Novel memory phenotype Tfh cells arise without overt antigen stimulation and are important for adaptive immune responses against viral infection

A Thesis Submitted for the Degree of Doctor of Philosophy

By

Zabreen Busharat

Biosciences, Brunel University London

2025

Declaration

I declare that the research presented in this thesis is my own work. Any work or images belonging to others have been appropriately referenced in the text, and where referencing was not possible, they have been acknowledged below. This thesis complies with the ethical requirements for the use of mouse models in research (UK Home Office Project License) and has not been submitted for any other degree. I confirm that I have completed all compulsory training required for this programme of study.

This research was a collaborative effort involving Dr. Alistair Symonds, Professor Ping Wang, Dr. Su-Ling Li, and myself. The Biological Services Unit at Brunel University, along with Dr. Su-Ling Li, were responsible for maintaining the GFP-Egr2/AmCyan-T-bet knock-in and CD2-Egr2/3$^{-/-}$/AmCyan-T-bet knockout mice and provided the ear puncture samples for genotyping.

Fluorescence-activated cell sorting (FACS) was performed by Dr. Gary Warnes, the Flow Cytometry Core Facility Manager, using the BD FACS Aria III Sorter at the Blizard Institute, Queen Mary University of London. Training for the use of the Cytek Aurora Spectral Analyser was provided by Dr. Gary Warnes, with data analysis support from Professor Ping Wang and Dr. Alistair Symonds. Viral administration and disease monitoring were carried out by Dr. Su-Ling Li at the Animal Facility, Brunel University.

T cell cultures for measuring interferon-gamma production and T cell proliferation were performed at the Blizard Institute, with support from Professor Ping Wang and Dr. Alistair Symonds. T cell stimulation, interferon-gamma (IFN-γ) production, and CellTrace™ Violet (CTV) proliferation assays were performed by Professor Ping Wang and Dr. Alistair Symonds, with additional contributions from myself. The *in vitro* Tfh functional assays and quantitative real-time PCR (qPCR) were conducted by Dr. Alistair Symonds.

The RNA sequencing library preparation and sequencing were carried out by UCL Genomics, UCL, UK, with bioinformatics training and guidance provided by Dr. Alistair Symonds. The immunohistochemistry procedures were conducted by my supervisor, Dr. Su-Ling Li, at Brunel University London.

Zabreen Busharat

Abstract

Pathogen-induced memory Tfh cells exert a Tfh effector response during reinfection, regulating the generation of high-affinity antibodies. Here, we define novel memory-phenotype Tfh cells which are generated from naïve T cells under homeostatic conditions. These MP Tfh cells are phenotypically and functionally similar to pathogen-induced Tfh cells. MP Tfh cells can be defined by Tfh cell specific markers, CXCR5, BCL6, and PD-1, and markers of pathogen-induced long lived Tfh cells, FR4. T-bet^{high} MP T cells exert an innate-like Th1 response against viral infections. The transcription factor EGR2 is a repressor of T-bet function, and we found that MP Tfh cells are distinct from T-bet^{high} MP T cells but express EGR2 highly. Previously, we found Egr2 is required for MP T cell homeostasis and inflammation. Here, we observed that, in Egr2/3$^{-/-}$ CD4^{+} MP T cells, MP Tfh cell development is impaired. FR4^{+} EGR2 $^{+}$ MP T cells upregulate genes related to homeostatic proliferation, Tfh cell development and metabolic pathways of pathogen-induced memory Tfh cells. MP Tfh cells can exert an adaptive function by regulating B cell-mediated IgG production *in vitro* whereas MP Tfr cells are involved in suppressing MP Tfh cell function, thereby preventing excessive inflammation. *In vivo*, MP Tfh cells support germinal centre formation and induce neutralising antibody production after infection with vaccinia virus. Thus, MP Tfh cells with similar characteristics to pathogen-induced memory Tfh cells are developed in absence of environmental antigens and to date are the only CD4^{+} MP T cell subset associated with an adaptive immune response against viral infection.

Acknowledgements

The most important part of this thesis is thanking the people who motivated, guided, and supported me through this challenging yet rewarding journey. This support didn't just start with my PhD—it began long before, in my childhood, by my late grandfather, Mohammad Abdul Ghani. He believed I was made for science, and without his excitement and love, I don't know what field of study I would be in today. I am very thankful to him, and I hope I've made him proud.

I would also like to thank our lab group members for welcoming me, putting up with me, and guiding me throughout my PhD. I'm especially grateful to my supervisors, Dr. Su-Ling Li and Professor Ping Wang, whose support, guidance, and motivation made this challenging journey so much easier.

A huge thank you to Dr. Alistair Symonds, our postdoc, for his incredible support—whether it was helping me with practicals or teaching me about RNA sequencing. His generosity with his time and expertise has meant so much to me, and I'm truly grateful.

I have to say, this was the best lab group I could have asked for. I will miss everyone dearly and will always be grateful for all their efforts, which went far beyond what I ever expected.

I would also like to thank my father, Busharat Ghani, and my mother, Nuzhat Busharat, for their unwavering support throughout my life. Without them, this journey wouldn't have been possible. Their constant encouragement, prayers, and belief in me kept me going, even during the toughest times. A heartfelt thank you to my siblings Ibrahim, Saba, Mahboob, and Zobia, Rehman and my sweetest baby brother, Ismail—for always cheering me on, supporting me and helping me with anything I need.

To my amazing friends, thank you for always supporting me, understanding my absence, and celebrating my achievements. I couldn't have done this without you.

And I want to thank myself for pushing through. Self-acknowledgment isn't shallow or selfish—it's a reminder that self-motivation is one of the greatest strengths we have.

I would like to thank the funders Medical Research Council UK and Barts charity. And I want to thank Jinfang Zhu, NIAID, NIH, USA for AmCyan-T-bet mice.

And finally, saving the best for last, I want to thank my husband, Muhammad Babar, for his unwavering love and support during the most challenging year of my PhD. I am glad to have him by my side.

Table of Contents

List of figures and tables

List of Abbreviations

Abbreviation	Definition
AmCyan	Aequorea macrodactyla Cyan Fluorescent Protein
AF	Antigen-free
APC	Antigen presenting cell
BCL6	B cell lymphoma 6
CD3	Cluster of differentiation 3
CD	Cluster of differentiation
CCR	C-C chemokine receptor
CSR	Class switch recombination
TCR	T cell receptor
EGR	Early growth response
tTregs	Thymus-derived T regulatory cells
CCR	C-C chemokine receptor
CSR	Class switch recombination
DC	Dendritic cells

DZ	Dark zone
FDCs	follicular dendritic cells
FOXP3	Forkhead box P3
FR4	Folate receptor 4
FACS	Fluorescence activated cell sorter
FBS	Fetal bovine serum
FCS	Fetal calf serum
GC	Germinal center
GF	Germ-free
GFP	Green Fluorescent Protein
HP	Homeostatic proliferation
pTregs	Peripherally derived T regulatory cell
Ig	Immunoglobulin
ILCs	Innate lymphoid cells
IL	Interleukin
IFN	Interferon
Knockin	Kin
Knockout	KO
LZ	Light zone

MP	Memory-Phenotype
MHC	Major histocompatibility complex
NK	Natural killer
OVA-VVWR	Ovalbumin – Vaccinia Virus Western Reserve
SHM	Somatic hypermutation
STAT	Signal Transducer and Activator of Transcription
SPF	Specific pathogen-free
T-bet	T-box expressed in T cells
TCR	T cell receptor
Tregs	T regulatory cell
Tfr	Regulatory T follicular
Tem	Effector-memory T cells
Th	T helper
Tfh	T follicular helper
Tcm	Central-memory T cells
VM	Virtual Memory
WT	Wildtype

Chapter 1

Introduction/ Literature review

1.1 Summary of thesis contribution

Memory phenotype (MP) T cells develop in the absence of foreign antigen stimulation. MP $CD8^+$ T cells, including Virtual memory (VM) and innate memory $CD8^+$ T cells, are well studied in this field and, are important for both innate and adaptive immunity (White, Cross and Kedl, 2017). However, knowledge of the functionality of $CD4^+$ MP T cells is still lacking. At present it is known that $CD4^+$ MP T cells are a heterogenous population and have an innate-like response during infection similar to NK and ILCs that bridge the innate and adaptive systems together (Kawabe *et al.*, 2017b, 2022).

Foreign antigen-induced memory T follicular (Tfh) cells (also known as antigen-experienced or conventional memory Tfh cells) play an important role in humoral immunity. Tfh cells are generated from naïve $CD4^+$ T cells during infection (Choi and Crotty, 2021). Currently it is unknown if MP $CD4^+$ T cells with Tfh-like functions can arise without pathogen encounter. The work presented in my thesis clearly defines the existence of MP Tfh cells, developed in the absence of overt antigen stimulation. MP Tfh cells display a characteristic phenotype of $BCL6^+$ $CXCR5^+$ $PD1^+$ $FR4^+$ $EGR2^+$. Similar to foreign antigen-induced memory Tfh cells, MP Tfh cells can effectively induce germinal center formation and support B cells responses to viral infection. So, MP Tfh cell subset of $CD4^+$ MP T cells is of great importance to adaptive immunity. The work of this thesis tremendously contributes to the emerging field of memory-phenotype $CD4^+$ T cells and to T follicular helper cells.

1.2 The immune system

The immune system functions to provide protection against pathogenic microorganisms such as bacteria, fungi and viruses, and toxins or allergens. It can also patrol the body and target killing of infected, dying and tumor cells. The immune system is branched into the innate and adaptive system which work together to provide the most effective immune response, thus defects in either system will result in host vulnerability to infections and an inadequate immune response such as allergic and autoimmune diseases (Chaplin, 2010). The innate system exists at birth and provides a rapid non-specific response against intruding pathogens. However, the adaptive response, which requires a longer period to develop, involves a more specific targeted response against a pathogen, establishing immunological memory for relatively faster response upon repeated exposure to a specific pathogen (Delves and Roitt, 2000).

1.2.1 The innate immune system

The innate immune system is activated during early hours of infection providing the first non-specific response to invading pathogens. The innate defence mechanisms include physical barriers such as the skin and mucous-lined membranes of body cavities such as the eyes, nose, mouth, gut, and reproductive organs. Pathogens that evade the physical barriers trigger the innate cellular response mechanism which produces an inflammatory response to rapidly clear off pathogens or activates the adaptive immune response, to collaboratively elicit a robust and effective immune response (Chaplin, 2010). The pathogens are recognised through the pathogen-associated molecular patterns (PAMPs) unique to microbes (e.g. viruses, bacteria, and fungi) by the germ-line encoded pattern recognition receptors (PRRs) associated with innate immune cells (Dempsey *et al.*, 2003).

Innate immune cells include:

Macrophages.

Macrophages are critical to maintain host protection, tissue homeostasis and tissue repair. In the steady state, macrophages exist in all tissues, including the lungs, liver and brain. These tissue-resident macrophages are either derived from the embryonic yolk sac or bone marrow-derived circulating monocytes (Figure 1.1). Embryonic-derived macrophages exhibit self-renewal and maintain tissue homeostasis. Whereas during an innate immune response, the circulating monocytes mature into macrophages within the infected tissue to initiate host

defence against pathogens. Macrophages can also eliminate infected, cancerous, and damaged cells through phagocytosis in which pathogens are recognised, engulfed and destroyed. Furthermore, macrophages serve as antigen-presenting cells (APCs) through displaying digested pathogen-derived antigens on its surface to trigger the adaptive immune response, thereby macrophages are crucial for communication between innate and adaptive immune systems (Figure 1.1, Hirayama *et al.*, 2017).

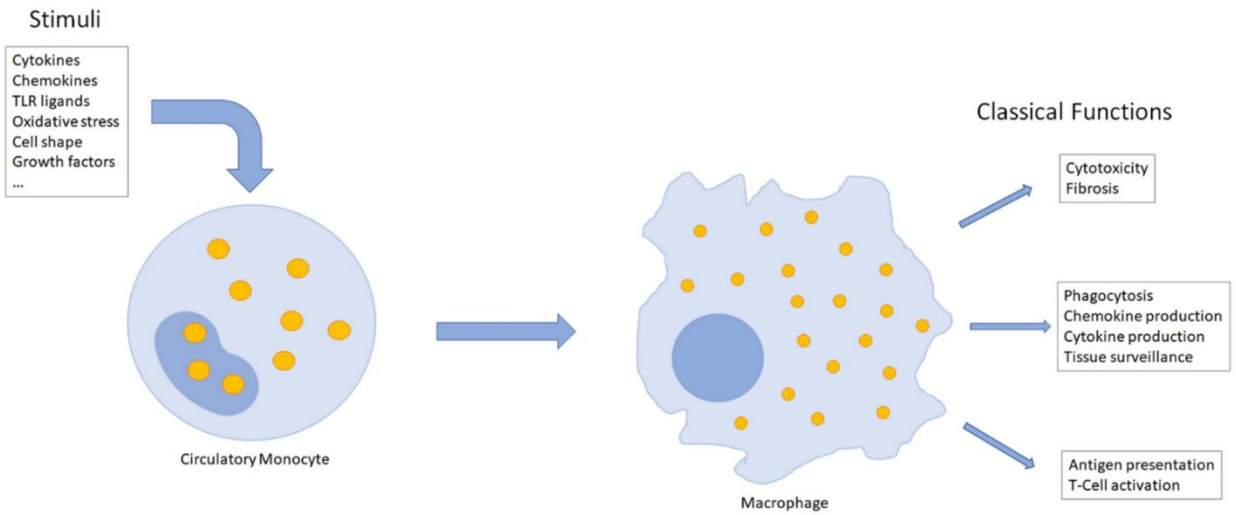


Figure 1.1. Macrophage development and function. Tissue-resident macrophages are derived from circulatory monocytes upon various stimuli and display multiple functions during an inflammatory response and in homeostatic tissue surveillance (Lendeckel *et al.*, 2022).

Dendritic cells.

Dendritic cells (DCs) are master regulators involved in coordinating a pathogen-specific immune response through antigen presentation, stimulatory cytokine production, and immune tolerance. Tissue-resident and circulatory DCs derive from hematopoietic stem cells from the bone marrow, whereas DCs accumulation due to an inflammatory response differentiates from circulating monocytes within the blood, and distinctively controls T cell responses dependent on the microbe identified (Mellman, 2013).

Unlike other APCs, such as macrophages, DCs are more efficient at initiating the adaptive immune response. DCs are also known as cellular communicators between the innate and adaptive systems rather than for involvement of direct killing and clearance of the invading pathogens. Thus, DCs are critical for capturing and presenting pathogen-derived antigens-major histocompatibility complex (MHC) complexes to cognate T cells for a pathogen-specific immune response. During an inflammatory response, pathogen-encounters, through PRRs, and proinflammatory signalling molecules, stimulate the immature DCs to engulf pathogens and

mature into antigen-presenting DCs. Mature dendritic cells can migrate from tissues into T cell zones within lymphoid organs for stimulation of naïve or memory T cells. Due to the nature of the pathogen and MHC molecule at the cell surface of DCs, the activated T cells then proliferate and differentiate into either cytotoxic T cells or T helper cells (Th1, Th2, Th17) (Mellman, 2013).

In addition to their role as APCs, DCs can enhance T cell responses and mediate T cell polarization. Upon maturation, DCs also express costimulatory molecules such as CD80 and CD86, that interact with costimulatory molecules on T cells for an effective response (Mellman, 2013). To further guide pathogen-dependent T cell differentiation, DCs produce a wide range of cytokines and chemokines such as IL-12 and IL-18 (inducing Th1 differentiation) or IL-4 and OX40L (Th2 differentiation) (De Jong *et al.*, 2005). DCs can also stimulate naïve B cells directly, initiating the humoral response (Heath *et al.*, 2019).

Along with their role in host defence, dendritic cells regulate immune tolerance preventing autoimmune diseases. Under standard conditions, DCs present self- and non-infectious environmental antigens to naïve T cells inducing T regulatory cell production (Mellman, 2013). Thus, DCs are key mediators of immune tolerance and link the innate and adaptive systems for effective pathogen clearance. Figure 1.2 illustrates the role of DCs in T-cell differentiation during tolerance and inflammatory responses against specific pathogens.

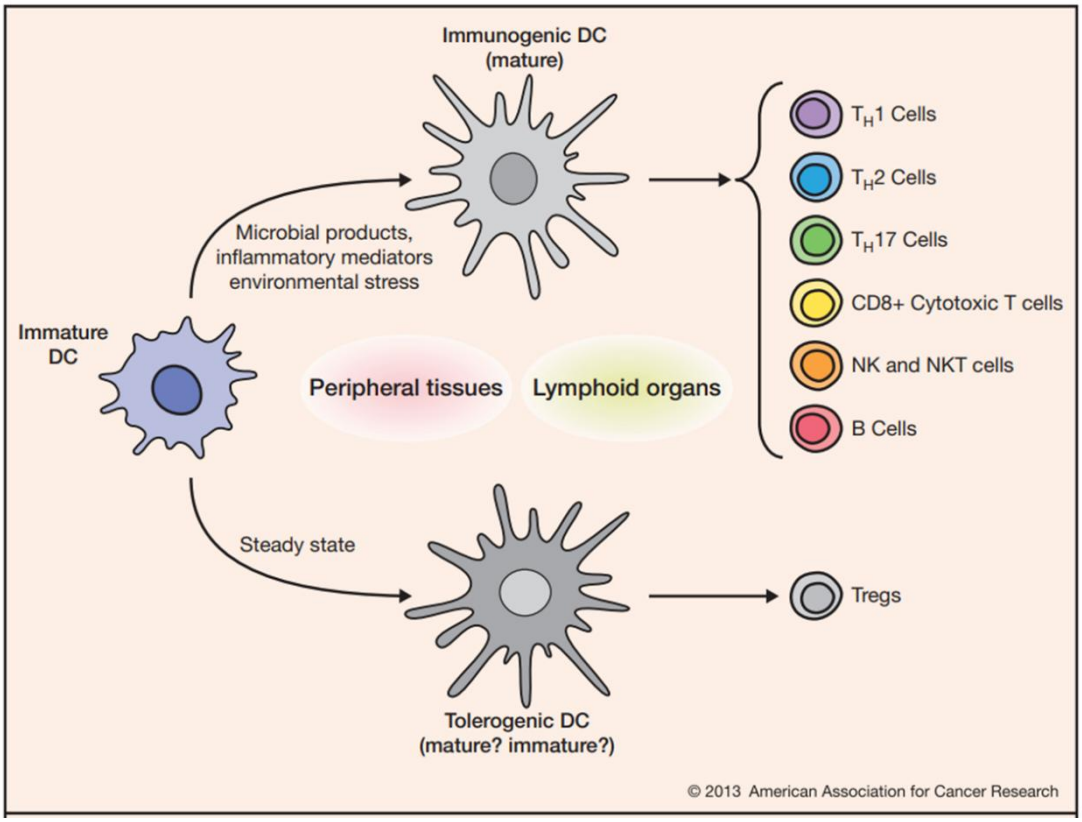


Figure 1.2. Dendritic cell (DC) maturation and immune functions. Diagram illustrating the maturation of immature dendritic cells to immunogenic DCs which play a role in the differentiation of naïve T cells into effector T cells; or into tolerogenic DCs which, in steady state, which differentiate naïve T cells to peripherally derived T regulatory cells (pTregs) (Mellman, 2013).

Granulocytes.

Granulocytic phagocytes such as neutrophils, basophils and eosinophils function as the innate defence against infections by destroying invading microbes and other foreign material (Krystel-Whittemore *et al.*, 2015). Neutrophils are the first effectors to arrive at the site of infection and function to destroy pathogens through phagocytosis and form neutrophil extracellular traps (NETs) to neutralise the infection (Kobayashi *et al.*, 2005; Papayannopoulos, 2017). Basophils play a role in allergic reactions by releasing histamine and other inflammatory mediators and are involved in the regulation of the immune system (Zhang *et al.*, 2021). Eosinophils participate in tissue homeostasis, remodelling and repair and can display cytotoxicity against parasites and in response to allergic reactions (Shamri *et al.*, 2011). Mast cells are tissue-resident myeloid cells which modulate inflammatory responses to bacteria, parasites, viruses and allergens as well as their role in regulating physiological functions such as those within the vascular system (Krystel-Whittemore *et al.*, 2015).

Lymphoid cells.

Natural Killer (NK) Cells.

NK cells are lymphocytes of the innate immune system which recognise and eradicate viral-infected and tumor cells via cytotoxicity by releasing cytokines and granules that induce apoptosis in the infected and cancerous cells (Yokoyama, 2005). NK cells play a critical role within the early defence system, providing a non-specific response against pathogens to control infections, and participating in immunosurveillance eliminating mutated cells (Saadh *et al.*, 2024).

Natural Killer T (NKT) Cells.

Natural killer T cells are a subclass of T cells originating from the thymus and aid the communication between the innate and adaptive immune systems. As well as their cytotoxic function to eliminate various pathogens and tumors, NKT cells play a direct role in inducing and influencing the type of T and B cell responses. NKT cells, found in lymphoid and non-lymphoid organs, can also regulate immune responses by interacting with cells of the innate and adaptive immune systems such as DCs and T cells. Thus, NKT cells are essential to balance the immune response and protect the host against an exaggerated immune response (Vivier *et al.*, 2008).

Innate Lymphoid Cells (ILCs).

ILCs are activated during the early inflammatory response and resemble T cells of the adaptive immune system. Much like T cells, ILCs are characterised as ILC1s (including NK cells), ILC2s and ILC3s dependent on their cytokine signatures. Upon stimulation by inflammatory signals, ILCs produce cytokines that influence the activation of adaptive immune cells. For instance, ILC1s respond to intracellular pathogens and initiate the Th1 immune response, while ILC2s play a role against parasitic infections and promote Th2 type response. Similarly, ILC3s are involved in the defence against extracellular microbes and contribute to Th17 response (Eberl *et al.*, 2015). Apart from their regulatory function, ILCs contribute to tissue repair and are found in mucosal barrier surfaces, where they are involved in the maintenance of barrier homeostasis and protection against mucosal pathogens. Additionally ILCs contribute to pathogenesis of mucosal tissues diseases such as allergies (Ryu *et al.*, 2023).

Gamma delta (γδ) T cells.

γδ T cells represent a unique population of T cells with innate cell-like characteristics. γδ T cells, originating in the thymus during fetal development, reside in epithelial tissues including the

epidermis and mucosal linings of the digestive and respiratory systems, where they are involved in epithelial cell homeostasis, tissue healing and regulation of organ function. Beyond their homeostatic functions, activated γδ T cells respond to stress-induced molecules and play a role in pathogen clearance, tumor surveillance and immune regulation. Activated γδ T cells secrete a range of cytokines that provide protection against various pathogens including viruses, bacteria, and parasites. Additionally, γδ T cells contribute to the clearance of pathogens through various mechanisms including induction of cell death of infected or cancerous cells, release of cytotoxic molecules, and indirectly through facilitating antibacterial functions of epithelial and other immune cells (Bonneville *et al.*, 2010). Overall, γδ T cells are important for innate and adaptive immunity and for tissue homeostasis and defence.

1.2.2 The Adaptive Immune System

The adaptive immune system is a secondary response mechanism established to control infection via communication with the innate immune system. Upon pathogen encounter it takes a couple of days to weeks for the adaptive immune response to be initiated. The adaptive immune system is made up of two types of cells, B and T cells. T and B cells develop within the bone marrow and the thymus (for T cells) circulate and reside in tissues and peripheral blood. Upon encounter with pathogenic antigens presented by APCs, these cells establish an immune response specific to the pathogen invading, referred to as an antigen-specific immune response. Importantly, B cells and T cells reactive to self-antigens are eliminated during development in the thymus and bone marrow. The adaptive immune system also functions to establish memory after pathogen clearance. Thus, the adaptive immune response is fundamental to vaccine development (Parkin and Cohen, 2001).

1.3 T cell

T cells are essential communicators with the innate immune system and play a role not only in T cell-mediated responses but also play a role in stimulating B cell-antibody responses against a variety of pathogen- and tumor-associated antigens. T cells are responsible for sustaining tolerance and establishing immunological memory. As they have a role in host protection and tolerance, T cells are associated with the pathogenesis of autoimmune disorders and inflammatory diseases (Kumar *et al.*, 2018).

1.3.1 T cell development in the thymus

In humans and mice, under homeostatic conditions, the T cell pool consists of naïve T cells, T regulatory cells, and the established long-term memory T cells against previously encountered pathogens. Naïve and natural T regulatory cells are developed from fetal liver and bone marrow lymphoid precursors during birth and after birth, respectively, that mature in the thymus to produce $CD4^+$ and $CD8^+$ naïve T cells, and T regulatory cells (Rothenberg *et al.*, 2008; Kumar *et al.*, 2018).

The thymus is a primary lymphoid organ that is responsible for T cell maturation, selection and export of newly generated naïve T cells. Thymic processes are highly regulated and active during childhood but decrease with age, generating fewer naïve T cells. The thymus structure consists of an outer compartment called the cortex and the central medulla, consisting of specialized cortical (cTECs) and medullary thymic epithelial cells (mTEcs) respectively. Once T cell precursors exit the bone marrow and enter the thymus, they interact with thymic epithelial cells and are referred to as thymocytes, which then migrate through the thymic microenvironment and undergo different stages of maturation in distinct subcompartments of the thymus. This process ensures the selection of (1) Functional T cells through positive selection and (2) Self tolerant T cells through positive selection, as illustrated in Figure 1.3 (Thapa and Farber, 2019). First, the thymocytes precursor enters at the corticomedullary junction and localize at the cortex for T cell receptor (TCR) rearrangement, TCR signaling and commitment to αβ and γδ T cell lineages. At this stage, the thymocytes are referred to double negative (DN) thymocytes lacking CD4 and CD8 mature T cell markers. The DN thymocytes undergo stages of maturation from DN1-DN4, identified by their distinct expressions of CD44, CD117, and CD25 surface markers exhibited within different regions of the thymus. DN1 thymocytes (ETPs) express $CD44^+$, $CD117^+$, $CD25^-$ which, upon transition to DN2 stage in the subcapsular cortical

region, results in the co-expression of all three markers ($CD44^+$, $CD117^+$, $CD25^+$). During DN2 and DN3 stages, T cells commit to either αβ and γδ lineages in order to escape cell death. The transition to DN2 triggers the rearrangement of the TCRγ, TCRδ and TCRβ gene loci. Transition to DN3 is distinguished by the loss of CD44 expression and is an important checkpoint for the expression of a functional TCR complex before transition into the DN4 stage. This includes the examination of a pre-TCR complex formed by a rearranged TCRβ with an invariant TCRα in a β-selection checkpoint, or of a fully assembled γδ TCR (γδ- selection) (Rothenberg, Moore and Yui, 2008; Hernandez, Newton and Walsh, 2010; Thapa and Farber, 2019; Parker and Ciofani, 2020). The γδ or αβ cell fates are decided by the intensity of the TCR signal influenced by the ERK (extracellular signal regulated kinase)-Egr (early growth response)-Id3 (inhibitor of differentiation 3) axis. DN2 thymocytes receiving a strong signal promotes the γδ lineage commitment, whereas a weaker signal directs the αβ cell fate (Kreslavsky *et al.*, 2010). The DN thymocytes expressing a functional pre-TCR complex transition to the DN4 stage for TCRα rearrangement and then become $CD4^+$ $CD8^+$ double positive (DP) thymocytes and continue to mature (Hernandez et al, 2010). In contrast, DN thymocytes expressing γδ TCR continue as double negative cells (Parker and Ciofani, 2020).

Following successful TCR rearrangement and TCR signalling, the DN thymocytes differentiate into DP, expressing both CD8 and CD4. DP thymocytes undergo the process of positive selection within the cortex by which DP differentiate into distinct CD4 and CD8 single positive thymocytes. TCRs which bind insufficiently to self antigen-MHC complexes on surface of cTECs undergo apoptosis by neglect, while adequate TCR signalling DP thymocytes survive the positive selection process. The commitment to CD4 and CD8 cell fates of the surviving DP thymocytes is dependent on the type of MHC molecule presenting self-antigens on cTECs (Thapa and Farber, 2019). Successful interaction of TCR with self antigen-MHC class I molecules triggers the downregulation of CD4, differentiating DP to CD8 single positive (SP) thymocytes. On the other hand, the binding of TCR to peptide-MHC class II complexes initiates the loss of CD8 expression and they commit to CD4 lineage (CD4 SPs) (Delaire *et al.*, 2004).

The resultant SP thymocytes then require screening for highly self-reactive SP thymocytes in a process known as negative selection. SP thymocytes migrate from the cortical region to the medullary compartment for negative selection, in a CCR7-dependent manner, in which the upregulation of CCR7 on SP thymocytes attracts them towards CCR7 ligands (CCL19 and CCL21) on the surface of mTECs. During the negative selection process, SP thymocytes are exposed to a variety of self-antigens displayed on the surface of mTECs. Highly reactive SP

thymocytes to self antigens are eliminated via apoptosis to avoid triggering autoimmunity once matured T cells exit the thymus. Through both positive and negative selection processes, around 95% of thymocytes fail to mature and undergo cell death. The remaining SP thymocytes following positive and negative selection are then released from the thymus as mature naïve $CD4^+$ and $CD8^+$ T cells which can then interact with invading antigen peptides to elicit a T cell-mediated immune response. Additionally, thymus-derived T regulatory cells (tTregs) are also exported from the thymus which undergo an additional selection process (Nitta *et al.*, 2009; Thapa and Farber, 2019).

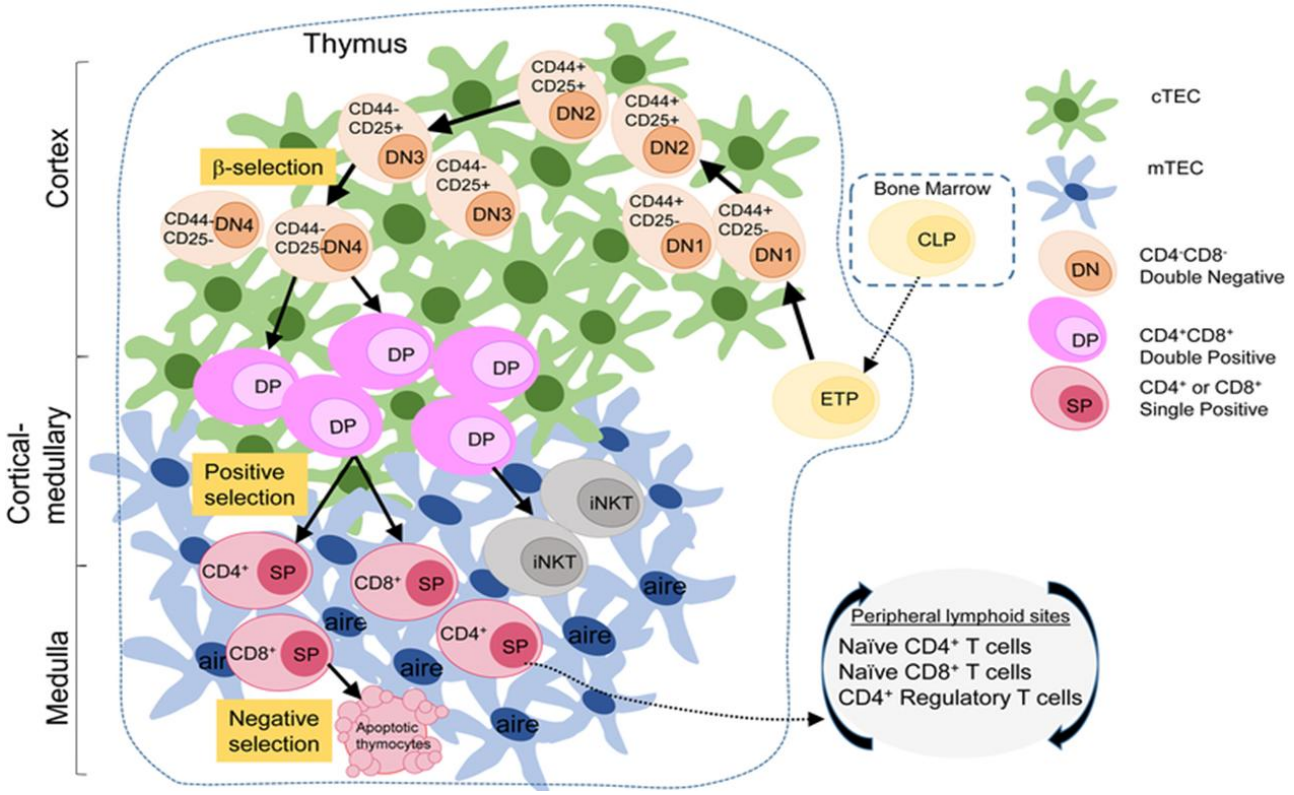


Figure 1.3. Schematic of T cell maturation within the thymus. Detailed diagram explaining the transition of bone-marrow-derived T cell precursors through the thymus to generate committed T cell lineages, including naïve $CD4^+$ T cells, naïve $CD8^+$ T cells and $CD4^+$ T regulatory cells. Thymocytes migrate through the thymus (as indicated by the arrows) and differentiate from DN (DN1-DN4 depicted in orange), DP (in pink) and finally to $CD4^+$ or $CD8^+$ SP (in red) thymocytes. There are three major selection process during T cell maturation. Firstly, the DN thymocytes undergo T cell receptor (TCR) β-chain rearrangement within the cortex. The second positive selection process involves the transition of DP thymocytes to either CD4 SP or CD8 SP thymocytes with functional TCRs. And finally, the CD4 SP or CD8 SP thymocytes migrate to the medulla and undergo a screening process in which autoreactive T cells are deleted (negative selection). The key indicates the cells within the thymus including the cortical and medullary thymic epithelial cells (cTEC and mTEC, respectively), $CD4^-$ $CD8^-$ double negative (DN), $CD4^+$ $CD8^+$ double positive and $CD4^+$ or $CD8^+$ single positive (SP) cells. The three substructures of the thymus (Cortex, Cortical-medullary junction, and the Medulla) are labelled on the diagram.

