cells also expressing the Vδ6.3 chain (Belles et al., 1996; Emoto et al., 1993; Matsuzaki et al., 1993). The Vγ1Vδ6.3 subset has a very restricted TCR repertoire with no diversity in junctional sequences, an effector phenotype (CD44+ CD62L-) and produces inflammatory cytokines (Azuara et al., 1997, 1998). The usage of the same chain pairing in both infections and their restricted TCR diversity points towards a broad response against multiple pathogens. It is likely that many of the γδ T cells responding in this chapter were reacting to a stress molecule or a PAMP. Whilst I have shown that the Vγ1Vδ6.3 pairing is often seen in our *S. typhimurium* model throughout infection (Figure 4.6), unfortunately the Vδ6.3 chain was not stained for in *L. monocytogenes.* The Vδ6.3 antibody should be included in all future work when characterizing these infections to help investigate a non-specific response.

However, whilst Vδ6.3 was not stained for, the delta chain usage in IFNγ+ responders was shown through single cell sequencing. In both infections, there was a sizable population of cells expressing the Vδ6 chain (Figures 4.5, 4.7). However, other delta chains were also

expressed at high levels. In *L. monocytogene*s there was a high usage of DV7 in IFNy+ responders in the infected spleen (Figure 4.5), which contrasts with effector yδ T cells in the healthy spleen which are mostly DV5 (Chapter 3). This change may be due to the inclusion of cm-like yδ T cells in the IFNy+ responders which were not included in the sequencing of the steady state spleen. The cm-like yδ T cells may have a high DV7 chain usage that skews the results, however this population was not sequenced in the healthy tissue so it remains unclear.

In the MLN of *S. typhimuirum* infected mice, the DV5 chain is most prominent within IFNy+ responders which is similar to the effector yδ T cells in steady state MLN. This similarity could be due to the effector yδ T cells taking a larger role in the IFNy response to this infection. This is in contrast to *L. monocytogenes* where the cm-like yδ T cells appear to dominate the IFNy+ responder compartment.

Alongside delta chain usage, the variable region of the CDR3 of individual IFNy+ responders was sequenced. As mentioned in Chapter 3, a conserved chain pairing and a clonal expansion of an identical sequence in a repertoire of an effector T cell population suggests a past clonal response to a stimulus. However some innate-like populations may also have an invariant and identical sequence in multiple cells. This is more likely due to the ease of the production of that sequence and its lack of junctional diversity, rather than the result of a TCR driven expansion. In naïve yδ T cells a broad repertoire with complex sequences indicates the potential to respond to a wide range of antigens (Chapter 3).

In the IFNy+ responders in both infection models there was little sign of clear clonal amplifications. In *L. monocytogenes*, there was no recurring delta chain sequences in the CDR3 region. The gamma chains also had no recurring sequences except the sequence CAVWPSGTSWVKIFAKGTKLV which was expressed by a 10th of cells. In *S. typhimurium* the gamma chains had no recurring sequences whilst the delta chains had one DV5 sequence

that occurred in a 5th of cells, CASGYLYGLLIGGIRATDKLVF. Both of these recurring sequences may at first glance suggest a form of clonal expansion, unfortunately neither of the recurrent sequences had any matched corresponding chain sequenced so it was not possible to determine if the sequence consistently matched with the same chain.

One indication that these recurring sequences are not true clonal expansions are the simplicity of the sequences themselves. Whilst sequenced IFNy+ responders in *L. monocytogenes* did not show a recurrent delta sequence, many of the sequences were very similar and differed only slightly from the GIRAT germline sequence. The sequence that is recurrent in *S. typhimurium* is also a GIRAT sequence and is also expressed in the IFNy+ responders to *L. monocytogenes,* reinforcing the lack of antigen specificity.

The lack of clonal expansions in the IFNy+ producers is not surprising due to their speed of response. Sequencing the IFNy+ producers at later timepoints in *L. monocytogenes* was not attempted as the levels of IFNy+ cells drop sharply within 5 days of infection. However in the chronic *S. typhimurium* it would be interesting in future work to sequence later IFNy+ producers to explore if any clonal expansions were generated by the infection.

Deep sequencing of the IL-17+ Vy6Vδ1 responders to *L. monocytogenes* showed the expansion of some non-canonical Vδ1 sequences which were not identified in previous sequencing of small number of cells in a publication from the same research group. They hypothesize that within the Vy6Vδ1 *L. monocytogenes* IL-17 response there are broadly reactive, public, canonical TCRs that are very common, whilst there are less common non-canonical TCRs, some of which are pathogen specific and others that are cross reactive with other bacterial antigens (Khairallah et al., 2021; Sheridan et al., 2013). Therefore to improve the characterisation of the IFNy+ responders to *L. monocytogenes*, bigger numbers of cells could be sequenced to increase the certainty of the data and bulk RNA sequencing could be

useful alongside the single cell sequencing to identify rarer sequences not picked up in smaller samples.

Besides sequencing the TCR repertoire, I also aimed to explore *in vivo* the role of the TCR in the activation of IFNy+ responders. It has previously been identified that Vy1+ T cells from *L. monocytogenes* infected mice produce IFNy *in vitro* without TCR stimulation. IL-12 was found to be required for the response (Matsuzaki et al., 2002). Macrophages in *L. monocytogenes* can produce IL-12 and IL-1 and the synergy of the two cytokines were thought to be optimal for IFNy production by yδ T cells (Skeen & Ziegler, 1995). In *S. typhimurium* yδ T cells in the gut responded quickly to infection without TCR stimulation as TCR blocking antibodies did not have an effect (van Konijnenburg et al., 2017b; Karin & Clevers, 2016).

Using the Nr4a3-TOCKY mouse model (Bending et al., 2018) I aimed to detect recent TCR signaling *in vivo* through the transcription of the Nr4a3 gene. Nr4a3 is within the family of Nra4 transcription factors and is rapidly upregulated following TCR activation. The Nr4a3 protein is bound to a fluorescent timer protein that becomes blue (half-life 4 hours) following transcription before decaying to red (half-life 5 days) (Bending et al., 2018).

In *L. monocytogenes*, the IFNy+ responders at day 1 post infection did not a Nr4a3-TOCKY signal suggesting that the TCR was not triggered (Figure 4.8). Hence it is very likely that the early IFNy production in both infection models is TCR independent. In other settings yδ T cells have also been observed to be activated through TCR-independent mechanisms such as NK receptors. Murine skin Vy5 T cells can be activated via stress molecules binding to their NKG2D receptors (Strid et al., 2008) and in humans Vy9Vδ2 T cells can also be actively independently of the TCR via NKG2D (Rincon-Orozco et al., 2005).

Interestingly the lack of TCR triggering contrasts with data from IL-17+ Vy6 Vd1 responders to *L. monocytogenes.* Nur77 is also a Nra4 transcription factor that becomes activated following

TCR activation (Moran et al., 2011). Using Nur77-GFP mice, a small proportion of IL-17+ responders in both primary and recall infections showed Nur77+ signal that lasted until contraction of the response around day 9 post infection (Khairallah et al., 2021). One explanation is that the IFNy+ producers are not TCR-driven, whilst a proportion of the IL-17 responders are. The Nur77 signal could also have been more sensitive than the Nr4a3-TOCKY system.

To validate the Nr4a3-TOCKY system I decided to run positive control experiments. Mice were infected with 2W1S-*L. monocytognes* or 2W1S-*S. typhimurium* and then the CD4 T cells were stained with a 2W1S-tetramer to detect antigen specific CD4 T cells. The expansion of the tetramer+ CD4 population following Listeria (at day 5) and Salmonella (at day 8) (Figures 4.9, 4.10) confirms that these populations recognised the antigen and expanded, producing IFNγ. However in both models, there was not a Nr4a3 signal that could be detected.

One explanation is that the Nr4a3-TOCKy signal was diluted by the amount of proliferation in CD4 T cells responding to the antigen. *In vitro* stimulation of Nr4a3-TOCKY cells showed that the strength of the signal is directly correlated to the proportion of TCR that are signalling and that each cell division halves (Bending et al., 2018) which may suggest the model is better used in tracking TCR triggering very soon after activation before clonal expansion. Previous characterisation of the CD4 Naïve response suggests the cells proliferate until 7.5 days post infection and are thought to have a proliferation rate of a doubling of the cells on each day (De Boer et al., 2003; Graw et al., 2012).

Another potential explanation is that the TCR binding interaction was not optimal for a Nr4a3-TOCKY response. This is not convincing however as Nr4a3 requires cognate peptide:MHC interactions for its expression and a full activation of the NFAT signalling pathway. This is unlike Nr4a1 which can be produced following tonic and weak signalling (Jennings et al, 2020).

The TCR of the antigen specific CD4 T cells is recognising and binding the 2W1S sequence that is expressed with the bacteria during infection. It could be that the 2W1S sequence induces a shorter or weaker signalling cascade than what would be detected in WT bacterial ligands. Alternatively it may be that the NFAT pathway is not switched on following stimulation with this antigen which may explain the lack of Nr4a3 expression.

Future work may examine the antigen specific CD4 T cells at an earlier time point in infection to ameliorate the effects of proliferation on the signal. Unfortunately this CD4 population is small at early days of infection whilst the cells undergo clonal expansion and therefore it may be hard to find a day that has a clear population of antigen specific cells but that the proliferation has not overwhelmed the TOCKY signal.

Unfortunately the lack of a clear Nr4a3 signal in our planned positive controls indicates that I cannot rule out conclusively that the TCR is not required for the activation of yδ T cells in our in-house infection models. Further work must be done to elucidate why the Nr4a3-TOCKY model is not working optimally and to fully understand the role of the TCR in the activation of immediate yδ T cell responders in *L. monocytogenes*.

4.5 Conclusion

In this chapter I characterised the response of lymphoid yδ T cells to both *L. monocytogenes* and *S. typhimurium*. A subpopulation of both effector and cm-like yδ T cells responded immediately to infection via IFNy and IL-17 production in infection-naïve mice. They are most likely involved in containing pathogen spread, before an adaptive response is generated to clear it. These rapid responders did not appear to recognise the antigens in a TCR specific manner as their TCR repertoire was diverse with few repeated sequences.

Conversely, the naïve lymphoid γδ T cells did not respond to either infectious stimuli immediately post infection. This was shown by their lack of cytokine production or proliferation and confirmed by adoptive transfer experiments. It may be that the naïve subset does not respond to these two particular pathogens or it could be that the naïve subset has an adaptive-like biology that does not have a pre-programmed, rapid response. In contrast, the effector and cm-like subsets may contain innate-like, preprogrammed effectors alongside cells generated from the naïve population in a more traditional adaptive-like response.

Chapter 5 – γδ T cell populations in human lymphoid tissue

5.1 Introduction

5.1.1 Innate γδ T cell biology in humans

Vγ9Vδ2 T cells are an innate-like γδ T cell population with a semi-invariant TCR repertoire with simple public germline sequences that have limited N-nucleotide addition during rearrangement (Davey et al., 2018). They respond rapidly en masse to phosphoantigens and produce cytotoxic molecules such as granzymes, perforin, TNFα and IFNγ (Cipriani et al., 2000; Morita et al., 2007; Poccia et al., 1997; Qin et al., 2012). Their rapid, effector-like response correlates with their phenotype that is heavily central memory-like (CD27+ CD45RA-) with a smaller subset of an effector memory phenotype (CD27low/neg CD45RA-) (Davey et al., 2018; Ryan et al., 2016).

5.1.2 Adaptive γδ T cell biology in humans

In the blood, Vδ1 and Vγ9-Vδ2 subsets have a diverse, private TCR repertoire with complex sequences suggesting an ability to detect a multitude of different antigens. From within this diverse repertoire, clones expand with age and pathogen exposure. However the expanded clones are not shared between donors reflecting an individualised response to pathogens and a donor's history of infections. The expanded effector clones are long lived and stable for several years, suggesting they may be required for long term memory against chronic infections such as CMV, although they can be found in CMV naive individuals so they must expand in other infections too. Accompanying the clonal expansion is a phenotypic transition from a naïve to an effector state that shares many features with αβ T cell clonal expansion and effector T cell development (Davey al., 2018; Kaminski et al., 2020). Hence blood Vδ1 and Vγ9-Vδ2 T cells exhibit hallmarks of an adaptive response and are thought to have an adaptive biology.

Previous work showed that naïve Vδ1 T cells in the blood express central homing markers, which may allow them to access secondary lymphoid organs (Davey et al., 2017). This correlates with work in this thesis that described a murine naïve yδ T cell population with central homing markers that recirculated throughout lymph nodes and spleen (Chapter 3).I wanted to explore this hypothesis in humans and so examined the Vδ1 population in human lymphoid tissue. Lymph nodes are difficult to source in humans and so tonsils were considered as an alternative way to assess the presence of Naïve and Effector Vδ1 and Vy9-Vδ2 T cells in a SLO.

I also wished to determine if differences could be observed between Vδ1 populations in lymphoid and peripheral tissue. Previous work in the liver showed the Vδ1 compartment had an effector phenotype with expanded clones that can respond to both TCR and innate stimuli. Shared clones were present in both the liver and the blood which suggests that some blood naive Vδ1 T cells will have undergone the transition to an effector phenotype and entered the liver following stimulation (Hunter et al., 2018). Therefore it would be interesting to determine if Vδ1 T cells in lymphoid tissue such as the tonsil were also dominated by an effector phenotype.

5.1.3 The immunology of the tonsil

The tonsil is a secondary lymphoid organ that protects the body against pathogens entering through the oral or nasal cavities (Sada-Ovalle et al., 2012). It is comprised of lymphoid follicles with memory B cells and extrafollicular regions where both αβ and yδ T cells reside (Banchereau et al., 1994; Sada-Ovalle et al., 2012). There is also a mucosal epithelium layer surrounding the tissue that has higher numbers of yδ T cells than in the lymphoid tissue (Gallo et al., 1992; Nave et al., 2001).

The tonsil is a site of antigen presentation and the initiation of immune responses as it is rich in dendritic cells (DCs) and macrophages that can process antigen and present it to T cells within the tissue (Brandtzaeg & Halstensen, 1992; Olofsson et al., 1998). Tonsil DCs have been shown to internalise antigens and express high levels of co-stimulatory molecules that initiates T cell proliferation and cytokine production in infection (Hallissey et al., 2014).

Many of the CD8 αβ T cells in the tonsil have an effector memory (CD45RA- CD62L-) or effector memory CD45RA+ (CD45RA+ CD62L-) phenotype and can degranulate upon stimulation indicating previous antigen exposure. However a significant amount of naïve αβ T cells are also present, suggesting antigen presentation and T cell activation may occur in the tissue (Sada-Ovalle et al., 2012).

In non-human mammals, T cells have been shown to continuously move between the blood and tonsil via high endothelial venules and back to the blood via the lymph (Girard & Springer, 1995; Nowara & Pabst, 1986; Westermann et al., 1996). This suggests that naïve T cells may enter the tonsil and are primed within and can then return to the blood or remain in the tonsil in the effector state. It is not currently known whether γδ T cells reside in the tonsil in significant numbers and whether these cells have a naïve or effector-like phenotype.

5.2 Aims of this chapter

In our working hypothesis, naïve Vδ1 T cells recirculate through lymphoid tissue in steady state conditions and in cases of inflammation or infection are activated in these tissues and clonally expand. The tonsil is thought to be a site of antigen exposure and presentation to T cells. The naïve Vδ1 T cells may enter the tonsil, clonally expand in response to their cognate antigen and transition to an effector phenotype.

The aims of this chapter are:

- To investigate the phenotype of Vδ1 T cells in the tonsil and compare it to naïve and effector Vδ1 T cells in the peripheral blood.
- To determine the effector potential and cytotoxic phenotype of Vδ1 T cells in the tonsil.
- To analyse the TCR repertoire of Vδ1 T cells in the tonsil and compare this to Vδ1 T cells in peripheral blood.

5.3 Results

5.3.1 γδ T cell populations in the human blood and tonsil

Blood PBMCs and tonsil samples were collected and analysed via flow cytometry. The human tonsil has a significant population of T cells (Sada-Ovalle et al., 2012). However, potentially because B cells are enriched in the tonsil, the blood has a greater T cell population with a significantly higher level of CD3+ cells (Figure 5.1a). In both the blood and the tonsil, αβ T cells formed the majority of the T cell compartment (Figure 5.1b).

When comparing the size of Vδ1 and Vδ2 populations in the blood and tonsil, there was generally a higher proportion of Vδ2 T cells in the blood than in the tonsil, although there was significant variation between donors. This recapitulates previous findings that Vδ2 T cells make up approximately 70% of peripheral blood γδ T cells on average, however this number is donor dependent (Figure 5.1c, (Davey et al., 2017)). However, in the tonsil there was a higher proportion of Vδ1 T cells than in the blood (Figure 5.1c). Within the tonsil itself however, there was no significant difference between the size of Vδ1 and Vδ2 T cell populations (Figure 5.1d).

The Vδ2 T cell population within the blood of many donors is mostly paired exclusively with a Vy9 chain (Morita et al., 2007). In some donors however, the Vδ2 population also has cells that are Vy9-, although this population is rare in CMV- adults (Davey et al., 2018). In the tonsil the Vδ2 T cells that paired with a Vy9- chain was significantly less prominent than the Vy9+ subset (Figure 5.1e), suggesting the Vy9-Vδ2+ cells do not access the tonsil in high numbers, or more likely that these healthy donors did not have appreciable population of Vg9-Vd2+ T cells which are not commonly found in the general population.

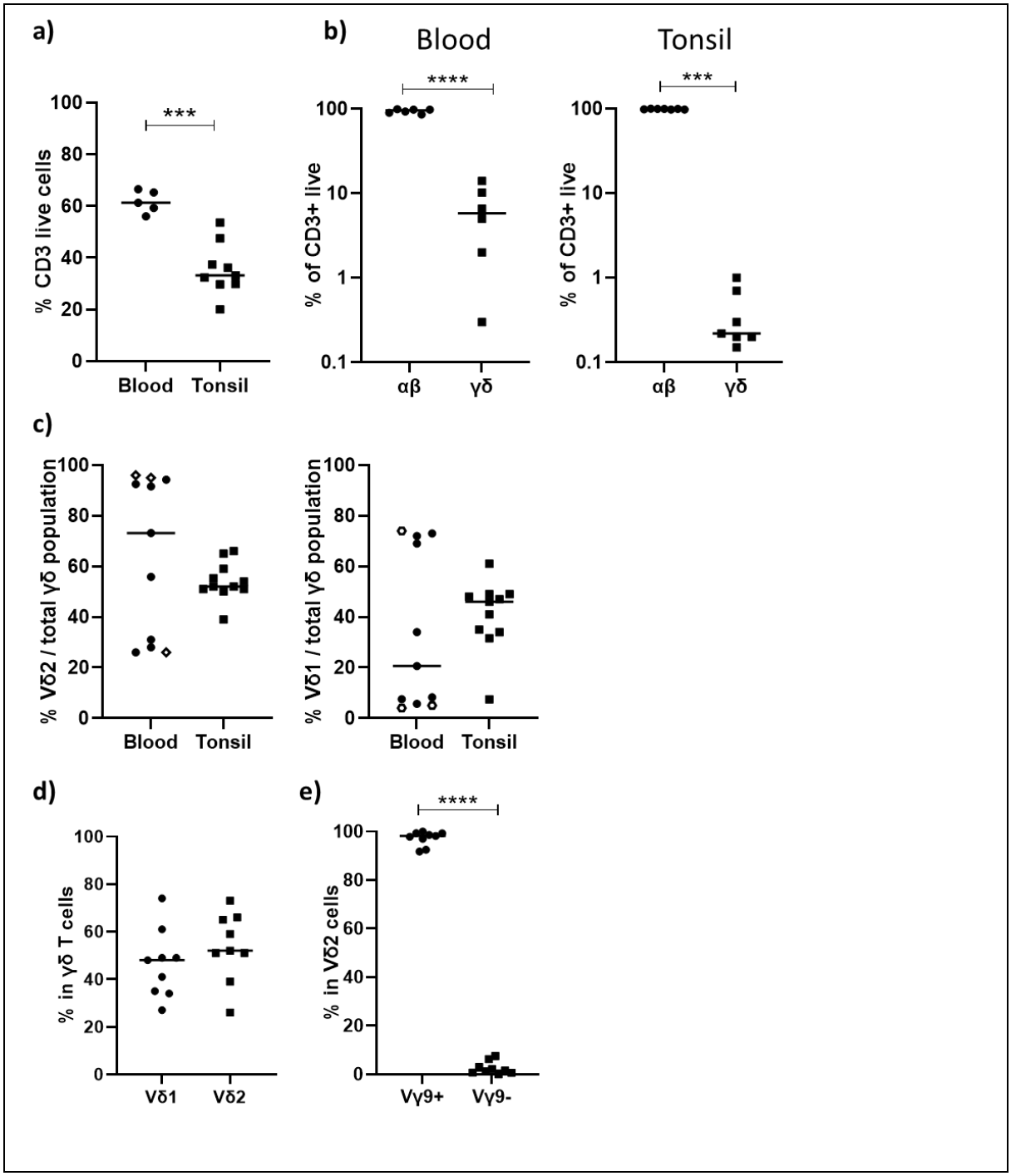

a)
b)
Blood
Tonsil
c)
d)
e)
***
****
% CD3 live cells
% of CD3+ live
% Vδ2 / total γδ population
% Vδ1 / total γδ population
% in γδ T cells
% in Vδ2 cells
Blood
Tonsil
αβ
γδ
Vδ1
Vδ2
Vγ9+
Vγ9-

Figure 5.1: T cell populations in human blood and tonsil. Fresh blood samples were prepared and PBMCs collected as described in the methods. Tonsil samples were processed as described in the methods and thawed immediately prior to staining. Samples were stained with antibodies and analysed with flow cytometry. Each experiment is a culmination of at least 3 repeats. Due to COVID-19 disruptions, samples acquisition was affected. Data points with clear centres were processed by colleagues in the Willcox lab and included to increase the power of the data. Each data point represents an individual donor and data were analysed with unpaired t-test or Mann Whitney test as required. * p0.05 **p0.01 *** p0.001 **** p0.0001

a) The percentage of CD3+ cells within the live compartment in the blood (n=5) and tonsil (n=9).

b) The percentages of αβ and yδ T cells in the blood (n=6) and tonsil (n=7).

c) The proportion of Vδ1 and Vδ2 cells within the blood (n=11) and tonsil (n=11).

d) The percentage of Vδ1 and Vδ2 cells within the yδ T cell compartment in the tonsil (n=9).

e) The percentage of Vy9+ and Vy9- chains within the Vδ2 T cell subset in the tonsil (n=9).

5.3.2 Naïve, cm-like and effector Vδ1 T cell populations in human blood and tonsil

In the tonsil, both CD8 and Vδ1 T cells were dominated by a naïve (CD27+ CD45RA+) population which correlates with the tissue's lymphoid function and the recirculation of these cells throughout it. There is also a sizable cm-like (CD27+ CD45RA-) phenotype and a small effector (CD27-) population (Figures 5.2b,c). These tonsil samples were acquired from donors that were healthy at the time of acquisition and had no current inflammation. They had previously had recurrent tonsilitis which may explain the presence of a resident effector population.

The presence of a cm-like population in most tonsil samples was surprising as it contrasts significantly with the majority of blood samples. In the previous phenotyping of more than 20 healthy donors, Blood Vδ1 T cells were mostly naïve (CD27+ CD45RA+) or a CD27low/neg CD45RA+ effector phenotype. Of the few cells that fell into the cm-like CD27+ CD45RA- gate, their TCR was diverse and mimicked the naïve population (Davey et al., 2017a). More recently I found two donors with a cm-like population in their blood, which is shown below, but this is extremely rare. Therefore the presence of a cm-like population in most tonsil donors is of great interest.

The naïve dominated phenotype in the tonsil is in contrast with matched blood samples which had a higher percentage of effector Vδ1 CD27- T cells (Figure 5.2d). This correlates with previous findings that blood Vδ1 and CD8 T cells have large effector CD45RA+ populations in most donors (Davey et al., 2017a; Hamann et al., 1997; McMurray et al, in preparation). Within the PBMC donors tested however, two had an unusual Vδ1 cm-like population (CD27+ CD45RA-) also (Figure 5.2a), similar to the population observed in the tonsil and which was analysed later in this chapter.

Therefore in the tonsil the Vδ1 T cell subset is dominated by naïve and cm-like cells compared to the blood which is dominated by effector Vδ1 cells in many donors. I set out to further characterise the phenotype and function of all three Vδ1 T cell populations present in the tonsil.

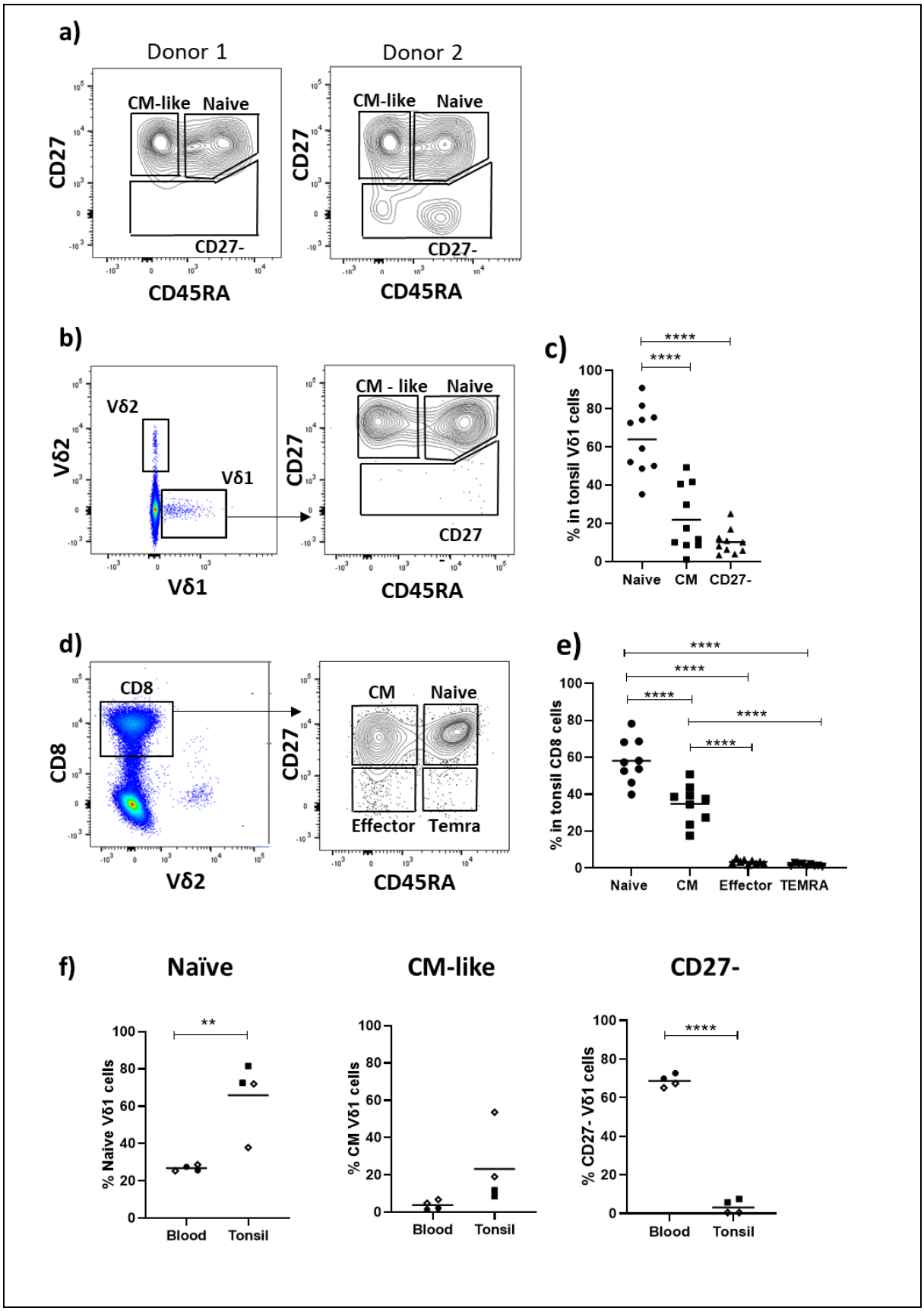

a)
Donor 1
Donor 2
CM-like
Naive
CD27-
CD27
CD45RA
b)
Vδ2
Vδ1
CM - like
Naive
CD27
CD45RA
c)
% in tonsil Vδ1 cells
Naive
CM
CD27-
****
d)
CD8
CM
Naive
Effector
Temra
e)
% in tonsil CD8 cells
Naive
CM
Effector
TEMRA
f)
Naïve
CM-like
CD27-
% Naive Vδ1 cells
% CM Vδ1 cells
% CD27- Vδ1 cells
Blood
Tonsil
**

Figure 5.2: Vδ1 T cell populations in human tonsil and blood. Fresh blood samples were prepared and PBMCs collected as described in the methods. Tonsil cells were collected as described in the methods and thawed immediately prior to antibody staining and analysis. Samples were stained with antibodies and analysed with flow cytometry. Due to COVID-19 disruptions, samples acquisition was affected. Data points with clear centres were processed and collected by colleagues in the Willcox lab and included to increase the power of the data. Each data point represents an individual donor and includes at least 3 repeats. Data were analysed with unpaired t-test or one way ANOVA as required. * p0.05 **p0.01 *** p0.001 **** p0.0001

a) Representative gating of naïve (CD27+ CD45RA+), cm-like (CD27+ CD45RA-) and effector (CD27- CD45RA-) Vδ1 T cells of two donors in the blood.

b) Representative gating of naïve (CD27+ CD45RA+), cm-like (CD27+ CD45RA-) and effector (CD27- CD45RA-) Vδ1 T cells in the tonsil.

c) The percentage of naïve (CD27+ CD45RA+), cm-like (CD27+ CD45RA-) and effector (CD27- CD45RA-) Vδ1 T cells in the tonsil (n=10).

d) Representative gating of naïve (CD27+ CD45RA+), central memory (CD27+ CD45RA-) , effector (CD27- CD45RA-) and Temra (CD27- CD45RA+) CD8 T cells in the tonsil.

e) The percentage of naïve (CD27+ CD45RA+), CM (CD27+ CD45RA-), Temra (CD27- CD45RA+) and effector (CD27- CD45RA-) CD8 T cells in the tonsil (n=9).

f) The percentage of naïve, cm-like and effector Vδ1 cells in matched blood and tonsil (n=4).