1.3.2 MHC restriction and antigen presentation

MHC molecules are critical for adaptive immune responses. In order for T cell activation, T cells need to interact with MHC molecules presenting antigenic peptides via the T cell receptor. Unlike innate cells, T cells cannot recognise pathogens unless they are processed and presented by MHC molecules, thus T cells are said to be restricted to peptide-MHC complexes. During T cell development, DP thymocytes with appropriate affinity for self MHC molecules are selected to undergo further maturation whereas T cells with high or low affinity undergo cellular death (Wieczorek *et al.*, 2017; La Gruta *et al.*, 2018).

There are two classes of MHC molecules, MHC I and MHC II molecules. Both MHC class molecules differ in terms of their structures and T-cell specificity. MHC class I molecules are made up of two chains, a heavy chain (α chain) and a light chain (β2-microglobulin), of which a closed antigen-binding cleft is created by the alpha helices formed within the heavy chain. The MHC class I molecule can only bind peptides of 8-10 amino acids in length. In comparison, the MHC class II molecules consist of two chains, α and β chains, which form an opened antigen-binding cleft capable of presenting peptides greater than 14 amino acids in length (Wieczorek *et al.*, 2017; La Gruta *et al.*, 2018). The MHC class I molecules are located on cells with a nucleus and present intracellular pathogenic peptides recognised by $CD8^+$ T cells, whereas MHC class II molecules found on antigen-presenting cells (e.g. dendritic cells) present extracellular peptides to $CD4^+$ T cells to initiate a T helper cell response (Wu *et al.*, 2021).

1.3.3 T cell receptor

The primary basis for naïve T cell activation and differentiation into effector cells is the signaling and communication with external stimuli via the TCR complex. The TCR complex is formed by the association of two TCR chains with 6 Cluster of differentiation 3 (CD3) chains. The TCR complex will consist of either TCRα and TCRβ chains or γTCR and δ TCR chains. The two TCR chains form an antigen recognition site at the extracellular region of the TCR complex for direct communication with the external environment. The TCR consists of either a TCRα and TCRβ heterodimer (αβTCR) or a γTCR and δ TCR heterodimer (γδTCR). The antigen recognition site is formed by the variable region of the TCR chains which undergoes recombination during T cell maturation in the thymus (Figure 1.4). The TCR chains also consist of a constant domain and a connecting peptide. The constant domain interacts with CD3 chains for TCR localization on the T cell surface to aid intracellular signaling upon antigen recognition. Each TCR complex consists

of 3 CD3 dimers, δε, γε, and ζζ. The CD3 chains consist of an intracellular immunoreceptor tyrosine activation motifs (ITAMs) which provide a docking site for association with proteins during TCR activation. CD4 and CD8 transmembrane coreceptors stabilise the TCR- peptide-MHC interaction via binding to the MHC class II or MHC class I molecules, respectively, enhancing T cell signalling (Figure 1.4, Mariuzza, Agnihotri and Orban, 2019; Shah *et al.*, 2021).

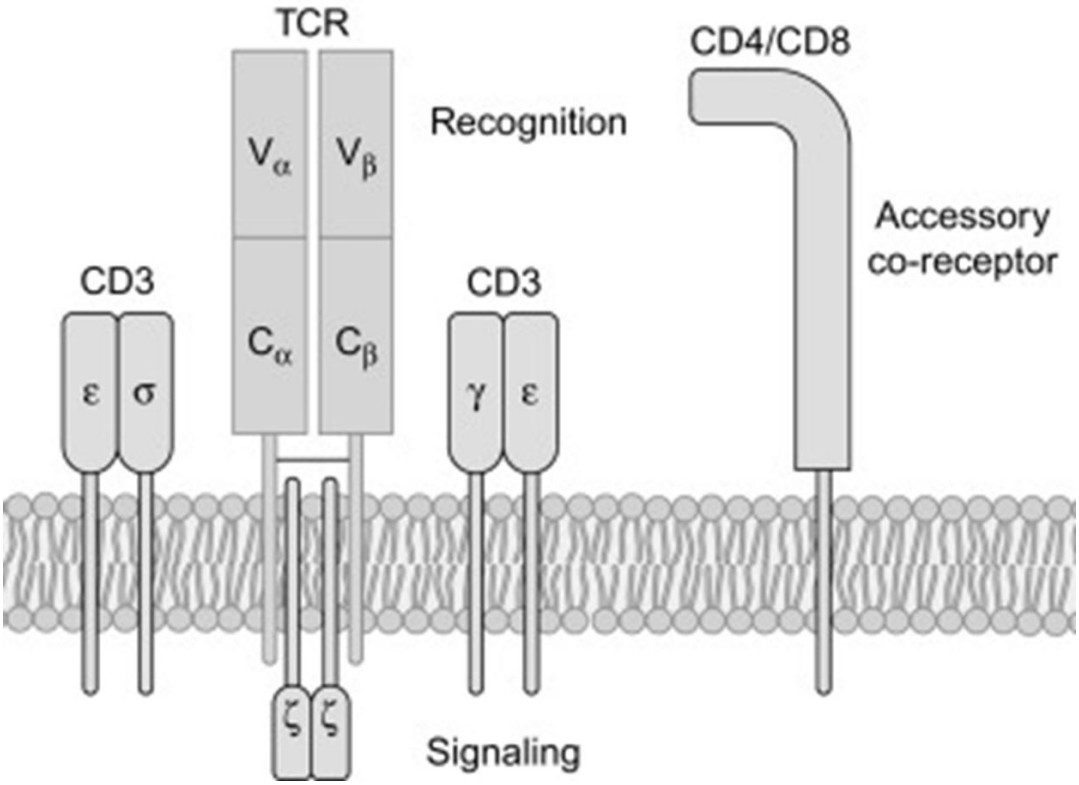


Figure 1.4. The T Cell Receptor (TCR) complex. Illustration of the TCR complex of $CD4^+$ T cells which is comprised of an α- and β-TCR chains held together by covalent bonds and a CD3 complex. Each TCR chain consists of a variable region and a constant region. The variable region of both α- and β-TCR chains forms the antigen binding site of the TCR complex and the constant regions of the TCR chains interact with the CD3 transmembrane proteins. The CD3 complex is made up of 6 polypeptide chains arranged in three dimers, δε, γε, and ζζ, required for intracellular signaling for T cell activation. The CD4 and CD8 coreceptors on the surface of T helper cells or cytotoxic $CD8^+$ T cells, respectively, strengthen the binding of the TCR complex to the antigen-MHC complex presented by antigen-presenting cells (APCs) (Actor, 2019).

1.3.3.1 T cell receptor development in thymus

Central to T cell responses is the TCR which is responsible for the binding and recognition of pathogenic antigens located on the surface of innate cells (APCs). Due to TCR specificity to antigen binding and subsequent T cell activation, a diverse repertoire of TCRs is generated which is essential for immune surveillance and recognition of a variety of pathogenic invaders

encountered by individuals during their lifetime. A diverse range of TCRs is achieved through a process known as TCR rearrangement during the DN stage of T cell development in the thymus. In this process, the TCR chain genes undergo recombination events to create a functional TCR signaling complex which is required for the subsequent stages of positive and negative selection during T cell development in the thymus (Krangel, 2009; Qi *et al.*, 2014).

The earliest development of the TCR complex is observed at the DN2/DN3 stage, in which DN thymocytes undergo a process called β-selection or γδ-selection, the earliest form of lineage commitment to either αβTCR or γδ T cells. As mentioned previously, there are four TCR chains, α, β, γ and δ. The variable region of the TCR chains is further divided either into Variable (V), diversity (D), and joining (J) segments (for β and δ chains) or Variable (V) and diversity (D) segments (for α and γ) thus undergo VDJ recombination and VJ recombination events, respectively. This process of somatic assembly is carried out by (Recombination-Activating Genes) RAG recombinases (RAG1 and RAG2) and DNA ligase, directed by recombination signal sequences and other genetic factors. During the DN3 stage, DN thymocytes undergo process of β-selection in which the TCR β chain undergoes somatic recombination and is assembled in a pre-TCR signaling complex along with a pre-αTCR chain. This triggers the downregulation of RAG genes and differentiation into DP thymocytes. During the DP stage, the αβ-committed DP thymocytes upregulate RAG genes and undergo somatic recombination of αTCR chain, forming a diverse TCR signaling complex. In contrast, γδ-selection occurs at the DN stage, promoting the complete assembly of a γδTCR complex (Krangel, 2009; BIO RAD, 2016).

1.3.3.2 T cell receptor signaling in T cell activation

TCR activation is initiated by the interaction of TCR with peptide-MHC complex which in turn stimulates a signaling cascade through CD3 signalling molecules. TCR activation involves the formation of a signaling complex. The cytoplasmic domain of CD4 and CD8 coreceptors recruit tyrosine kinases, LCK and Fyn which can then phosphorylate the ITAMs of CD3 chains. Phosphorylated ITAMs can then bind with ZAP-70 which is activated by phosphorylation through LCK, forming the TCR signalling complex. ZAP-70 can then phosphorylate downstream signalling molecules. This downstream signalling includes components of positive and negative signalling pathways. The positive signaling pathways include the mitogen-activated protein kinase (MAPK), Ca^{2+}-calcineurin and nuclear factor-κB (NF-κB) signalling pathways. These

pathways play a role in activating transcription factors that regulate transcription of genes for T cell activation, immune tolerance, and differentiation. As well as positive TCR signalling regulatory pathways, phosphatases, DAG kinases and Ubiquitination pathways negatively regulate TCR signalling to prevent hyperactivation, defected immune tolerance and inflammatory and autoimmune diseases. Thus, T cell activation is strictly controlled by TCR signalling which, if defective, can cause T cell autoimmunity or anergy (Cantrell, 1996; Courtney, Lo and Weiss, 2017; Shah *et al.*, 2021).

1.3.3.3 Costimulatory molecules in T cell activation

The binding of the TCR with its cognate antigen alone is insufficient for T cell activation but a second signal through the CD28 surface receptor strengthens downstream TCR signaling, thus T cell activation. Without a secondary signal, the weak TCR signal induces T cell anergy, which means the T cells enter a non-functional state or, it can undergo cellular death. Thus, CD28 is an important costimulatory molecule in T-cell activation (Shah *et al.*, 2021).

CD28 is found on all mouse T cells and the majority of human $CD4^+$ T cells (80%) but only expressed on around half of the human $CD8^+$ T cell population. Its ligands, CD80 (B7-1) and CD86 (B7-2) are expressed on APCs. Upon ligation, CD28 amplifies downstream TCR signaling via intracellular communication through its cytoplasmic tail which ultimately results in the proliferation and differentiation of naïve T cells into either effector helper $CD4^+$ T cells or cytotoxic $CD8^+$ T cells (Esensten *et al.*, 2016). Although CD28 is not involved in the initial activation of TCR signaling pathways, it is required to sustain T cell activation for an adequate immune response. Through recruiting phosphatidylinositol-3-kinase (PI3K) to its cytoplasmic tail, CD28 subsequently activates AKT. AKT facilitates the multiple signaling cascades involved in the downstream signaling pathways such as NF-κB, NFAT, and PI3K–AKT–mTOR, and the Ca^{2+} response (Porciello and Tuosto, 2016; Shah *et al.*, 2021). For instance, AKT assists in activating the NF-κB signaling pathway resulting in the enhanced transcription and secretion of IL-2, which in turn binds to the IL-2 receptor on the surface of T cells initiating T cell proliferation upon TCR ligation. CD28 is also required to boost the transcription of other proinflammatory cytokines/chemokines as well promoting T cell proliferation and survival (Porciello and Tuosto, 2016).

1.4 $CD4^+$ T cells

$CD4^+$ T cells are essential to ‘help’ initiate an immune response by activating immune cells such as B cells, cytotoxic T lymphocytes (CTLs), innate immune cells by releasing cytokines and chemokines as well as its role in regulating the suppression of immune responses. The antigen and specific cytokine milieu influence activation of transcription factors that drive differentiation of $CD4^+$ T cells into the appropriate T helper cell subset required to eradicate the pathogen. Cytokine-receptor interaction initiates a signalling cascade through the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway involved in specific T helper cell differentiation. The JAK-STAT signalling pathway is associated with transmitting signals from cytokine bound receptors into the nucleus, through the phosphorylation of STAT proteins, which then either activate or repress transcription of specific cytokine-inducible genes by directly binding to their recognition sites in the promoter regions. Cytokine-ligand interaction consequently activates JAK proteins which are involved in recruiting inactive STATs for signal transmission, and activation of lineage specific transcription factors. These transcription factors can act as both activators and repressor to drive differentiation and prevent expression of another lineage associated factors (Seif *et al*., 20127; Sun *et al*., 2023). As shown by Figure 1.5 $CD4^+$ Naïve T cells can differentiate into T helper 1, Th2, Th17, Tregs and Tfh cells and produce pathogen-specific effector functions as described below.

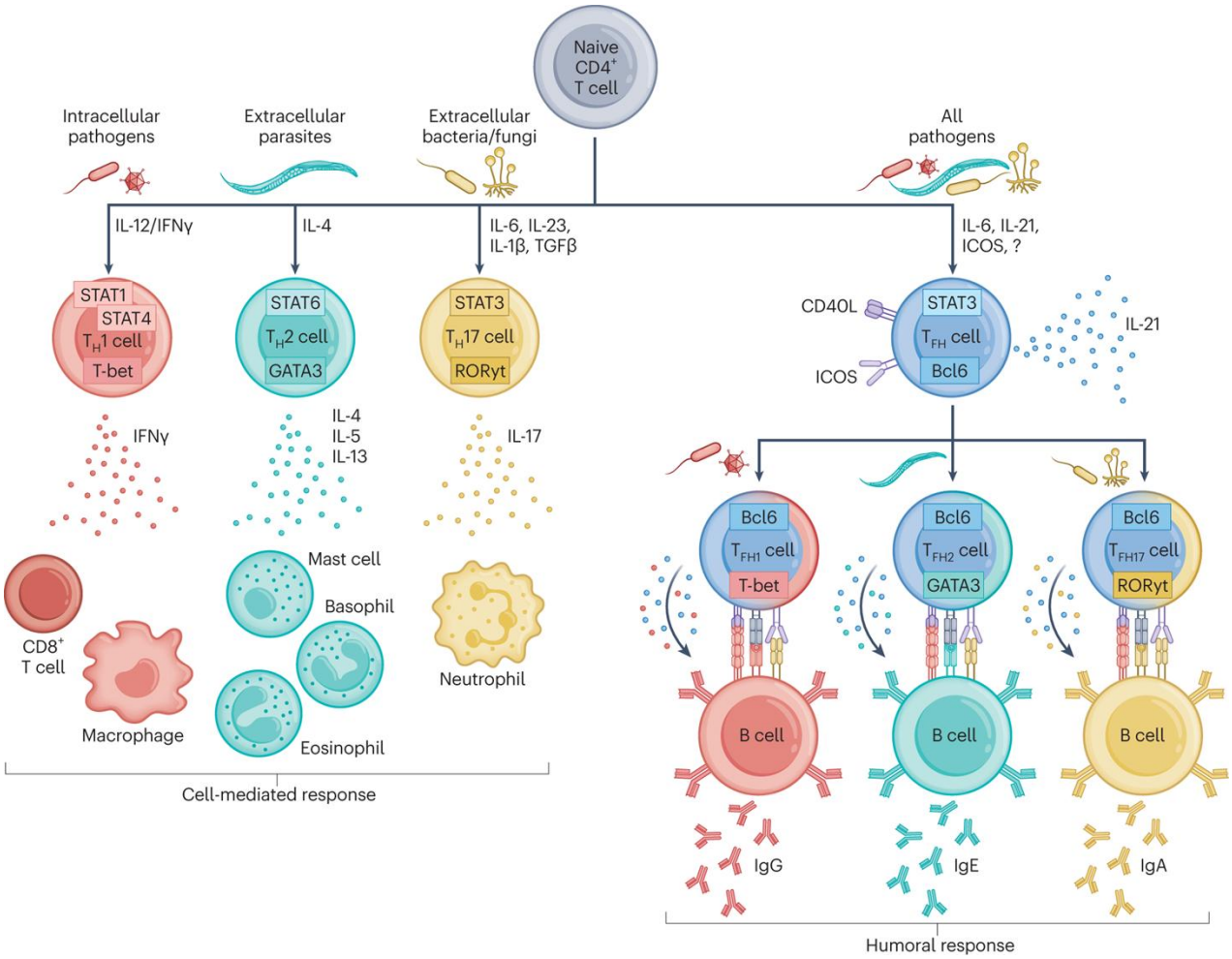


Figure 1.5. CD4⁺ T cell differentiation and T helper functions. In the presence of specific pathogens (as depicted in the diagram) and distinct cytokine milieu, activated naïve CD4⁺ T cells (in blue) differentiate into either T helper cell 1 (Th1), Th2, Th17, and T follicular (Tfh) cells shown in red, green, yellow and blue, respectively. The cytokines that drive T effector cell differentiation are shown above the arrow and the cytokines produced by each effector T cell are shown below the arrow. The secreted cytokines by the effector T helper cells recruit and activate other immune cells as shown in the illustration. In parallel to the cell-mediated response, the Tfh cells are involved in activating the humoral response. Tfh cells receive B cell help and participate in germinal center B cell response resulting in antibody-secreting plasma cells and memory B cells. Tfh cells may differentiate into subtypes depending on their low expression of Th1- (T-bet), Th2- (GATA3), or Th17- (RORγt) lineage-specific transcription factors along with the high expression of the Tfh-lineage specific transcription factor, BCL6 (Künzli and Masopust, 2023).

1.4.1 Th1 cells

Th1 cells are required for the effective clearance of intracellular pathogens. Drivers of Th1 differentiation program in naïve T cells include the recognition of viral- and intracellular bacteria-associated peptides on APCs by TCR complex and the presence of cytokines, interleukin-12 (IL-12), and IFN-γ. IL-12 and IFN-γ are produced by innate cells which sense pathogenic invaders during the innate immune response. There are two signaling pathways involved in driving Th1 differentiation program and effector responses, the IFN-γ/STAT1 and IL-12/STAT4 (Luckheeram *et al.*, 2012).

IFN-γ is a proinflammatory cytokine produced during the innate response by cells including dendritic cells and natural killer cells. Upon recognition by naïve T cells, bound IFN-γ drives the activation of JAK1/2 which leads to the phosphorylation and dimerization of STAT1. STAT1 translocate to the nucleus and activates T-bet, known as the master regulator of Th1 differentiation. In turn, T-bet regulates the gene expression of Th1-associated genes, including the production of IFN-γ, whilst suppressing other T helper cell lineages. The further induction of IFN-γ mediated by STAT1 activation then creates an autocrine loop leading to further activation of JAK-STAT signaling and an increase in T-bet-mediated IFN-γ production in Th1 polarising cells. Thus, enhancing the Th1 responses against intracellular pathogens. The second pathway involves the binding of IL-12 to its receptor on naïve $CD4^+$ T cells. IL-12/STAT4 mediated signaling activates T-bet in differentiating cells, independent of IFN- γ/STAT1 signaling pathway. In the absence of TCR signaling, IL-12/STAT4-mediated IL-18 enhances IFN-γ production for continuous IFN-γ production by Th1 cells (Luckheeram *et al.*, 2012; Basu *et al.*, 2021; Spinner and Lazarevic, 2021) .

T-bet regulated Th1 signature chemokines and cytokines include CXCR3, CCR5, IL-2, IFN-γ, and lymphotoxin-*α* (LT-α) (also known as Tumor Necrosis Factor- β (TNF-β)) which are involved in Th1 cell recruitment and helper responses (Spinner and Lazarevic, 2021). The expression of chemokines such as CXCR3 allows the recruitment of effector Th1 cells into inflammatory sites stimulated by the expression of its ligands, CXCL9, CXCL10 and CXCL11 expressed by numerous cell types including macrophages, neutrophils, endothelial and epithelial cells (Groom and Luster, 2011; Marshall *et al.*, 2017). After migration to the inflamed tissue, Th1 effector cells produce hallmark cytokines such as IL-2, IFN-γ, LT-*α* and Tumor Necrosis Factor- α (TNF-α) (Szabo *et al.*, 2000). IFN-γ, TNF-α and IL-2 produced by Th1 cells stimulate the phagocytosis and intracellular killing of phagocytosed pathogens by activating macrophages (Romagnani, 1999). Activated macrophages also increase expression of MHC class II molecules thereby

leading to further antigen-presentation to T cells. In addition, IL-2 and IFN-γ produced by Th1 cells amplify the responses of cytotoxic $CD8^+$ T cells, NK cells, and group 1 innate lymphoid cells, shaping both innate and adaptive immune responses to intracellular pathogens (Sun *et al.*, 2023). IFN-γ and LT-α are also involved in immune cell recruitment at the site of inflammation by promoting endothelial cell retraction and vascular leakage (Spellberg and Edwards, 2001).

Apart from their protective role during acute infection, Th1 cells are involved in pathogenesis of chronic autoimmune diseases such as multiple sclerosis, rheumatoid arthritis and Crohn's disease (Ramponi, Brunetta and Folci, 2022).

1.4.2 Th2 cells

Th2 cells are responsible for an immune response against parasites such as helminthic parasites by producing Th2-specific inflammatory cytokines, IL-4, IL-5, and IL-13. Th2 cells are also defined by their expression of surface molecules, IL-33R and CCR8, and lineage-specific transcription factors, GATA3 and STAT6 (Walker and McKenzie, 2017; Stark, Tibbitt and Coquet, 2019). GATA3 and STAT6 are the drivers of Th2 transcriptional program in naïve T cells in response to IL-4 cytokine produced by innate cells (e.g. basophils, NKT cells) and by antigen-bound $CD4^+$ T cells. Once bound to the IL-4 receptor on the surface of T cells, the JAK-STAT signalling pathway is activated (Kokubo *et al.*, 2022). JAK1/3 are involved in recruiting and phosphorylating STAT6 which then dimerizes and translocates to the nucleus inducing the expression of Th2 master regulator, GATA3. GATA3 further induces the expression of Th2-lineage genes such as *IL4* thereby increasing the production of GATA3 in a positive feedback loop, driving Th2 phenotype in naïve $CD4^+$ T cells (Bertschi, Bazzini and Schlapbach, 2021). Other Th2 specific genes induced by GATA3 include *Il3, Il5, Il10, Il24,* and *Ccr8.* Apart from positively regulating the expression of Th2- associated genes, GATA3 inhibits the Th1-associated *Tbx21*, *Ifng* and *Stat4* and *Il12rb2* gene expression (Spinner and Lazarevic, 2021). Another pathway involved in driving Th2 differentiation includes the IL-2/STAT5 signalling pathway. IL-2 is expressed by activated T cells and IL-2-mediated STAT5 activation induces expression of the IL-4Rα thereby increasing the cellular responsiveness to IL-4 signalling driving Th2 differentiation (Ho, Tai and Pai, 2009).

During the Th2 responses, IL-4, IL-5, IL-9 and IL-13, are secreted and involved in multiple Th2 effector mechanisms. These cytokines drive humoral responses by promoting the secretion of

immunoglobulin E by B cells, recruitment and activation of eosinophil and mast cell, and induce macrophage polarisation towards the M2-phenotype (Walker and McKenzie, 2017). IL-13, in particular, plays a key role in further Th2 polarisation within lymph nodes by recruiting dendritic cells and increasing Th2 cytokine production. This mechanism is supported by IL-4 produced by cells like basophils, reinforcing Th2 responses (Ho, Tai and Pai, 2009).

Pathogenic Th2 responses are involved in driving allergic diseases such as Allergic Asthma and food allergies. Ongoing antigen exposure triggers the pathogenic Th2 phenotype which enhances Th2 effector function and innate responses (Bertschi, Bazzini and Schlapbach, 2021).

1.4.3 Th17 cells

Th17 effector cells drive the response against extracellular fungi and bacteria at mucosal surfaces. A range of cytokines drive Th17 differentiation including TGF-β, IL-21, IL-6, and IL-23, with RORγt as the master regulator of Th17 differentiation. TGF-β, in the presence of IL-6, induces RORγt driven Th17 differentiation whereas TGF-β alone drives Foxp3-driven peripherally derived T regulatory cells (pTregs). differentiation. In addition, IL-6 signaling activates STAT3 which induces RORγt and IL-21 production and promotes the transcription of other TH17-specific cytokines, IL-17A and 1L-17F, thereby driving the Th17 differentiation program (McAleer and Kolls, 2011; Luckheeram *et al.*, 2012). In turn, the IL-21 produced by TH17 cells further enhances Th17 differentiation, in a positive feedback loop. Th17 cell proliferation and maintenance are regulated by the binding of IL-23, produced by APCs, to IL-23R induced by IL-21 and IL-6 signalling (Luckheeram *et al.*, 2012).

During an immune response, Th17 cells secrete IL-21, IL-22, IL-17A and IL-17F cytokines (Luckheeram *et al.*, 2012). Both IL-17 and IL-22 are involved in protecting the mucosal surfaces whilst eliciting an immune response for example through coordinating the migration of inflammatory cells to the inflamed mucosal tissue by inducing proinflammatory cytokines (Luckheeram *et al.*, 2012; Valeri and Raffatellu, 2016). Lastly, IL-21, in addition to enhancing Th17 cell differentiation, plays multiples roles including the activation of NK cells and inducing the humoral response (Luckheeram *et al.*, 2012)

Although they play a role in protective immunity, during chronic inflammation, Th17 cells are involved in the pathogenesis of autoimmune (including rheumatoid arthritis and systemic lupus

erythematosus) and inflammatory (such as inflammatory bowel disease and asthma) diseases (Maddur *et al.*, 2012).

1.4.4 Tregs

Tregs are immunosuppressive, preventing autoimmunity and are involved in the prevention of excessive inflammatory responses to foreign pathogens, by suppressing immune responses upon pathogen clearance, preventing immunopathology (Workman *et al.*, 2009; Luckheeram *et al.*, 2012). Tregs exist in two forms, thymus-derived Tregs (tTregs) and peripherally derived Tregs (pTregs), both characterised by their expression of the transcription factor forkhead box P3 (Foxp3) (Figure 1.6, Workman *et al.*, 2009; Abbas *et al.*, 2013) . The main difference between tTregs and pTregs is the process of their development. tTregs are formed in the thymus and account for ~10% of the $CD4^+$ SP thymocytes expressing both CD25 and FOXP3 (Kumar, Connors and Farber, 2018). In the presence of IL-2, thymocytes with high affinity for self-antigens mature into tTregs within the thymus with elevated expression of TNFR2, GITR and OX40 surface receptors (Sjaastad *et al.*, 2021). Outside of the thymus, tTregs make up the majority of Tregs within the intestine and the skin of animals, and of adipose tissues (Khantakova, Bulygin and Sennikov, 2022). In contrast, pTregs are generated from $CD4^+$ $CD25^-$ naïve T cell, within secondary lymphoid organs, in response to foreign antigens such as commensal microbes thus present in barrier tissues such as the gut (Workman *et al.*, 2009; Khantakova, Bulygin and Sennikov, 2022). The pTregs are described as a heterogeneous population. In the presence of IL-10, a subtype of type 1 regulatory T (Tr1) cells is induced whereas, TGF-β1 induces Th3 subset of pTregs during differentiation. Although Th3 subset expresses FOXP3 induced via TGF-β1 signalling, Tr1 do not express FOXP3. However, all Tregs, including pTregs and tTregs, express CD25, GITR, CD62L, CTLA-4 (Workman *et al.*, 2009). Both Tr1 and Th3 cells play important roles in mucosal immunity and are also found in tissue transplants. For instance, IL-10-producing Tr1 cells transferred in IL-10 deficient mice prevents the development of inflammatory bowel disease. And the TGF-β1-producing Th3 cells were identified by their role in oral tolerance (Gol-Ara *et al.*, 2012).

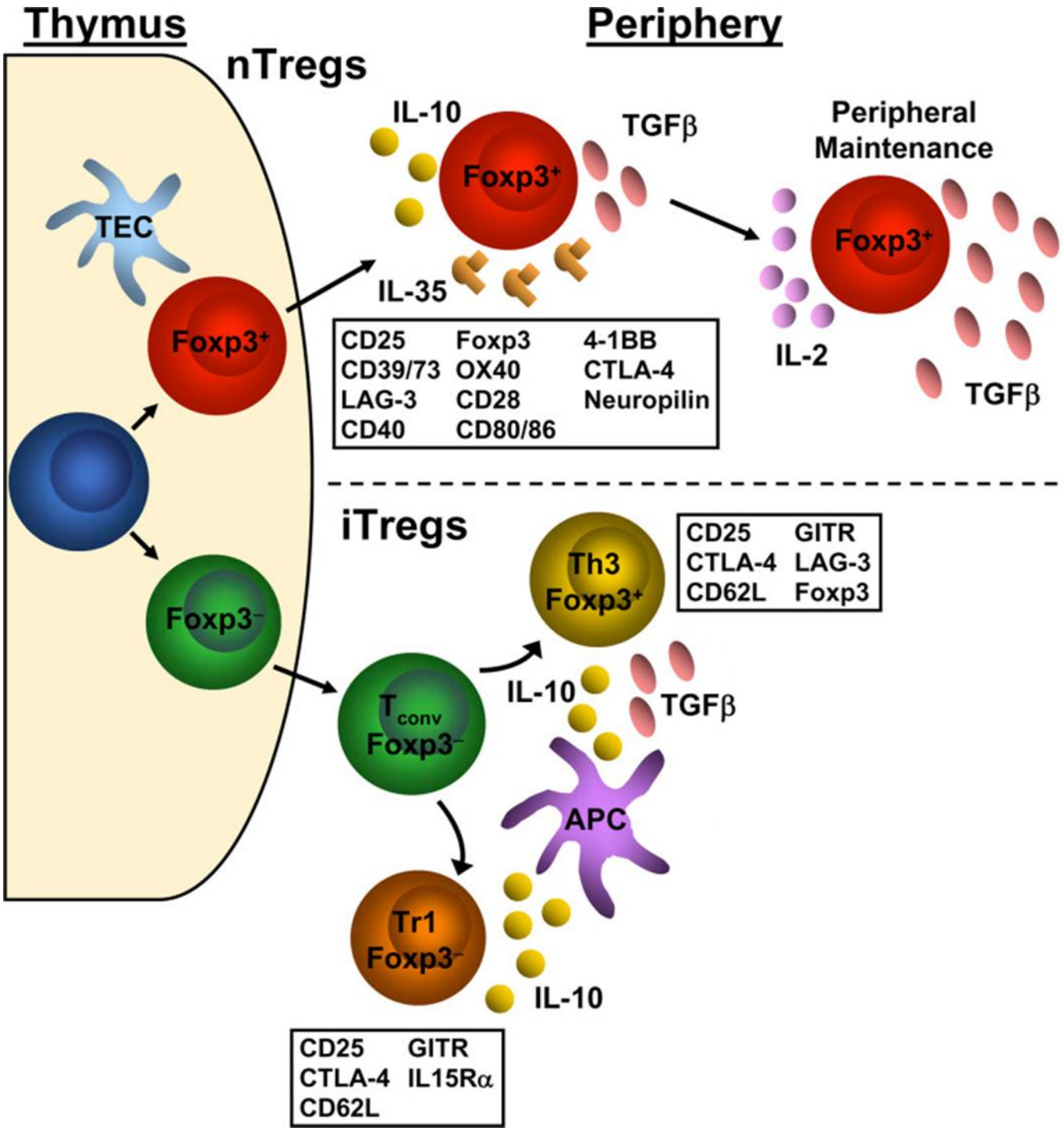


Figure 1.6. The comparison of thymic-derived Tregs (tTregs) and peripherally derived Tregs (pTregs) development and marker expression. The top section of the diagram describes the development of tTregs within the thymus of T cell precursors which then released into the periphery and express a range of surface markers (shown in the box) and exert their suppressive function through the release of cytokines such as IL-10, IL-35 and TGFβ. Along with FOXP3, required for tTregs stability, IL-2 and TGFβ play a role in Treg maintenance. The bottom half of the diagram represents the differentiation of FOXP3$^-$ naïve T cells into either Th3 or Tr1 subsets of Tregs in the presence of IL-10 or TGFβ or both released by antigen-presenting cells. the surface markers associated with Th3 and

Tr1 cells are depicted in the boxes. This diagram was adapted from Workman *et al.*, 2009, with updated nomenclature: tTregs (previously nTregs) and pTregs (previously iTregs) (Workman *et al.*, 2009; Abbas *et al.*, 2013).