5.3.3 The phenotype of naïve, effector and cm-like Vδ1 T cells in the blood and tonsil

Following the identification of cm-like, naïve and effector Vδ1 subsets in the tonsil, further characterisation of these cells was needed to determine whether these cells had hallmarks of traditional αβ T cell memory, naïve and effector populations. The Vδ1 T cells were phenotyped and compared to CD8 T cells where possible.

IL-7Rα is required for naïve and memory cell survival and homeostasis in the periphery (Carrette & Surh, 2012; Parretta et al., 2008; Schluns et al., 2000; Tough & Sprent, 1994). Within the tonsil, the naïve and cm-like Vδ1 cells had significantly higher population of IL-7Rα+ cells than in the effector Vδ1 subset (Figure 5.3a). This coincides with naïve Vδ1 cells in the blood that express significantly higher IL-7Rα than the effector Vδ1 population (Davey et al., 2017a).

CCR7 was also expressed on a larger population of naïve Vδ1 cells than on cm-like and effector Vδ1s, however the difference did not reach significance (Figure 5.3a). In CD8 T cells however the CCR7+ naïve T cells were significantly higher than effector and CM cells (Figure 5.3a), recapitulating previous data (Sada-Ovalle et al., 2012). CCR7 is expressed on both naïve cells and central memory cells in the blood to allow access to lymphoid tissue, whilst not expressed on effector memory cells that do not require access to the lymphatic system (Campbell et al., 2001; Davey et al., 2017a; Sallusto et al., 1999).

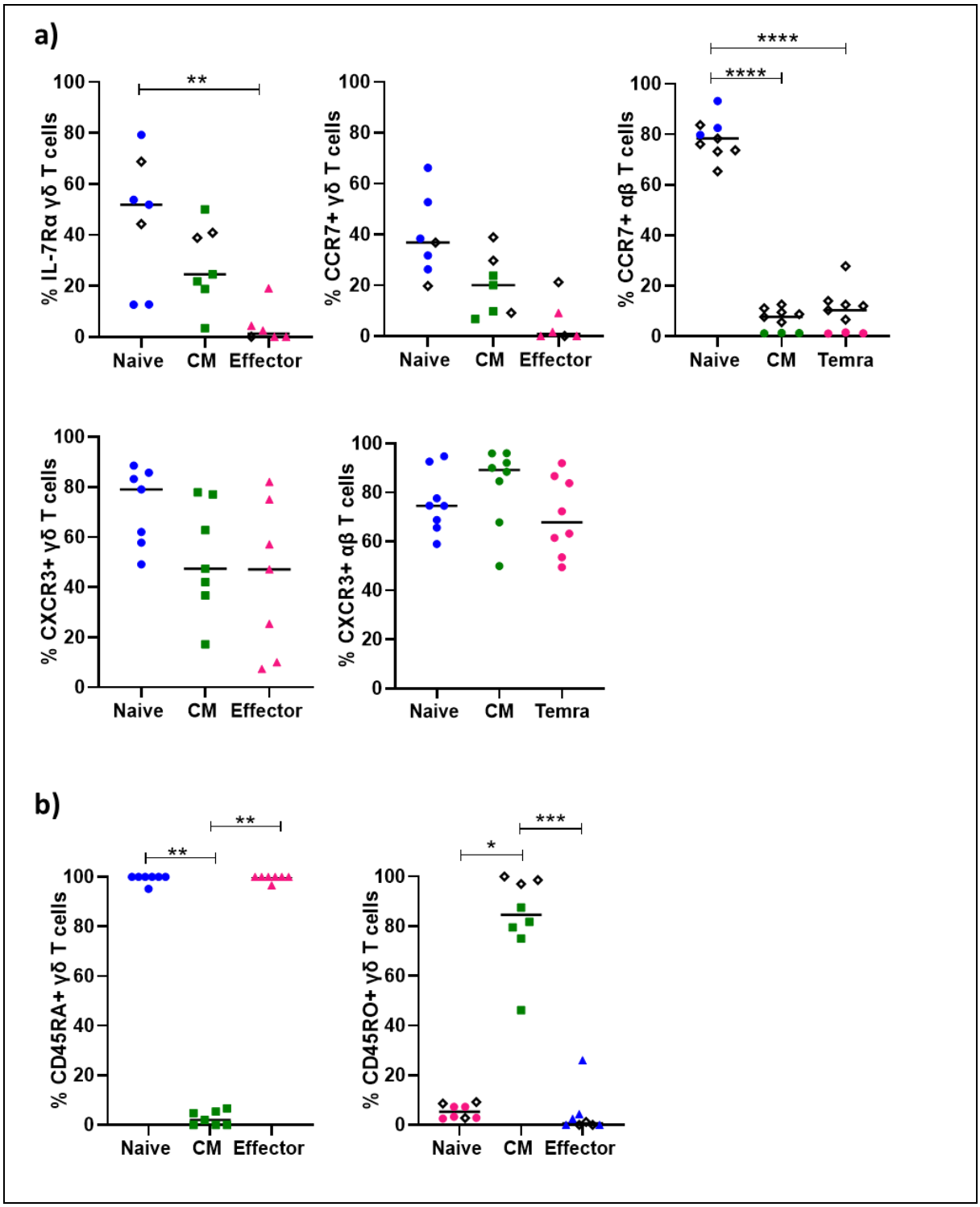
CXCR3 was previously thought to be upregulated on T cells following activation and is thought to guide cells to inflamed tissue (Hu et al., 2011; Luster et al., 2005). However, the situation may be more complex. CXCR3 may be expressed on a subset of naïve CD8 T cells with enhanced effector function *ex vivo* (De Simone et al., 2019). Recently, naïve Vδ1 T cells were

shown to express CXCR3 (McMurray et al, in preparation), and it has been found to be expressed on naïve αβ T cells and yδ T cells in children (Syrimi et al., 2020). In the tonsil, CXCR3 was expressed on the majority of the naïve population and at high levels on cm-like and effector cells in both Vδ1 and CD8 T cells (Figure 5.3a), which reiterates findings that T cells in the tonsil and Vδ1 T cells within the blood express it (Poggi et al., 2004; Rabin et al., 2003).

The three Vδ1 T cell populations in the tonsil also share characteristic CD45RA and CD45RO expression noted in αβ T cells and yδ T cells in the blood. Different CD45 allotypes are created through variation in splicing of exons and the extracellular domain, with each allotype associated with different cell subsets and activation states. Naïve T cells express the high molecular mass allotype CD45RA whilst central memory cells express the low molecular mass CD45RO (Akbar et al., 1988; Dawes et al., 2006; Merkenschlager et al., 1988). CD45RO expression is thought to correlate with a lower threshold for TCR driven activation (McKenney et al., 1995; Novak et al., 1994). CD45RA was expressed on significantly lower numbers of cells in the cm-like population, whilst CD45RO was significantly higher in the cm-like population than in Naive and Effector Vδ1 T cells (Figure 5.3b).

Resting Effector Vδ1 T cells had significantly higher levels of Granzyme B+ and Perforin+ cells than naïve populations (Figure 5.3c), which is consistent with their phenotype in the peripheral blood (Davey et al., 2017a). Alongside the expression of cytotoxic molecules, pro-inflammatory transcription factors were also highest in the effector Vδ1 population. T-bet+ cells were significantly higher in the effector population compared to naïve cells (Figure 5.3d). T-bet is a master transcription factor for driving effector function and differentiation (Kallies & Good-Jacobson, 2017). Eomes is also linked to effector differentiation and can drive cytotoxic cytokine production and release and expressed most frequently by effector Vδ1 cells (Figure 5.4d, (Pearce et al., 2003, McMurray et al, in preparation). However the higher levels in the

effector population did not reach significance due to a substantial level of Eomes in the naïve Vδ1 population (Figure 5.3d). In the CD8 T cells naïve cells did have much lower expression of Eomes and it was expressed by the effector and memory compartment (Figure 5.3d).


a)
% IL-7Rα γδ T cells
% CCR7+ γδ T cells
% CCR7+ αβ T cells
% CXCR3+ γδ T cells
% CXCR3+ αβ T cells
Naive
CM
Effector
Temra
b)
% CD45RA+ γδ T cells
% CD45RO+ γδ T cells

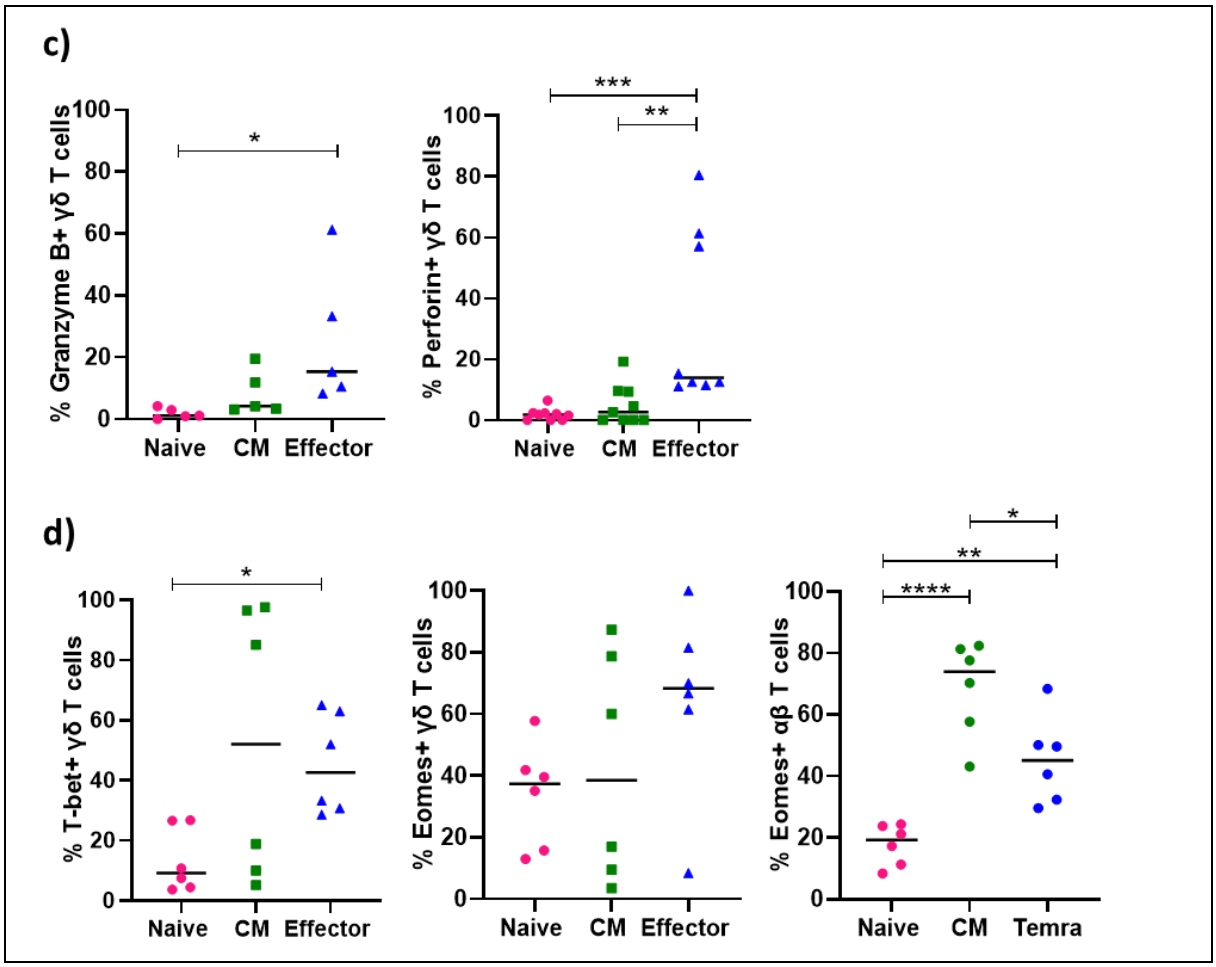


Figure 5.3: The phenotype of Naïve, Effector and cm-like Vδ1 cells in the tonsil. Tonsil PBMCs were collected previously as described in the methods and thawed immediately prior to antibody staining and analysis. Samples were stained with antibodies and analysed with flow cytometry. Due to COVID-19 disruptions, sample acquisition was affected. Data points with clear centres were processed by colleagues in the Willcox lab and included to increase the power of the data. Each data point represents an individual donor and data was analysed with one-way ANOVA and a Tukey's post hoc test or Kruskal Wallis with Dunn's post hoc test as required. * p0.05 **p0.01 *** p0.001 **** p0.0001

a) The expression of CCR7, IL-7Rα and CXCR3 on naïve, cm-like and effector Vδ1 T cells (n=7) and CD8 T cells in the tonsil (n=9).

b) The expression of CD45RA and CD45RO on naïve, cm-like and effector Vδ1 T cells in the tonsil (n=7).

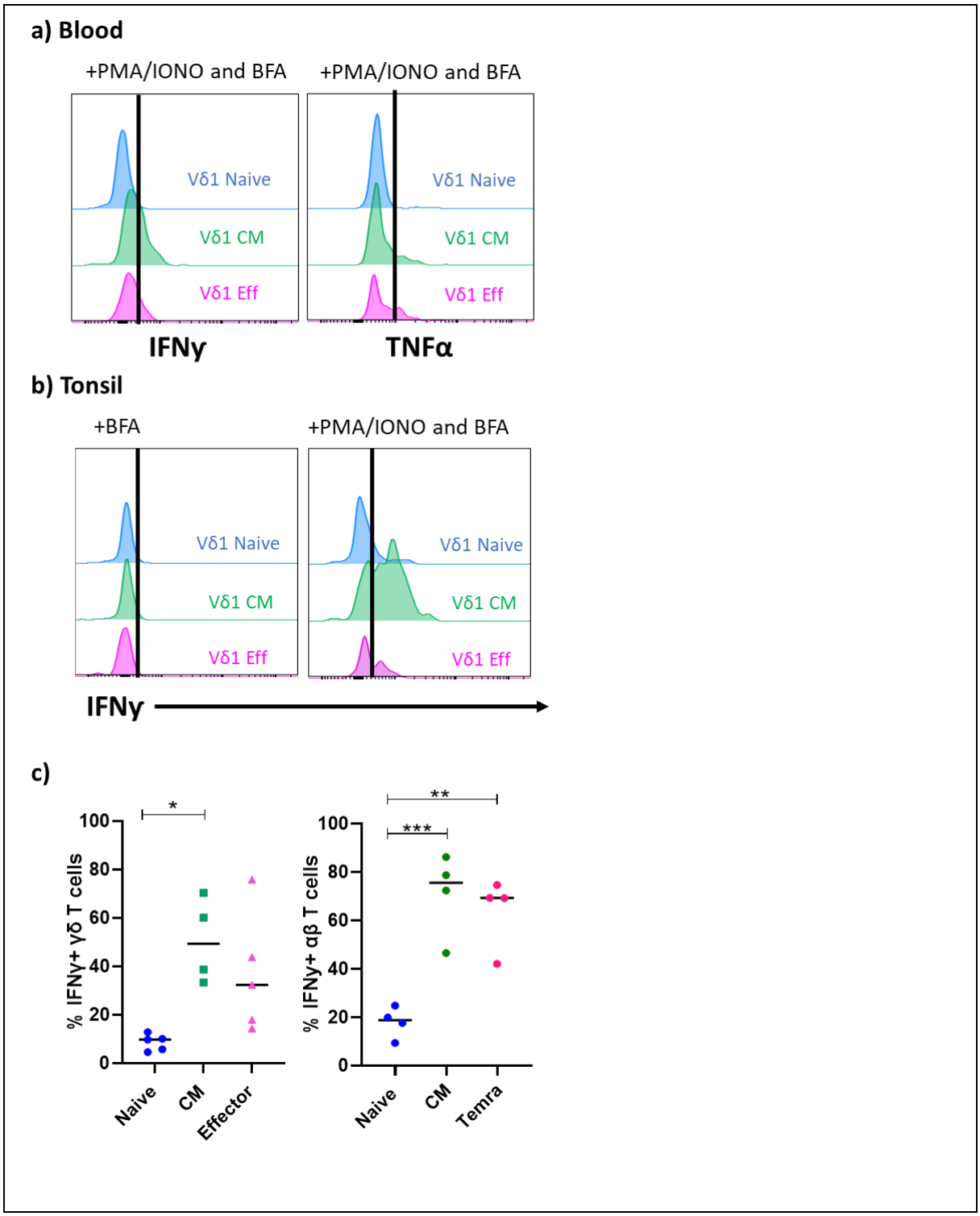
c) The expression of Granzyme B (n=5) and Perforin (n=8) on naïve, cm-like and effector Vδ1 T cells in the tonsil.

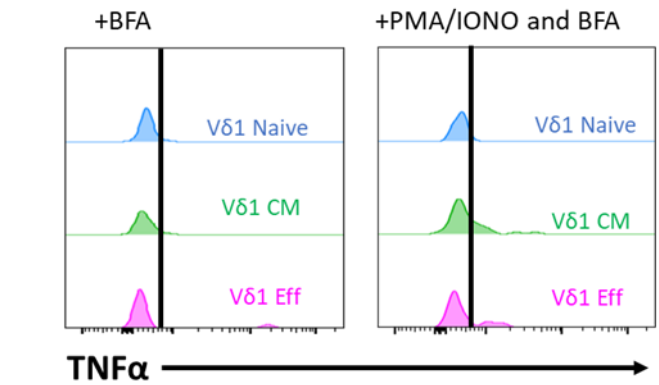
d) The expression of T-bet on naïve, cm-like and effector Vδ1 cells (n=6) in the tonsil and the expression of Eomes on naïve, cm-like and effector Vδ1 cells (n=6) and CD8 T cells in the tonsil (n=6).

5.3.4 The response of tonsillar naïve, effector and cm-like Vδ1 T cells to stimulation

We also wished to determine if the tonsillar Vδ1 T cell subsets responded to TCR-independent stimulation. Effector CD27low/neg Vδ1 T cells have previously been shown to produce greater amounts of pro-inflammatory cytokines and are rapidly activated by both TCR-dependent (anti-CD3/CD28) and independent signals (such as IL-15) compared to the naïve CD27+ CD45RA+ Vδ1 compartment which requires TCR stimulation (Davey et al., 2017a).

The ability of effector and cm-like Vδ1 T cells to respond to stimulation was found in the blood. Both the effector and cm-like Vδ1 T cells have a higher percentage of IFNγ+ cells and TNFα+ cells than the naïve subset following PMA/Ionomycin stimulation (Figure 5.4a). This is also observed in the tonsil with a higher number of IFNγ+ and TNFα+ cells in the cm-like and effector Vδ1s than in the naïve cells (Figure 5.4c,e). These patterns are also present in CD8 T cells with effector and CM T cells dominating the IFNyand TNFα release.


a) Blood
+PMA/IONO and BFA
+PMA/IONO and BFA
Vδ1 Naive
Vδ1 CM
Vδ1 Eff
Vδ1 Naive
Vδ1 CM
Vδ1 Eff
IFNγ
TNFα
b) Tonsil
+BFA
+PMA/IONO and BFA
Vδ1 Naive
Vδ1 CM
Vδ1 Eff
Vδ1 Naive
Vδ1 CM
Vδ1 Eff
IFNγ
c)
% IFNγ+ γδ T cells
100
80
60
40
20
0
*
Naive
CM
Effector
% IFNγ+ αβ T cells
100
80
60
40
20
0
**
***
Naive
CM
Temra



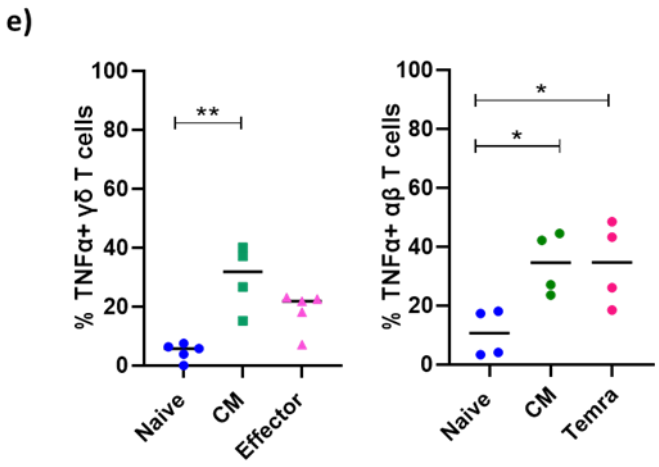


Figure 5.4: The response to stimulation by naive, effector and CM CD8 and Vδ1 T cells in the blood and tonsil. Fresh blood samples were prepared and PBMCs collected as described in the methods. Tonsil PBMCs were collected previously as described in the methods and thawed immediately prior to antibody staining and analysis. Samples were stained with antibodies and analysed with flow cytometry. Cells were stained with PMA and Ionomycin for 6 hours. BFA was added 1 hour into the stimulation. Each data point represents an individual donor and was analysed with one-way ANOVA and a Tukey's post hoc test or Kruskal Wallis with Dunn's post hoc test as required. * p0.05 **p0.01 *** p0.001 **** p0.0001

a) Histograms of the expression of IFNyand TNFa and on naïve, cm-like and effector Vδ1 cells in the blood post stimulation.

b) Histograms of the expression of IFNyin naïve, cm-like and effector tonsil Vδ1 cells following stimulation.

c) The expression of IFNyon naïve, cm-like and effector Vδ1 T cells (n=5) and CD8 T cells in the tonsil following stimulation (n=4).

d) Histograms of the expression of TNFα in naïve, cm-like and effector tonsil Vδ1 cells following stimulation.

e) The expression of TNFα on naïve, cm-like and effector Vδ1 T cells (n=5) and CD8 T cells in the tonsil following stimulation (n=4).

5.3.5 The TCR repertoire of naïve, cm-like and effector Vδ1 T cells in the blood and tonsil

Previous work had found that Vδ1 naïve CD27+ cells in peripheral blood had a diverse TCR repertoire whilst the effector CD27- compartment in the blood and liver was highly clonal (Davey et al., 2017a; Hunter et al., 2018). Because cm-like Vδ1 T cells are relatively rare, the TCR repertoire of this population has not been examined to explore whether cm-like Vδ1 T cells are clonal, and if so, are they expressing distinct clonotypes to effector Vd1 in the same donor. Therefore, the TCR repertoire of naïve, effector and cm-like populations were sequenced from the blood in one donor that had all three populations (Figure 5.2a).

Single cell sequencing of Vδ1 chains from Vδ1+ T cell subsets from this donor recapitulates previous data as their naïve Vδ1+ T cells expressed diverse CDR3 delta sequences, whilst their effector Vδ1+ T cells expressed a monoclonal CDR3 delta sequence. A single instance of the clonal delta sequence is also seen in the naïve population. This has been observed before in other donors (Davey et al., 2017a) although the significance is unclear, and could represent a stem-like population of the clone, although these cells are so rare they are difficult to study. Interestingly the same monoclonal TCR was expressed in the cm-like population, suggesting that a single clonotype can exist in multiple differentiation states (CD45RA+CD27low and CD45RA-CD27+) (Figure 5.5a).

Within the effector compartment the clonal CDR3 delta sequence is paired with 2 different Vy4 sequences and both of which appear functional (Figure 5.5b). This has been observed before, albeit rarely, in Vδ1 T cell clones from other donors (McMurray et al, in preparation), and suggests that allelic exclusion is not absolute in yδ T cells. It remains unclear which Vy4 chain is preferentially expressed or if both Vy4 sequences can be expressed on the cell surface paired with the same TCRδ. This would be intriguing to follow up, although technically difficult.

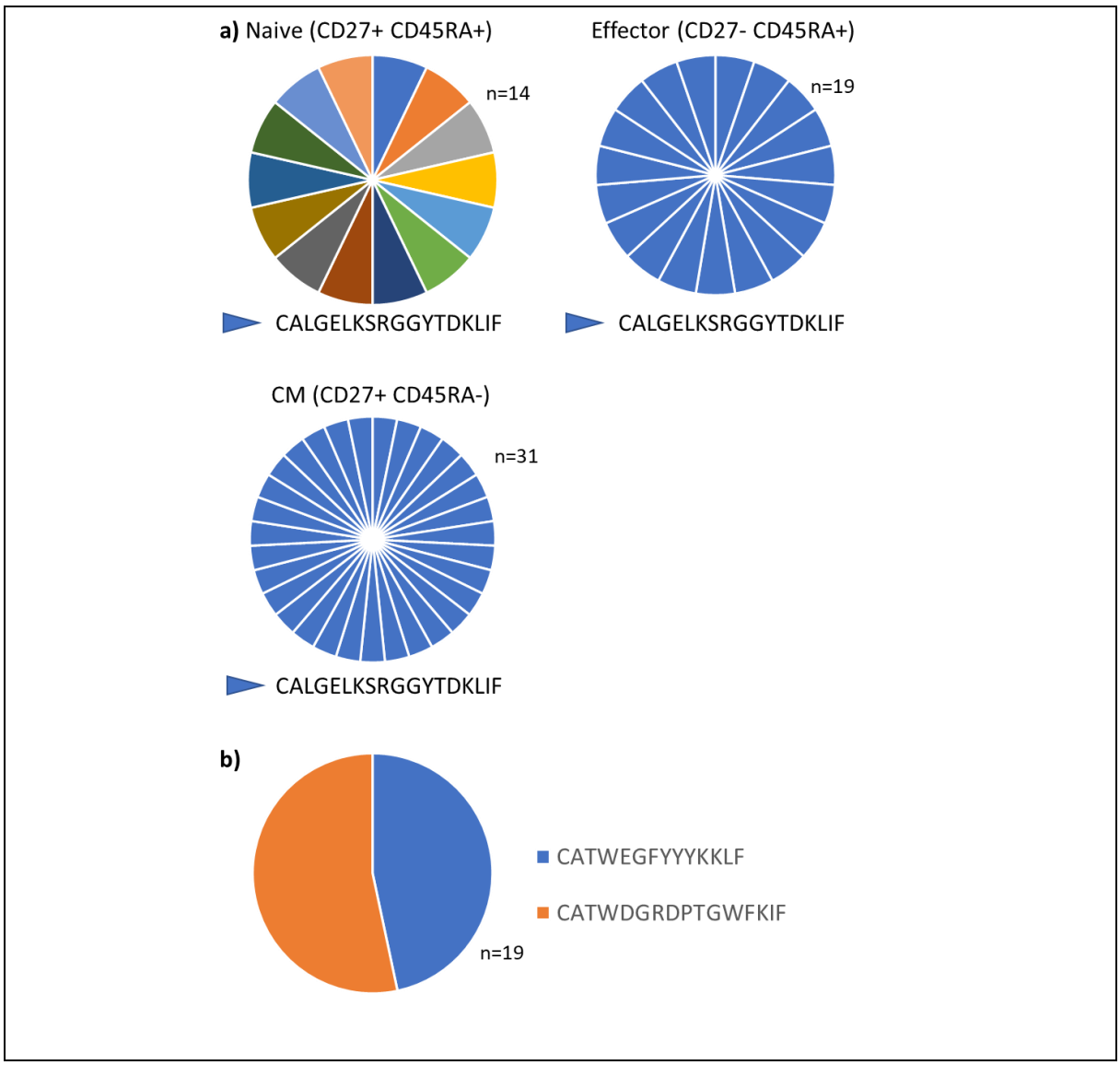

a) Naive (CD27+ CD45RA+)
n=14
CALGELKSRGGYTDKLIF
Effector (CD27- CD45RA+)
n=19
CALGELKSRGGYTDKLIF
CM (CD27+ CD45RA-)
n=31
CALGELKSRGGYTDKLIF
b)
CATWEGFYYYKKLF
CATWDGRDPTGWFKIF
n=19

Figure 5.5: The TCR repertoire of naïve, cm-like and effector Vδ1 T cells in the blood. PBMC were harvested from a donor that has the three Vδ1 populations in their blood (CD27/CD45RA phenotype in Figure 5.2a). Single Vδ1 T cells from each subset were then FACS sorted based on their CD27/CD45RA expression and their CDR3 sequences sequenced. Sequencing data was visualised and analysed by the Sequencher software.

a) The proportion of each delta chain CDR3 sequence in the naïve (n=14), effector (n=19) and cm-like (n=31) Vδ1 population in the blood.

b) The delta chain clone expressed by effector cells was paired with 2 different Vy4 sequences. This pie chart represents the proportion of this clonal effector CDR3 delta sequence (n=19) that is paired with each Vy4 chain

The CDR3 sequences of Vδ1 T cell naïve, cm-like and effector subsets from the tonsil of two donors were also sequenced to analyse the TCR repertoire differences. In one donor, the cm-like subset had two clones, as well as another sequence that was present once in the cm-like subset, and once in the naïve subset (Figure 5.6a). Unfortunately this donor did not have a substantial effector population that could be sequenced. In the second donor there were three clones present in the cm-like subset, interestingly one of these was also present in the naïve subset, and the effector subset, suggesting some phenotypic heterogeneity in this clone (Figure 5.6c).