Apart from the subsets of Tregs previously described, there are reports of other Tregs subsets in which FOXP3 is co-expressed with either Th1-lineage transcription factor (T-bet), Th17-lineage transcription factor (RORγt) or Tfh-lineage transcription factor (BCL6) in different disease contexts (Sjaastad *et al.*, 2021; Khantakova, Bulygin and Sennikov, 2022) (Figure 1.7). In addition, there are also $CD8^+$ Tregs. Uniquely, all Treg subsets are defined by the expression of FOXP3 for their differentiation and maintenance with the exception of the previously described, Tr1 cells, and a subset of IL-35-secreting T cells (Khantakova, Bulygin and Sennikov, 2022).

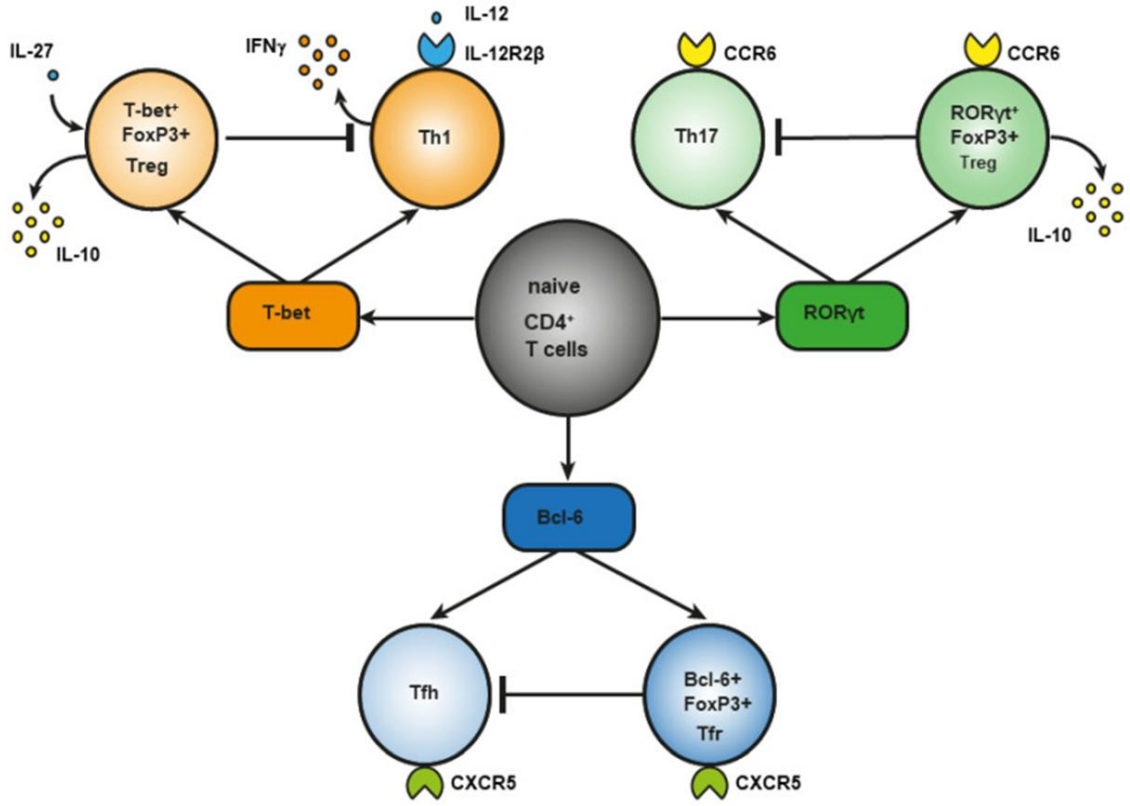


Figure 1.7 Phenotype of $CD4^+$ T regulatory cell subsets with the master regulators of effector $CD4^+$ T cells. The diagram illustrates the differentiation of Tregs into multiple subsets from naïve T cells dependent on the expression of transcription factors. For instance, the upregulation of T-bet drives differentiation of both $CD4^+$ Th1 effector cells and T-bet^+ Tregs (Khantakova, Bulygin and Sennikov, 2022).

As Tregs play a vital role in maintaining tolerance, impaired Treg development, maintenance or suppressive function defined by mutations in FOXP3 converts immunosuppressive Tregs into pathogenic Tregs which leads to autoimmune diseases such as type 1 diabetes, rheumatoid arthritis and multiple sclerosis (Bednar, Lee and Ort, 2022).

1.4.5 Tfh cells

Tfh cells are a specialised subset of $CD4^+$ T cells, defined by the expression of BCL6 lineage-specific transcription factor, that play a crucial role in antibody responses against viral, parasitic, bacterial, and fungal infections. The effector mechanism of Tfh cells involves the secretion of cytokines such as IL-21 and directly communicate with B cells to initiate B cell proliferation and maturation, and antibody production (Crotty, 2014).

1.4.6 Memory $CD4^+$ T cells

The immune system has the ability to recall its memory of past pathogen encounters through infection or vaccinations resulting in a rapid secondary response to re-exposure. This phenomenon is known as immunological memory (Ratajczak *et al.*, 2018). After the primary immune response and infection clearance, which can last around 14 days, majority of the activated T cells undergo apoptotic cell death. The remaining antigen-specific effector T cells (around 5%–10%) form long-lived memory T cells which produce a faster secondary response and provide instant protection against reinfection than naïve T cells (Sallusto *et al.*, 2010). These memory T cells, alike to effector T cells, express the T cell activation surface marker, CD44, which distinguishes them from $CD44^{low}$ naïve T cells (Lam, Lee and Farber, 2024). In the steady state, memory T cell maintenance is driven by homeostatic proliferation at a slow rate without the need for TCR signaling (Kaech, Wherry and Ahmed, 2002; Pepper and Jenkins, 2011). This is distinct from naïve T cell maintenance, as naïve T cells are maintained in the periphery via weak TCR signaling from self-ligands (Surh and Sprent, 2000). This form of memory T cells maintenance provides life-long immunity. This life-long protective immunity provided by memory T cells forms the basis of vaccinations whose purpose is to reduce disease severity or entirely stop reinfection (Kaech, Wherry and Ahmed, 2002).

There are three types of memory $CD4^+$ T cell populations including effector-memory (Tem) cells and central-memory (Tcm) present within the circulation and tissue-resident memory (Trm) cells located within peripheral tissues. $CD4^+$ Tcm cells are defined by their expression of chemokine/homing receptor C-C chemokine receptor type 7 (CCR7) and adhesion marker CD62L which assist homing to secondary lymphoid organs at the site in which they exert their secondary immune response. In contrast, Tem cells that are also found within the circulation downregulate CD62L and CCR7 so they remain in the blood. However, during an immune response, Tem cells can enter inflamed tissues by expressing homing receptors to non-

lymphoid tissues (Pepper and Jenkins, 2011; Gray, Westerhof and MacLeod, 2018). There are also differences in the response time of Tcm and Tem cells during secondary infections (figure 1.8). After antigen re-exposure, Tcm cells activate and rapidly proliferate, whereas Tem cells have a low proliferative potential but higher effector cytokine production, providing a more immediate effector response, at the site of infection, upon activation. Tcm cells can also differentiate into Tem cells, if required, and provide a more rapid effector response (Gray, Westerhof and MacLeod, 2018; Künzli and Masopust, 2023). During the primary response, T cells commit to a specific effector T cell lineage driven by the cytokine microenvironment and the pathogen encountered (Künzli and Masopust, 2023). However, Tcm cells are uncommitted to T helper cell-specific cytokine production but produce IL-2 upon activation which is involved in T cell proliferation (Gray, Westerhof and MacLeod, 2018). On the other hand, Tem and Trm cells are a heterogeneous population of Th1, Th2, and Th17 effector memory T cells which produce high amounts of IFN-γ, IL-4, and IL-17 inflammatory cytokines, respectively, and can recruit other immune cells to the inflamed tissue (Figure 1.8; Gray, Westerhof and MacLeod, 2018; Künzli and Masopust, 2023). The highly efficient response of memory T cells compared to naïve T cells is due to changes that occur upon memory T cell lineage commitment. For instance, memory T cells undergo demethylation of chromatin regions which encode for effector cytokines so their promoters are more accessible for transcription (Lam, Lee and Farber, 2024). In the steady state, the memory T cells lie in the G1 phase of the cell cycle in which protein synthesis occurs. Compared to naïve T cells, memory T cells have greater RNA/protein content and thus are more ready to expand and produce effector response than naïve T cells which are resting in a more quiescent G1 phase. In addition, mRNA for effector cytokines is transcribed before secondary stimulation rather than being initiated after TCR stimulation. Thus, the readiness of memory T cells correlates to their rapid effector function upon secondary activation to specific antigen re-exposure (Berard and Tough, 2002). Other factors contributing to the rapid responsiveness of memory T cells include the fact that memory T cells do not require costimulation, which is essential for naïve T cell activation and possess higher avidity for APCs due to increased expression of adhesion molecules in memory T cells. These biological changes acquired during memory T cell differentiation underlie their enhanced responsiveness compared to naïve T cells (Berard and Tough, 2002; Lam, Lee and Farber, 2024).

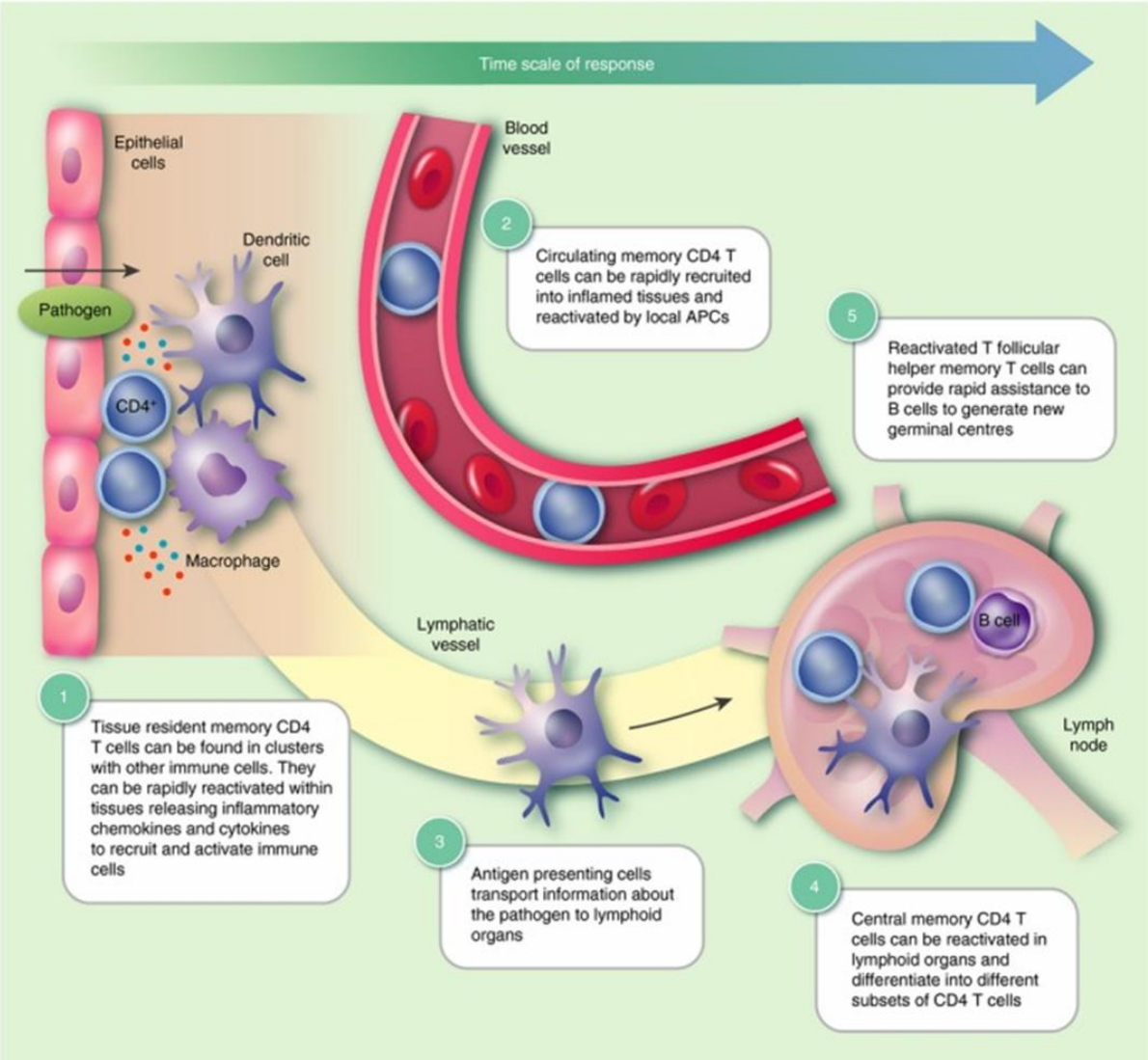


Figure 1.8- Responses of CD4+ memory T cells during the secondary response. The illustration represents the function of the $CD4^+$ memory T cell subsets during a secondary immune response. (1) The encounter of pathogens within specific tissues first elicits the activation of tissue-resident memory $CD4^+$ T cells (Trm cells). The Trm cells engage with other immune cells by secretion of inflammatory chemokines/cytokines. (2) Upon activation, the circulating $CD4^+$ memory T cells, also known as effector memory cells (Tem cells), can enter the inflamed tissue and provide an immediate cytokine response with low proliferative potential. (3/4) Central memory $CD4^+$ T cells (Tcm cells) are found within the secondary lymphoid organs such as the spleen and lymph nodes. Antigen-presenting cells that enter the secondary lymphoid organs activate the Tcm cells. As Tcm cells are not committed to a specific T helper cell lineage unlike other memory T cell subsets, Tcm cells proliferate and differentiate into specific T helper cell subsets to exert a specific Th response dependent on the pathogen present. Thus, the response time of Tcm cells is longer than the more immediate inflammatory response provided by Tem and Trm cells. (5) simultaneously, memory Tfh cells and memory B cells become reactivated and produce antibody-mediated response through formation of germinal centres (Gray, Westerhof and MacLeod, 2018).

The memory T cell differentiation model is yet undefined, as multiple differentiation models have been proposed over the years (Figure 1.9). The traditional linear model, based on differentiation being an irreversible process, states that a subpopulation of effector T cell precursors further

differentiates into Tcm and Tem cells, whilst the majority undergo cellular death. The Tem cells can then convert into Tcm cells over time. Whilst there is evidence to support this model, the lack of Tcm effector-like functionality raises the question as to whether these memory T cells are derived from effector T cell precursors during an immune response. This question is answered through the bifurcative/divergent model which illustrates that these central memory T cells are not descendants of antigen-specific effector T cell precursors but of activated naïve T cells (Ahmed *et al.*, 2009; Lam, Lee and Farber, 2024). The memory T cells then remain after pathogen clearance as a protective T cell compartment able to generate secondary effector T cell progeny upon re-exposure to the same antigen once again (Kaech, Wherry and Ahmed, 2002). This model is understood through the mechanism of asymmetric cell division of activated naïve T cells by which the daughter cells possess different properties, leading to either effector or self-renewing memory T cell fates (Lam, Lee and Farber, 2024). The third progressive model proposes that during the immune response, the naïve T cells follow a progressive path through intermediate differentiation stages in which cells become either Tcm or Tem or terminally differentiated effector T cells which are short-lived. In this model, the naïve T cell fate is decided by the degree of activation. A prolonged stronger TCR signal drives terminal effector T cell differentiation which undergo apoptosis after pathogen clearance. However, intermediate TCR signal strength promotes long lived memory cell differentiation (Gasper, Tejera and Suresh, 2014).

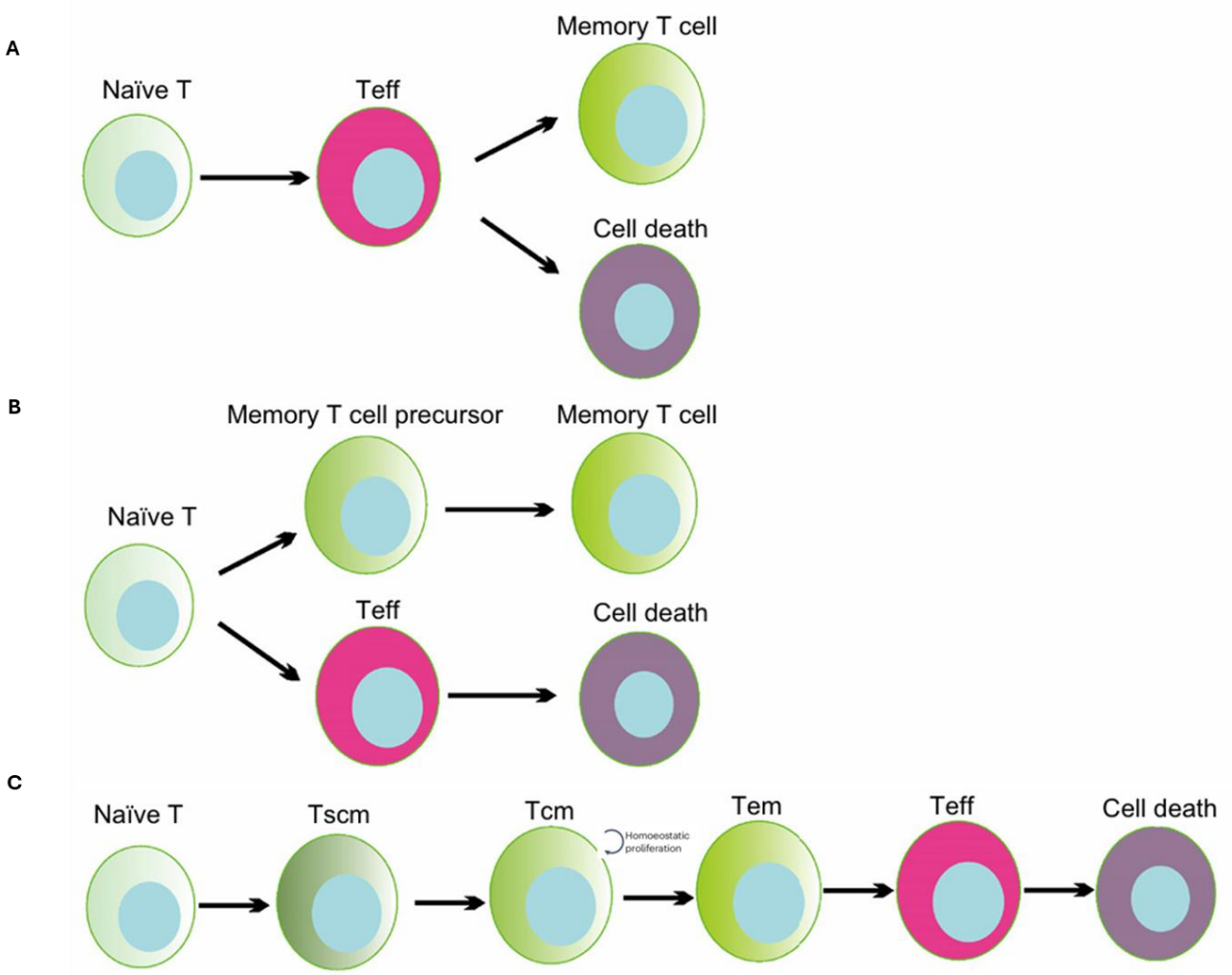


Figure 1.9- CD4$^+$ memory T cell differentiation models. Three main models have been proposed to explain the mechanism of memory CD4$^+$ T cell generation during a primary immune response. (A) The linear model proposes that, upon activation, naïve T cells differentiate into effector T cells and induce an immune response. Upon pathogen clearance, the effector T cells either further differentiate into memory T cells or undergo cell death. (B) The Bifurcative model explains that activated naïve T cells undergo asymmetric cell division producing either memory T cell precursors which differentiate further into memory T cells or effector T cells that undergo apoptosis upon pathogen clearance. (C) The progressive model illustrates that naïve T cells undergo intermediate differentiation states during an immune response, generating central memory T cells, effector memory T cells, and terminally differentiated effector T cells which undergo cell death.

The memory T cell pool includes memory Tregs originating from pTregs induced by an immune response. Memory Tregs cells play a role in suppressing an immune response against commensal bacteria (Khantakova, Bulygin and Sennikov, 2022). In addition, memory Tfh cells exist in both mice and humans defined by the expression of FR4, PD-1 and CXCR5, but reduced expression of the Tfh-lineage master regulator, BCL6. During an immune response, activated memory B cells induce BCL6 re-expression in memory Tfh cells which then become effector Germinal center (GC) Tfh cells (Crotty, 2014).

1.5 $CD8^+$ T cells

$CD8^+$ T cells, also known as cytotoxic T lymphocytes (CTLs), are generated in the bone marrow and mature within the thymus as previously described. Upon activation, $CD8^+$ effector T cells kill target host cells infected with intracellular pathogens and cancer cells through their cytotoxic activity. Once the pathogen has cleared, $CD8^+$ T cells further differentiate into memory $CD8^+$ T cells which prevent severe infection upon re-exposure to the same pathogen; whilst the majority of the effector $CD8^+$ T cells undergo apoptotic cell death (Koh *et al.*, 2023).

CTLs reside within secondary lymphoid organs where they become activated through TCR signaling and cytokines such as IL-12 produced by APCs for their differentiation into short-lived effector CTLs during infection (Zhang and Bevan, 2011). The differentiation of naïve $CD8^+$ T cells into CTLs involves multiple transcription factors such as EOMES, T-bet and STAT4. Once activated, the CTLs function to eliminate infected or tumor cells through multiple mechanisms of cytotoxic killing, including perforin and granzyme-dependent pathway or Fas/FasL interaction-dependent pathway, in absence of perforin, both of which require cell-to-cell contact (Osińska, Popko and Demkow, 2014; Koh *et al.*, 2023). The former involves channel formation by perforin molecules to allow entry of granzymes into the cytosol for inducing apoptotic events. The latter pathway induces apoptosis through interaction of Fas ligands expressed on CTLs with Fas receptors on the infected target cell (Osińska, Popko and Demkow, 2014). Another mechanism of cytotoxic killing, with direct cell-to-cell contact involves cytokines (e.g. IFN-γ and TNF-α) secreted by CTLs for the duration of infection (Andersen *et al.*, 2006).

Besides the cytotoxic killing by CTLs (also known as Tc1), other $CD8^+$ T cell subsets are found within different disease contexts. These $CD8^+$ T cell subsets differ from the classic CTLs because they have a lower cytotoxic function. However, the non-cytotoxic $CD8^+$ T cell subsets exert a cytokine-mediated function, with similar cytokine and lineage-specific transcription factor profiles as their $CD4^+$ counterparts, as illustrated by Figure 1.10. For instance, the $CD8^+$ T cell subset, Tc2, much like Th2 cells secrete IL-4 and IL-5 cytokines upon activation, which promote IgE secretion from B cells and eosinophil activation during allergy (Koh *et al.*, 2023). The function of $CD8^+$ T cell subsets in diseases are shown in Figure 1.11.

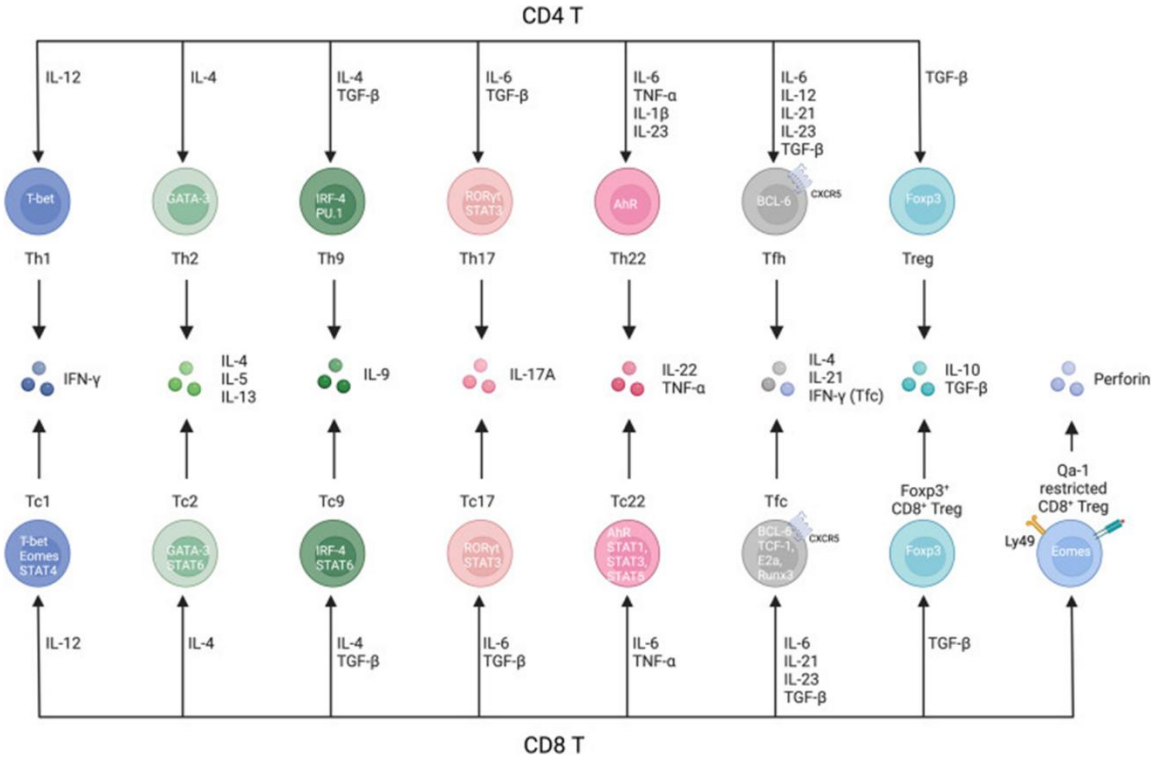


Figure 1.10. Lineage Differentiation of CD4⁺ and CD8⁺ T Cell Subsets. This schematic illustrates the differentiation pathways of CD4⁺ and CD8⁺ T cell subsets in response to cytokine signaling. At the top, naïve CD4⁺ T cells differentiate into Th1, Th2, Th9, Th17, Th22, Tfh, and Treg subsets under the influence of specific cytokines, transcription factors, and signaling molecules. Each subset is characterized by key transcription factors (e.g., T-bet for Th1, GATA3 for Th2, RORγt for Th17) and produces distinct cytokines (e.g., IFN-γ for Th1, IL-4 for Th2, IL-17A for Th17). The corresponding CD8⁺ T cell subsets, including Tc1, Tc2, Tc9, Tc17, Tc22, Tfc, and regulatory CD8⁺ T cells, share similar differentiation cues and functional profiles. The role of regulatory T cells (Tregs) in immunosuppression is highlighted, along with Qa-1-restricted CD8⁺ Tregs. Arrows indicate the cytokine-driven differentiation pathways for each subset (Koh *et al.*, 2023).

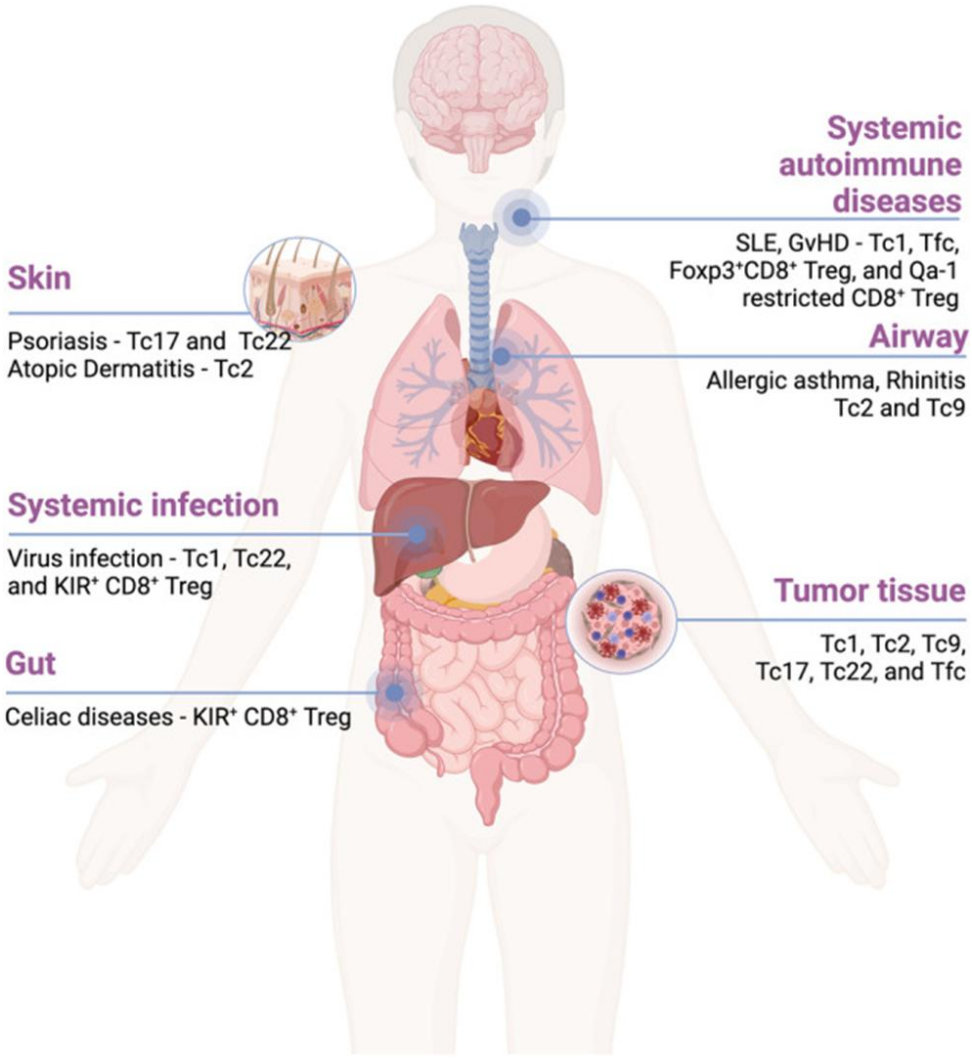


Figure 1.11. The function of Tc subsets in diseases. The illustration shows the types of Tc subsets which have a pathogenic function in different diseases (Koh *et al.*, 2023).