We wanted to explore the TCR repertoire in matched tonsil and blood to examine if clones found in the tonsil were also present in the blood, and if the phenotype of these clones was the same in different tissues. I obtained two such matched samples, however these samples were not healthy upon thawing and yielded very few TCR sequences, suggesting the RNA was degraded. In the future I would like to investigate this if I could obtain further matched samples.

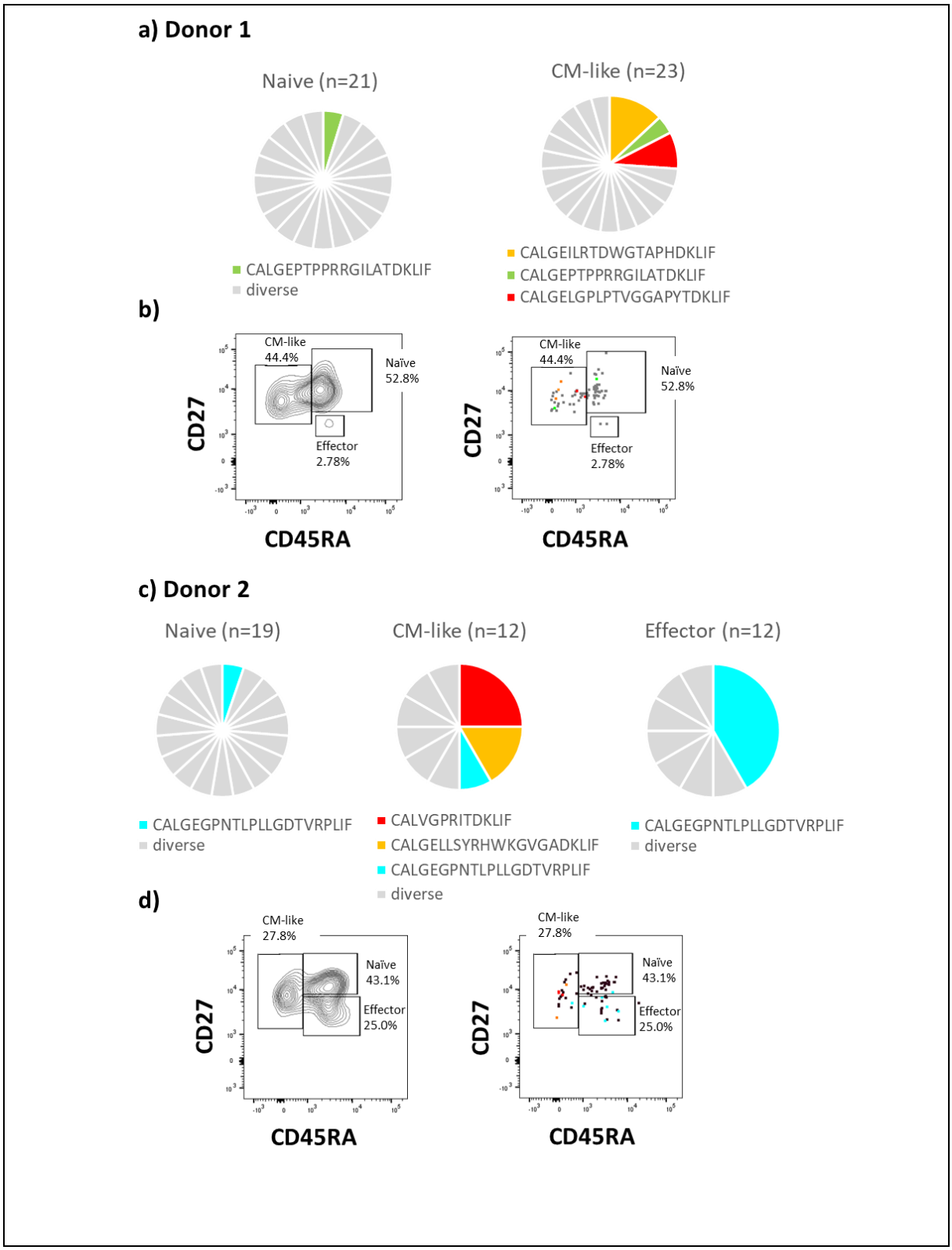

a) Donor 1
Naive (n=21)
CALGEPTPPRRGILATDKLIF
diverse
CM-like (n=23)
CALGEILRTDWGTAPHDKLIF
CALGEPTPPRRGILATDKLIF
CALGELGPLPTVGGAPYTDKLIF
b)
CM-like
44.4%
Naïve
52.8%
Effector
2.78%
CD27
CD45RA
c) Donor 2
Naive (n=19)
CALGEGPNTLPLLGDTVRPLIF
diverse
CM-like (n=12)
CALVGPRITDKLIF
CALGELLSYRHWKGVGADKLIF
CALGEGPNTLPLLGDTVRPLIF
diverse
Effector (n=12)
CALGEGPNTLPLLGDTVRPLIF
diverse
d)
CM-like
27.8%
Naïve
43.1%
Effector
25.0%
CD27
CD45RA

Figure 5.6: The TCR repertoire of naïve, cm-like and effector Vδ1 T cells in the tonsil. T cells were harvested from the tonsils of two donors. Single Vδ1 T cells from each subset were then FACS sorted based on their CD27/CD45RA expression and their CDR3 region sequenced. All sequencing data was analysed via Sequencher.

a) The proportion of each delta chain CDR3 sequence in the naïve (n=21) and cm-like (n=23) Vδ1 population in the tonsil of donor 1. Each section of the pie chart corresponds to a sequence.

b) The flow cytometry plots showing the gates drawn for FACS in each of the populations from donor 1.

c) The proportion of each delta chain CDR3 sequence in the naïve (n=19) and cm-like (n=12) and effector (n=12) Vδ1 population in the tonsil of donor 2. Each section of the pie chart corresponds to a sequence.

d) The flow cytometry plots showing the gates drawn for FACS in each of the populations from donor 2.

5.4 Discussion

The aim of this chapter was to investigate Vδ1 T cell populations in the tonsil and characterise their phenotype, effector function and TCR repertoire. In our donors with matched samples, the tonsil had a significantly higher numbers of naïve Vδ1 T cells in the tonsil than in the blood, which had a significantly higher percentage of Vδ1 effector T cells. In some donors there was also a significant population of Vδ1 T cells in the tonsil with a CD27+ CD45RA- cm-like phenotype. Through further analysis of these three populations I determined that they had some hallmarks of naïve, CM and effector T cells but also diverged from traditional characteristics of these three subsets in other areas.

5.4.1 The phenotype of naïve, effector and cm-like Vδ1 T cells in the blood and tonsil

Whilst the αβ T cell population was larger in both the blood and the tonsil, a clear population of γδ T cells was present (Figure 5.1). The size of the γδ T cell population (as a proportion of total CD3+ T cell) within the tonsil was smaller than what had previously been detected in the blood, and in peripheral tissues (Di Marco Barros et al., 2016; Hunter et al., 2018), suggesting γδ T cells are less prominent in some human lymphoid tissue. This recapitulates previous findings showing human γδ T cells are found most frequently at mucosal sites and peripheral tissues (Gentles et al., 2015; McCarthy & Eberl, 2018).

Vδ1 T cells in the blood can be separated into naïve and effector subsets through their CD27 and CD45RA expression in contrast to αβ T cells which are frequently delineate by CCR7 and CD45RA. However, transcriptional analysis, stimulation assays and antigen recall responses have shown CD27 can also be used on αβ T cell populations (de Jong et al., 1992; Hamann et al., 1997). CD27 was found to closely correlate with CCR7 expression on Vδ1 T cells whilst

also being more highly expressed and clearer to use in delineating subsets (Davey et al., 2017a).

Blood Vδ1 T cells are mostly CD27+ CD45RA+ (Naïve) or CD27low/neg CD45RA+ (Effector) T cells (Davey et al., 2017a; Dieli et al., 2003; Pitard et al., 2008). When applied to the tonsil, there was a significantly higher proportion of CD27+ naïve CD8 and Vδ1 T cells compared to the blood which had significantly more CD27- effector T cells. This was observed in both matched and unmatched samples (Figure 5.2). However it is important to remember that some donors had high levels of naïve Vδ1 T cells in their blood samples and so the blood Vδ1 T cell population is variable between donors and contains both effector and naïve subsets.

The high proportion of naïve Vδ1 T cells in the tonsil corroborates the higher levels of naïve αβ T cells in lymphoid tissue. In the mouse this pattern also emerges; naïve CD44+ CD62L+ γδ T cells are present within the lymphoid tissue and are not present within peripheral tissues such as the liver and gut (Chapter 3, (Ugur et al., 2018)). Naïve T cells remain a significant proportion of T cells within lymphoid tissue even after thymic involution and retain their functionality even in older age (Kumar et al., 2018; Thome et al., 2016).

In the blood of some donors there was also a cm-like CD27+ CD45RA- population but it was rare. I identified this population in only two out of >40 healthy donors. However in the tonsil a much higher proportion of donors had a cm-like population, and this population was found to varying degrees in most donors (Figure 5.3). The presence of a cm-like γδ T cell population within the tonsil aligns with previous work on αβ T cells where CD45RO+ CD27+ CD45RA- αβ T cells were higher in the tonsil and lymph nodes than in peripheral blood (Rosenmann et al., 1998; Sada-Ovalle et al., 2012; Thome et al., 2016).

The high proportion of cm-like Vδ1s in the tonsil could be due to naïve T cells being activated within the tonsil, transitioning into an effector cell and then into a cm-like population to retain

memory ability following antigen clearance. The tonsil is a site of antigen exposure and allows CM and naïve T cells to interact with multiple stimuli (Nave et al., 2001). CM and naïve T cells also have the ability to circulate through SLOs via their expression of CD62L and CCR7 (Sallusto et al., 1999) so it may be that the cm-like Vδ1 T populations are simply in transit and passing through the tonsil as they circulate for antigen.

Future work could entail attempting to delineate between donors with different proportions of cm-like Vδ1 T cell compartment in their tonsil. Their medical history and their exposure to pathogens may have a link to the formation of a cm-like compartment. Viral infections such as CMV and EBV have been shown to drive expansion of Vδ1 T cells in the blood and the liver alongside a clonal focusing of the Vδ1 TCR repertoire and the emergence of long term clones (Davey et al., 2017a; Farnault et al., 2013; Hunter et al., 2018; Pitard et al., 2008; Ravens et al., 2017). In EBV, expanded CD8 EBV-specific T cells are mostly CM with CD27+ CD45RA-CD45RO+ phenotype. In CMV the majority of blood T cells have a TEMRA phenotype however there is a CMV-specific CM compartment in bone marrow (Derhovanessian et al., 2011; Kuijpers et al., 2003; Letsch et al., 2007). Hence pathogen specific CM T cells may exist within tissues and be linked to a patient's infection history.

CD45RO and CD45RA phenotyping was used to further establish the identities of the populations. In T cell biology CM cells express CD45RO whilst CD45RO- CD45RA+ cells are naïve or effector cells dependent on CD27 expression (Golubovskaya & Wu, 2016). The expression of the low molecular mass allotype CD45RO allows for the rapid activation and proliferation of memory cells compared to naïve cells (Dawes et al., 2006). In the tonsil the cm-like Vδ1 T cell population had significantly higher numbers of CD45RO+ cells and significantly lower CD45RA+ cells compared to naïve and effectors Vδ1 T cells (Figure 5.3). αβ CD45RO+ T cells increase in the tonsil with age (Bergler et al., 1999) so it would be interesting in future work to investigate the Vδ1 T cell cm-like populations in donors of different ages.

By staining for surface marker expression I saw that naïve and cm-like Vδ1 T cells within the tonsil expressed high levels of CCR7 and IL-7Rα compared to effector Vδ1 T cells (Figure 5.3). CCR7 is a chemokine receptor commonly used as a naïve and CM T cell markers as it is required to enter SLOs. It binds to CCL21 on endothelial cells within high endothelial venules and transmigrates into lymphatic structures (Campbell et al., 1998; Campbell et al., 1998b; Gunn et al., 1998; Sallusto et al., 1999) The fact that some naïve and cm-like T cells do not express it within the tonsil could be due to cryopreservation of the cells prior to staining which can cause the loss of chemokine receptor signals (Brodie et al., 2013).The ability to stain fresh sample would have been preferable to retain expression. Another possibility is that they downregulate expression once within the tonsil. The low expression on the Effector population could be that it is not recruited by CCR7, they lose it following entry into the tissue or that they are tissue resident and do not recirculate through lymphoid tissue.

IL-7 receptor alpha (IL-7Rα) is required for IL-7 signalling that regulates T cell survival and turnover. IL-7 is thought to be produced within SLOs and remain within the tissue, bound to the extracellular matrix (Surh & Sprent, 2008; Wrenshall & Platt, 1999). This ensures that CM and naïve T cells must enter SLOs to access the IL-7 signal needed for their homeostasis. On terminally differentiated effector cells such as the CD8 effector CD45RA+ subset, the IL-7R expression is much lower than naïve and CM cells (Verma et al., 2017; Willinger et al., 2005). The higher amount of IL-7Ra+ on cm-like Vδ1 T cells in the tonsil compared to the effector compartment could be due to their potentially higher turnover, which is seen in αβ T cell biology (Geginat et al., 2003). Their higher proliferation requires more IL-7 and may be the cause of the differential expression between the cm-like and effector cells (Figure 5.3).

CXCR3+ cells were most prominent in the naive Vδ1 cells but also present effector and cm-like populations (Figure 5.3). This was surprising as CXCR3 increases following activation and is traditionally expressed on effector and memory cells. It allows the cells access to peripheral

tissues (Hu et al., 2011; Luster et al., 2005; Sallusto et al., 1998). In the liver Vδ1 effector T cells expressed high levels of it (Hunter et al., 2018) and it was expressed on effector Vδ2 cells in the blood (Poggi et al., 2004).

However recent work suggests it can be expressed on naïve cells also. CD4 naïve T cells can express it after short term TCR stimulation and a subset of naïve CD8 T cells in the blood, tonsil and lymph nodes express it at steady state. CXCR3+ naïve cells proliferate more and it has been suggested that it is a marker of homeostatic proliferation. CXCR3+ naive cells are also more likely to become effectors (De Simone et al., 2019; Rabin et al., 1999). Hence, the presence of CXCR3+ naïve Vδ1 T cells may indicate their high proliferation capability, however it remains not fully conclusive.

5.4.2 The cytotoxic potential of effector and cm-like Vδ1 cells within the tonsil

The Vδ1 effector T cells within the tonsil had a greater proportion of Granzyme B and perforin+ cells than naïve or cm-like Vδ1 T cells (Figure 5.3). These markers are hallmarks of a cytotoxic effector T cell and not expressed at significant levels in either resting memory or naïve cells. They are stored in cell granules until their rapid release upon antigen recognition and have toxic effects on target cells. Perforin creates holes in the cell membrane and granzyme B induces apoptosis pathways (Catalfamo & Henkart, 2003; Curtsinger et al., 2005; Nowacki et al., 2007; Shresta et al., 1998; Trambas & Griffiths, 2003; Trapani & Smyth, 2002; Trapani & Sutton, 2003).

The presence of a cytotoxic effector Vδ1 subset in the tonsil recapitulates the Vδ1 effector T cell subset in the blood that has significantly higher levels of both Granzyme A and B and perforin compared to naïve CD27+ Vδ1 cells (Davey et al., 2017a). Effector Vδ1 T cells in the

liver and gut also expressed high levels of granzyme B. In the liver these cells shared a phenotype with the effector tonsil subset, CD27- CD45RA+ (Hunter et al., 2018; Mikulak et al., 2019).

The high expression of cytotoxic markers in the effector Vδ1 T cell population in the tonsil was also reaffirmed by its expression of transcription factors that drive cytotoxic function (Figure 5.3). T-bet and Eomes are both from the T-box transcription factor family that share a DNA-binding domain within the genome. They act to increase the production of IFNγ, granzymes and perforin (Cruz-Guilloty et al., 2009; Herrmann, 1992; Hersperger et al., 2010; Knox et al., 2014; McLane et al., 2013; Pearce et al., 2003).

Interestingly one tonsil donor had high T-bet expression but low levels of IFNy following stimulation. This is contrary to expectations as T-bet is an essential transcription factor for the maturation of Th1 subsets and IFNy and T-bet are thought to be intrinsically tied. Whilst one study reported a αβ T cell population that could produce IFNy in T-bet -/- mice, it did still express high levels of Eomes which may act in the place of T-bet (Kannan et al., 2017). It is most likely that the cells have high T-bet and a potential to produce IFNy but they may be unhealthy after cryopreservation and unable to respond to stimulation in this assay.

Both T-bet and Eomes are expressed at high levels in αβ and yδ T cell effectors in the blood, whilst naive cells lack expression of either (Dimova et al., 2015b; Knox et al., 2014, McMurray et al, in preparation). In the tonsil, this was the same with T-bet higher in the effector than the naïve T cell subset (Figure 5.3). High Eomes expression was present in the effector populations in both Vδ1 and CD8 T cells in the tonsil, whilst lower in the naïve populations (Figure 5.3). The lower levels of Eomes in naïve CD8 T cells compare to naïve Vδ1 T cells, recapitulates the Eomes expression in these populations in the blood (McMurray et al, in preparation).

The reason for presence of variable Eomes and T-bet expression in cm-like populations remains unclear, and may be due to donor variation. Further donors would help to strengthen the data and determine a better average for Eomes levels. CM T cells are thought to have intermediate Eomes levels in yδ T cells (Knox et al., 2014) and hence the current median on the graph may be quite representative. Many of the studies analysing T-bet and Eomes expression however are in the blood due to the ease of tissue access and so it is unclear how representative it is of CM T cells within solid tissue.

The stimulation profile of the three populations were also investigated to test their cytotoxic potential. CD8 T cells within the tonsil have previously been shown to produce both IFNy and TNFα following stimulation (Sada-Ovalle et al., 2012). In blood, liver and tonsil there was an increase in IFNy and TNFα in effector and cm-like Vδ1 cells following stimulation. However in the liver the highest IFNy and TNFα responses were in a tissue resident CD45RA- CD27- Vδ1 population ((Hunter et al., 2018) ,Figure 5.4). This Vδ1 population was not present in any tonsil tissue but is also prominent in the gut Vδ1 (Mayassi et al., 2019). Hence this tissue resident subset with high cytotoxic responses may only be in peripheral tissue and not in lymphoid organs.

In the tonsil and the blood the cm-like Vδ1 T cell populations had the highest levels of IFNy and TNFα following stimulation, however there was not a significant difference with the effector Vδ1 T cell subset (Figure 5.4). The high levels of cytokine production was surprising given their supposed memory role. It may be that this subset has a higher cytokine production whilst the effector subset is more cytotoxic. It puts into question whether the cm-like subset is a memory population or whether it is a different effector population. Further difficulty in delineating the populations arise due to the ability of CM T cells to become effector CD45RA+ T cells upon stimulation and the presence of different subsets within the CM compartment with different proliferative and cytokine potential (Geginat et al., 2003). Staining the cells for proliferation

following stimulation would help to determine this question as CM T cells would have a higher proliferative rate.

Interestingly however, the same pattern was observed in CD8 αβ T cells with the CM subset dominating the cytokine response. As the CD45RA+ CD27+ CM population has been characterised by others as a memory population (Geginat et al., 2003; Golubovskaya & Wu, 2016), it is likely that the effector population is underperforming in our samples rather than the CM T cell subset releasing exceptionally high levels of cytokine.

The poor response of both effector αβ and yδ T cells may also be due to a reduced survival of the subset following cryopreservation. Long-term storage of more than a year can reduce IFNy responses following stimulation and lead to a loss of effector T cells (Owen et al., 2007). Many donors also only have small populations of effector T cells and hence a loss of cells would be more problematic than from the larger naïve or CM Vδ1 T cell compartments. If the cells were not healthy they may have continued to die following stimulation.

The method of stimulation could also have played a role in the poor effector response. Stimulating cells with PMA/ionomycin or with anti-CD3/anti-CD28 can drive different cytokine production levels even from within the same donor and cell sample (Olsen & Sollid, 2013). Future work could examine the effects of a TCR driven stimulation by stimulating the cells with anti-CD3 and anti-CD28 or with an anti-γδ TCR antibody, alongside also testing further non-TCR signals such as IL-2/IL-15. It would be interesting to determine whether the Vδ1 T cells within the tonsil responded to these innate signals by proliferating or through effector function or whether they require TCR stimulation for cytotoxic responses. Alongside the release of cytokines following stimulation, the rate of proliferation could also be used as a measure for the size of the response as CM T cells turnover rapidly during a response.

5.4.3 The diversity of naïve Vδ1 TCR repertoires in the blood and tonsil

The TCR repertoire allows the examination of the function and biology of a subset of T cells. A naïve T cell subset would be predicted to have a diverse TCR repertoire, which would become clonal following the activation of specific TCRs with their cognate antigen (Thome et al., 2014). Within the blood the Vδ1 naïve T cells have a diverse, private repertoire that undergoes some clonal expansions with antigen experience and accompanied differentiation into effector cells. The Vδ1 effector subset however has a clonal TCR repertoire with the expansion of private, long lived clones (Davey et al., 2017a). This finding was recapitulated with a sequenced blood donor that had diverse delta chain sequences in their naïve T cells and a single delta chain that was expressed by one cell in the naïve population whilst being the only sequence expressed by effector and cm-like subsets (Figure 5.5).

Interestingly within the effector compartment the single delta chain was paired with two different Vy4 chains, both of which appear functional (Figure 5.5). Through sequencing alone I can't determine if either of these chains are preferentially expressed on the surface or if there are two clones that share a delta chain but differ with their gamma chain usage. Unfortunately the gamma chain was not sequenced at high quality in the cm-like population and so I was unable to determine if the same delta chain expressed in the cm-like subset also pairs with the identical Vy4 sequences.

It is interesting that the same delta sequence is expressed in both effector and cm-like blood Vδ1 populations, this was also seen with one clone in the tonsil (Figure 5.6). This has been shown previously with CMV, EBV and Influenza A reactive TCRαβ clones seen in both TCM and effector compartments that were stable and long lived (Baron et al., 2003; Hislop et al., 2001; Oakes et al., 2017). The presence of a clone in both subsets may also be due to cells moving between the two populations following a change in their phenotype over time. In CMV+

donors CMV responsive CD8 T cells with a CD27- CD45RA+ effector phenotype transformed into a TCM population phenotype with CD45RO expression following stimulation with CMV peptides. And in later stages of infection and latency, CD27 expression was lost and CD45RA expression was gained, allowing the cells to transition to an effector memory population (Wills et al., 2002). A reactivation of a latent infection could cause continuous movement between the compartments.

It would have been interesting to sequence further donors with cm-like populations in their blood to confirm if there is any overlap between the expanded sequences across donors. This is unlikely however as the Vδ1 blood repertoire is incredibly private (Davey et al., 2017a). Also it would have been useful to determine if other donors also have shared sequences between their effector and cm-like compartments. Unfortunately donors with blood cm-like compartments are rare and more samples could not be collected before the completion of my studentship.

In the tonsil, single cell sequencing of Vδ1 T cells from two donors showed that the naïve compartment in tonsil had a diverse delta chain repertoire whilst some clones were observed in the cm-like or effector compartments (Figure 5.6). Unfortunately these two donors had few surviving effector T cells to analyse and FACS sort following thawing. Their effector populations were not large even in the steady state and effector Vδ1 T cells could have been fragile and did not survive their extraction from tissue, cryopreservation and thawing overnight before FACS and sequencing.

For future work, it would also be useful to sequence naïve, cm-like and effector Vδ1 T cells from matched blood and tonsil to examine whether any clones were shared across the two tissues. Previous work has found that Vδ1 effector T cells in the liver share clones with blood Vδ1 effector T cells (Hunter et al., 2018). If matched clones were found in the tonsil and the

blood, it would indicate that Vδ1 cells are circulating between the tissues. If not then it may suggest, particularly in the effector Vδ1 cells, that these cells have a tissue resident biology and remain within the tissue to respond immediately following activation.

5.5 Conclusion

In summary, within the tonsil, there are naïve, cm-like and effector Vδ1 T cells. In the matched samples tested, the tonsil had a significantly higher percentage of naïve and cm-like cells compared to the blood which had higher levels of effector Vδ1 T cells in most donors. The phenotype of these cells within the tonsil was characterised by surface marker and transcription factor expression, response to stimulation and TCR repertoire.

The high concentration of naïve and cm-like Vδ1 T cells within the human tonsil and within lymph nodes in the mouse (Chapter 3) suggests that just as with αβ T cells, potentially adaptive-like γδ T cells can circulate around the SLOs. These populations are also present at much lower levels in peripheral and mucosal tissues such as the gut and liver in both species (Chapter 3,(Hunter et al., 2018; Mikulak et al., 2019)) which may suggest that predominantly effector γδ T cells can access these tissues. Future work in other human lymphoid tissue would further strengthen this hypothesis and address the adaptive-like function of these cells.

Chapter 6 – Concluding remarks

This project explored whether an adaptive-like γδ T cell biology, already identified in humans, could be detected in murine γδ T cells. Exploring similarities across both species is important as it helps us to determine which collections of murine data can be extrapolated to humans. Murine models will prove essential in exploring key γδ questions such as their migration, response to different infections, development and tissue localisation.

6.1 The difficulty in defining adaptive-like and innate-like paradigms

Whilst this thesis aimed to explore an adaptive-like γδ paradigm, this remains challenging as there is some variability in the definition of adaptive and innate cells. In this thesis, an adaptive response was characterised as naïve T cells expressing a diverse TCR repertoire to allow for antigen recognition and clonal expansion following activation. The clonal focussing would be accompanied by a transition from a naïve to an effector phenotype. However, innate-like biology has pre-programmed responses, with an semi-invariant/invariant TCR repertoire and cell subsets that are produced only in fixed developmental windows (Willcox et al., 2020).

Alongside variable definitions of innate and adaptive biology, identifying an identical T cell population across research groups can be challenging. Many different phenotyping methods are used with a wide range of discreet identification markers. Therefore an identical γδ T cell population could be described in two publications completely differently. This is further complicated by the constant flux of T cells between different sub-populations, and the alteration of their phenotype in response to both infection and homeostatic activation (Goldrath et al., 2000; Pennock et al., 2013).

Identifying adaptive-like γδ T cells can also be challenging due to the wide range of responses exhibited in γδ biology. Murine γδ T cells are mostly closely aligned with an innate-like biology, as they often exhibit pre-programmed and fixed responses with little TCR repertoire diversity

(Carding & Egan, 2002). However some populations are thought to demonstrate adaptive or quasi-adaptive biology, having both innate and adaptive characteristics for example, Vy6Vδ1 responses to *L. monocytogenes* (Khairallah et al., 2021; Sheridan et al., 2013) and Vy1Vδ6.3 responses to *P. falciparum* (Mamedov et al., 2018). Therefore some murine yδ T cells appear to exist on a spectrum of immune responses rather than fixed innate and adaptive populations.

6.2 Mapping of murine yδ T cell populations to key adaptive traits

6.2.1 The ability of naïve and central memory T cells to home to lymphoid tissues

A crucial component of adaptive biology is the ability of naïve and central memory T cells to traffic through the lymphoid system in search of antigen. In healthy mice we were able to identify higher numbers of naïve and cm-like yδ T cells in lymphoid tissue than in the gut (Chapter 3). This coincides with data from human lymphoid tissue, the tonsil, where there was substantially more naïve Vδ1 cells than in matched blood samples (Chapter 5).

Using the Kaede mouse model we were able to confirm the higher migration of both naïve and cm-like cells through lymphoid tissue, with the majority egressing from a set lymph node within 24 hours. It coincides with the CD62L expression seen on both subsets and which is lacking on the effector population. This suggests that in both species naïve and cm-like cells may preferentially enter lymphoid tissue compared to the effector compartment.

6.2.2 Naïve T cells transition to an effector phenotype following activation

Another essential trait of an adaptive biology is the ability of naïve T cells that are produced throughout life, to transition to an effector phenotype following activation. Using RAG-GFP mice we determined that the naïve population is continuously produced even in adults at

surprisingly high levels, with up to 50% of naïve γδ T cells in the spleen undergoing VDJ recombination within 2 weeks of the experiment, whilst the effector subset had much lower levels of RAG expression (Chapter 3).

Interestingly, some lymphoid effector γδ T cells were seen very early in life as found in young, unweaned mice (Chapter 3). Their generation in these very young, healthy mice did not appear to be microbially driven as the cells are seen even in GF mice. This could imply that the effector cells are specific for self-antigens or that their differentiation is stochastic and not dependent on antigen. It is possible that within the effector population there are subpopulations that are present from birth and others that are generated from the naïve compartment throughout life after antigen recognition and expansion of naïve cells in a more adaptive-like paradigm.

Whilst no direct, tracked conversion was seen between the naïve and effector γδ T cells in this thesis, due to difficulties in labelling/tracking the naïve population, the proportion of effector γδ T cells did increase following *L. monocytogenes* infection. However, this may have been due to an expansion from within the effector population rather than the transition of naïve to effector T cells. Tracking naïve cells through adoptive transfer experiments in infections models known to generate a vigorous γδ T cell response such as MCMV, may showcase a clearer transition if present.

6.2.3 The clonotypic expansion of an antigen-specific TCR sequence from within a diverse repertoire following activation

Traditionally in an adaptive response, a naïve cell from a pool of cells with diverse TCR sequences will recognise an antigen and clonally expand. There was not extensive clonal focusing in the lymphoid effector γδ T cell compartment in mice. Therefore it is likely that some of the effector T cells in lymphoid tissues are produced through stochastic mechanisms, rather

than through TCR-driven recognition. Also many of the effector population express high levels of activatory NKRs (Chapter 3), which may provide a way of TCR-independent activation. This is in contrast to humans where the transition of naïve to effector Vδ1 T cells is correlated to clonal focusing ((Davey et al., 2017a), Chapter 5), suggesting TCR triggering is more involved in that transition.