1.6 The function of the transcription factors EGR2 and T-bet in T cells

1.6.1 T-box protein expressed in T cells (T-bet)

The T-box protein family was derived from the discovery of the Brachyury (T) gene in mice by Dobrovolskaı¨a-Zavadskaı¨a in 1972. The T protein plays a role in embryonic development, where a mutation in the T gene results in the death in homozygous mice embryos but only causes short tail development in the heterozygous mice. The T protein is a nuclear protein termed as a DNA-binding protein (Papaioannou and Silver, 1998). The T protein binds to DNA through its unique and specific T-box domain sequence of around 180-200 amino acids, which is conserved in homologs and orthologs of the T protein discovered later in a range of species such as humans, zebrafish, chicken and Xenopus, giving the name of T-box protein family (Bollag *et al.*, 1994; Papaioannou, 2014). The T-box family is divided into 5 subfamilies referred to as T (T and TBX19), Tbx1 (TBX1,TBX10, TBX15,TBX18,TBX20 and TBX22), Tbx2 (TBX2,TBX3,TBX4 and TBX5), Tbx6 (TBX6 and Mga) and Tbr1 (TBR1, TBR2 and TBX21) which function either a transcriptional activators or repressors, or both (Papaioannou, 2014b; Chang *et al.*, 2016). T-box proteins are involved in development of organs and cancer development as they have roles in cell lineage specific differentiation, proliferation, epithelial-mesenchymal transition, and tissue integrity (Papaioannou, 2014). These development-related roles include T-box protein regulation in the brain (Eomes and TBR1), craniofacial (Tbx10, Tbx1, Tbx22 and Tbx15), pituitary gland (Tbx19 and Tbx3), mammary gland (Tbx2 and Tbx3), liver (Tbx3), lung (Tbx2, Tbx4 and Tbx5), limb (TBX5 and tbx4), and in the thymus (Tbx1). Further roles of T-box proteins have been identified in pigmentation (TBX15)and the immune system (T-bet) (Papaioannou, 2014). As the mouse species is biologically alike to humans, human orthologs of 17 mouse T-box genes have also been discovered (Papaioannou, 2014). In humans, mutations in t-box proteins are associated with hereditary diseases such as DiGeorge syndrome (TBX1), Cousin syndrome (TBX15) and Holt–Oram syndrome (TBX5), and cancers (Stein, 2009; Papaioannou, 2014).

T-bet (also known as TBX21) was originally discovered by Szabo and colleagues in a study focusing on the isolation of transcriptional regulators of Th1-specific cytokines (Szabo *et al.*, 2000). T-bet initiates Th1 cell development through transactivating IFN-γ production and repressing IL-12 expression. Intracellular cytokine analysis of stimulated and retrovirally transduced T-bet^{+} Th2 clones, under IL-2 conditions *in vitro*, illustrated a reduction in Th2 specific IL-4 and IL-5 cytokines. This shows that T-bet plays a negative role in inhibiting the Th2

program through direct regulation of GATA3. Itself, T-bet is regulated by IL-12 and STAT4 signalling, which are required for driving the Th1 program in naïve T cells (Szabo *et al.*, 2000). Following studies have identified further roles of T-bet in T helper cell differentiation, innate immune cells, B cells, autoimmunity, and resistance to infection.

1.6.1.1 The structure of T-bet and DNA binding

Murine T-bet (T-box expressed in T cells), also named TBX21, is a transcriptional regulator composed of 530 amino acids with a 189-amino acid T-box domain located between 138-327 amino acid residues (Szabo *et al.*, 2000). In comparison, the human T-bet ortholog is a 535 amino acid protein (Oh and Hwang, 2014). However, both mouse and human T-bet proteins share 88% homology (Szabo *et al.*, 2000). The relatively close homology of the T-box domains within TBR1, TBR2 and TBX21 lies them into the same T-box subfamily, Tbr1 (Szabo *et al.*, 2000). In 2016, the crystal structure of the T-bet T-box domain and a 24bp DNA with two palindromic consensus T-box binding sequences was established. Two T-bet T-box domain monomers form a dimer that can simultaneously bind to either two promoter recognition elements of different genes or, a promoter and distal enhancer sites of a single gene in a DNA looping model. This supports T-bet's function in driving Th1 differentiation by activating multiple Th1 specific genes whilst simultaneously suppressing Th2-specific gene transcription programs (Liu *et al.*, 2016).

1.6.1.2 T-bet in T cell differentiation

T-bet is an important regulator in $CD4^+$ T helper cell differentiation by acting as both activator and repressor of multiple Th lineage specific genes. Positive regulatory roles of T-bet include its ability to directly promote transcription of Th1 cell-specific genes including cytokines like *Ifng* (Szabo *et al.*, 2000), and chemokine such as *Ccl3* (Jenner *et al.*, 2009) and *Xcl1* (Thieu *et al.*, 2008), and chemokine receptors such as *Cxcr3* (Lord *et al.*, 2005). Interestingly, murine T-bet deficient $CD4^+$ T cells also expressed significantly reduced levels of EGR2 and EGR3 transcription factors, whose expression is restricted to Th1 cells and required for the expression of Fas Ligand that binds to Fas apoptosis inducer (Rengarajan *et al.*, 2000; Thieu *et al.*, 2008). Thus, T-bet and STAT4, which is also required for activation of other Th1 cell-specific genes, are essential for effective Th1 programming (Thieu *et al.*, 2008).

T-bet also acts as a repressor. In late stages of Th1 differentiation, highly expressed T-bet physically interacts with available low-levels of Tfh-lineage specific BCL6 repressor, for directing T-bet-dependent repression of Th2 promoting genes such as *Socs1*, *Socs3*, and *Tcf7* (Oestreich *et al.*, 2011). Oestreich and colleagues also observed that the T-bet and BCL6 repressor complex inhibits IFN-γ overproduction, preventing over inflammation in response to pathogens (Oestreich *et al.,* 2011). The restricted expression of BCL6 in Th1 cells, preventing Tfh differentiation program, relies on T-bet itself (Nakayamada *et al.*, 2011).

As well as preventing Tfh differentiation, induction of Th2 and Th17 gene programs are also negatively regulated by T-bet. In Th1 cells, T-bet directs Th2 specific lineage factor, GATA3, to promote transcription of Th1 specific genes. Thus, T-bet is responsible for blocking GATA3-dependent induction of Th2 gene programs (Hwang *et al.*, 2005; Kanhere *et al.*, 2012). The direct silencing of IL-4, by T-bet and RUNX3 complex could be a result of GATA3 supression by T-bet (Zhuang *et al.*, 2009). In addition, T-bet expression also represses the expression of NFAT1-mediated Th2 cytokines such as IL-2, IL-4, IL-5, and IL-13. Alike to GATA3, NFAT1 is a transcription factor which drives Th2 gene programming (Jang *et al.*, 2021). Furthermore, T-bet can also indirectly inhibit the expression of the Th17 lineage specific transciption factor, RORγt. T-bet interaction with RUNX1, a transactivator of *Rorc* gene, blocks RUNX1-mediated expression of RORγt required for driving Th17 phenotype (Lazarevic *et al.*, 2011). Apart from Th1 differentiation, T-bet also regulates differentiation of cytotoxic effector $CD8^+$ T cells from naïve $CD8^+$ T cells. Thus, T-bet plays a major role in type 1 immune responses (BM *et al.*, 2003).

1.6.1.3 T-bet in T cell responses during infection and autoimmunity

T-bet mediated IFN-γ production in numerous immune cells including CD4, CD8 and NK cells is required for both innate and adaptive immune responses for host resistance to various intracellular pathogens such as Salmonella, Mycobacterium tuberculosis, lymphocytic choriomeningitis virus (LCMV) and vaccinia virus (BM *et al.*, 2003; Matsui *et al.*, 2005; Ravindran *et al.*, 2005; Sullivan *et al.*, 2005). In *in vivo* studies of murine infection models of salmonella and Mycobacterium tuberculosis, which requires Th1 cells and IFN-γ prduction for pathogen clearance, T-bet deficiency resulted in uncontrolled bacterial burden, impaired IFN-γ production by pathogen-specific Th1 cells but an elevated IL-10 production, a cytokine which represses T cell proliferation and Th1 activity. Thus, T-bet not only positively regulates IFN-γ but

also represses IL-10 secretion in $CD4^+$ T cells in response to certain pathogens (Ravindran *et al.*, 2005; Sullivan *et al.*, 2005). Another study by Sullivan and colleagues on the function of T-bet in *in vivo* $CD8^+$ T cell driven immune responses against lymphocytic choriomeningitis virus illustrated how T-bet did not solely affect IFN-γ production in $CD8^+$ T cells but also reduced cytotoxic activity of T-bet deficient LCMV-specific $CD8^+$ cells (BM *et al.*, 2003). Furthermore, vaccinia virus infection murine model clearly demonstarted that T-bet deficient mice are more prone to infection than control littermates in conjunction with the function of T-bet in; 1) VV-specific $CD8^+$ T cell cytotoxicity and proliferation, 2) NK cell-mediated lysis, and 3) repression of Th2 program for Th1 response. The latter confirms the role of T-bet in preventing Th2 differentiation program in $CD4^+$ T cells within a disease context (Matsui *et al.*, 2005). Other studies mentioned previously did not observe a alternative Th2 cell specific cytokine production, in T-bet deficient mouse strains, in response to infections reliant on Th1 cells for protection (Ravindran *et al.*, 2005; Sullivan *et al.*, 2005).

Even though T-bet is significant in resistance for various disease conditions, there are conflicting findings in disease models such as Toxoplasma gondi and Listeria Monocytogenes infection (Way and Wilson, 2004; Pritchard *et al.*, 2015). Host defence against Listeria Monocytogenes infection relies on IFN-γ production during both early innate and late adaptive immune responses. Way and Wilson observed that T-bet deficiency in mice during innate response to Listeria Monocytogenes infection did not alter IFN-γ production in NK cells. However, upon antigen stimulation, there was an approximately 50% reduction of IFN-γ producing $CD4^+$ T cells was observed with enhanced levels of IFN-γ producing $CD8^+$ T cells in T-bet deficient mice compared to control. Thus, IFN-γ production by immune cells, including $CD4^+$ T cells, is not only reliant on T-bet in certain circumstances (Way and Wilson, 2004). The most applauding notion is that T-bet-dependent or independent resistance to infection solely relies on the pathogen context (Matsui *et al.*, 2005; Pritchard *et al.*, 2015).

Th1/2 cytokine imbalance due to virally-induced exaggerated type 1 inflammatory responses can initiate development of autoimmune diseases such as multiple sclerosis, herpes stromal keratitis, or type 1 diabetes (T1D). Autoagressive destruction of insulin-producing beta-pancreatic cells in type 1 diabetes, correlates with levels of type 1 cytokines such as IFN-γ and IL-12, and cytotoxicity of $CD8^+$ T cells (Christen and Herrath, 2004). Using LCMV infection induced T1D model, Juedes and colleagues identified a critical pathogenic function of T-bet in the generation of IFN-γ producing anti-LMCV effector $CD8^+$ T cells *in vivo*. In T-bet deficient mice infected with LMCV, the antigen-specific effector/memory $CD8^+$ T cells developed 8 days

after viral clearance were significantly reduced by day 14 after infection. This was accompanied by reduction in IFN-γ compared to control mice, indicating a decrease in autoagressive behaviour of LCMV-specific $CD8^+$ T cells in T-bet deficient mice, without influencing primary IFN-γ production during viral clearance (Juedes *et al.*, 2004). Even though type 1 cytokines results in autoimmunity, type 2 cytokines such as IL-10 or IL-4 prevent progression of type 1 diabetes (Christen and Herrath, 2004). A recent study identified an immunomodulatory function of engineered T-bet lacking Th2 cells which shifts Type 1 towards a type 2 cytokine milieu which is sufficient to supress the activation and proliferation of LCMV-specific effector $CD8^+$ T cells for protection from autoimmune type 1 diabetes (Muñoz *et al.*, 2021). Overall, T-bet is a therapeutic target not only to control inflammation in T cells, but also for humoral and innate immune responses that were briefly described earlier (Ji, Sosa and Forsthuber, 2011). Although T-bet mediated IFN-γ production in T cells is required for resistance against multiple infections, T-bet suppression is enough to prevent autoimmunity not only in T cells but in other innate immune cells as described above. The mechanisms of T-bet regulation which lead to these contradictory outcomes is not clear.

1.6.2 Early growth response (EGR) proteins in T cells

EGR transcription factors, induced by environmental stimuli such as growth factors and hormones, are involved in cell growth, proliferation, differentiation and apoptosis (Chavrier *et al.*, 1988; Lee *et al.*, 1996; Levkovitz and Baraban, 2001; Punetha *et al.*, 2020). EGR2 protein expression was primarily observed during the early GO/GI transition in proliferating fibroblasts *in vitro* (Chavrier *et al.*, 1988). A role for EGR2 has been associated with hindbrain maturation in vertebrates, as EGR2 is found to be differentially expressed in rhombomeres 3 and 5 (Wilkinson *et al.*, 1989). EGR2 mediates the expression of *Hoxb2* gene through EGR2-binding sites on the enhancer region of *Hoxb2* gene, whose expression is required for hindbrain segmentation (Sham *et al.*, 1993). Egr2 homozygous mice, with deletion of the zinc finger domain, displayed defects among trigeminal and facial ganglia required for motor functions such as eating which contributed to early death after birth (Swiatek and Gridley, 1993). Further roles of EGR2 in the nervous system have also been described. Topilko and colleagues observed absence of myelinated axons due lack of expression of late myelin genes in Schwann cells of Egr2-deficient mice, possibly by the blockage of Schwann cell differentiation (Topilko *et al.*, 1994). Further study by Zorick and colleagues established that EGR2 acts as a transactivator of myelination

genes required for terminal differentiation of myelin-forming Schwann cells (Zorick *et al.*, 1996). EGR2 defects in myelination are associated with peripheral nerve diseases such as Congenital Hypomyelinating Neuropathy (Baloh *et al.*, 2009), Charcot-Marie-Tooth disease type 1 (Mikešová *et al.*, 2005) and Dejerine–Sottas syndrome (Timmerman *et al.*, 1999).

In 1991, EGR3 was isolated as an result of its expression upon mitogenic stimulation of human and mouse fibroblasts, similar to EGR2 discovery, and of monkey kidney epithelial cell line (Patwardhan *et al.*, 1991). Tourtellotte and Milbrandt further identified a characteristic expression of EGR3 important for muscle spindle formation (Tourtellotte and Milbrandt, 1998). Muscle spindles are specialised receptors which detect stretching and the adjustments in the length of a muscle (Kröger and Watkins, 2021). Homozygous Egr3-deficient mice displayed a loss of basic motor skills such as coordinated movement, associated with the absence of muscle spindles (Tourtellotte and Milbrandt, 1998). A couple of studies have investigated the importance of EGR3 in the sympathetic nervous system, which controls homeostatic mechanisms of tissues and organs (Eldredge *et al.*, 2008; L. Li *et al.*, 2011; Quach *et al.*, 2013). EGR3, whose expression is regulated Nerve Growth Factor (NGF), in turn regulates genes required for sympathetic neuron axon and dendrite development (Eldredge *et al.*, 2008; Quach *et al.*, 2013). Furthermore, multiple studies have investigated the role of EGR proteins, particularly EGR1, induced in synaptic activity related to learning and memory processes (Li *et al.*, 2007; Poirier *et al.*, 2008; Cheval *et al.*, 2012; Duclot and Kabbaj, 2017).

So far, two studies have also explored a role for EGR2 in apoptosis programmed cell death (Unoki and Nakamura, 2003; Yokota *et al.*, 2010). Unoki and Nakamura identified EGR2 as a mediator of growth inhibitory signals of the PTEN pathway, a tumor supressor gene found in different types of cancers. Using adenovirus vector for EGR2 expresssion, they found that EGR2 induces apoptosis by directly upregulating *Bnip3l* and *Bak* transcription (apoptosis-related genes), in cancerous cell lines including colon, ovarian and prostate cancers (Unoki and Nakamura, 2003). Further conserved binding sites for the p53 and other p53 family protiens (p63 and p73) were found within the *Egr2* gene, upregulating its expression downstream of the DNA damage-induced p53 signalling pathway; promoting apoptotic cell death (Yokota *et al.*, 2010). As EGR2 obstructs growth of cancer cells, EGR2 could be a possible therapeutic target for cancer treatment in the near furture (Unoki and Nakamura, 2003; Yokota *et al.*, 2010).

1.6.2.1 EGR proteins and DNA binding

The EGR protein family consists of 4 members including EGR1 (other names: NGFI-A, zlf268, TIS8 and krox-24), EGR2 (other name: krox-20), EGR3 (other names: PILOT), and EGR4 (other names: pAT133 and NGFI-C) (Beckmann and Wilce, 1997). The EGR proteins are a group of transcriptional regulators with high similarity of amino acid sequence (~90% homology) in the DNA-binding zinc finger domains (Shao *et al.*, 1997). EGR protein contains three Cys_2-His_2 type zinger finger structural motifs, which are a common elements for protein-DNA interactions in eukaryotes (Beckmann and Wilce, 1997; Leon and Roth, 2000). The Cys_2-His_2 type zinger finger domain consists or two anti-parallel β-sheets and an α-helix, which adopts a folded structure following the interaction of the zinc ion with two cysteines and two histidines from the β-sheet and α-helix, respectively (Pavletich and Pabo, 1991; Fedotova *et al.*, 2017). Pavletich and Pabo reported a crystal structure for explaining the interaction between EGR1 zinc finger domains and DNA (Pavletich and Pabo, 1991). Interaction occurs by the binding of zinc fingers to the GC-rich promoter sequences (5'- GCGTGGGCG-3'), where each zinc finger binds to 3 base pairs on the binding site, as shown below (Christy and Nathans, 1989; Pavletich and Pabo, 1991; Anand *et al.*, 2013). Thiel and colleagues have also reported an activation domain of EGR1 transcription factor (Thiel *et al.*, 2000). The EGR proteins, excluding EGR4, also contain a repressor domain (R1) for the binding of NAB proteins which inhibit their activity (Russo *et al.*, 1995; Sevetson *et al.*, 2000). The differences between the EGR proteins outside the zinc finger domain accounts for the disparate interactions and functions (Duclot and Kabbaj, 2017).

1.6.2.2 EGR proteins in T cell development

Early studies explored the role of the EGR transcriptional regulators in thymocyte maturation (Miyazaki, 1997; Shao *et al.*, 1997b; Carter *et al.*, 2007). EGR1, EGR2 and EGR3, but not EGR4, zinc finger proteins are expressed in immature thymocytes dependent upon MHC-TCR interaction induced TCR signalling (Shao *et al.*, 1997b; Carleton *et al.*, 2002). Carleton and colleagues demonstrated that progression through β-selection checkpoint of defected TCR thymocytes is induced by forced overexpression of EGR 1,2 and 3 *in vitro* (Carleton *et al.*, 2002). EGR3 deficiency partially inhibits transition of DN thymocytes through the β-selection phase, and a study by Bettini and colleagues identified no influence of EGR1 deficiency in inhibiting DN thymocyte maturation through to the DP stage (Bettini *et al.*, 2002; Xi and Kersh,

2004). Thus, it is highly possible that other closely related EGR proteins (EGR2), whose expression is induced at similar times during selection, can compensate for EGR1 and EGR3 deficiency in DN3 thymocyte maturation through the β-checkpoint (Bettini *et al.*, 2002). A similar observation was observed from our group that found no difference in thymocyte development in Egr2-deficient mice (Zhu *et al.*, 2008). However, apart from the suggested compensatory roles of EGR proteins in differentiation through the β-checkpoint, EGR proteins regulate different processes involved in thymocyte development. For example, Xi and Kersh demonstrated that even though deficiency of EGR3 did not significantly affect differentiation through β-selection checkpoint, EGR3-deficient thymocytes have reduced proliferative capability in response to TCR signalling (Xi and Kersh, 2004). Furthermore, Carter and colleagues observed a combined role of EGR1 and EGR3 in positively regulating thymic survival as double deletion of EGR1 and EGR3 proteins resulted in severe thymic atrophy due to impaired proliferation and increased apoptosis of DN4 thymocytes (Carter *et al.*, 2007).

As well as their expression in DN thymocytes, EGR proteins are also induced in DP and CD4/CD8 SP thymocytes through TCR signalling during positive and negative selection (Shao *et al.*, 1997). For instance, EGR2 positively regulates ID3 and BCL2 expression during positive selection of CD4/CD8 SP thymocytes (Lauritsen *et al.*, 2008). Our group also highlighted the importance of EGR2, not only in DP to SP thymocyte maturation, but in development of mature B cells from immature B cells in the bone marrow (Li *et al.*, 2011). Interestingly, Lauritsen's group observed a reduction in the number of mature splenic $CD4^+$ T cells in Egr2 deficient mice, implicating a role for EGR2 in growth or survival of mature $CD4^+$ T cells (Lauritsen *et al.*, 2008).

The EGR2 zinc protein, but not EGR1/3, regulates NKT cell development (Lazarevic *et al.*, 2009). NKT cells regulate the protective immune responses against viral, bacterial, fungal pathogens as well as protozoa and parasites (Wu and Kaer, 2011). Using mice lacking calcineurin, Lazarevic and colleagues identified that calcineurin-NFAT signalling is crucial for NKT cell development (Lazarevic *et al.*, 2009). Calcineurin-NFAT signalling pathway is induced upon antigen-TCR interaction, in which calcineurin influx activates nuclear factor of activated T cells (NFAT) protein-mediated transcription of genes associated with thymocyte maturation and naïve T cell differentiation (Macian, 2005). Without NFAT-mediated EGR2 expression, Lazarevic and colleagues observed increased apoptosis and proliferation of Egr2 deficient-NKT precursors, highlighting the importance of EGR2 in survival and development of NKT cell in the thymus. In contrast to T cell maturation, compensation of EGR proteins was not observed in

NKT cell development as EGR1 and EGR3- deficient NKT cell precursors normally transition into NKT cells (Lazarevic *et al.*, 2009).

1.6.2.3 EGR proteins in T cell responses, self-tolerance and autoimmunity

Over the past years, EGR2 and EGR3 have emerged as key regulators of T cell-mediated immune responses, to avoid occurrence of excessive inflammation and autoimmunity. Harris and colleagues first described the association of EGR2 transcription factor with induction of T cell anergy, an immune tolerance mechanism (Harris *et al.*, 2004). *In vitro* induction of anergy, in a murine A.E7 $CD4^+$ T cell clone, highly increased the expression of EGR2 (NFAT-target gene) with phenotypic downregulation of IL-2 production. However, the expression of EGR1 was not maintained after TCR stimulation, without costimulation, in non-proliferating anergized cells. Anergic function of EGR2 was further supported as, in absence of EGR2, T cell responsiveness was restored and prevented anergy induction (Harris *et al.*, 2004). Consistent with these findings, Safford and colleagues, identified not only EGR2, but EGR3 zinc finger protein was also expressed in anergized cells in a similar pattern and inhibited T cell activation. The group illustrated that, the mechanism of T cell activation inhibition by EGR2/3 may involve transcriptional activation of inhibitory genes to induce anergy, as the expression of inhibitory factor E3 ligase c-Cbl was reduced in $Egr3^{-/-}$ mice compared to wild type T cells in anergic conditions (Safford *et al.*, 2005). This group was unable to study the effect of EGR2 deficiency *in vivo*, as EGR2 is associated with hindbrain maturation, causing early death in $Egr2^{-/-}$ mice (Swiatek and Gridley, 1993). Our group overcame this by generating mice with $CD2^+$ lymphocyte-specific Egr2 deletion (Egr-2 cKO mice), demonstrating a role for EGR2 in self-tolerance of T cells and autoimmunity (Zhu *et al.*, 2008). Our group illustrated that EGR2-deficient T cells exhibit normal primary T cell responses upon stimulation but persistent TCR signalling, which is associated with immune responses against chronic infections, caused exaggerated proliferation. This was further evident *in vivo*, as EGR2 deficiency caused spleen enlargement in mice of 15 month due to elevated number of $CD4^+$ $CD44^{high}$ T cells, associated with a late onset of T cell driven Lupus-like autoimmune disorder. In addition, the group found that in absence of EGR2, the expression of p21cip1, which is associated with T cell proliferation and T cell self-tolerance, significantly decreased in EGR2 deficient $CD4^+$ $CD44^{high}$ T cells compared to wildtype (Zhu *et al.*, 2008). This further supports the idea that EGR2 maintains the expression of factors associated with mechanisms of T cell tolerance, consistent with previously described E3 ligase c-Cbl (Safford *et al.*, 2005). In contrast to previous studies that indicated a

inhibitory function of EGR2 in T cell activation by repression of IL-2 signalling *in vitro* (Harris *et al.*, 2004; Safford *et al.*, 2005), our group found that EGR2 did not inhibit TCR signalling but it does control proliferation and expression of T cell tolerance mediators in effector $CD4^+$ $CD44^{high}$ T cells *in vivo* and the *in vitro* production of Th1(IFN-γ)/Th17(IL-17)-specific cytokines (Zhu *et al.*, 2008). The latter suggests EGR2 may regulate T cell effector functions. But a study by Ramon and colleagues did not observe a similar increase in IFN-γ production nor a defective immune response by hyperproliferative EGR2 deficient $CD4^+$ $CD44^{high}$ T cells against minor histocompatibility antigens, lymphocytic choriomeningitis virus and *Toxoplasma gondii* infection in young CD4-specific Egr2 KO mice. It is likely that once the late onset of autoimmunity develops, T cell immune responses will not be as effective or EGR3, with similar functions, compensates for EGR2 deficiency (Ramón *et al.*, 2010).

Following these findings, our group established the first model of CD2-$Egr2^{-/-}$$Egr3^{-/-}$ mice by breeding of the previously described Egr-2 cKO mice with Egr3 KO mice (Li, Miao, Sebastian, Bhullar, Ghaffari, Liu, Alistair L.J. Symonds, *et al.*, 2012). In this new mouse model, the group observed an early onset of systemic autoimmune disease at 2 months of age resulting in lethality in mice around 8 months of age with enlarged spleen and lymph nodes, multiorgan inflammation (mainly B cells, IL-17- and IFN-γ-producing $CD4^+$ T cells), glomerulonephritis and increased levels of anti-self antibodies. In contrast to the findings of the function of EGR2 in inhibition of TCR signalling through E3 ligase c-Cbl activation in anergic conditions, full stimulated EGR2/EGR3-deficient naïve B and $CD4^+$ T cells displayed impaired proliferation and IL-2 signalling *in vitro*. Suggesting alternate regulatory mechanisms of EGR2/3 in anergic cells compared to naïve. The group further observed upregulated expression of inflammatory cytokines including IL-17, IL-4, IL-21, GM-CSF and IFN-γ *in vivo*, suggesting a role for EGR2/3 in differentiation. Our group further illustrated hyperactivation of STAT1 and STAT3 correlating reduced expression of their suppressors SOCS1 and SOCS3, respectively. The group presented a regulatory mechanism by which EGR2 and 3 directly induces SOCS1 and SOCS3 expression to maintain the production of STAT1- and STAT3-mediated Th1 and Th17 cell-derived cytokines. Thereby EGR2 and 3 are required to prevent inflammatory pathology during antigen-specific immune responses and prevent autoimmune disorders (Li, Miao, Sebastian, Bhullar, Ghaffari, Liu, Alistair L.J. Symonds, *et al.*, 2012).

Further studies focused on understanding this regulation of cytokine expression, thus T cell differentiation, by EGR2 and 3 in T cell responses. For instance, EGR2 controls BAFT-mediated Th17 differentiation and BCL6-mediated Tfh differentiation (T *et al.*, 2013; Ogbe *et al.*, 2015). Du

and colleagues demonstrated a positive regulatory function of EGR2 in T cell differentiation against TCR stimulation *in vitro* and T-cell mediated immune responses to influenza virus *in vivo* (Du *et al.*, 2014). The expression of Th1/Th17-specific cytokines was significantly reduced upon TCR stimulation and influenza infection in absence of EGR2. Using RNA-seq, the group identified 36 directly bound target genes of EGR2 including the Th1- and CD8-lineage specific transcription factor T-bet whose expression is reduced in absence of EGR2 (Du *et al.*, 2014). A more recent study further supports a positive regulatory function of EGR2 in Th1 cell differentiation in mice with lupus. Silencing of EGR2 downregulated IFN-γ production and Th1 cell differentiation in mice prone to lupus after TCR stimulation *in vitro*. However, in resting lupus-prone mice, EGR2 expression is significantly upregulated in $CD4^+$ T cells compared to non-autoimmune controls. Thus, EGR2 is implicated in promoting differentiation of IFN-γ producing $CD4^+$ T cells resulting in excessive inflammation in lupus autoimmune disease (Dai *et al.*, 2020).

In contrast, a recent study by our group identified a suppressive role of EGR2/3 in Th1 differentiation (Singh *et al.*, 2017). In response to Vaccinia virus infection, Egr2/3-deficient $CD44^{high}$ $CD4^+$ and $CD8^+$ T cells produced excessive amounts of IFN-γ production, indicating a role of EGR2 in maintaining IFN-γ production in effector T cells during adaptive immune responses. Our group also observed that expression of EGR2 was downregulated by Th1-inducing cytokines such as IFN-γ, increasing Th cell polarization toward Th1-phenotype. The group further identified a physical association of EGR2 with the T-box binding domain of T-bet, thereby repressing T-bet mediated differentiation of IFN-γ producing $CD8^+$ and Th1 cells but not its expression, as suggested by Du and colleagues (Singh *et al.*, 2017). The difference in findings between the two groups is unknown but could be due to differences in mouse models as a single CD4-Egr2 conditional knockout model was used by Du's group whereas our group used a double Egr2/3 knockout mouse model (Du *et al.*, 2014; Singh *et al.*, 2017). Further contradictions, between a positive and negative regulatory role of EGR2 in IFN-γ producing cells, could be due to mouse models and different functions of EGR2 in different context (Singh *et al.*, 2017; Dai *et al.*, 2020). However, the findings of our group are significant as T-bet has been implicated in immunopathology, and this is the first study to describe a suppressive mechanism to maintain T-bet mediated differentiation of IFN-γ producing effector T cells preventing T-bet mediated autoimmunity whilst producing an effective immune response (Singh *et al.*, 2017). Both studies by our group indicated that the STAT1/IFN-γ pathway which regulates T-bet mediated IFN-γ production in effector T cells is controlled by EGR2 in such a

way that it can repress STAT1 activation and T-bet function (Li, Miao, Sebastian, Bhullar, Ghaffari, Liu, Alistair L.J. Symonds, *et al.*, 2012; Singh *et al.*, 2017).

1.7 B cells

B cells are the lymphocytes of the adaptive immune system recognised for producing antibodies against pathogens and vaccinations (Haberman *et al.*, 2019). B cells develop in the bone marrow and mature B cells reside in secondary lymphoid organs and are activated upon antigen recognition by the B cell receptor (BCR) on its surface (Wen *et al.*, 2019). Much like the TCR, the antigen-recognition site on the BCR complex is unique for each B cell facilitating the recognition of the diverse range of pathogenic antigens invading the body (Lebien and Tedder, 2008). The BCR complex consists of a membrane-bound immunoglobin (Ig) linked to Igα/Igβ heterodimer. Igα and Igβ are transmembrane proteins that covalently bind to form a heterodimer and mediate intracellular signaling through ITAMs located on their cytoplasmic domain, during antigen recognition through the BCR. Antigen recognition by the BCR complex initiates phosphorylation of ITAMs by lymphocyte-specific tyrosine kinases, namely Lyn, which then recruits Syk, the signal transducer through which downstream signaling pathways are initiated (Wen *et al.*, 2019). The Ig or antibody is responsible for antigen-binding and is either of class IgM, IgA, IgD, IgE, or IgG (Dong *et al.*, 2022). Igs are made up of two identical heavy chains and two identical light chains, both form the constant and variable structural domain. The constant regions are different between the classes of Igs and exert effector functions such as activating complement proteins (Janda *et al.*, 2016). The variable region contains the antigen-binding domain which functions to bind to antigens with high antigen-specificity established through somatic recombination events during B cell maturation (Eibel *et al.*, 2014; Janda *et al.*, 2016). Upon activation, B cells can differentiate into plasma cells which secrete non-membrane-bound form of Ig antibodies into the bloodstream and can bind to cognate antigens to neutralise pathogens, remove microbes or infected cells (Vinuesa and Chang, 2013; Dong *et al.*, 2022).

The processes of B cell development and maturation, activation and formation of germinal centre B cell is described in the following sections.

1.7.1 B cell development and maturation

Unlike T cells which mature in the thymus, B cell development and maturation occur within the bone marrow. As shown in Figure 1.12, stages of B cell maturation include progenitors, pre-B cells, immature, and then mature B cells which all differ by their phenotype and, heavy and light chain gene rearrangements (Carsetti, 2000; Wen *et al.*, 2019). At first, hematopoietic stem cells commit to the B cell lineage and become B cell progenitor (Pro-B) cells. Phenotypically, progenitor cells are characterized by surface expression of CD43, c-kit, and BB20. In addition, pro-B cells undergo gene rearrangement of heavy Ig chains. After the successful rearrangement of Ig heavy chains, pro-B cells differentiate into pre-B cells (Carsetti, 2000). pre-B cells express a non-functional pre-BCR complex formed with the rearranged heavy chain and a surrogate light chain, required for further pre-B cell development (Eibel *et al.*, 2014; Wen *et al.*, 2019). These cells lose the expression of c-kit and CD43 (Carsetti, 2000). The light chain genes then undergo recombination to establish a complete Ig molecule at the next immature B cell stage(Wen *et al.*, 2019). During this stage, immature B cells expressing highly self-reactive BCR are removed through apoptosis, whereas non-self-reactive B cells transform into transitional B cells (Eibel *et al.*, 2014). Transitional B cells exit the bone marrow and undergo 2 stages of differentiation (T1 and T2) before forming mature B cells (Lebien and Tedder, 2008). During the transitional B cell stage, another screening process for autoreactive transitional B cells occurs within the spleen. The remaining non-self-reactive B cells can then enter the mature naïve B cell compartments within the spleen (Chen *et al.*, 2022). These naïve B cells express both IgM and IgD on their surface and both have the same antigen specificities (Wen *et al.*, 2019). The transitional B cells can mature into different B cell subsets such as marginal zone, follicular or B1 B cells (Vinuesa and Chang, 2013). Marginal zone B cells are the early responders to blood-borne antigens which enter the spleen and differentiate into short-lived extrafollicular plasma cells which produce IgM or IgG antibodies in a T cell-independent manner. Marginal zone B cells reside in the marginal zone of the spleen, between the red and white pulp (Lopes-Carvalho and Kearney, 2004; Vinuesa and Chang, 2013). B1 B cells are mainly found in the peritoneal and pleural cavities of the spleen (Vinuesa and Chang, 2013). Unlike conventional B cells (marginal zone and follicular B cells), B1 B cells are generated during fetal development and are capable of self-renewal maintaining their population in adulthood (Lebien and Tedder, 2008). These cells are referred to as innate-like B cells as they provide the first line of defence against bacterial infection by differentiating into extrafollicular plasma cells and producing natural IgM antibodies, which are antibodies present in the body naturally without prior infection (Lebien and

Tedder, 2008; Smith and Baumgarth, 2019). Both of these B cells are responsible for maintaining homeostasis as well as their role in primary humoral responses for host protection against infection and vaccinations (Lopes-Carvalho and Kearney, 2004). The predominant subset of B cells, B follicular cells, reside in B cell follicles of the spleen and lymph nodes. Follicular B cells respond in a T cell-dependent manner, producing short-lived plasma cells or long-lived plasma and memory B cells. The latter are formed once activated follicular B cells enter germinal centers and differentiate into germinal center B cells which undergo processes such as somatic hypermutation and affinity maturation producing long-lived plasma and memory B cells (Hamel, Liarski and Clark, 2012; Vinuesa and Chang, 2013; Eibel *et al.*, 2014).

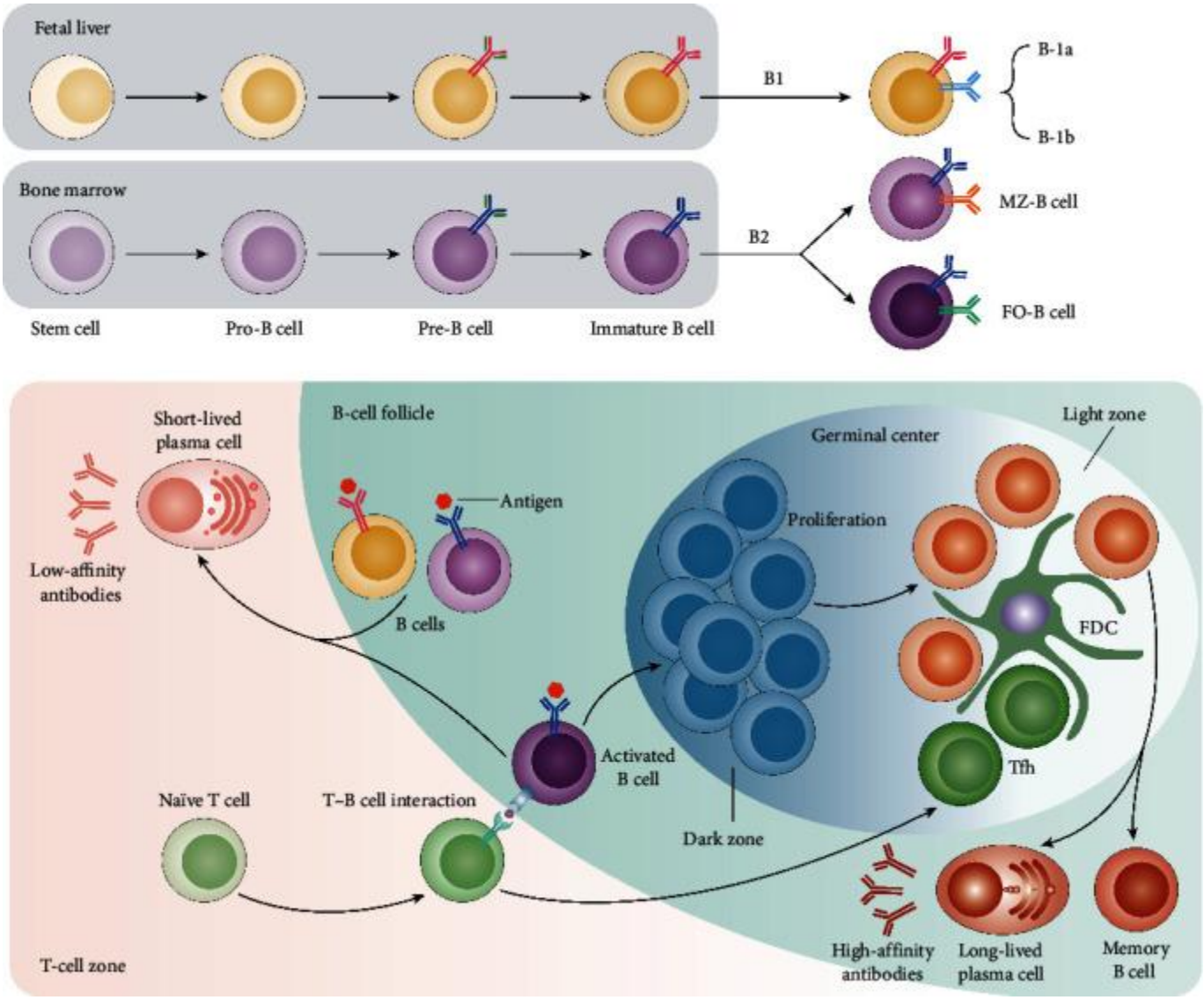


Figure 1.12 The developmental stages of B cell lineages and their differentiation during an immune response. (A) B1 B cells originate during fetal development in sequential stages of maturation. There are two subsets of B1 B cells including B-1a and B-1b. (B) The second subset of B2 B cells follow the same developmental stages as the B1 subset but within the bone marrow. The immature B2 B cells then mature into either follicular (FO) B cells or marginal zone (MZ) B cells. (C) B1 and MZ B cells are activated independently of T cells and differentiate into short-lived plasma cells. On the other hand, the FO B cells are activated in a T cell-dependent manner and their cell fates are decided upon by the strength of the B cell-to-T cell interaction. If sufficient help signals are provided by the T cell then the FO B cell will enter the germinal centers (GCs) and further mature into long-lived plasma cells which secrete high-affinity antibodies and high-affinity memory B cells. However, if the help provided by T cells is weak the activated FO B cells mature into low-affinity antibodies producing short-lived plasma cells outside the GCs. The germinal center consists of a dark zone and a light zone which includes follicular dendritic cells and T follicular helper (Tfh) cells (Dong *et al.*, 2023).

During a response, all B cells proliferate and differentiate into plasma cells and secrete antibodies. Each plasma cell secretes one type of Ig molecule with antigen-binding specificity. In addition, a small fraction of B cells differentiate into long-lived memory B cells, lose their IgD expression, and are capable of differentiating into plasma cells at a faster rate upon encountering the same antigen again (Wen *et al.*, 2019). Although follicular B cells are associated with the development of memory B cells in a T cell-dependent manner, Marginal zone and B1 B cells can also form memory B cells in specific immune challenges (Lopes-Carvalho and Kearney, 2004; Smith and Baumgarth, 2019). Following this section, the thesis will only focus on follicular B cells and their functions.

1.7.2 Germinal center B cells

Germinal center B cells produce high-affinity, class-switched antibodies during host protection. Germinal centers (GCs) are microanatomical structures that form within B cell follicles of secondary lymphoid organs such as the spleen and lymph nodes, during infection and after vaccinations (Young and Brink, 2021). Once the antigen clears, the germinal centre reactions terminate and the GC undergoes shutdown. However, dysregulated GC reactions can lead to autoimmunity, chronic infections, and the generation of autoreactive B cells leading to B cell lymphomas. Thus, GC reactions need to be tightly regulated (Gatto and Brink, 2010; Arulraj *et al.*, 2021).

GCs are divided into two compartments: the dark zone (DZ) and the light zone (LZ). In the light zone, B cells interact with follicular dendritic cells (FDCs) and T cells (Gatto and Brink, 2010). The LZ is positioned closer to the marginal zone and subcapsular sinus of the spleen and lymph nodes, respectively (Mesin, Ersching and Victora, 2016). This positioning facilitates exposure to the blood-borne antigens which enter the tissue into the marginal zone and allows interaction with antigens and immune cells from the lymphatic drainage (Haberman *et al.*, 2019). The DZ is packed with proliferating B cells and is located closer to the T cell zone. Within the dark zone, FDCs are absent, and proliferating B cells undergo somatic hypermutation (SHM) of their Ig variable region genes (Gatto and Brink, 2010). Once the process of somatic hypermutation is completed, the B cells migrate to the LZ for positive selection of high affinity GC B cells (Gatto and Brink, 2010; Hamel, Liarski and Clark, 2012; Vinuesa and Chang, 2013). The structural overview of the B cell follicle, T cell zones, and GCs are illustrated in Figure 1.13. These

processes of GC B cell formation and subsequent GC reactions are described in the following sections.

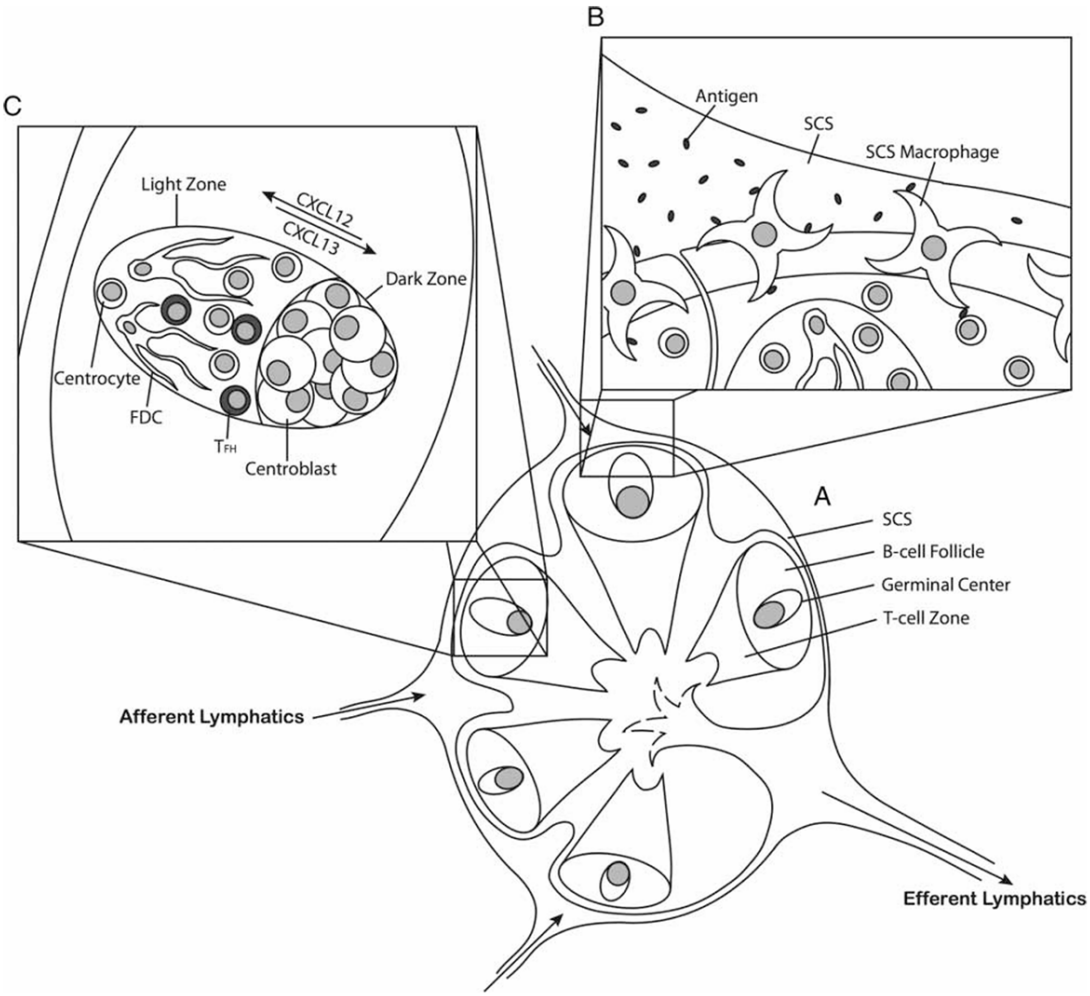


Figure 1.13 Antigen transport and the organisation of the lymph nodes. (A) Anatomical view of the lymph node compartments. Below the outer collagen-rich capsule of the lymph nodes lies the subcapsular sinus (SCS), the B cell follicle which contains germinal centers (GCs), and the T cell zones. (B) Antigens are transported into the SCS of the lymph nodes from the efferent lymphatic vessels. The SCS macrophages present the antigens to cognate follicular B cells. (C) Activated B cells engage with T cells at the T cell-B cell boundary. The T cells provide the help signals required for the entry of B cells into GCs. The GC consists of a dark zone which consists of proliferating centroblasts and is located closer to the T cell;B cell border. The light zone of the GC is positioned closer to the SCS and consists of follicular dendritic cells (FDCs), T follicular helper (Tfh) cells, and centrocytes. The migration of GC B cells between the light and dark zones of the GCs are against a chemokine gradient (Hamel, Liarski and Clark, 2012).

1.7.2.1 B cell activation and formation of GC B cells

Following infection or vaccinations, naïve follicular B cells are activated through the BCR by engagement with antigens. BCRs can bind to antigens presented on macrophages (Figure 1.13), or FDCs, or soluble antigens (Hamel, Liarski and Clark, 2012; Young and Brink, 2021). BCR- antigen complex triggers downstream BCR signaling resulting in B cell activation, antigen-presentation on MHC class II molecule, and the upregulation of CCR7 which facilitates the migration of the B cells to the T zone-B zone border. At the T cell zone-B cell zone border, the B cells interact with $CD4^+$ T cells by presenting antigens on their MHC class II molecule. As a result, T cells produce signals required for B cell proliferation and differentiation (Gatto and Brink, 2010). B cells then proliferate at the T cell-B cell border and this T cell-dependent activation of B cells can drive differentiation into three different B cell fates, as shown in figure 1.14. Activated B cells can become short-lived plasma cells with low antigen affinity providing the initial antibody response until GC response is achieved, and a short lifespan lasting the duration of infection. Alternatively, the B cells could differentiate into memory B cells independent of the germinal centre. Consequently, memory B cells formed outside of the GC do not exhibit somatic hypermutation and isotype switching thus they are relatively distinct from GC B cells and cannot differentiate into long-lived plasma cells. The third B cell lineage activated B cells can exhibit through T cell-dependent interactions is differentiation into GC B cells. GC B cells produce memory B cells and long-lived plasma cells once GC formation is complete. As the GC formation can take several days, GC-independent short-lived plasma and memory B cells are important for the initial antibody-mediated defence against infections (Akkaya, Kwak and Pierce, 2019).

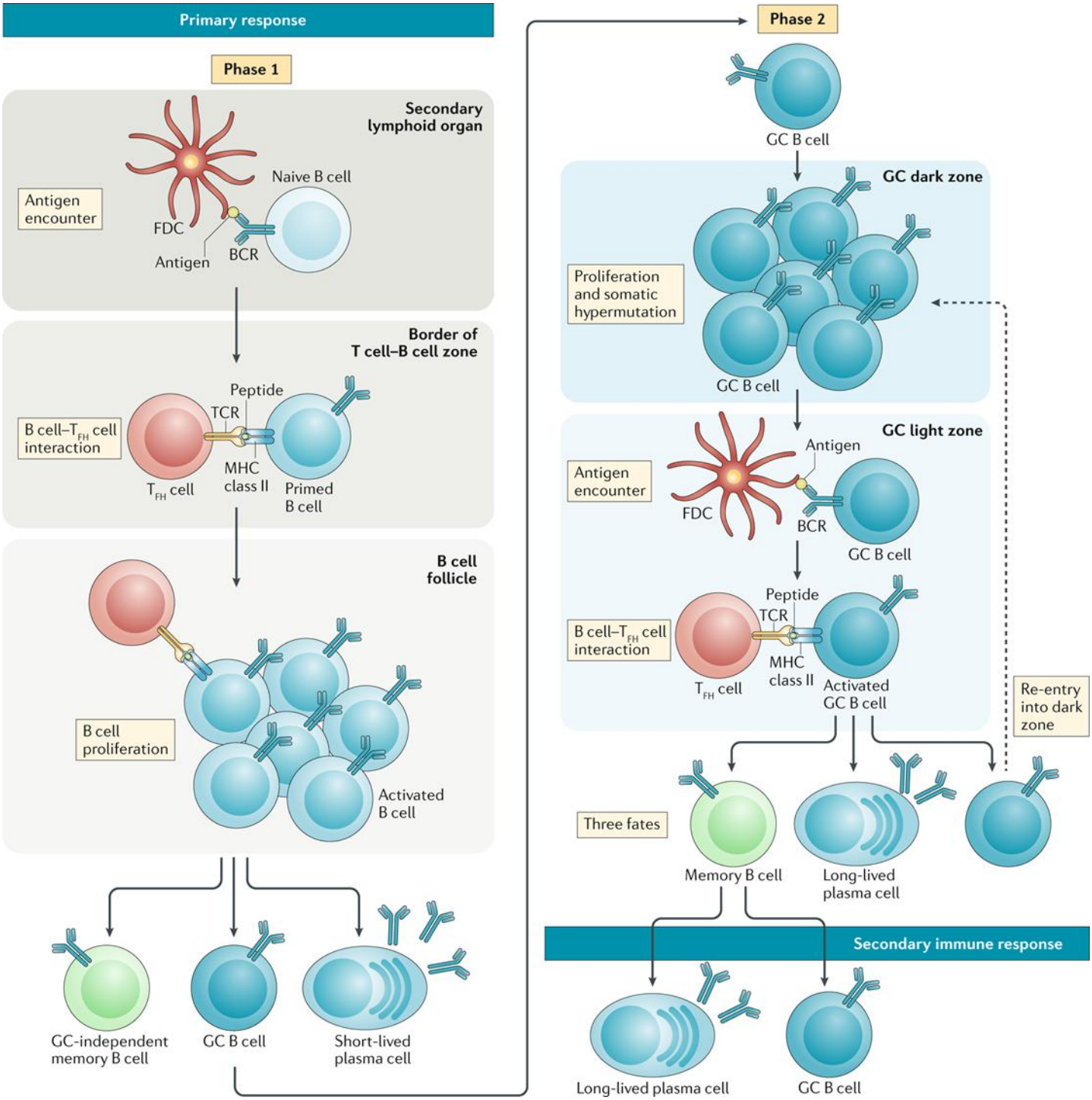


Figure 1.14. Primary and secondary B cell responses. In phase 1 (left), the B cells are activated by antigen-presenting follicular dendritic cells (FDCs) in secondary lymphoid organs. The primed B cells present antigenic peptides to T cells. This initiates B cell proliferation and differentiation into one of the three B cell lineages, short-lived plasma cells, germinal center (GC)- dependent B cells, or GC-independent memory B cells. Phase 2 (right) of the primary response illustrates the further maturation of GC B cells into high affinity memory B cells and long-lived plasma cells. The GC B cells first enter the dark zone. In the dark zone, the B cells proliferate and undergo somatic hypermutation of the Ig variable region. The mutated B cells then enter the light zone in which they encounter antigens of the FDCs through their mutated BCR and are selected by T follicular helper (Tfh) cells. During a secondary

immune response (presented at the bottom), the memory GC B cells rapidly response to the previously encountered pathogen and differentiate into long-lived plasma cells which secrete high affinity antibodies or enter new GCs as GC B cells.

The fate decision of activated naïve B cells which is dependent upon both BCR-antigen and T cell interaction, is influenced by multiple factors including migration and strength of BCR-antigen engagement. GC B cells and short-lived plasma cells exhibit distinct expression of chemokine receptors which determines their migration into different anatomical positions of the spleen and lymph node which selectively express specific receptor ligands. For example, follicular B cells display expression of EB12 for migration to the periphery of B cell follicles and express CXCR5 for localization in B cell follicles. Upon differentiation into short-lived plasma cells downregulate the expression of EB12 and CXCR5, and upregulate the expression of CXCR4 for localization into the splenic red pulp and the medullary cord in the lymph nodes. In comparison, activated B cells differentiating into GC B cells retain CXCR5 expression, for localization into B cell follicles but downregulate EB12 expression to facilitate their movement to the centre of B cell follicles for GC formation which originates at the centre of B cell follicles (Gatto and Brink, 2010). In addition, fate decisions also rely on the B cell's affinity to antigens. B cells which bind with higher affinity to their cognate antigens during B cell activation tend to differentiate into short-lived plasma cells. Whereas, B cells which require lower affinity differentiate into GC B cells which undergo affinity maturation within GCs (Hamel, Liarski and Clark, 2012).

Other factors influencing fate decisions include the transcriptional regulation by the B cell lymphoma 6 (BCL6), the master regulator of GC formation, influencing GC B cell and Tfh cell differentiation programmes. BCL6 expression is tightly regulated upon B cell activation, whereby its distinctly expressed in proliferating B cells committing to GC B cell lineage but absent in B cells undergoing differentiation into short-lived plasma cells (Gatto and Brink, 2010). Along with its role in GC B cell differentiation, BCL6 mediates the migration of GC B cells into the dark zone of germinal centres, by upregulating CXCR4 expression and downregulating S1PR1. Other roles of BCL6 are associated with germinal centre responses including B cell proliferation and resistance to DNA damage sensing pathways essential for SHM reactions occurring in the germinal centre (Recaldin and Fear, 2016).

1.7.2.2 Germinal centre responses

The GC responses play a pivotal role in the development of high-affinity antibody secreting plasma cells and memory B cells to clear of the pathogenic invaders. The GC responses involve rapid proliferation of GC B cells, isotype switching, somatic hypermutation and affinity maturation (Lebien and Tedder, 2008). Cell types such as FDCs and Tfh cells facilitate high-affinity B cell differentiation and help avoid autoreactive B cells from exiting the GCs (Gatto and Brink, 2010). While Tfh cells play a crucial role in GC responses (See section on Tfh cells), this section will focus on the intrinsic and functional properties of GC B cells. The processes that B cells undergo are highlighted in the following sections and illustrated in figure 1.15.

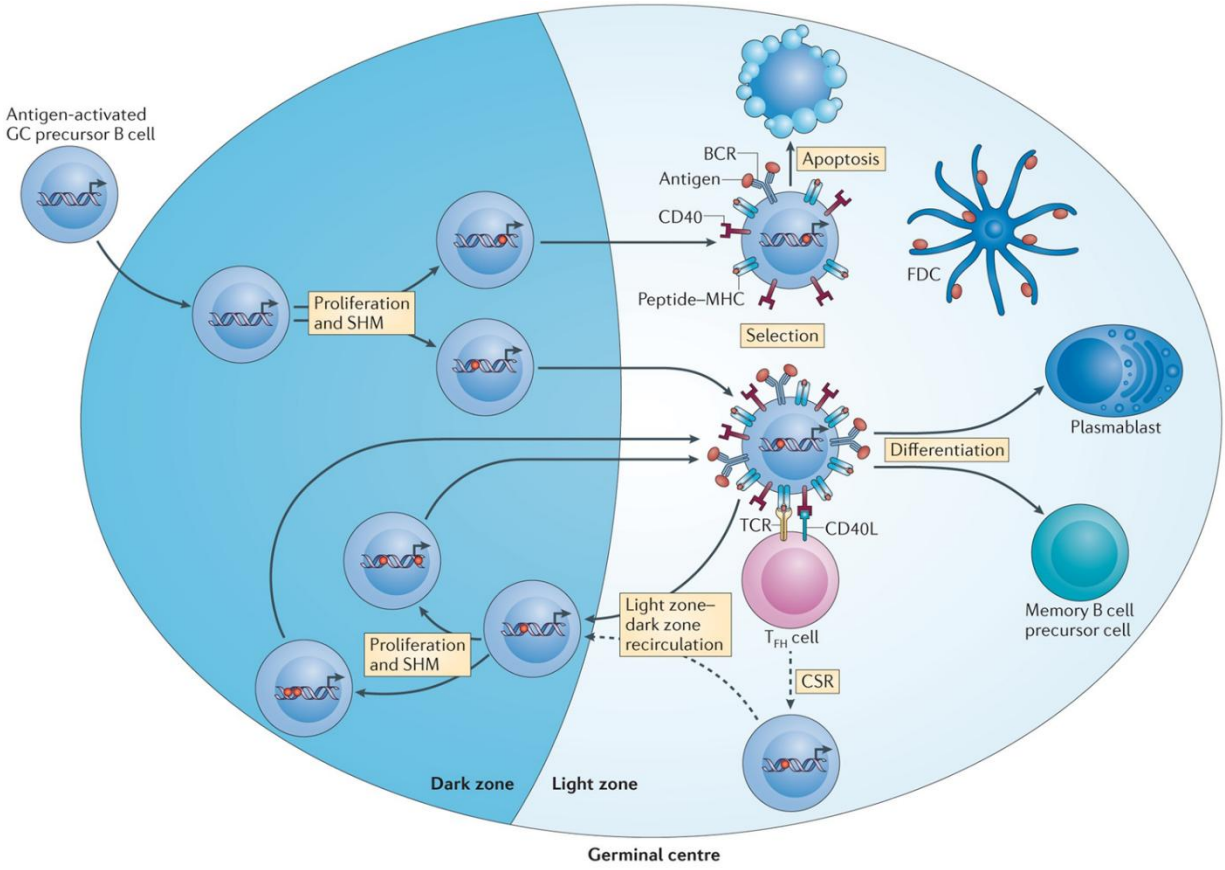


Figure 1.15. Overview of Germinal center (GC) reactions. The diagram summarises the GC reactions and migration (via arrow) of GC B cells throughout the dark (DZ) and light zone (LZ) of the GCs. Antigen-dependent B cell activation occurs outside the GCs after which GC B cells enter the germinal center into the dark zone. In the dark zone, the GC B proliferates and undergoes further changes in the rearranged variable region of the immunoglobulin (Ig) molecule through a process called somatic hypermutation (SHM). The red dots on the DNA molecules within the cells indicate this. The DZ B cells then migrate to the light zone, where they are selected by T follicular helper (Tfh) cells based on the strength of antigen binding of the altered B cell receptor (BCR). These high-affinity LZ B cells effectively capture more antigen which means there is increased presentation of peptide-MHC complexes on the B cells. This results in stronger interaction with Tfh cells and in turn positive selection of high affinity B cells. If the mutated LZ B cells cannot bind to antigens presented by follicular dendritic cells, this triggers apoptosis. Also within the GC, the high affinity B cells

undergo Ig class switch recombination (CSR) and then either differentiate into plasma and memory B cell precursors or recirculate between the DZ-LZ. After several rounds of SHM and positive selection, the GC B cells differentiate into memory B cell precursors and plasmablasts which differentiate into high affinity memory B cells and long-lived plasma cells, respectively (De Silva and Klein, 2015).

1.7.2.2.1 Somatic hypermutation and affinity maturation

SHM is a process which further introduces diversity to the BCR repertoire to ensure that B cells can recognise a range of antigens. After early GC B cell commitment, the GC B cells migrate to form the GC, and localize and proliferate rapidly within the DZ of the germinal centre. These B cells are termed centroblasts and exhibit high expression of CXCR4 thus, attracted to the CXCL12 ligand dominant DZ (Hamel, Liarski and Clark, 2012). The proliferating centroblasts undergo SHM which introduces a point mutation within the previously rearranged Ig variable region during B cell maturation. This process is catalysed by the enzyme activation-induced deaminase (AID). The resultant mutated B cells have altered antigen affinity and predominantly migrate to the LZ of the GC for Tfh cell-mediated selection of high-affinity B cells (Gatto and Brink, 2010; Hamel, Liarski and Clark, 2012).

The mutated B cells downregulate CXCR4 and retain CXCR5 expression, which facilitates their migration into the CXCL13-rich region of the LZ. These GC B cells are called centrocytes. The LZ consists of many FDCs and a limited number of Tfh cells (5-20%) required for affinity maturation (Gatto and Brink, 2010). Affinity maturation is a GC response by which centrocytes are positively selected determined by the antigen affinity of their BCRs (Mesin, Ersching and Victora, 2016). Within the LZ, the centrocytes bind via their BCRs to opsonised antigens presented by the FDCs (Eibel *et al.*, 2014). Since there is a limited T cell-help available, the high-affinity B cells outcompete the low-affinity B cells in binding to the Tfh cells. The Tfh cells provide the essential signals for the survival of the selected high-affinity B cells which can then cycle back and forth between the DZ and the LZ for several rounds of SHM and positive selection, generating effective B cell clones with high-affinity for the cognate antigen. Alternatively, the generated B cells can directly differentiate into plasma cells without further rounds of BCR refinements (Gatto and Brink, 2010; Hamel, Liarski and Clark, 2012; Young and Brink, 2021). The B cells with lower antigen affinity do not receive the survival signals from Tfh cells, thus undergo apoptosis (Eibel *et al.*, 2014).

1.7.2.2.2 Isotype switching

Isotype switching, also referred to as class switch recombination (CSR), is an event that occurs upon upregulation of AID enzyme in activated B cells. Thus, this event begins before B cells enter GC, at the T cell-B cell boundary, and continues within the LZ of the GCs, where the centrocytes encounter Tfh cells (De Silva and Klein, 2015; Chi, Li and Qiu, 2020). After antibody class switching, the GC B cells may differentiate into B cells and plasma cells or re-circulate through the DZ and LZ of the GC (De Silva and Klein, 2015). In comparison to SHM which determines the antigen specificity of GC B cells, isotype switching alters the effector function of the plasma and memory B cells generated (Chi, Li and Qiu, 2020). The antibody classes differ by the arrangement of the constant region of their heavy chains. The heavy chains of the different antibodies, IgM, IgA, IgD, IgE, or IgG are μ, α, δ, ε, and γ chains, respectively (Charles A Janeway *et al.*, 2001). During isotype switching, the constant regions of the initial IgM or IgD heavy chains are switched by recombination with downstream constant region sequence of another antibody isotype (Kotnis *et al.*, 2008; Hamel, Liarski and Clark, 2012). The activation of B cells by interaction with Th cells at the T cell-B cell boundary provide the cytokine signals that influence the type of isotype switching that occurs for an effective immune response against specific pathogens (Gatto and Brink, 2010). Similarly, to SHM, this process is mediated by the enzyme AID. AID introduces mismatched bases at the 5' end switch recombination sequences of the IgM heavy chain constant region genes (*Ighm*) and at the downstream region gene (either *Igha*, *Ighe*, or *Ighg*) by deaminating cytidines that convert it to uracils (Kotnis *et al.*, 2008; Gatto and Brink, 2010; Hamel, Liarski and Clark, 2012). This chemical reaction initiates DNA repair mechanisms. The Uracil is then excised by uracil DNA glycosylase which introduces DNA double-stranded breaks at the targeted switch recombination regions (Gatto and Brink, 2010). The DNA is then repaired by a process called non-homologous end joining (NHEJ) in which the switch recombination sequences of the *Ighm* constant region and the alternative Ig constant region ligate in such a way that the *Ighm* constant region sequence is excised and the downstream constant region of either *Igha*, *Ighe*, or *Ighg* replaces the position of *Ighm*. This results in the generation of IgA-, IgG- or IgE-expressing B cells (Kotnis *et al.*, 2008; Gatto and Brink, 2010; Chi, Li and Qiu, 2020). IgD recombination event only occurs within a limited number of B cell subsets that reside in lymphoid tissues such as mesenteric lymph nodes (Chi, Li and Qiu, 2020).