It is likely therefore that within a lymphoid effector population in mice there is an innate-like population that develops early in life, is not dependent on microbial signals for its development and gains an effector phenotype without TCR-driven activation. Yet there may also be a subpopulation of effector γδ T cells that showcase a clonal expansion. A recent publication observed that following *L. monocytogenes* the responding Vγ6Vδ1 population contained canonical, public sequences and non-canonical, pathogen-specific sequences (Khairallah et al., 2021). Therefore there is potential for a duality of response within a population of γδ T cells.

6.3 Future directions

In this thesis I identified lymphoid naïve, effector and cm-like γδ T cell populations in mice and characterised their phenotype and responses in healthy and infected mice. Whilst in humans, I explored the Vδ1 subset in lymphoid tissue, as the Vδ1 T cells were previously shown to have an adaptive biology in the blood of some donors. Whilst the human tonsil showed a high proportion of naïve and cm-like cells, further lymphoid tissues could be explored to determine if this is a tonsil specific result. Techniques such as immunohistochemistry & imaging for human γδ T cells are improving and could allow the identification of γδ T cell subsets within varied lymphoid organs.

Future work in mice would further characterise adaptive-like populations within both lymphoid and non-lymphoid tissue. Infections such as MCMV or Malaria which previously have been

suggested to generate an adaptive-like/quasi-adaptive response (Mamedov et al., 2018; Sell et al., 2015) could be used. The Kaede model (Chapter 3) would address the migratory response of different yδ T cells following infection. Whilst an adoptive transfer model of naïve yδ T cells (Chapter 3) pre-infection would allow us to track the naïve response. By tracking the transferred cells at later timepoints of infection, we could allow them the time required to respond and proliferate.

Overall, the use of mice as a model to investigate the adaptive biology seen in human yδ T cells is attractive due to their ease of use, alongside the wide range of experimental procedures and genetically modified mice available. In addition, as highlighted by Butyrophilin-yδ T cell biology recently (Di Marco Barros et al., 2016), there is perhaps closer alignment of human and mouse yδ biology than previously appreciated. However, based on the results in this thesis, more data is needed on TCR repertoire, phenotypic changes and functional responses in different infection models before a definitive paradigm of adaptive biology in mice is established. Hence it remains to be determined whether murine yδ T cell subsets can be definitively labelled as innate or adaptive T cells due to their varied phenotype but also due to their potential for a duality in physiological responses.

Appendices

Appendix 1: Single cell sequencing of the CDR3 region in the TCR of naïve and effector yδ T cell subsets in steady state mice

Gamma chain			
Spleen Naïve population	**Spleen Effector population**	**MLN Naïve population**	**MLN Effector population**
CAVWIIGTSWVKIF	CAVWINWGTSWVKIF	CAVWIPGTSWVKIFAEKGTKLVVIPP	CAVWVSGTSWVKIFAKGTKLVVIPP
CAVWIPSGTSWVKIF	CAVWXAXWVKIF	CAVWPS?T?WV?IFAKGTKLVVIPPDKR	CAVWIRSSSGFHKVFAEGTKLIVIPS
CAVWXNGPXTSXVKIF	CAVWINPGTSWVKIF	CAVWMGGTSWVKIFAKGTKLVVIPP	CAVWIAGTSWVKIFAKGTKLVVIPP
CAVWTGTSWVKIF	CAVWVRSGTSWVKIF	CAVWGYSSGFHKVFAEGTKLIVIPS	CAVWRGTGTSWVKIFAKGTKLVVIPP
CAVWIKGTSWVKIF	CAVWMEGTSWVKIF	CAVWYCTSWVKIFAKGTKLVVIPP	CSYGHLYSSGFHKVFAEGTKLIVIPS
CAVWMSGTSWVKIF	CAVWMWXXTXXVXIF	CAVWRSGTSWVKIFAKGTKLVVIPP	CSYGDSSGFHKVFAEGTKLIVIPS
CAVWRSGTSWVKIF	CAVWGNRSGTSWVKIF	CAVWISGTSWVKIFAKGTKLVVIP	CSYYSSGFHKVFAEGTKLIVIPS
CAVQSGTSWVKIF	CAVWSLSGTSWVKIF	CAVWIVSGTSWVKIFAKGTKLVVIPP	
CAVAGGTSWVKIF	CAVWVRSGTSWVKIF	CAVWSGTSWVKIFAKGTKLVVIPP	
CAVWSGTSWVKIF	CAVWRSGTSWVKIF		
CAVWIKGSGTSWVKIF	CAVWIPGTSWVKIF		
CAVWRSGTSWVKIF	CAVWVGPSGTSWVKIF		
CAVWINRSGTSWVKIF	CAVWIKGTSWVKIF		
CAVWGSGXSWVKIF	CAVWSGTSWVKIF		
CAVWISGTSWVKIF	CAVWRSGTSWVKIF		
CAVWRSGTSWVKIF	CAVWINPRSGTSWVKIF		
CAVWPPGTSWVKIF	CAVWGSGTSWVKIF		
CAVWIRSGTSWVKIF	CAVWKASGTSWVKIF		
SYGSYSSGFHKVFAEGTKLIVIPS	CAVWRYSSGFHKVF		
SYGLYSSGFHKVFAEGTKLIVIPS	CSYGLYSSGFHKVFAEGTKLIVIP		
SYGAGGSGFHKVFA?GTKLIVIPS	CSYGVYSSGFHKVFAEGTKLIVIPSDKR		
SYGDSSGFHKVFA?GTKLIVIPS	CSYGXYSSXFHKVFAXXTXLIVIPS		
YGYPYSSGFHK?FAEGTKLIVIPS	CSYGXYSSXFHKVFAXXTXLIVIPS		
SYGSYSSGFHKVFAEGTKLIVIPS	CSYGXYSSGFHKVFAEGTKLIVIPS		
SYGHSSGFHKVFA?GTKLIVIPS	CSYGLYSSGFHKVFAEGTKLIVIPS		
YGQGSSGFHKVFAEGTKLIVIPS	CSYGRYSSGFHKVFAEGTKLIVIPSDK		
CAMRDGTYRRDT?P?TDKLVF	CSYGLYSSGFHKVFAEGTKLIVIPS		
CAMERRDGGI?PTDKLVL	CSYGRYSSGFHKVFAEGTKLIVIPS		
CASGSWHIGGGFATDKLVF	CSYGLYSSGFHKVFAEGTKLIVIPS		
CASGYIISEG?ELTDKLVL	CSYGSYSSGFHKVFAEGTKLIVIPS		
CALIWPHSIGGIRGKLVLDKEPK	CSYSSGFHKVFAEGTKLIVIPS		
CAFPLYRRDPATDKLVFGQG	SYGLYXSGFHKVFAXGTXLIXIP		
CALYRRDTADKLVL	CSYGLYSSGFHKVFAEGTKLIVIPS		
CSYGSYSSGFHKVFAEGTKLIVIPS	CSYGLYSSGFHKVFAEGTKLIVIPSDKR		
CSYGQRGLYSSGFHKVFAEGTKLIVIPS	CSYGVYSSGFHKVFAEGTKLIVIP		
CSYDVGYSSGFHKVFAEGTKLIVIPS	CSYGLYSSGFHKVFAEGTKLIVIPS		
CSYGRYSSGFHKVFAEGTKLVIPS	SYGYSSGFHKVFAXGTKLIVIP		
CSYGRYSSGFHKVFAEGTKLIVIPS	CSYGEYSSGFHKVFAEGTKLIVIPS		
CSYGASSGFHKVFAEGTKLIVIPS	CSYGSYSSGFHKVFAEGTKLIVIPSDKR		
CSYGPYSSGFHKVFAEGTKLIVIP	CSYGYSSGFHKVFAEGTKLIVIPSDKRL		
CSYGFNSSGFHKVFXEGTKLIVIP	CSYGYQYSSGFHKVFAEGTKLIVIPSDKRLD		
SYGLLYSSGFHKVFAEGTKLIVIP	CSYGTYXSGFHKXFAXGTXLXXXPSDKRL		
CSYGHSSGFHKVFAEGTKLIVIPS	CSYGLYSSGFHKVFAEGTKLIVIPS		
CSYGEYSSGFHKVFAEGTKLIVIP	CSYGLYSSGFHKVFAEGTKLIVIP		
	CSYGSYSSGFHKVFAEGTKLIVIPSDKR		
	CSYGLYSSGFHKVFAEGTKLIVIPSD		
	CSYGLYSSGFHKVFAEGTKLIVIPS		

Delta chain			
Spleen Naïve population	**Spleen Effector population**	**MLN Naïve population**	**MLN Effector population**
CAMEQGMHSYXRDTATDKLVF	CALMERAPEGYGPLPTDKLVF	CALMERYRRDRSDKLVF	CALWELLYRRDTVADKLVF
CAAIRGHTPSGGATDKLVF	CGGKSPSIGGIREGTDKLVF	CALWELYIGGIRAGADKLVF	CASGTSMASTPATDKLVF
CGGKYGISEGYELTDKLVF	CAMEYGIWGSEGSDKLVF	CGVVSSYGEGYERDDKLVF	CALMERADRRDTRHGADKLVF
CALWEDMAYGRIRATDKLVF	CAMERPEGYELGDKLVF	CGGRSEGYELRTDKLVF	CALMEPLSEGYELRTDKLVF
CALMERGGGIRNDKLVF	CGGKGISEGYELWRTDKLVF	CGGKEGKYRRDTDKLVF	CALMEREGTTDKLVF
CGGKEWPYRRDTSPSTDKLVF	CALWELGRDTSPATDKLVF	CGGKGAEGYPATDKLVF	CGGKVRRDTSSTDKLVF
CALSEPGGHIGDTRGSWATDKLVF	CGGKEEWHIPAIGGIRTSTDKLVF	CALWEPWPLYRREATDKLVF	CASPIRWGDKLVF
CAMEVAYRRDTRADKLVF	CILIGGIRKATDKLVF	CALWELGNIGGSTDKLVF	CALWEPDMASRRDTSSDKLVF
CASGGIRASTDKLVF	CALWELNMAIGGTTDKLVF	CALWEPYIGGIRTIDKLVF	CALSEWPPDIGGIRATDKLVF
CALMERGRRDKATDKLVF	CASGYIGGIRATDKLVF	CALSEPHMALYRRIGGYRTDKLVF	CASGSRIISEGYEATDKLVF
CGGKVISEGYESDKLVF	CASGVYGISLSEGYGTD	CGGKRHERPATDKLVF	CGGKAAGGIRATDKLVF
CAASSIGGIRATDKLVF	CASGYIGGIRATDKLVLDK	CALGPSYRRDTTDKLVF	CGGIWHMKGDTATDKLVF
CALMERQGYDPDKLVF	CASGYMVGGIR?T?KLVF	CALWELEGIWHMRDTSSDKLVF	CASGYMASIGGIRRATDKLVF
CALWGWREGYEPPDKLVF	CASGYIGGIRTDKLVF	CALSYMAYRRDTSTDKLVF	CALWELGGSEGYEADKLVF
CAIRNRRDMGDKLVF	CASGYIGGIRATNKLVF	CALWERRDATDKLVF	CASGYGIHI?EGH?LATDKLVF
CALMERASEGYADKLVF	CASGYIGGIRATDKLVLDK	CAMGDRRGPATDKLVF	CAMREGGAEGYELADKLVF
CGGSDRRDTTDKLVF	CASGYIGGIR?TDKLVF	CAIPYPYRR?ATDKLVL	CGGKAGGLLLSEGYERADKLVF
CAMGIRATDKLVF	CASGYIGGIRATDKLVF	CASGYIGGIRATDKLVF	CASGYIWHIPYRRDTIPAADKLVL
CASGGELADKLVF	CAS?YVGGIRATDKLVF	CAPYRRDTDKLVF	CASGKVMAWDIGGIAATDKLVF
CGGKHIGGIATDKLVF	CASGYIGGIRATDKLVF	CASGPNIGGIRAGSKLVF	CALWEPNMAISEGYVATDKLVF
CALMERPGGGRYRRDTRATDKLVF	CASGYIGGIRA?DRLVF	CALSVSEGYTDKLVF	CASGPRDKATDKLVF
CALMERWHISEGYGPTNSS	CAIGG?RAKLVF	CASGLYIGGIRATDKLVF	CASGYPIWHIGGIRTDKLVF
CALMERGGTGGIRATDKLVF	CASGYIGGIRATDKLVL	CASGYIGLIGGIRAPDKLVF	
CAMERRRD?TDKLVFGQGTQ	CASGYKRRIRA?DKLVF	CASGYILGGIRTTDKLVF	
CASGYMGIRTTDKLVF	CASGYMAPQPEGF?TDKLVL	CASGPIGGIRLLVF	
CASGYGFGGILSDKLVF	CASGYIGGIRATDKLVLDK	CASGLIWPTYRRDTGKLVF	
SYGRISSGFHKVFA?GTKLIVIPS	CASGYIGGIRATDKLVLDK	CASGYIDGISEGYESSSTDKLVF	
SYGRYSSGFHKVFA?GTKLIVIPS	CASGYIGGIRATDKLVLDK	CASGSDMACRPISEGPATDK?V	
CASGSYMAPYRRATDKLVLDKE		CASGYGLSEGYELRTDKLVF	
CALMDYRRDTSL?DKLVLDK		CAMEYRRVLTDKLVF	
CALWELAGGIRATDKLVL		CAMPLLSEGYTLVF	
CGGKAAIGGIRATDKLVLDKE		CAMERRRDTSKLVF	
		CAMGYIGGIRARTTDKLVF	
		CASGYIGGIRATDKLVF	
		CALMERVGGIRATDKLVF	
		CAMVGIYKGIRADKLVF	
		CASGYIGGIRATDKLVF	

Appendix 2: Single cell sequencing of the CDR3 region in the TCR of IFNy+ yδ T cell subsets in infection