1.7.2.2.3 GC B cell differentiation into memory B cells and long-lived plasma cells

GC B cells undergo several rounds of SHM and affinity maturation before differentiating into long-lived plasma cells and memory B cells and then exit the GCs to exert their effector and memory functions, respectively (Mesin, Ersching and Victora, 2016). The fate decisions are made within the LZ influenced by the signaling through Tfh cells, the level of BCR affinity and the expression of specific transcription factors. GC B cells that undergo extensive SHM are prone to differentiate into long-lived plasma cells which have higher antigen affinity. Whereas GC B cells with limited rounds of SHM and lower antigen affinity differentiate into memory B cells with cross-reactive BCRs providing broader protection to a variety of antigens. These GC B cells with lower affinity are referred to as memory precursor B cells and are marked by the expression of CCR6 (Akkaya, Kwak and Pierce, 2019). In addition to the BCR affinity, transcription factors such as BACH2 induce differentiation into memory B cells whereas IRF-4 is required for plasma cell differentiation (Akkaya, Kwak and Pierce, 2019; Palm and Henry, 2019). Once memory B cells exit the GC, they remain in a quiescent state until antigen exposure after which they differentiate into antibody-secreting long-lived plasma cells or GC B cells (figure 1.14) (Akkaya, Kwak and Pierce, 2019).

1.8 T follicular helper cells

Tfh cells are a specialized subset of $CD4^+$ T helper cells that mediate germinal center formation, high-affinity antibody production, and the generation of long-lived plasma cells and memory B cells during the humoral response. Thus, Tfh cells are key drivers of protective antibody responses during both vaccine-induced and pathogen-induced immune reactions (Choi and Crotty, 2021).

In the early 2000s, Tfh cells were identified from other T helper cells and naïve T cells in human tonsils due to their elevated expression of CXCR5 and decreased expression level of the T cell zone homing receptor, CCR7. CXCR5 expression is a unique feature of Tfh cells for their migration into B cell follicles (Zhu, Zou and Liu, 2015). T helper cell fate decisions are made early during naïve $CD4^+$ T cell proliferation. Within the first two cell divisions, the choice of becoming a Tfh cell or a non-Tfh cell is made. If CXCR5 is induced then the $CD4^+$ T cells adopt a Tfh cell fate migrate to the T cell-B cell border, mature into GC Tfh cells and provide help to B cells in the germinal center. However, if the naïve T cell receives signals to differentiate into non-Tfh helper T cells, such as Th1 or Th17, it follows a different path. These T helper cells will exit the lymphoid tissue and migrate toward the site of infection or injury by following inflammatory chemokine gradients, where they exert their effector functions (Crotty, 2014, 2019).

The discovery of BCL6 as the master regulator of Tfh cells in 2009 was paramount in advancing Tfh cell research. Several studies have reported the importance of BCL6 in the differentiation and maintenance of Tfh cells to allow them to provide continuous signaling for GC maintenance during an immune response (Choi and Crotty, 2021). In mice with BCL6-deficient $CD4^+$ T cells, Tfh cell differentiation is impaired but can be restored by enhancing BCL6 expression through retroviral transduction (Ogbe *et al.*, 2015; Johnston *et al.*, 2009). Another study showed that temporal deletion of BCL6 in $CD4^+$ T cells induced by tamoxifen revealed a transition of committed Tfh cells into Th1 cells during LCMV infection in mice. This temporal deletion was achieved using a tamoxifen-inducible *Cre-ERT2* system in which tamoxifen-activated Cre recombinase deletes floxed *Bcl6* gene, enabling controlled deletion of BCL6 (Alterauge *et al.*, 2020). Thus BCL6 transcription factor is described as the Tfh lineage-specific transcription factor used to distinguish Tfh cells from other T helper cell fates (Figure 1.5) (Yu et al., 2009).

Additional phenotypic markers that can be used to identify Tfh cells from $CD4^+$ T cells through flow cytometry include the high expression of PD-1, ICOS, in conjunction with BCL6 and

CXCR5 (Deenick and Ma, 2011). Further explanation of Tfh cell differentiation, function, and regulatory T follicular (Tfr) cells are detailed in the following sections.

1.8.1 Tfh cell activation and differentiation

Tfh cell differentiation is described as a multistep process influenced by TCR signaling, costimulatory molecules, the cytokine milieu, and transcription factors as shown in figure 1.16 (Crotty, 2014, 2019). Naïve CD4⁺ T cells with high-affinity TCRs for non-self-antigens are prone to Tfh differentiation. In addition, prolonged TCR signaling which is first mediated by dendritic cells and then by B cells, initiates GC-Tfh cell differentiation (Deenick and Ma, 2011).

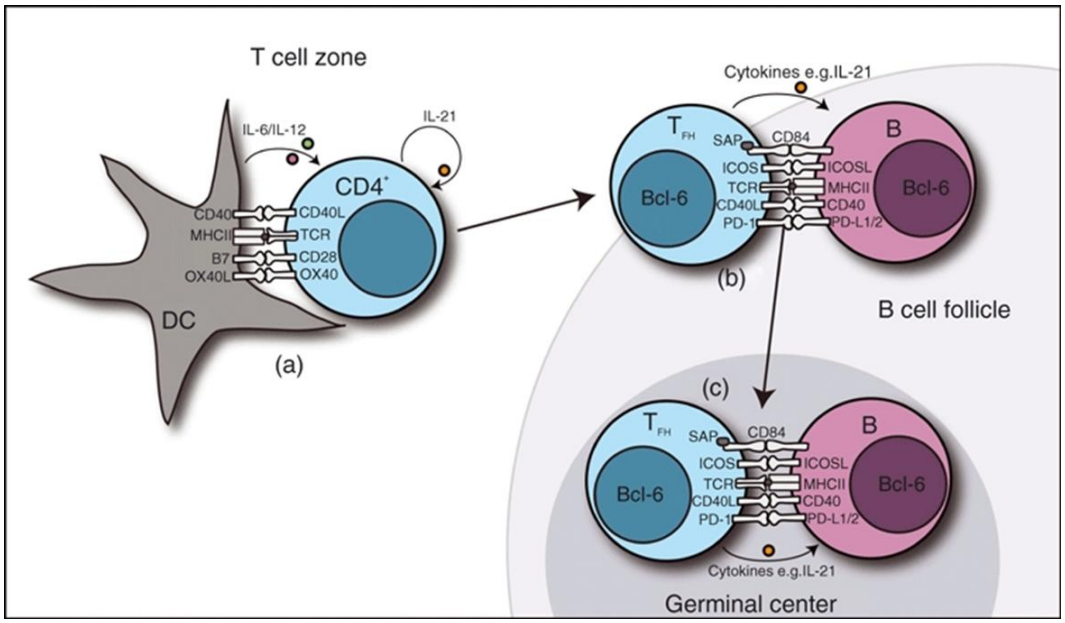


Figure 1.16. Overview of T follicular helper (Tfh) cell differentiation. The diagram summarises the three stages of Tfh cell differentiation during an immune challenge. During stage 1 (a) in the T cell zone, the naïve CD4⁺ T cells interact with antigen-presenting dendritic cells (DCs), which initiate TCR signalling and induce the pre-Tfh cell differentiation program with the upregulation of Tfh cell specific genes such as *Cxcr5*, *Bcl6* and the secretion of IL-21. The pre-Tfh cells then migrate to the B cell follicle for stage 2 (b) for communication with B cells which promote further TCR signalling for the maturation of pre-Tfh cells into terminally differentiated Tfh cells. The signalling between Tfh and B cells facilitates the formation of germinal centres and differentiation of pre-Tfh cells into GC Tfh cells (c). The signalling molecules involved in the cross talk between Tfh cells with DCs and B cells are mentioned in the diagram (Deenick and Ma, 2011).

During the first step of Tfh cell differentiation, the naïve CD4⁺ T cell interacts with the antigen-presenting DC which is sufficient to drive a precursor (pre-) Tfh cell fate by upregulating the expression of CXCR5 and the Tfh lineage-specific transcription factor, BCL6, and

downregulating CCR7 (Hardke *et al.*, 2005; Nurieva *et al.*, 2009; Crotty, 2014). Apart from TCR signaling, the pre-Tfh cell fate is regulated by CD28, CD40L, and the cytokine environment, mainly IL-6 and IL-2 (Deenick and Ma, 2011; Crotty, 2014). For $CD4^+$ T cell activation, costimulatory signaling via CD28 is essential. CD28 is a costimulatory molecule expressed on the surface of $CD4^+$ T cells which binds to its ligands, CD80 and CD86, expressed by DCs (Rudd *et al.*, 2009). The DCs are then activated through the binding of the CD40 receptor on the surface of DCs with the CD40 ligand (CD40L) expressed on T cells. Both CD28 and CD40 have crucial roles in the function of Tfh cells. Inhibition of CD28 signaling or the lack of CD40, inhibits the upregulation of CXCR5, a receptor essential for homing to B cell follicles. Thus, this leads to impaired migration of pre-Tfh cells into B cell follicles which is important to provide B cell help (Deenick and Ma, 2011). ICOS is another co-stimulatory molecule in the CD28 family that plays an important role in Tfh cell differentiation and, the production of IL-21. However, ICOS signaling plays distinct roles during both the differentiation and maintenance of these cells. During differentiation, ICOS-mediated interactions between T cells and dendritic cells promote the upregulation of the Tfh cell–specific marker CXCR5. In contrast, the maintenance of mature Tfh cells relies on continuous ICOS signaling through interactions with B cells, to preserve the $CXCR5^+$ $CCR7^-$ Tfh cell phenotype (Weber *et al.*, 2015; Lee *et al.*, 2015). IL-6 produced by DCs also contributes to the expression of BCL6. IL-6 interacts with its receptor, IL-6R, on $CD4^+$ T cells which in turn induces STAT1/STAT3 signaling and promotes BCL6 expression in the newly activated T cells (Choi *et al.*, 2013). IL-6 signaling also induces the expression of IL-21 in Tfh cells, which is predominantly secreted by Tfh cells for their function (Eto *et al.*, 2011). In addition, IL-21 also regulates BCL6 expression in Tfh cells (Ozaki *et al.*, 2004). However, in humans, IL-12 and Transforming growth factor beta (TGF-β), rather than IL-6, is responsible for IL-21 production by Tfh cells (Schmitt *et al.*, 2009, 2014).

Other factors involved in regulating the expression of Bcl6 include Blimp-1 and IL-2. Upon T cell activation and subsequent proliferation, *in vivo*, the fate of T cells toward becoming Tfh cells or other T helper (non-Tfh) cells is determined as early as the second cell division (Choi *et al.*, 2011). IL-2 acts very early during T-cell activation and acts as an inhibitor of Tfh cell differentiation. IL-2 signaling induces the expression of Blimp-1 and STAT5 which represses BCL6 expression (Johnston *et al.*, 2012). Conversely, BCL6 can directly repress the gene *Prdm1*, which encodes Blimp-1, with the help of other transcription factors such as Tcf1. Thus, $BCL6^+$ $Blimp\text{-}1^-$ $CD4^+$ T cells differentiate into pre-Tfh cells whereas $Blimp\text{-}1^+$ $BCL6^-$ $CD4^+$ T cells differentiate into non-Tfh cell lineages including Th1, Th2 and Th17 cells (Johnston *et al.*,

2009; Choi *et al.*, 2015). In addition, the early expression of CXCR5 also determines T cell fate as CXCR5$^+$ pre-Tfh cells will migrate to the B cell follicle border, along the CXCL13 gradient, to become terminally differentiated GC-Tfh cells (Breitfeld *et al.*, 2000; Crotty, 2014). Other inhibitors of Tfh cell differentiation include Cytotoxic T-lymphocyte associated protein 4 (CTLA-4). The mechanism of early Tfh cell differentiation, withstanding inhibition by IL-2 and CTLA-4 produced by Tfh , Tfr and Tregs is not entirely clear (Jogdand, Mohanty and Devadas, 2016).

Many transcription factors are involved in driving Tfh cell differentiation program including BCL6, c-Maf, Tcf1 and others which play a role in inducing the expression of Tfh cell specific genes and repressing other non-Tfh cell fates. The most prominent transcription factor, known as the Tfh-lineage specific transcription factor is BCL6. BCL6 is a transcriptional repressor that regulates not only Tfh cell differentiation but also its function (Yu et al., 2009; Hatzi *et al.*, 2015). In the absence of BCL6 in CD4$^+$ T cells, there is defective Tfh cell and GC formation which can be restored upon enforcing BCL6 expression in CD4$^+$ T cells (Yu *et al.*, 2009). BCL6 induces the expression of Tfh cell-specific genes including *Cxcr5*, *Icos*, and *Pdcd1* whilst repressing the expression of non-Tfh cell genes such as *Ccr7*, *Il2r*, *Selplg* (encoding PSGL1) (Yu et al., 2009; Johnston *et al.*, 2009; Poholek *et al.*, 2010). The repression of genes encoding CCR7 and PSGL1 while upregulating CXCR5 expression supports the migration of pre-Tfh cells to the B cell follicle border (Poholek *et al.*, 2010; Yu *et al.*, 2009). In addition, BCL6 represses the expression of other T helper cell lineage-specific genes such as *Tbx21*, *Rorc*, *Stat5* and *Gata3*, in both mouse and human CD4$^+$ T cells (Kusam *et al.*,2003; Yu *et al.*, 2009). However, this remains controversial as BCL6 expression with alternate lineage-specific transcription factors has been observed in Tfh cells under pathological conditions (Choi and Crotty, 2021). It has also been shown that BCL6 can directly bind to non-Tfh cell specific cytokine receptors (e.g. IL-17) to facilitate Tfh differentiation program (Yu *et al.*, 2009).

The next stage of Tfh cell maturation and lineage commitment is crucial for developing functional and terminally differentiated Tfh cells. This requires additional TCR signaling which is then provided by B cells to drive further differentiation of pre-Tfh cells into GC-Tfh cells. This is an essential step in GC-Tfh cell development. The initial differentiation stage driven by DC priming induces a Tfh program which is reversible in the absence of further signaling by B cells at the T-B cell border (Krishnaswamy *et al.*, 2018). Pre-Tfh cells with elevated CXCR5 expression migrate to the T-B cell border, where they interact with B cells, which also function as antigen-presenting cells (Crotty, 2014). Similarly to DCs, B cells provide signals for Tfh maturation through peptide-MHC complex, and CD40L, CD80/CD86, and ICOSL ligands

(Deenick and Ma, 2011; Crotty, 2019). Apart from its costimulatory function, ICOS-ICOSL interaction also directs the positioning of pre-Tfh cells to the B cell follicles (Deenick *et al.*, 2010). In addition, activated B cells produce IL-6 required to drive Tfh cell development (Karnowski *et al.*, 2012).

The final stage of Tfh cell differentiation involves the formation of GC-Tfh cells (Zhang *et al.*, 2021). Committed pre-Tfh cells can migrate into the B cell follicle, and mature into GC Tfh cells within GCs phenotypically characterised as $CXCR5^{high}$ $PD1^{high}$ $BCL6^{high}$ MAF^{high} and exert effector functions mediated by the secretion of IL-4, CXCL13, and IL-21 (Crotty, 2014; Zhang *et al.*, 2021). GC Tfh cells also have elevated expression of SAP. SAP binds to the cytoplasmic tail of adhesion molecules, namely, the SLAM family receptors such as SLAMF6, CD84 and SLAM expressed on GC Tfh cells. This is crucial for GC Tfh cell formation and the communication between Tfh and B cells for the production of long-lived plasma cells and memory B cells as a single mutation in SAP is powerful enough to lose Tfh cells within the GCs (Deenick and Ma, 2011; Crotty, 2014). Other factors such as reduced EBI2 expression on GC Tfh cells and GC B cells promotes their localisation specifically within GCs (Gatto *et al.*, 2009; Suan *et al.*, 2015).

Tfh cell-mediated antibody responses are generated in all immune challenges in contrast to responses by other T helper cell subsets which are dependent on the type of virus or infection present (Olatunde, Hale and Lamb, 2021). Thus, apart from the 3 stages of Tfh differentiation, Tfh cells exhibit a more complex differentiation pathway based on the cytokine microenvironment which polarises pre-Tfh cells to differentiate into either Tfh1, Tfh2 or Tfh17 cells with dual expression of BCL6 with either T-bet, GATA3 or RORγt, respectively (Powell *et al.*, 2019; Glatman *et al.*, 2009; Liu *et al.*, 2017; Olatunde, Hale and Lamb, 2021). In turn, this promotes the production of pathogen-specific antibody production by B cells. For example, Tfh17 cells facilitate the generation of IgG and IgA by B cells (Zhang *et al.*, 2021).

In summary, Tfh cell biology is complex and influenced by multiple factors which skew Tfh cell differentiation to adapt to various immune challenges (Wellford and Schwartzberg, 2024).

1.8.2 Tfh cell function

Tfh cells function to induce antibody responses against variety of infections caused by bacteria, fungi, parasites and viruses (Crotty, 2019). Tfh helper cells function is well known within GCs (Crotty, 2014). Fully differentiated Tfh cells reside in the light zone of the GCs, termed GC Tfh

cells. The interactions between GC Tfh cells and GC B cells serve a dual function: (1) B cell signaling induces terminal differentiation of GC Tfh cells and, (2) Tfh cells provide help signals for B cell proliferation, selection, and the generation of memory B cells and plasma cells (Crotty, 2019). GC B cells undergo multiple rounds of proliferation and SHM and high-affinity selection driven by Tfh cells. First within the LZ of the GC Tfh cells interact with antigen-presenting GC B cells through their TCR and the GC Tfh cells transmit the help signals for B cell survival and proliferation. These activated B cells migrate to the DZ for proliferation and SHM and then the mutated B cells return to the LZ for high-affinity B cell selection by Tfh cells which then further provide signals for their survival and entry into the DZ for another round of SHM and proliferation. This process generates high affinity antibody production (Zhu, Zou and Liu, 2015).

The Tfh cell help provided within the GCs relies on competitive binding due to limited amount of GC Tfh cells. Thus, only GC B cells with higher affinity receive costimulatory and cytokine signalling through Tfh cells required for complete BCR signalling that is not achievable by antigen presentation alone (Crotty, 2014, 2019). Tfh cell help signals involve positive and negative signalling which ensures that Tfh cells exert adequate effector functions to promote the differentiation and survival of the higher affinity B cells to the pathogen encountered (Crotty, 2014; Zhu, Zou and Liu, 2015). The major positive signalling is provided by CD40L expressed on GC Tfh cells, and IL-21 and IL-4 secreted by Tfh cells that play roles in B cell survival, proliferation, differentiation and isotype switching (Zotos *et al.*, 2010; Weinstein *et al.*, 2016). The negative signals are provided by for example, PD-1-PDL1/PDL2 signalling and SLAMF6. SLAMF6 prevents excessive Tfh cell help to B cells ensuring higher antigen affinity antibodies are produced during an immune response (Crotty, 2014). In addition, PD-1-PDL1/PDL2 signalling reduces Tfh cell numbers but is required for Tfh-B cell interaction and plasma cell formation (Good-Jacobson *et al.*, 2010; Hams *et al.*, 2011). Controlling Tfh cell proliferation through high expression of PD1 on Tfh cells, serves the purpose of GF Tfh cells in regulating B cell responses without excessive Tfh cell proliferation (Cubas *et al.*, 2013). Thus, a balance between negative and positive signalling is required for effective Tfh cell functions in mediating the humoral immune response (Zhu, Zou and Liu, 2015).

Once GC Tfh cells have facilitated the responses of GC B cells, they can exit the GCs. GC Tfh cells that exit the GC can either go into a new GC or cycle back into its original GC once again. Alternatively, the GC Tfh cell can form memory Tfh cells (Crotty, 2014).

1.8.3 Memory Tfh cells

Pathogen-induced Tfh cells are essential for supporting B cell responses during chronic infection and secondary encounters with pathogens (Crotty, 2014). Tfh cells also develop into memory Tfh cells, with the ability to recall their preserved Tfh cell effector functions in response to reinfection (Hale and Ahmed, 2015). These memory Tfh cells have been found in both humans and mice (Crotty, 2014; Schmitt, Bentebibel and Ueno, 2014; Asrir *et al.*, 2017). Memory Tfh cells exhibit a less functional and polarised Tfh cell phenotype with downregulated BCL6 expression but sustaining CXCR5 expression. These cells also upregulate IL-7Rα expression required for differentiating into resting memory cells with a similar characteristic to central memory cells (Kitano *et al.*, 2011). Tfh cells can reside in secondary lymphoid organs such as the spleen and lymph node, and bone marrow and are found in the circulation. Memory Tfh cells do not need to originate from GC Tfh cells (Crotty, 2014).

There are two subsets of memory Tfh cells: 1) local memory Tfh cells and 2) circulatory memory Tfh cells. The local memory Tfh cells are sustained in the draining lymph compartment due to consistent TCR signaling whereas blocking this *in vivo* causes the local memory Tfh cells to enter the circulatory compartment. Asrir and colleagues demonstrated that the memory Tfh cells can be detected 30 days after infection both locally and within the circulation of C57BL/6 mice (Asrir *et al.*, 2017). Although Tfh cells have a characteristic set of markers, memory Tfh cells carry a distinct set of markers that have not been completely discovered (figure 1.17). Memory Tfh cells are well-defined as $CXCR5^+$ memory Tfh cells, however they lack the expression of many Tfh cell markers such as the Tfh-lineage specific marker, Bcl6 (Kitano *et al.*, 2011). In addition to CXCR5, memory Tfh cells carry high expression of folate receptor 4 (FR4), which is upregulated on effector Tfh cells and is maintained on Tfh memory cells. However, not all $CXCR5^+$ memory T cells polarise to the Tfh phenotype upon reinfection, reflecting phenotypic and functional heterogeneity within the $CXCR5^+$ memory compartment. Memory Tfh cells and central memory T cells share expression of CXCR5 and other markers such as CCR7 and ICOS. Künzli and colleagues found that the expression of FR4 distinguishes memory Tfh cells from central memory T cells. In addition, this group showed that memory Tfh cells have a unique glycolytic metabolic profile often associated with T cell activation and proliferation. However, Tfh memory cells do not require ongoing antigen stimulation and activation for their survival. In addition, these memory Tfh cells also expressed Tcf1, required for self-renewal (Künzli *et al.*, 2020).

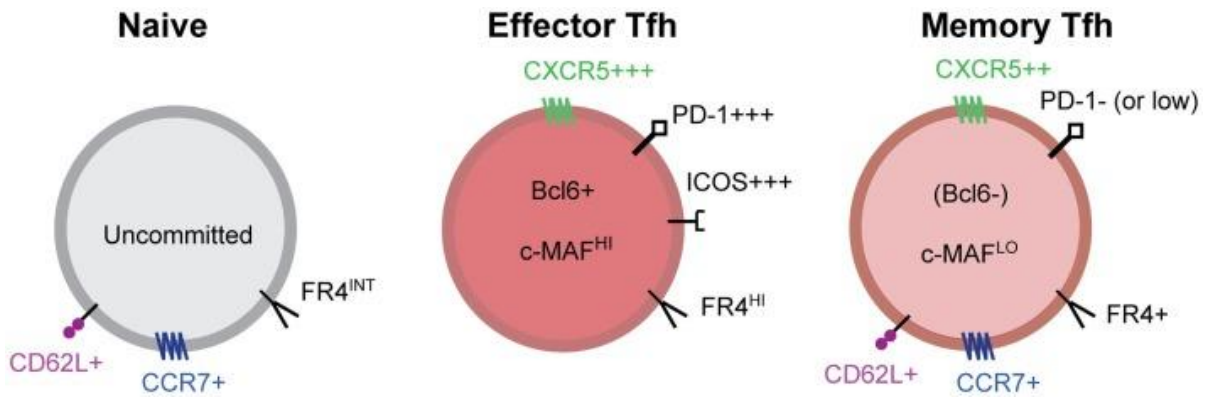


Figure 1.17. Distinct marker expression of naïve T cells, T follicular helper (Tfh) effector cells and memory Tfh cells.

In humans, memory Tfh cells make up around 20% of the $CD4^+$ memory T cell compartment and upon TCR signaling, these cells become Tfh cells and GC Tfh cells (Hale *et al.*, 2013). Upon reinfection and memory B cell activation, memory Tfh cells reinforce BCL6 expression and GC localization (Ise *et al.*, 2014). Memory Tfh cells, particularly in human blood, display heterogeneity with some subsets (e.g. PD-1^+ memory Tfh cells) displaying greater polarization, defined as stronger commitment to the Tfh cell lineage and an enhanced capacity to provide B-cell help, compared to other subsets during the secondary immune response (Figure 1.18; He *et al.*, 2013; Crotty, 2014; Schmitt, Bentebibel and Ueno, 2014).

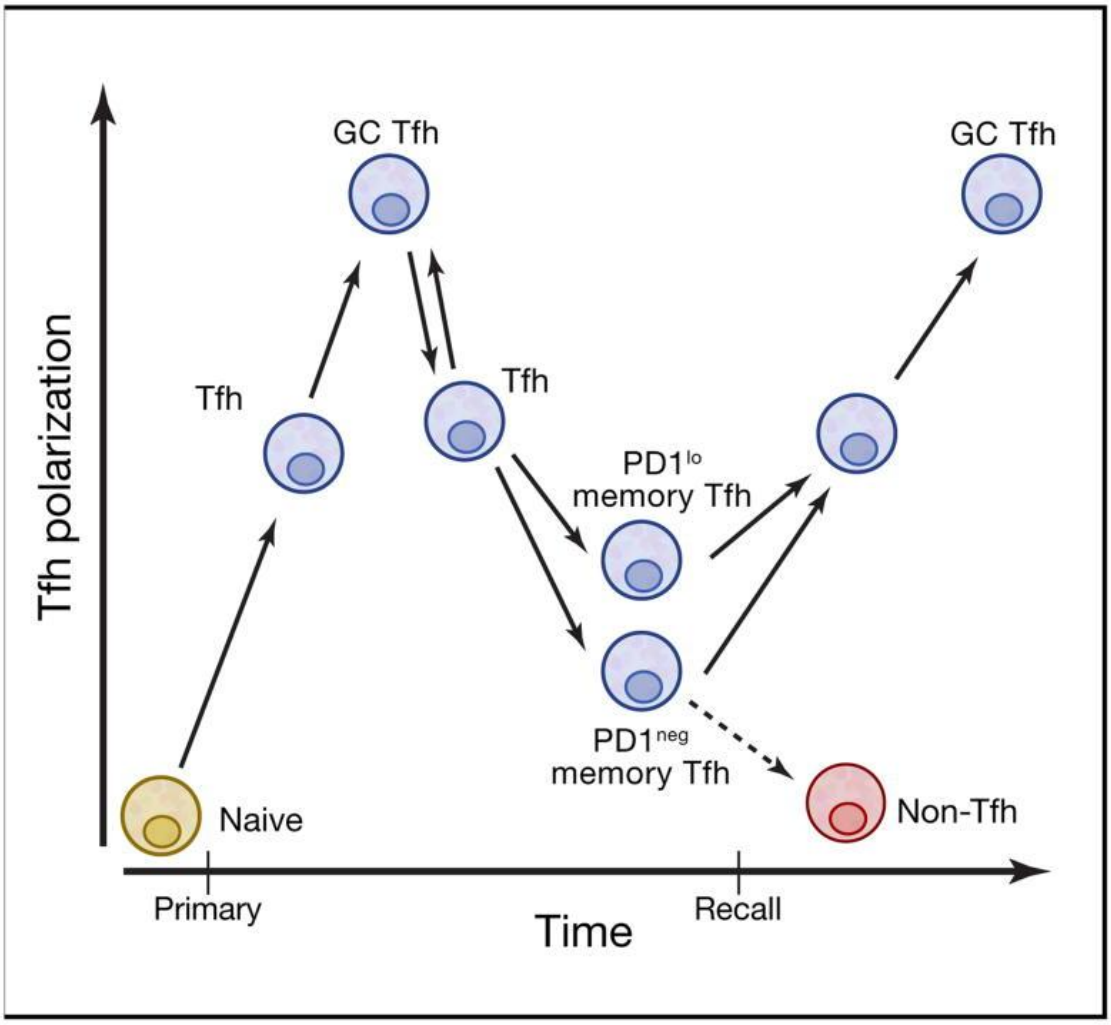


Figure 1.18 Polarisation of memory Tfh cells. Precursor Tfh cells are generated from naïve T cells and can differentiate into GC Tfh cells and then memory Tfh cells. memory Tfh cells can enter GCs and develop into GC Tfh cells. The memory Tfh cells are a heterogenous population with differential expression of PD-1. PD-1^{low} Memory Tfh cells are more functional than PD-1^{-} Memory Tfh cells (Crotty, 2014).

Human memory Tfh cells in the circulation with expression of CXCR5, induce isotype switching and antibody production (Morita *et al.*, 2011). Alike to Tfh cells, $CXCR5^{+}$ Tfh memory cells exhibit heterogeneity and can be further characterized into subsets of Th1-like, Th2-like, and Th-17-like cells with differential expression of CXCR3 and CCR6 (Morita *et al.*, 2011). The $CXCR3^{+}$ $CCR6^{-}$ Th1-like subset did not provide help to B cells, whereas $CXCR3^{-}$ $CCR6^{-}$ Th2 induced production of IgG, IgM, IgE and IgA antibodies and, whereas $CXCR3^{-}$ $CCR6^{+}$ Th17-like cells promoted IgG, IgM, and IgA antibodies. In addition, these memory Tfh cells do not express

CD69 expression which suggests these memory Tfh cells are resting memory cells in the absence of antigen stimulation (Morita *et al.*, 2011).

Another study identified $CXCR5^+$ $CXCR3^-$ resting memory T cells, divided into two subsets based on the expression of PD-1. The study demonstrated that the $CXCR5^+$ $CXCR3^-$ $PD\text{-}1^+$ resting memory T cell compartment exhibits Tfh characteristics and represents a circulatory memory Tfh cell-like population. Transcriptional profiling showed that $CXCR5^+$ $CXCR3^-$ $PD\text{-}1^+$ resting memory T cells have similar gene expression signatures with tonsillar Tfh cells (Locci *et al.*, 2013). In addition, T: B cell coculture experiments demonstrated that $CXCR5^+$ $CXCR3^-$ $PD\text{-}1^+$ cells were superior in their capacity to provide B cell help, inducing plasma cell formation and IgG secretion invitro compared to other $CXCR5^+$ T cell subsets. Overall, these findings indicate that $CXCR5^+$ $CXCR3^-$ $PD\text{-}1^+$ resting memory T cells represent a highly functional memory Tfh cell population, consistent with previous findings by Morita and colleagues that CXCR3- memory Tfh subsets, including $CXCR3^-$ $CCR6^-$ Th2-like and $CXCR3^-$ $CCR6^+$ Th17-like cells, are providers of B-cell help (Morita *et al.*, 2011; (Locci *et al.*, 2013)).

1.8.4 Regulatory Tfr cells

T follicular regulatory (Tfr) cells are a distinct population of Tregs that constitute around 10-25% of $CXCR5^+$ $CD4^+$ T cells found within the germinal center and are involved in the regulation of Tfh cell-driven germinal centre reactions. Tfr cells are a subset of Tregs, originating from natural thymic $FOXP3^+$ precursors, with an induced Tfh developmental program to enable their localisation in the GC. Similar to Tfh cells, Tfr cells express BCL6, CXCR5, ICOS, PD-1. However, Tfr cells lack expression of Tfh-specific cytokines, IL-4 and IL-21. In comparison, Tfr cells express FOXP3, CTLA-4, GITR, ICOS and IL-10 similar to effector Treg cells. This suggests that Tfr cells function as suppressors of Tfh cell-dependent GC reactions and the induced Tfh program enables them to localisation in the GCs to carry out their function during an immune response (Linterman *et al.*, 2011).

During an immune response, Tfh cells regulate the selection of high-affinity GC B cells which compete for Tfh cell interactions (Zhu, Zou and Liu, 2015). In turn, Tfr cells regulate the number of Tfh cells within GCs. In the absence of Tfr cells, the germinal centre size increases with an increased number of GC-Tfh cells. This results in a higher chance of low-affinity B cell selection and accumulation and, production of autoreactive antibodies. Thus, Tfr cells are important to

limit Tfh cell numbers and maintain self-tolerance during an immune response (Linterman *et al.*, 2011).

In summary, this section highlights that the regulation of Tfh cell-dependent responses by Tfr cells is essential to prevent GC-derived autoimmune responses.

1.9 Memory-phenotype T cells

Antigen-specific immunological memory is one of the most important functions of the immune system to provide long-lasting protection against pathogens. Protective immunity has led to the establishment of effective vaccine development. Thus, it is the most crucial mechanism of the adaptive immune system (Gray *et al.*, 2018). Conventionally, memory is said to be acquired after birth due to exposure to a diverse range of environmental pathogens. However, it is now understood that T cells with a memory phenotype exist in mice and humans without overt antigen stimulation, further expanding the complexity of the immune system. Thus we can appreciate that, under homeostatic conditions, the T-cell pool consists of naïve T cells, regulatory T cells, foreign antigen-experienced memory T cells, and foreign-antigen-inexperienced MP T cells (Seok *et al.*, 2023).

The following sections discuss the generation/maintenance and functions of $CD8^+$ and $CD4^+$ MP T cells during infections, using the current literature available.

1.9.1 Virtual memory $CD8^+$ T cells

1.9.1.1 The generation and maintenance of virtual memory $CD8^+$ T cells

Foreign-antigen inexperienced $CD8^+$ T cells with a memory-phenotype, have been extensively studied compared to their $CD4^+$ counterparts. The detection of MP T cells in the absence of external stimuli was first uncovered through T cell adoptive transfer experiments in lymphopenic hosts, inducing homeostatic proliferation (HP) (Goldrath *et al.*, 2000, 2004). T cell homeostasis is a mechanism that maintains consistent T cell numbers during an individual's lifetime, which is restored to standard levels either by apoptosis of effector T cells after an immune response or by spontaneous proliferation of naïve T cells triggered by lymphopenia (Surh and Sprent, 2000). This spontaneous HP generates T cells with a memory phenotype expressing elevated levels of CD44, CD62L, Ly6C, CD122, and LFA1 similar to pathogen-induced central memory $CD8^+$ T cells. Although it was proposed that these memory $CD8^+$ T cells are driven by foreign antigens within immunodeficient hosts, Haluszczak and colleagues proved that antigen-inexperienced MP T cells are generated free of immunisation, environmental antigens and without specific pathogens, in unimmunised wildtype (WT), germ-free (GF) and specific pathogen-free (SPF) mice, respectively (Haluszczak *et al.*, 2009). Thus, foreign-antigen inexperienced MP $CD8^+$ T

cells are induced in endogenous lymphoreplete conditions with limited antigenic stimulation (Haluszczak *et al.*, 2009). The authors further deduced that the adhesion molecule, CD49d, which is upregulated on memory $CD8^+$ T cells, is lowly expressed on MP T cells (Haluszczak *et al.*, 2009). This phenotypic difference between foreign-antigen inexperienced MP $CD8^+$ T cells and memory $CD8^+$ T cells further supports the idea of the lack of foreign-antigen stimulation required for the development of MP $CD8^+$ T cells.

Studies of MP $CD8^+$ T cells in different mouse strains have found two major types of memory-phenotype $CD8^+$ T cells, that is virtual memory (VM) and innate memory $CD8^+$ T cells which have overlapping phenotypes and functions within the periphery but are distinct by their origin, as illustrated by figure 1.19 (White *et al.*, 2017; Thiele *et al.*, 2020; Seok *et al.*, 2023). Due to their phenotypic and functional similarities, these cells are often characterised as a unified population by most groups (Thiele *et al.*, 2020; Seok *et al.*, 2023) whereas some researchers underscore their distinct developmental features (White *et al.*, 2017). Innate memory $CD8^+$ T cells are characterised by the high expression levels of IL4R, CD5 and, CD49d. However, once in the periphery, innate memory $CD8^+$ T cells downregulate CD49d and become indistinguishable from virtual memory $CD8^+$ T cells (Savid-Frontera *et al.*, 2022). A significant number of $CD122^{high}$ $CD44^{high}$ innate memory $CD8^+$ T cells were found within the thymus of IL-4-inducing mouse knockout strains (e.g mice deficient in Krueppel-like factor 2 (KLF2), CREB-binding protein (CBP) and, ID3). Whereas, in the absence of IL-4 or IL-4-producing-thymic NKT cells, there is a reduction in the innate memory $CD8^+$ T cell numbers. These cells are also observed in wildtype mouse strains (Weinreich *et el.*, 2010; Verykokakis *et al.*, 2010; White *et al.*, 2017).

In comparison to innate memory T cells, VM $CD8^+$ T cells develop in the periphery from naïve T cell precursors through spontaneous proliferation (figure 1.19) (White, Cross and Kedl, 2017). At the ‘resting’ state, naïve T cells are maintained in the periphery from ongoing weak TCR signaling by self-antigens and this interaction induces HP of naïve T cells into foreign-antigen inexperienced MP T cells under cytokine signaling (Surh and Sprent, 2000). The reliance on self-antigens for TCR signaling of naïve T cells is evident as naïve T cells expressing high levels of CD5, a marker of the strength of TCR self-reactivity, are found to generate VM $CD8^+$ T cells in the periphery (White *et al.*, 2017). During maturation in the thymus, the $CD5^{high}$ T cell precursors upregulate Eomes, which then induce the expression of CD122 on virtual memory $CD8^+$ T cells (Miller *et al.*, 2020). As CD122 has a high binding affinity for IL-15, VM $CD8^+$ T cells exhibit cytokine sensitivity to IL-15, produced by $CD8\alpha^+$ DCs, for their development (Sosinowski *et al.*,

2013; Miller *et al.*, 2020). Even though virtual memory CD8⁺ T cells do not develop in the thymus, it is evident that the VM CD8⁺ T cell fate is decided during maturation in the thymus by the increased CD5 levels and the upregulation of Eomes on their T cell precursors (Miller *et al.*, 2020). The phenotype of VM T cells is illustrated in figure 1.20.

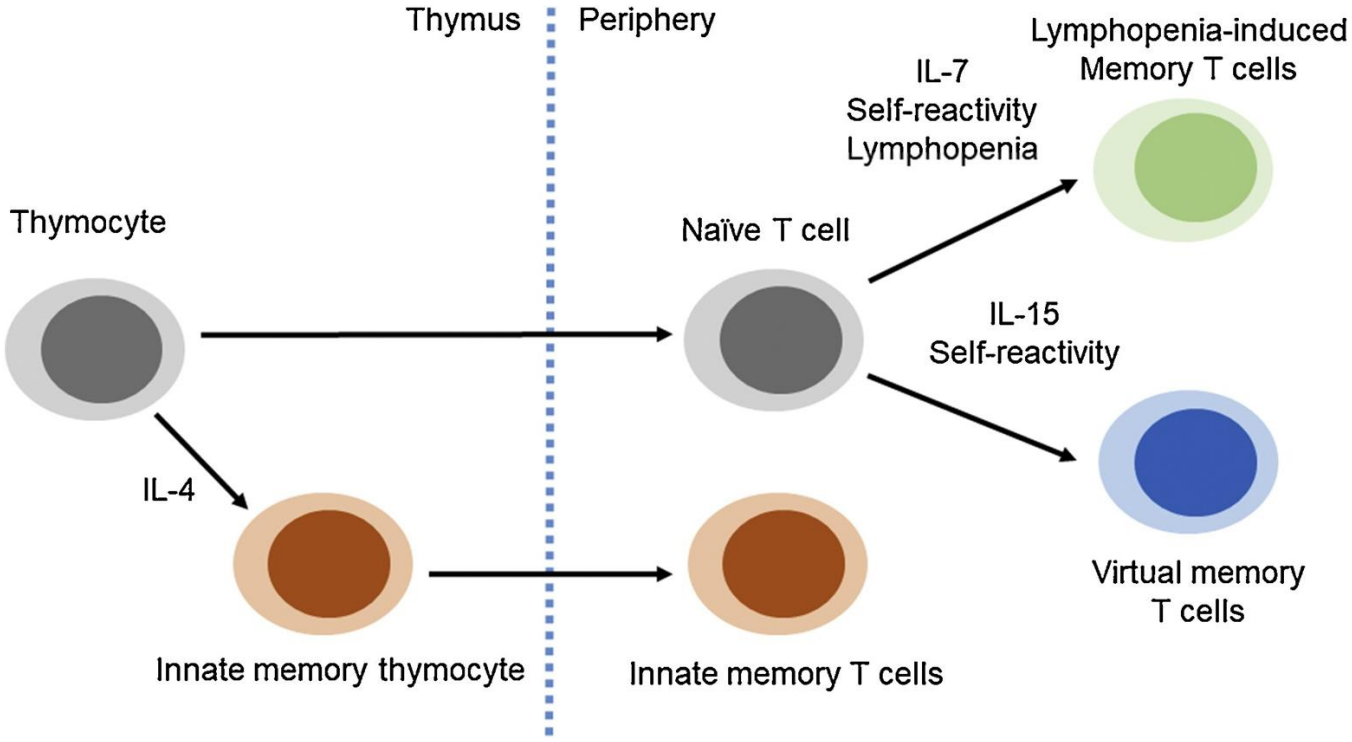


Figure 1.19. Generation of innate memory and virtual memory (VM) CD8⁺ T cells. In the thymus (on the left), thymocytes differentiate into innate memory CD8⁺ T cells in the presence of IL-4. In the periphery (on the right), naïve T cells undergo homeostatic proliferation and either acquire a virtual memory phenotype, in the presence of IL-15 and self antigen stimulation, or become lymphoenia-induced memory T cells through IL-7 and self-reactivity in lymphopenic conditions (Pribikova, Moudra and Stepanek, 2018).

1.9.1.2 VM CD8⁺ T cell functions in young and aged environment

As mentioned previously, apart from the developmental differences between innate memory and VM CD8⁺ T cells, distinguishing the heterogeneous population of MP CD8⁺ T cells phenotypically is challenging. Therefore, the following section will discuss the functions of these cell types, which are broadly referred to as VM CD8⁺ T cells by several researchers (Van Kaer, 2015; Thiele *et al.*, 2020; Savid-Frontera *et al.*, 2022; Seok *et al.*, 2023).

The innate immune system provides a non-specific response against infection (Chaplin, 2010). It has been reported that, much like innate lymphoid cells (e.g. NKT and ILCs) that bridge the innate and adaptive immune systems together, VM CD8⁺ T cells exhibit characteristics of the adaptive T cells but exert an early innate-like response against pathogens (Joyce, 2022; Seok *et al.*, 2023). VM T cells rapidly produce IFN-γ in an antigen-independent manner, during the early primary immune response. During an infection, PAMPs stimulate IL-18, IL-12 and, IFN-α/β cytokine production by macrophages and dendritic cells which then indirectly triggers IFN-γ production by VM T cells (Figure 1.20; White, Cross and Kedl, 2017; Seok *et al.*, 2023). These cells have been reported to express NKG2D and granzyme B which mediate the bystander cytotoxic killing by VM T cells (Figure 1.20; Chu *et al.*, 2013).

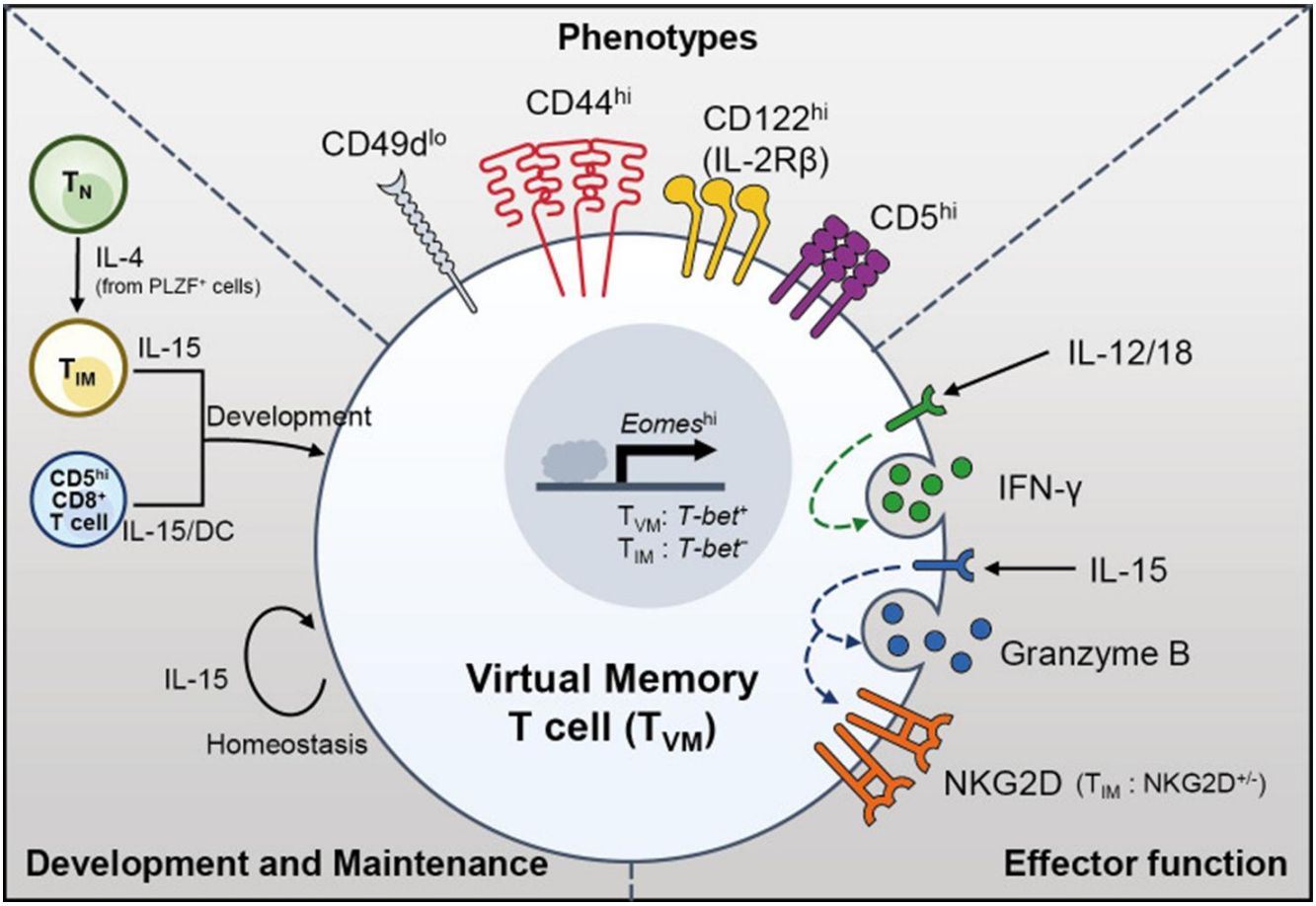


Figure 1.20. An overview of Virtual memory (VM) CD8⁺ T cell development and maintenance, phenotype and effector functions. VM T cells are indistinguishable from IL-4 driven innate memory CD8⁺ T cells thus innate memory CD8⁺ T cells are mainly referred to as VM T cells in the periphery. VM T cells are also generated in the periphery from $CD5^{high}$ CD8⁺ T cell precursors driven by IL-15, produced by dendritic cells (DCs), which is also required for their maintenance. Phenotypically, VM T cells display high expression of CD44, CD122 (IL-2Rβ), and CD5, but low expression of CD49d. In addition, VM T cells highly express Eomes and T-bet whereas T-bet expression is reduced in innate memory CD8⁺ T cells in the thymus. VM T cells exert bystander effector

functions mediated by cytokines such as IL-12/IL-18 or IL-15, resulting in innate-like IFN-γ production and cytotoxicity, respectively (Seok *et al.*, 2023).

In addition to their effector function mediated in a cytokine antigen-independent manner, VM $CD8^+$ T cells also exert pathogen-specific responses similar to conventional memory $CD8^+$ T cells (Su *et al.*, 2005; Lee *et al.*, 2013). Lee and colleagues showed that VM T cells possessed elevated expression of T-bet and Eomes, required for effector $CD8^+$ T cell responses, and provided protective immunity against *Listeria monocytogenes* infection. To confirm responses of VM $CD8^+$ T cells against *Listeria monocytogenes* are antigen-specific, cytokine-dependent stimulation was inhibited with blocking antibodies against IL-12 and IL-18 *in vitro*, resulting in an IFN-γ response (Su *et al.*, 2005). In addition, helminth infection and IL-15 induce an increase of VM $CD8^+$ T cell proliferation either from proliferation of VM T cells (seen in C57BL/6 mice) or by the conversion of naïve T cells into VM T cells (in BALB/c mice), dependent on species-specific response (Rolot *et al.*, 2018; Hussain *et al.*, 2023). Overall, VM $CD8^+$ T cells can exert an innate-like response by bystander activation and provide conventional memory T cell-like antigen-specific protection during the primary response. Thus, VM $CD8^+$ T cells are vital for both innate and adaptive immunity.

VM T cell population constitutes around 10-20% of the memory population in unprimed young mice. However, their frequency increases with age (Sprent and Surh, 2011). Thus, their function in the aged immune system is worth noting. The decline of naïve T cell emigrants from the thymus due to age-associated thymic involution, can be a contributing factor to the accumulation of VM T cells in an aged immune system. This is because, the remaining naïve T cells trigger increased HP to maintain the T cell numbers. As mentioned in the previous section, this process, driven by cytokines such as IL-15, leads to the conversion of naïve T cells to VM T cells in the periphery. Although there is no direct evidence within the literature highlighting thymic involution as a causative factor of VM T cell increase in aged mice, it is indeed a contributing factor (Nikolich-ˇ Zugich, 2014; Seok *et al.*, 2023). In young mice, VM T cells rapidly proliferate in response to homeostatic cytokines, increasing VM T cell numbers. Although the number of VM T cells increases, the effector functions of VM $CD8^+$ T cells (e.g. proliferative capacity) in response to TCR stimulation declines with age (Quinn *et al.*, 2018; Seok *et al.*, 2023).

1.9.1.3 VM CD8$^+$ T cells in humans

In mice, VM CD8$^+$ T cells have been well investigated compared to humans. Nevertheless, a phenotypically and functionally similar VM CD8$^+$ T cell population has been identified in the human peripheral and cord blood (Sosinowski *et al.*, 2013; Jacomet *et al.*, 2015). Human VM CD8$^+$ T cells are marked with the expression of CD45RA, NK receptors (e.g. NKG2A and KIRs), and Eomes (White *et al.*, 2016; Jacomet *et al.*, 2015). The phenotype of VM CD8$^+$ T cells is similar to that of terminally differentiated CD8$^+$ memory T cells which re-express the naïve T cell marker CD45RA called TEMRA (Van Kaer, 2015). Thus, isolation of TEMRA would typically include VM CD8$^+$ T cells. However, VM CD8$^+$ T cells can be distinguished from TEMRA with regards to their higher proliferative capacity and antigen-independent development (Quinn *et al.*, 2018; Guo *et al.*, 2023; Sosinowski *et al.*, 2013). Furthermore, human VM CD8$^+$ T cells possess a similar function to that of mice VM CD8$^+$ T cells. Human VM CD8$^+$ T cells exert an innate-like IFN-γ response to IL-18 and IL-12 cytokines and display cytotoxicity and rapid proliferation (Jacomet *et al.*, 2015; Drobek *et al.*, 2018; Quinn *et al.*, 2018). However, with age, human VM T cells become dysfunctional in response to TCR stimulation (Thiele *et al.*, 2020).

1.9.2 Memory-phenotype CD4$^+$ T cells

1.9.2.1 MP CD4$^+$ T cell development/ maintenance

MP T cells, including both CD62L^{low} CD44^{high} CD8$^+$ and CD4$^+$ MP T cells, are found in GF, Antigen-free (AF) and SPF mice (Haluszczak *et al.*, 2009; Kawabe and Sher, 2021). CD8$^+$ MP T cells are described as a heterogeneous population based on their developmental origin. Innate memory CD8$^+$ T cells are generated in the thymus whereas VM CD8$^+$ T cells are generated in the periphery through naïve T cell precursors (White, Cross and Kedl, 2017). In contrast, CD4$^+$ MP T cells originate in a similar manner to VM CD8$^+$ T cells. Within the periphery, CD4$^+$ MP T cells develop from CD5^{high} naïve T cell precursors due to rapid HP through self-antigen driven TCR signalling within physiological environments of both lymphopenic neonatal and lymphosufficient adult mice (figure 21) (Min *et al.*, 2005; Kawabe, 2023). As these MP T cells do not express CD25 and CD69, they indeed possess a memory-phenotype rather than effector T cell phenotype (Kawabe, 2023). In addition to TCR stimulation, costimulatory signalling through CD28 and OX40 is also required for fast HP (Kawabe and Sher, 2021). These self-reactive clones generated through naïve T cell precursors, if not regulated by Tregs and mechanisms

which fine-tune the TCR activation threshold, then could potentially lead to the development of autoimmunity due to the inflammatory nature of $CD4^+$ MP T cells (Kawabe, 2022).

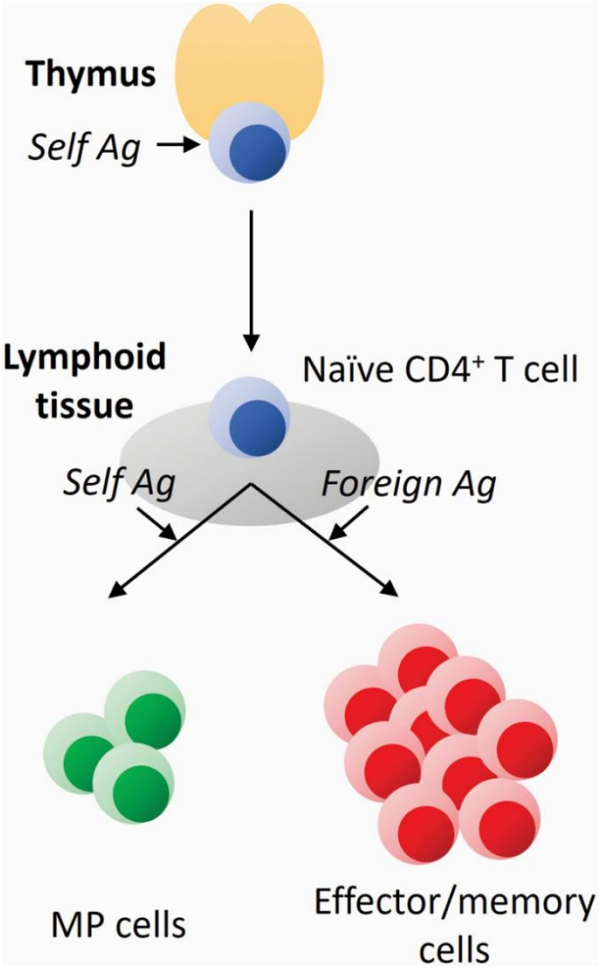


Figure 1.21. Comparison of memory-phenotype (MP) and pathogen-induced memory $CD4^+$ T cells. The diagram shows that naïve T cells are generated in the thymus through self-antigen presentation and then within lymphoid tissues MP T cells are generated in response to self-antigen stimulation whereas pathogen-induced effector/memory cell are generated through foreign antigen stimulation (Kawabe and Sher, 2021).

1.9.2.2 $CD4^+$ MP T cell maintenance, differentiation and function in host protection and autoimmunity

$CD4^+$ MP T cells are a heterogeneous population in terms of their maintenance, differentiation and function. MP T cell maintenance, alike to their generation, is mainly studied by $CD4^+$ MP T cell transfer into lymphopenic recipients but recent studies have explored the maintenance of $CD4^+$ MP T cells in normal unimmunised mice as well as their functions during an immune response (Kawabe *et al.,* 2021).

Long-lived pathogen-induced memory $CD4^+$ T cells are found in a quiescent state under homeostatic conditions and are maintained through IL-7/IL-15 mediated TCR signalling in a non-specific manner, once the infection is cleared (Gasper, Tejera and Suresh, 2014). Pathogen-induced memory $CD4^+$ T cells proliferate at a slower rate than MP T cells under homeostatic conditions (Younes et al, 2011). $CD4^+$ MP T cells exhibit two modes of HP for their maintenance upon transfer to lymphopenic hosts, including slow and fast HP (Kawabe and Sher, 2021). Slow proliferation is mediated by cytokine signalling through IL-7, whereas fast proliferation is MHC II-dependent and requires TCR engagement with peptide–MHC II complexes and co-stimulatory signalling provided by dendritic cells (Kawabe and Sher, 2021).

Within normal unimmunised mice i.e. non-lymphopenic mice, $CD4^+$ MP T cell proliferation is not well studied. Two studies by similar groups suggest that MP T cell pool consists of Ki67+ MP T cells and $Ki67^-$ MP T cells which gradually lose their Ki67 expression over time (Younes *et al.*, 2011; Kawabe *et al.*, 2022). Ki67 is a marker of cellular proliferation and its expression is reduced in quiescent cells (Sobecki *et al.*, 2017). The proliferation of MP T cells in normal unimmunised mice and GF mice is not similar to the burst-like proliferation (i.e. generating many daughter cells from a single T cell precursor) observed in lymphopenic hosts. Thus, MP T cells in steady state do not proliferate as rapidly as lymphopenia-induced MP T cells but at much faster rate than foreign-antigen specific memory T cells (Younes *et al.*, 2011). In addition, single cell RNA sequencing of $CD44^{high}$ $CD62L^{low}$ $CD4^+$ MP T cells, support slow and fast proliferation of $CD4^+$ MP T cells in normal unimmunized SPF mice without MP T cell transfer experiments. This study showed that $CD4^+$ MP T cells are divided into two populations, $BCL2^{low}$ $CD127^{high}$ $Sca1^{high}$ and $BCL2^{low}$ $CD127^{low}$ $Sca1^{low\sim high}$. The former population is $Ki67^-$ suggesting a more matured quiescent slow proliferative state whereas the latter are $Ki67^+$ and undergo MHCII and CD28-dependent fast HP, as shown in figure 1.22 (Kawabe *et al.*, 2022). Thus, this demonstrates that the mechanism of MP T cell maintenance in normal and lymphopenic

conditions is indeed similar but lymphopenia-induced MP T cells undergo more rapid proliferation, due to the extreme environment, to restore the T cell pool (Kawabe, 2023).

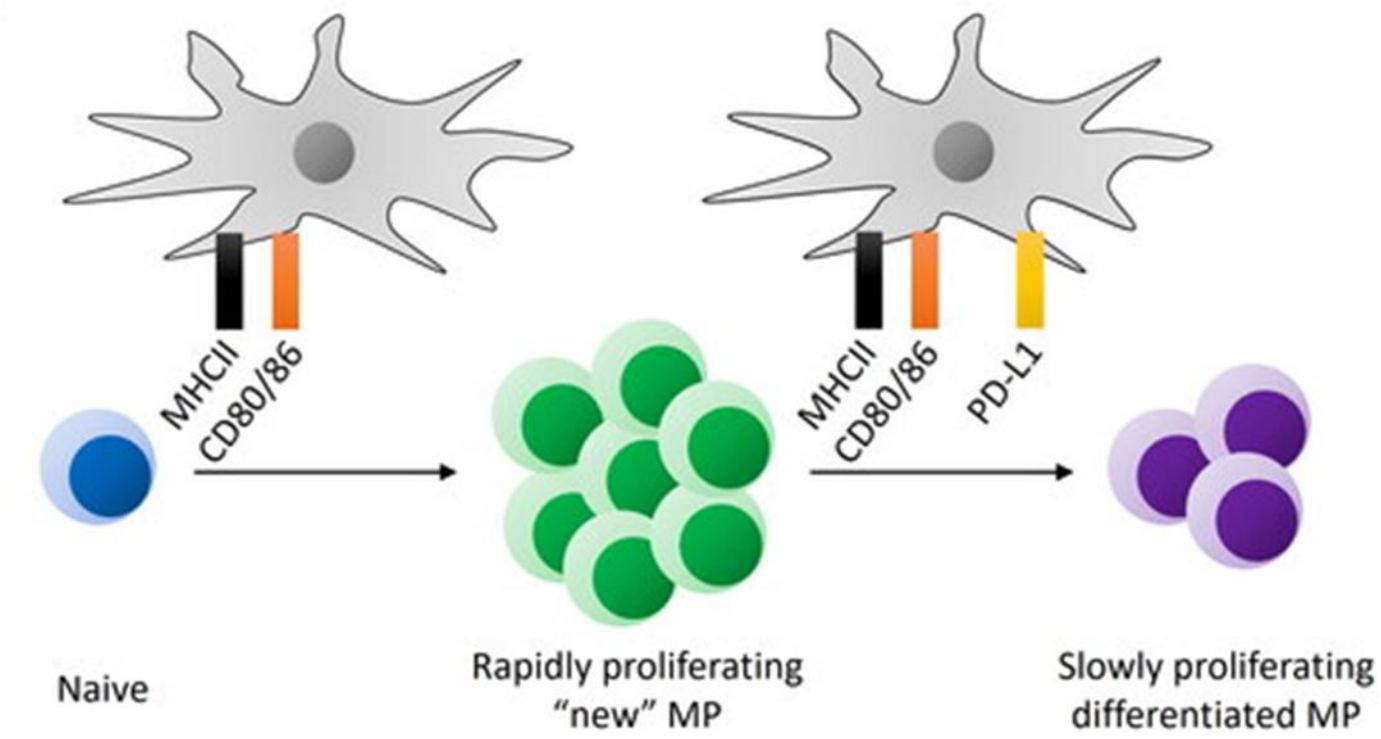


Figure 1.22. MP T cell generation and proliferation in the steady state. Newly generated MP T cells, from naïve T cell precursors induced TCR signalling, are highly proliferated but then differentiate and mature into slow proliferating MP T cells which requires TCR signalling, costimulatory signalling and PDL1/PD-1 signalling (Kawabe, 2023).

Once fast HP is depleted, the $CD4^+$ MP T cells enter a more mature quiescent state which acquires further heterogeneity in terms of differentiation and function. At least two subpopulations of MP T cells have been described similar to the conventional pathogen-induced Th1 and Th17 (Kawabe *et al.*, 2017b; Cho *et al.*, 2023). A recent study found that much like pathogen-induced Th1 cells defined by the expression of T-bet, there is a T-bet^{high} MP T cell population at steady conditions which generates a bystander Th1-like innate immune response against different infectious settings including Toxoplasma infection and Mycobacterium Tuberculosis (Kawabe *et al.*, 2017). The generation and maintenance of this subset is illustrated in figure 1.23. In steady state, TLR-MyD88 signalling activates DC1 cells to produce tonic IL-12

resulting in the differentiation of naïve T cell precursors to T-bet^{high} MP T cells. This MP T cell subset is maintained by IL-12 produced through CD40-CD40L signalling (DC-T cell interactions) (Kawabe *et al.*, 2020). In addition, Lo and colleagues present findings of a naïve-like T-bet^{+} CD4^{+} T cell population which may be the naïve T cell precursors for the generation of T-bet^{+} MP T cells (Lo *et al.*, 2022). However, whether these naïve-like T-bet^{+} CD4^{+} T cells directly give rise to T-bet^{+} MP T cells has not been studied yet.