CDR3 sequences of IFNy+ yδ T cells in *S. typhimurium*	
Gamma chain	**Delta chain**
CAVWIEGTSWVKIF	CALWEPYMAPISEGYSATDKLVF
CAVWKYSSGFHKVFAEGIK	CALWEPNIGGIRASDKLVF
CAVWMAGTSWVKIF	CASMRHLL?EGYAADKLVF
CAVWIPGTSWVKIF	CASGYLYGLLIGGIRATDKLVF
CAVWIAGTSWVKIF	CASGYLYGLLIGGIRATDKLVF
CAVFGTSWVKIF	CASGPMAYP?IGGIRAHKLVF
CAVWTGTSWVKIF	CASGYIRYRRAAIDKLVF
CAVWSGTSWVKIF	CASGIPIIGGIRPFTDKLVF
CAVWIGNTGTSWVKIF	CAMPGGIRATDKLVF
CAVWIPGTSWVKIF	CASGISEGYGATDKLVF
CAVWGSGTSWVKIF	CAIRIRSTDKLVF
CAVWKSGTSWVKIF	CAIDIGGIRGDKLVF
CAVWVGGTSWVKIF	CAMVWPQYRRDTGATDKLVF
CAVWMSTSWVKIF	CALWELAPDKLVFGQG
CAVWPGSGTSWVKFF	CGGQISEGWTDKLVF
CAVWIVISWVKIF	CASGYMSISEGFDKLVF
CAVWTSGTSWVKIF	CGGKAPGAYRRDRATDKLVF
CAVWNSSGFHKVFAEGTKLIVIPS	CAIMAYKVGGIRATDKLVF
CAVWHGSGFHKVFAEGTKLIVIPS	CASGYLWHSEGCPSTDKLVF
CAVWITFTSWVKIFAKGTKLVVIPP	CASGPHPDKLVF
SYGPYSSGFHKVFAEGTKLIVIPS	CAMVLLAEGYVKLVF
SYGSGFHKVFAEGTKLIVIPS	CASGVSEGYEFSTDKLVF
SYGLYSSGFHKVFAEGTKLIVIPS	CASGYIGGIRADKLVF
SYGRGGSGFHKVFAEGTKLIVIPSDKR	CAHGISEGYPSTDKLVF
SYGGYSSGFHKVFAEGTKLIVIPSDKR	CASGSPPMGIWFFIPSSTDKLVF
SYGYSSGFHKVFAEGTKLIVIPS	CASGYSEGYPATDKLVF
SYGHSSGFHKVFAEGTKLIVIPS	CAIDRRDRTTDKLVF
SYGHSSGFHKVFAEGTKLIVIPS	CAIYRRDATDKLVF
SYGVYSSGFHKVFAEGTKLIVIPS	CAMVDMAYVRRDKGDKLVF
SYGKGSSGFHKVFAEGTKLIVIPS	CASPLSEGYADKLVF
SYGSYSSGFHKVFAEGTKLIVIPS	CASGYSEGYPDKLVF
SYGQYSSGFHKVFAEGTKLIVIPS	CASGYTPPISEGYELTDKLVF
SYGSHHKVFAEGTKLIVIPS	CASGYLACQSEEATDKLVF
SYGYSSGFHKVFAEGTKLIVIPS	CALMERGFIWIEGPATDKLVF
SYGPYSSGFHKVFAEGTKLIVIPS	CGGKVGGGIRSTDKLVF
SYGLYSSGFHKVFAEGTKLIVIPS	CASGYRRAATDKLVF
SYGPGG?S?FHQVLFEGTKLI?IPS	CALMELPDKLVF
SYGRYSSGFHKVFAEGTKLIVIPS	CASGYRVAPGYELSTDKLVF
SYLNSSGFHKVFAEGTKLIVIPS	CALWELVGPHIGGNATDKLVF

SYGPYSS?FHKVFA?	CA?MERGGGIRATDKLVF
SYGLYSS?FHKIFA?	CGGEGYELPDKLVF
SYGSGSSGFHKVFA	CASGPVSEGYELSDKLVF
SYGDSSGFHKVFAE	CAMEQGGGPPRDKLVF
SYGDSSGFHKVFAE	CALSELKAEGYERGDKLVF
SYGPQYSSGFHKVFAE	CALMERGYETDKLVF
SYGPQYSSGFHKVFAE	CALWELAGPFPRRDTDKLVF
	CGGVWHAHIGGIDATDKLVF
	CALSEPGRAISEGYPIDKLVF
	CGGKLALATDKLVF
	CALMEREGGMAERDTSRKLVF
	CALMEPRHMANYRREATDKLVF
	CASGE?GISEGYELPDKLVF
	CASGYGISEGYEDATDKLVF
	CALMERYRRDTPATDKLVF
	CASGLIWHMREGYETDKLVF
	CASGYLYGLLIGGIRATDKLVF
	CASGYLYGLLIGGIRATDKLVF
	CASGYPMAIGGIRPDKLVF
	CASGYLYGLLIGGIRATDKLVF
	CASGYLYGLLIGGIRATDKLVF
	CASGYLYGLLIGGIRATDKLVF
	CASGYLYGLLIGGIRATDKLVF
	CASGYLYGLLIGGIRATDKLVF
	CASGYLYGLLIGGIRATDKLVF
	CASGYLYGLLIGGIRATDKLVF
	CASGPMAYP?IGGIRAHKLVF

CDR3 sequences of IFNy+ γδ T cells in *L. monocytogenes*	
Gamma chain	**Delta chain**
CAVWPSGTSWVKIFAKGTKLVVIPPDKR	CAKRDTATDKLVF
CAVWIPAGTSWVKIFAKGTKLVVIPPDKR	CAMGYRRDRDKLVF
CAVWSSGTSWVKIF	CALWELDGGIRATDKLVF
CAVWMGTSWVKIF	CALWELGGGIRATDKLVF
CAVWMGSSGFHKVFAEGTK	CAMVRRDTRVPATDKLVF
CAVWIKEAG?KIFAKGTKLAEF	CAMPYRRGATDKLVF
CAVWGSGTSWVKIF	CGGYRREATDKLVF
CAVWMN?SGFHKVFA	CAIDLGGILVF
CAVWIARLSQGICRRN?AH?	CALPYRRDPATDKLVF
CAVWMSYSSGFHKVFAEG	CAMEGRDTATDKLVF
CACWDLAQV???VFAEGTK?IVIPPP	CILRVALGIRATDKLVF
CSYGEEYSSGFHKVFAEGTK	CAMVGGIRATDKLVF
CASGWGD?C	CALWEHIGGIRATDKLVF
CSYGYSSGFHKVFAEGT	CAMRSEGYESPDKLVF
YGDMA?DTRTDKLVLD	CALWELSISEGYELKTDKLVF
CSYGYSSGFHKVFAETA	CGGKPGGIPPTDKLVF
CSYGEYSSGFHKVFAEGTKLI	CAMELGIWRRDTSWADKLVF
CSYGLGSS?FHKVFAEGTKL	CALWELRGGISTDKLVF
CASGYIGGI??TDKLVF	CAMKGGINIGGIRATDKLVF
CALWEHIGGI?VTDKLVLDK	CAMKYRRDTDKLVF
YGYSSGFHKVFAKGTKLIVIPS	CALSELMVTDKLVF
YGLYSSGFHKVFAEGTKLIVIPS	CGGKAPEGWATDKLVF
YSSGFHKVFE?GTKLIVIPS	CASGYIGGIRATDKLVF
YSSGFHKVFA?GTKLIVIPS	CALMERDRRDTSSDKLVF
YGGYSSGFHKVF?EGTKLIVIPSDK	CASGYIGGIRATDKLVF
YSSGFHKVFAKGTKLIVIPS	CASGYLYGLLIGGIRATDKLVF
CAVWPSGTSWVKIFAKGTKLVVIPPDKR	CGGIWPHIGGIRAVDKLVF
CAVWPSGTSWVKIFAKGTKLVVIPPDKR	CASGYWGASIGGIRATDKLVF
	CASGYNRRDTDKLVF
	CGGPPHRRDTSWGDKLVF

Appendix 3: CDR3 sequences of the gamma and delta chain of naïve, effector and cm-like Vδ1 T cells in human blood and tonsil.

Blood – Donor 1

CDR3 gamma chain sequences effector
CATWEGFYYYKKLF
CATWEGFYYYKKLF
CATWEGFYYYKKLF
CATWEGFYYYKKLF
CATWEGFYYYKKLF
CATWEGFYYYKKLF
CATWEGFYYYKKLF
CATWEGFYYYKKLF
CATWDGRDPTGWFKIF
CATWDGRDPTGWFKIF
CATWDGRDPTGWFKIF
CATWDGRDPTGWFKIF
CATWDGRDPTGWFKIF
CATWDGRDPTGWFKIF
CATWDGRDPTGWFKIF
CATWDGRDPTGWFKIF
CATWDGRDPTGWFKIF
CATWDG?DPTGWFKIF
CATWDGGDPTGWFKIF

CDR3 delta chain sequences		
naïve	**effector**	**cm-like**
CALGETPRVLGGHTDKLIF	CALGELKSRGGYTDKLIF	CALGELKSRGGYTDKLIF
CALGDIRFWGIRYTDKLIF	CALGELKSRGGYTDKLIF	CALGELKSRGGYTDKLIF
CALGDPASYGPGWGSHTDKLIX	CALGELKSRGGYTDKLIF	CALGELKSRGGYTDKLIF
CALGASSWGIRFADKLIF	CALGELKSRGGYTDKLIF	CALGELKSRGGYTDKLIF
CALGEPLVRGIRTDKLIF	CALGELKSRGGYTDKLIF	CALGELKSRGGYTDKLIF
CALGGVPDSGELGAYTDKLIF	CALGELKSRGGYTDKLIF	CALGELKSRGGYTDKLIF
CALGKHSLPKIGGLYTDKLIF	CALGELKSRGGYTDKLIF	CALGELKSRGGYTDKLIF
CALGELQVLAGNTDKLIF	CALGELKSRGGYTDKLIF	CALGELKSRGGYTDKLIF
CALGELKSRGGYTDKLIF	CALGELKSRGGYTDKLIF	CALGELKSRGGYTDKLIF
CALGWFGLPTKVLGAYKLIF	CALGELKSRGGYTDKLIF	CALGELKSRGGYTDKLIF
CALGGRLDWGIPSTDNSS-FGKG	CALGELKSRGGYTDKLIF	CALGELKSRGGYTDKLIF
CALGTTWVTGGYLYKLIF	CALGELKSRGGYTDKLIF	CALGELKSRGGYTDKLIF
CALGLTKGRRPEYTDNSS-FGKG	CALGELKSRGGYTDKLIF	CALGELKSRGGYTDKLIF
CALGEPIARPKHWSPRGKLIF	CALGELKSRGGYTDKLIF	CALGELKSRGGYTDKLIF
	CALGELKSRGGYTDKLIF	CALGELKSRGGYTDKLIF
	CALGELKSRGGYTDKLIF	CALGELKSRGGYTDKLIF
	CALGELKSRGGYTDKLIF	CALGELKSRGGYTDKLIF
	CALGELKSRGGYTDKLIF	CALGELKSRGGYTDKLIF
	CALGELKSRGGYTDKLIF	CALGELKSRGGYTDKLIF
		CALGELKSRGGYTDKLIF
		CALGELKSRGGYTDKLIF
		CALGELKSRGGYTDKLIF
		CALGELKSRGGYTDKLIF
		CALGELKSRGGYTDKLIF
		CALGELKSRGGYTDKLIF
		CALGELKSRGGYTDKLIF
		CALGELKSRGGYTDKLIF
		CALGELKSRGGYTDKLIF
		CALGELKSRGGYTDKLIF
		CALGELKSRGGYTDKLIF
		CALGELKSRGGYTDKLIF

Tonsil – Donor 1

CDR3 delta chain sequences	
naïve	**cm-like**
CALGELSPYWGMRGFSDPNTDKLIF	CALGEYRRRGLWGIPECLKLIF
CALGEITGLFALVWGTTYTDKLIF	CALGELVGEDLLGDLDKLIF
CALGDPLVLRGYTDKLIF	CALGELYDPTGGYSTYTDKLIX
CALGEMVISPLVLGDIPWLRPNHPDKLIF	CALGELVRWGDKYTDKLIF
CALGELTSYWGIPSDKLIF	CALGGLSPTPVLGDTHRTDKLIF
CALGSPAPGGTPYTDKLIF	CALGEILRTDWGTAPHDKLIF
CALGERGSFLPGGYKDNTDKLIF	CALGEPTPPRRGILATDKLIF
CALGEHGLPTLGIRADKLIF	CALGHGLLTALGDPVPDKLIF
CALGELSPRLAPGRGGGDDKLIF	CALGDQLPSHTDKLIF
CALGALRAWGIPADKLIF	CALGELGKELLYWGIHYTDKLIF
CALRPGITDKLIF	CALGELPTLREIKLIF
CALGELVPPFDGGLGADKLIF	CALGEKPILPRMWGIQGDTDKLIF
CALGELGPTGDTKADYRSTDKLIF	CALGEFPQTYKLKSKLIF
CALGAPYGGWGIDKLIF	CALGEHLGDTPDKLIF
CALGELYPAMGWGLTDKLIF	CALGALNPAYPSTGLIF
CALGEPTPPRRGILATDKLIF	CALGHLWPPPGPNGVLGDHTDKLIX
CALGVVPPSSFRLGELKLIF	CALGGHFLLWDTADKLIF
CALVRGGWGSDKLIF	CALGEPSQEATDKLIF
CALGEEGVGYAYPETDKLIF	CALGVSLGDYGYKLIF
CALGTTTTGQLPYWGIQGKLIF	CALGGLSPTPVLGDTHRTDKLIF
CALGGKPPAVLGAPKLIF	CALGELGPLPTVGGAPYTDKLIF
	CALGELGPLPTVGGAPYTDKLIF
	CALGEILRTDWGTAPHDKLIF
	CALGEILRTDWGTAPHDKLIF

Tonsil – Donor 2

CDR3 delta chain sequences		
naïve	**cm-like**	**effector**
CALGEGPNTLPLLGDTVRPLIF	CALVGPRITDKLIF	CALGEGPNTLPLLGDTVRPLIF
CALGELGPPNRLGVLYDTDKLIF	CALGELLSYRHWKGVGADKLIF	CALGEGPNTLPLLGDTVRPLIF
CALGELSWLPWGIRGTDKLIF	CALGEQLYWGIKGPDKLIFG	CALGEGPNTLPLLGDTVRPLIF
CALGGRIDNWGRPKLIF	CALGELTLLLRYWGVALTAQLFF	CALGEGPNTLPLLGDTVRPLIF
CALGANTFPTLTAQLFF	CALGDDFLRFAVLTAQLFF	CALGEGPNTLPLLGDTVRPLIF
CALGELAWGDTSTDKLIF	CALGEECPDGFYWGILDKLIF	CALGELVTPPHVLLSSDKLIF
CALGELGGYVVPWGTIYRRYTDKLIF	CALGETPQGWGISDKLIF	CALGEPREDKTYTDKLIF
CALGDPIQGRGGDTDKLIF	CALGELCASFLPYWGIRGTDKLIF	CALGDPGVGDTRALLTDKLIF
CALGEPTFGGFVLGDRPDKLIF	CALGELLSYRHWKGVGADKLIF	CALGERASLTGGPYTDKLIF
CALELSYLAGGPDYTDKLIF	CALVGPRITDKLIF	CALGEGALYWRSTDKLIF
CALGELRRLPMVIGILTPRVYTDKLIF	CALVGPRITDKLIF	CALGELSTRWGILVETDKLIF
CALGEPIRVLGDNKLIF	CALGEGPNTLPLLGDTVRPLIF	CALGELAPPTRTGGYGNDKLIF
CALGDLANTVKLIF		
CALGTPPPTSPHRRYLSDKLIF		
CALGELENFLLPGRPWGRRGKLIF		
CALGERAGLGDISEHKLIF		
CALGEFSENLWRTGGRGHTDKLIF		
CALGEPRSYWRGLIF		
CALGIPSSWALGDTGTDKLIF		
CALGEGPNTLPLLGDTVRPLIF		

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