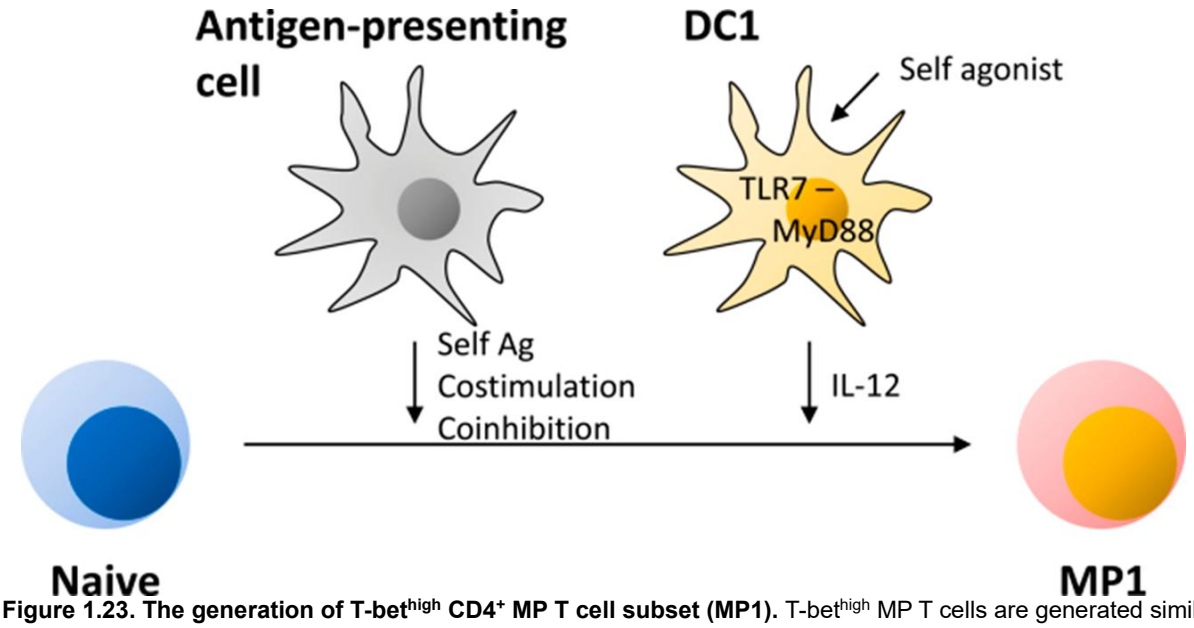


Figure 1.23. The generation of T-bet^{high} CD4^{+} MP T cell subset (MP1). T-bet^{high} MP T cells are generated similar to CD4^{+} MP T cells, through naïve CD4^{+} T cells, however their differentiation required IL-12 signalling by DC1 subset (Kawabe, 2022).

Apart from the Th1-like CD4^{+} MP T cell subset, Cho and colleagues described a subset of Th17-like CD4^{+} MP T cells which contributed to the development of autoimmune neuroinflammation through bystander activation. Through single cell RNA sequencing they observed CCR6^{high} CD4^{+} MP T cells that, when stimulated with IL-23 and IL-1β, exert innate-like effector functions in the Experimental Autoimmune Encephalomyelitis (EAE) mouse model (Cho *et al.*, 2023). The effector function is exerted through the production of the pathogenic Th17 proinflammatory cytokines (IL-17A and GM-CSF) mediated by the transcription factor, Bhlhe40, which amplifies the inflammatory response, similar to autoantigen-specific Th17 cells but in absence of TCR stimulation (Lin *et al.*, 2014; Cho *et al.*, 2023). Kawabe and colleagues illustrated that a minor population of T-bet^{-} RORγt^{+} CD4^{+} MP T cells are found in the T-bet-AmCyan RORγt-E2Crimson double reporter mice, indicating a Th17-like phenotype at homeostasis (Kawabe *et al.*, 2020). However, their differentiation and maintenance in steady state as well as a potential role in host protection against infection is not known.

Recently, our group found that the transcription factor, EGR2, is highly expressed in PD-1^{high} memory phenotype T cells and controls their homeostasis and inflammatory responses (Symonds *et al.*, 2020). EGR2 and EGR3 are key transcriptional regulators of T cell-mediated immune responses, acting by inducing SOCS1 and SOCS3 to limit STAT1/3-mediated IFN-γ and IL-17A, and by controlling AP-1 activation (a key transcription factor regulated in T cell tolerance) through inhibition of its suppressor, BATF, thereby preventing excessive T cell activation, uncontrolled cytokine production, and autoimmunity (Li et al., 2012). We showed that the expression of T-bet was upregulated in EGR2/3$^{-/-}$ PD1^{high} CD4^{+} MP T cells which are associated with autoimmunity (Symonds *et al.*, 2020).

Overall, the existence of inexperienced, memory-like CD4^{+} T cells have been widely recognized. Rather than requiring strong antigenic stimulation, these cells are thought to develop in response to weak self-antigen interactions and homeostatic proliferation. This suggests that CD4^{+} MP T cells can serve as therapeutic targets for autoimmune diseases (Marusina *et al.*, 2016; Kawabe, 2022). Thus, extensive research is required to elucidate the mechanisms involved in differentiation and function of unknown CD4^{+} MP T cell subsets.

1.9.3 MP T cell section conclusion

MP T cells are considered similar to NK cells and ILCs that bridge the innate and adaptive immune systems. MP T cells can exert a pathogen-specific response, and an innate bystander activated response due to cytokine sensitivities mainly IL-15, IL-12 and IL-18 (White, Cross and Kedl, 2017; Seok *et al.*, 2023). Compared to VM and innate memory CD8^{+} T cells, CD4^{+} MP T cells are less well studied and require further functional insights (Kawabe et al., 2021). Understanding the functions and regulation of MP T cells is of importance due to their responsiveness to endogenous signals which suggests a pathogenic role in chronic inflammation and autoimmune diseases.

1.10 Conclusion

In conclusion, previous studies have characterised $CD4^+$ MP T cells, but the mechanism of development and function in different disease contexts and autoimmunity is still unclear. This thesis aims to address some of these limitations by investigating the function of the newly discovered MP Tfh cells under homeostatic conditions, providing novel insights into the other T helper-like MP $CD4^+$ T cell subsets developed in pathogen-free conditions. In addition, we show that the $CD4^+$ MP T cell pool consists of a regulatory compartment of MP Tfr cells. The works of this thesis show that the MP $CD4^+$ T cell pool is indeed quite similar to the conventional T cell-mediated immune responses against infections. This thesis provides the avenue to further explore potential implications of MP Tfh cells in autoimmune diseases and therapeutics.

1.11 Aims of this study

The aim of this thesis is to investigate the function of the newly found MP Tfh cell during an immune response against vaccina virus infection *in vivo* and to reveal the transcriptional profile of MP Tfh cells under homeostatic conditions, using our recently established experimental models, GFP-Egr2 AmCyan-T-bet knockin mice and CD2-Egr2/3 AmCyan-T-bet conditional knockout mice.

Specific objectives:

- Objective 1: To validate the GFP-EGR2 AmCyan-T-bet knockin mice and CD2-Egr2/3 AmCyan-T-bet mice and characterise the phenotype of $CD4^+$ MP T cells including their differential expression of EGR2 and T-bet.
- Objective 2: To determine the phenotype of MP Tfh cells which reside in the $CD4^+$ MP T cell population based on their expression of Tfh-specific markers of conventional T cells.
- Objective 3: To analyse the function of MP Tfh cells in providing help to B cells *in vitro* by measuring the B cell mediated-IgG production after stimulation with anti-CD3 and anti-IgM in culture with and without MP Tfr cells.
- Objective 4: To study the function of MP Tfh cells in supporting germinal centre formation *in vivo* and production of anti-viral antibodies in response to viral infection.
- Objective 5: To reveal the transcriptional programming of MP Tfh cells further exploring their biology, homeostasis and functional characteristics.

Chapter 2

Materials and Methods

2.1 Mice

The GFP-Egr2 /AmCyan-T-bet mice were generated by crossing the AmCyan-T-bet reporter mice (Yu *et al.*, 2015) with the GFP-Egr2 homozygous mice, as reported in our previous publication (Miao *et al.*, 2017) . To summarise, the GFP sequence was inserted at the start site of the *Egr2* gene to monitor and visualise the expression of EGR2 by flow cytometry. The second mouse line, CD2-Egr2/3$^{-/-}$ /AmCyan-T-bet mice, was established by crossing the AmCyan-T-bet mice (Yu *et al.*, 2015) with the CD2-Egr2-/-Egr3-/- mice, a line with the deletion of EGR2 and EGR3 in CD2^{+} lymphocytes as described in our earlier studies (Li *et al.*, 2012) . The representative genotyping results are illustrated in Figure 3.1 and Figure 3.2 (See Chapter 3, Section 3.1) and the primer sequences for PCR are shown below (Table 2.1). The AmCyan-T-bet reporter mice were obtained from J.Zhu, NIAID, NIH USA (Yu *et al.*, 2015) and were constructed as reported in their previous publication (Zhu *et al.*, 2012).

All the mice used for this study were housed at the Biological Services Unit, Brunel University, and studied according to institutional guidelines permitted under the UK Home Office Project License obtained by my supervisor, Dr Su-ling Li. All mouse lines are on the C57BL/6 background and, were bred and maintained under Specific Pathogen Free (SPF) conditions at room temperature. Wildtype LY5 mice were used for the colour compensation controls. Both male and female mice, aged around 10-12 weeks or older based on experiment required, were randomly used for this study.

2.2 Genotyping

Genotyping was used to determine the excision of EGR2/3 and the incorporation of GFP-EGR2 in the CD2-Egr2/3$^{-/-}$/AmCyan-T-bet mice and GFP-Egr2/AmCyan-T-bet mice, respectively, as well as the addition of AmCyan-T-bet in both of the experimental mouse models.

2.2.1 DNA Extraction

The mice ear tissues were punched, and the ear clippings were collected in an eppendorf tube by a member of the Biological Services Unit, at Brunel University. The DNA from the ear clipping tissues was extracted using the REDExtract-N-Amp™ Tissue PCR Kit (Sigma-Aldrich), according to the manufacturer's guidelines. First, 20 µL of the extraction buffer was added to the eppendorf tubes and vortexed to disrupt the cell membrane. Then 5 µL of the tissue preparation solution was added and, briefly vortexed and centrifuged for 1 minute at 13,000 rpm. After centrifuging, the eppendorf tubes were incubated on a heating block for 30 minutes at 55°C to catalyse the breakdown of the cellular proteins by the protease enzyme in the buffers. The incubation temperature was then increased to 95°C for 3 minutes to aid the denaturation of the protease enzyme. To stop the reaction, 20 µL of neutralisation buffer was added. The samples were then vortexed and centrifuged for 1 minute, prior to dilution of both GFP-Egr2/AmCyan-T-bet and CD2-Egr2/3$^{-/-}$/AmCyan-T-bet ear punch samples with 30 µL of distilled water. The samples were then stored at 4°C and transferred for further analysis.

2.2.2 Polymerase Chain Reaction (PCR)

PCR was performed for the amplification of DNA from the mice ear samples within a cyclic thermal enzymatic reaction which requires gene-specific (sense and anti-sense) PCR primers and, the Thermus aquaticus (Taq) polymerase. The samples were further diluted with 800- 1000 µL of distilled water. Then the PCR master mixtures were prepared as follows: 5 µL of 2X MyTaq HS Red master mix (Bioline), 2.5 µL of PCR-grade water and, 0.5 µL of sense and antisense primer**.** Then, 2 µL of the DNA ear sample was added into the PCR tubes. The commercial 2X MyTaq HS Red master mix consists of the Taq polymerase, dNTPs, MgCl2, and buffer with pre-optimized concentration for PCR. Due to the sensitive nature of this procedure, the PCR products were validated for false positives via contamination (negative controls), and to ensure the correct DNA sequence has been amplified (positive controls). For the GFP-Egr2/AmCyan-T-bet knockin mice, gene-specific primer sequences were used to determine the

incorporation of AmCyan-T-bet and GFP-EGR2. Whereas the primers T-bet-AmCyan, Egr3-WT allele, Egr3-Neo allele, Cre and Egr2-LoxP alleles were used to detect for true CD2-Egr2/3$^{-/-}$ /AmCyan-T-bet mice. The details of the sense and anti-sense primer sequences are recorded in Table 2.1.

Locus	Primer	Band size
Egr2 LoxP	Sense- TCA GCA TGC GTG TAT GTG Antisense- GAA GCT ACT CGG ATA CGG	Wildtype = 145bp LoxP = 179bp
CD2-Cre	Sense- CCA ACA ACT ACC TGT TCT GCC G Antisense- TCA TCC TTG GCA CCA TAG ATC AGG	133bp
Egr3	Sense- CTA TTC CCC CCA GGA TTA CC WT Antisense- TCT GAG CGG GCT GAA ACG KO Antisense- GAT TGT CTG TTG TGC CCA GTC	Wildtype = 360bp KO = ~700bp
GFP-Egr2	Sense- GCT CAG TTC AAC CCC TCT CC Antisense- GGA TTT TGT CTA CGG CCT TG	Wildtype = 119bp GFP-Egr2 = 854bp
T-bet-AmCyan	Sense- GAC AAG AGA CTT ACA CTT AGG AGT G Antisense- GTA GGT GAA GGT TCT CTC GTA G	~600bp

Table 2.1. List of primer sequences and their PCR product size.

The samples were then subjected to amplification for 1 hour using the Rotor Gene 3000 real-time PCR system. The temperature settings used to run PCR on this system are illustrated in the diagram below (Figure 2.1). The temperature variations facilitate the 3-step amplification process, consisting of 35 repeat cycles, to yield enough PCR amplicons for subsequent analysis on an agarose gel. The initial step involved subjecting the DNA double helix to the highest temperature of 95°C, causing denaturation and revealing single-stranded DNA. At the temperature of 60°C (or 55°C for AmCyan-T-bet PCR), gene-specific sense and antisense primers bind to complementary single DNA strands. In the final step, at the optimal temperature of 72°C, Taq polymerase catalysed the addition of nucleotides using the dNTPs, resulting in the formation of new DNA strands. The initial denaturation and final extension steps are held for 5 minutes to compensate for incomplete DNA amplification. Once the PCR is completed, the PCR products are examined on the agarose gel by using electrophoresis.

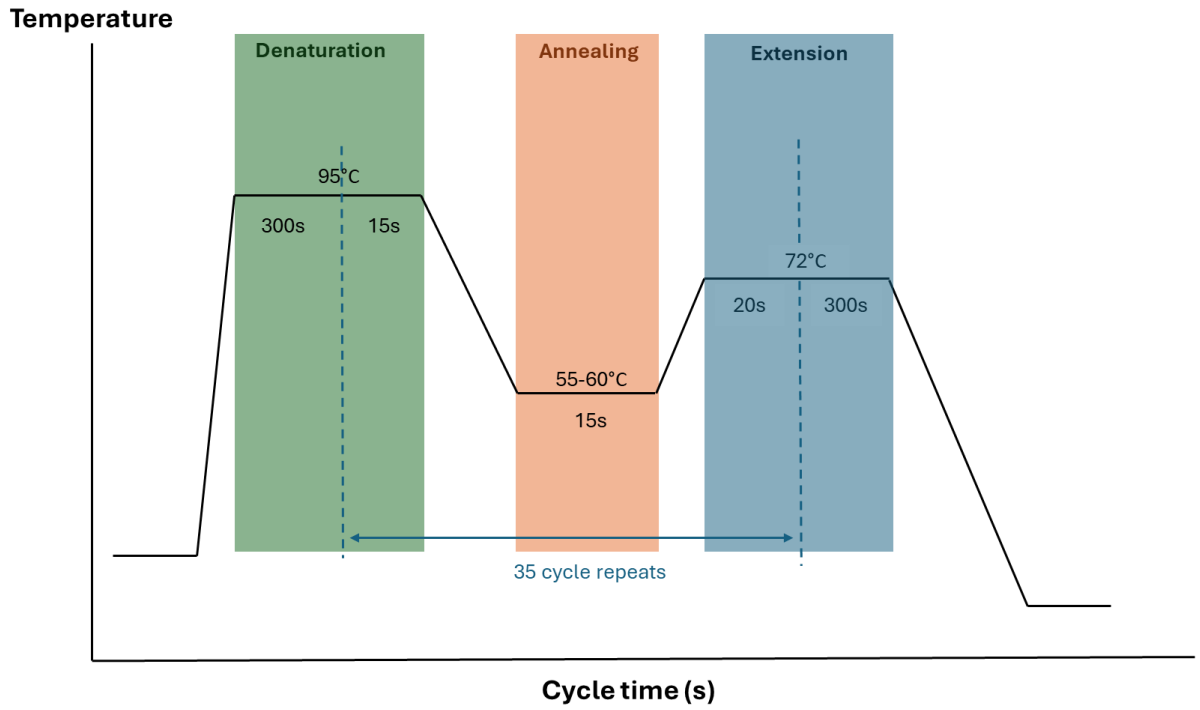


Figure 2.1. Graphic representation of the PCR protocol. The diagram illustrates the temperature changes the samples are subjected to per cycle of the 3-step amplification process. The three steps of PCR (Denaturation, Annealing, and Extension) are represented by the green, orange, and blue colour-coded rectangles, respectively. The start and the end of a PCR cycle are shown by the dashed-blue line and this cycle is repeated 35 times in this PCR protocol. The temperatures (y-axis) and the cycle times (x-axis) are included for each step of the amplification process.

2.2.3 Agarose Gel Electrophoresis and Analysis

2% Agarose Gel Preparation

To prepare 2% agarose gel, first 0.5% Tris-Acetate-EDTA (TAE) buffer was made from 50x TAE stock solution (Fisher BioReagents). Then 2g of agarose powder (Sigma-Aldrich) was dissolved in 100 mL of 0.5% TAE buffer and mixed with 6 µL of SYBR Safe DNA Gel Stain (Invitrogen), a safer and effective alternative to the widely used carcinogenic ethidium bromide. The agarose gel was poured into the assembled gel casting tray and left to solidify for 30 minutes at room temperature.

Loading the Gel and Running Gel Electrophoresis

The PCR products and 6 µL of the reference Hyper Ladder 50 bp mark (Meridian Bioscience) were gently loaded onto the wells of the 2% agarose gel. The DNA hyper ladder enhances the

reliability of the PCR experiment by verifying the amplification of the target sequence based on its size. These sizes are included in Table 1.

Next, the gel electrophoresis system was assembled. The gel was placed in the electrophoresis tank and submerged in 0.5% TAE buffer. After this the cathode and anode were connected to the Power pack 300 electrophoresis power supply (Bio-Rad). Once the current of 120V was applied, the negatively charged DNA migrated to the positive charged anode, a movement facilitated by the TAE buffer which acts as a conductive medium. This process takes around 20-30 minutes.

2.2.4 Gel imaging and processing

After the gel electrophoresis procedure, the gel was then visualised using an Ultra-violet (UV) transilluminator. The DNA stained by the SYBR Safe dye fluoresces under the UV light, thereby revealing the migration patterns of the amplified genes. The image was captured on the GeneSys software for analysis. The images were processed and annotated using the GIMP graphic editor (version 2.10.24). Processing of the images involved adjustments to brightness/contrast and various transformations, including image cropping and rotating. Additional annotation, such as labelling the band sizes, was completed using Microsoft PowerPoint.

2.3 Cell Culture

2.3.1 The Isolation of Lymphocyte from the Spleen and Lymph Nodes

The spleen and lymph nodes were surgically removed from the euthanized mice. The spleen and lymph nodes were then collected in 10 mL of Dulbecco's Phosphate Buffered Saline (PBS) (Sigma-Aldrich). The tissues were first meshed and strained into single cells and then centrifuged for 5 minutes at 13,000 rpm at room temperature. After centrifuging, the supernatant was removed, and the cells were resuspended in 5 mL of 0.8% ammonium chloride solution for a 5-minute incubation period at the environmental temperature. The ammonium chloride solution acts as an inducer of red blood cell lysis by disrupting the osmotic equilibrium and causing the swelling and bursting of the red blood cells. Meanwhile, the structural integrity of the remaining non-red blood cell populations, including white blood cells (e.g., B cells, T cells), remains unaffected. After 5 minutes, the ammonium chloride buffer was diluted with 5 mL of PBS to neutralise the cell lysis process to prevent further damage to other cell types, including lymphocytes. The cells were then centrifuged again for another 5 minutes, and the supernatant was discarded.

Control cells

White blood cells were extracted from the spleen of Ly5 mice, as described above. Following the red blood cell lysis, the remaining cells were resuspended in freezing medium (10% dimethyl sulfoxide (DMSO) + 90% Fetal bovine serum (FBS)) of 50 µL per sample, with the assumption that 5 samples could be aliquoted per spleen. The cell aliquots were then stored at -80°C until use. These cells were used as controls for single colour compensation for flow cytometry.

2.3.2 The Isolation of $CD4^+$ T cells

$CD4^+$ T cells were separated from the white blood cells, from both CD2-Egr2/3$^{-/-}$/AmCyan-T-bet and GFP-Egr2/AmCyan-T-bet mice samples, using Magnetic-activated cell sorting (MACS). In this procedure, the cells were resuspended in 1 mL of cold PBS supplemented with 5% Foetal calf growth serum (FCS) to prevent cellular loss during multiple wash steps of $CD4^+$ T cell isolation and further staining during flow cytometry. Then the cells were mixed with 30 µL of CD4 (L3T4) MicroBeads (Miltenyi Biotec), per spleen. The samples were kept on ice for 20 minutes, with the tubes being gently agitated every 5 minutes during the incubation period. After

20 minutes, 10 mL of the cold PBS/5% FCS buffer was added to the sample tube to wash away unbound CD4 microbeads which were discarded as supernatant after the samples were centrifuged for 5 minutes at 1300 rpm. The cells were then resuspended in 500 µL – 1 mL of cold PBS/5% FCS solution, dependent on the number of spleens. The cells bound to the magnetic anti-CD4 antibodies were separated through the MACS column matrix. The magnetic columns were calibrated using 2 mL of cold PBS/5% FCS buffer, after which the cells were left to pass through the column matrix. As the cell suspension flows through the column, cells bound to the CD4 microbeads are selectively retained within the column by the magnetic field, while other cell types and debris are discarded. Once the cell suspension had passed, the column was washed twice with 2 mL of the cold PBS/5% FCS buffer. The $CD4^+$ cells were then suspended in 1 mL of the cold PBS/FCS solution and injected into a collection tube, with high level of purity.

2.4 Flow cytometry

Flow cytometry was widely used throughout this thesis from characterizing the mouse models to the phenotypic and functional analysis of MP Tfh cells. Flow cytometry is a commonly used technique in immunology, cancer, microbiology and, for the detection of communicable diseases. This technique relies on the flow of fluorescently labelled single cells through a beam of laser light of which the specific wavelength of fluorescence emitted, and the scatter of visible light are detected and computed as fsc files for analysis. The movement of cells through a flow cytometer is shown in Figure 2.2. Through the fsc files, the data can be analysed to characterise the cells and to isolate cells based on markers through a process called Fluorescence-Activated Cell Sorting (FACS). In addition, the visible light scatter provides information about the sizes (Forward Scatter) and granularity (Side Scatter). Therefore, flow cytometry allows the characterization of cell populations dependent on their phenotype and cellular characteristics such as shape and internal complexity (McKinnon, 2018).

In this thesis, we used the Cytek-aurora flow cytometer. The Cytek Aurora is a highly sensitive spectral flow cytometer which consists of up to 5 lasers and can detect up to 40 colours per panel (CytekBiosciences). Unlike conventional flow cytometers that isolate specific peak wavelengths of fluorescence light to be read by specific detectors, spectral flow cytometers use multiple detectors to produce a spectrum profile for the corresponding fluorophore signal unrestricted to predefined wavelengths. Thus, a spectral flow cytometer can distinguish closely related fluorophores through their unique reference spectrum profile. In comparison, this degree of discrimination is not possible using a conventional flow cytometer which is dependent on spillover compensation to correct for spectral overlap (Nolan, 2022). Figure 2.3 illustrates the optical system of the Cytek Aurora.

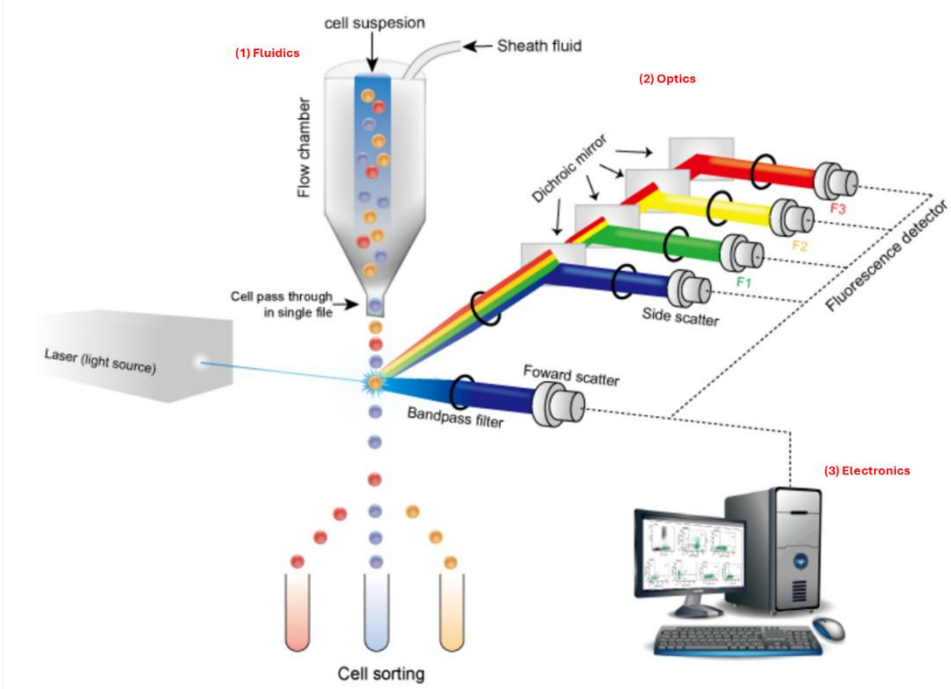


Figure 2.2. Flow cytometer schematic (image adapted from CreativeDiagnostics)**.** The diagram illustrates the complex structure of a flow cytometer which includes three major parts: the fluidics, the optical system, and the electronic system. (1) The fluidic system includes the sample passing through a nozzle in a single file suspended in the flow sheath in the direction of the laser beam. After passing through the laser the sample is discarded, or the cells are sorted and collected in a tube. (2) The optics system works by directing the movement of light and ensuring that the fluorescence emission signal is read by the suitable detector dependent on the wavelength of the light emitted. (3) The photocurrent generated by detectors is then passed through the electronics system which computes and processes the data for analysis (ThermoFisher).

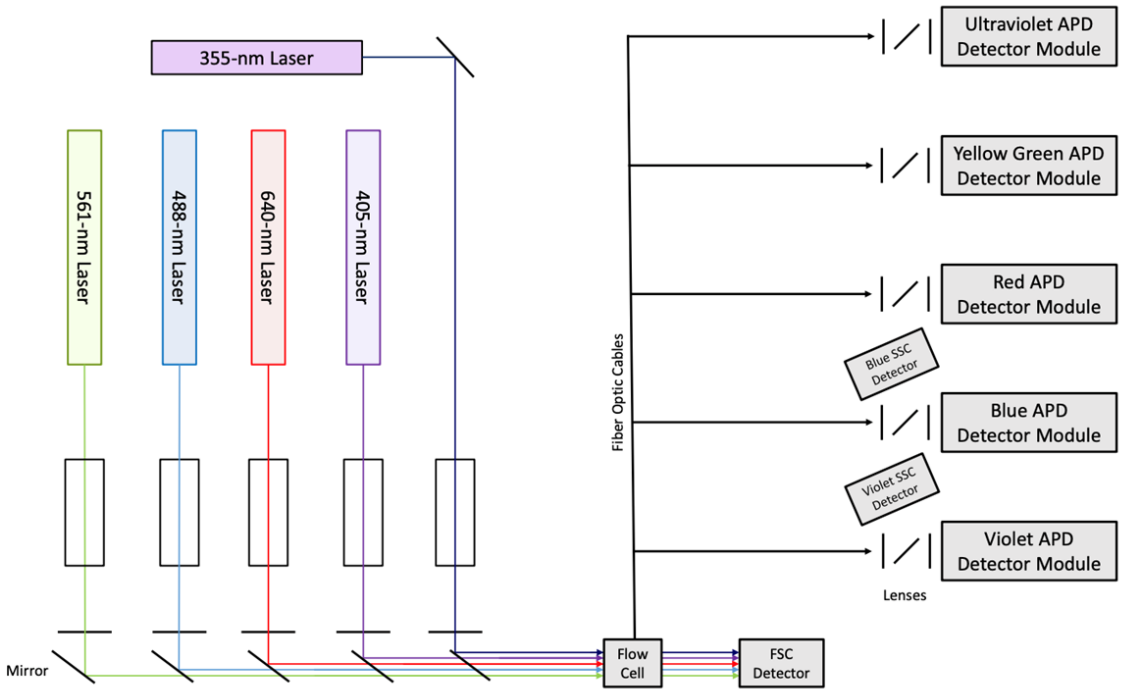


Figure 2.3. Optical design of the Cytek Aurora spectral flow cytometer (image obtained from CytekBiosciences)**.** This diagram illustrates the key features of the Cytek Aurora which includes five excitation lasers (355 nm, 405 nm, 488 nm, 561 nm, and 640 nm) which excite the fluorescently labelled cells passing through the flow cell. The emitted fluorescence signal is transmitted to multiple avalanche photodiode (APD) detector modules via fiber optic cables. The ADP detectors capture the full emission spectrum which is then computationally unmixed to distinguish between fluorophores with overlapping spectral profiles (CytekBiosciences).

Antibody/Markers panel

The antibody panels used per experiment are listed below.

Company	Antibody for	Clone	Fluorescent conjugate(s)	Intracellular, Cytokine or Surface Staining
eBiosciences	CD44	IM7	APC-eFluor780	Surface
	CD62L	MEL-14	PE-Cy7	
	CD25	PC61.5	APC	
Biolegend	CD4	GK1.5	AlexaFluor700	Surface
	Ki67	16A8	PE	Intracellular

Table 2.2. Antibody panel used for phenotyping memory-phenotype (MP) $CD4^+$ T cells at steady state.

Company	Antibody for	Clone	Fluorescent conjugate(s)	Intracellular, Cytokine or Surface Staining
eBiosciences	CD44	IM7	APC-eFluor780	Surface
	CD25	PC61.5	APC	
Biolegend	CD4	GK1.5	AlexaFluor700	Surface

Table 2.3 Antibody panel used to investigate the expression of GFP-EGR2 and AmCyan-T-bet in $CD4^+$ MP T cells at steady state.

Company	Antibody for	Clone	Fluorescent conjugate(s)	Intracellular, Cytokine or Surface Staining
eBiosciences	CD62L	MEL-14	PE-Cy7	Surface
Biolegend	CD3	145-2C11	BV605	Surface
	CD4	GK1.5	AlexaFluor700	
	CXCR5	L138D7	BV421	
	CD44	IM7	BV510	
BD Biosciences	BCL6	K112-91	PE	Intracellular

Table 2.4 Antibody panel used to investigate the expression of T follicular helper (Tfh) cell-specific markers within $CD4^+$ MP T cell population.

Company	Antibody for	Clone	Fluorescent conjugate(s)	Intracellular, Cytokine or Surface Staining
eBiosciences	PD1	J43	SuperBright780	Surface
Biolegend	CD4	GK1.5	AlexaFluor700	Surface
	CD44	IM7	APC	
	CXCR5	L138D7	BV421	
	FR4	12A5	APC/Fire750	
BD Biosciences	BCL6	K112-91	PE	Intracellular

Table 2.5 Antibody panel used for phenotyping MP Tfh cells.

Company	Antibody for	Clone	Fluorescent conjugate(s)	Intracellular, Cytokine or Surface Staining
eBiosciences	PD1	J43	SuperBright780	Surface
	FOXP3	FJK-16s	PerCP-Cy5.5	Intracellular
Biolegend	CD4	GK1.5	AlexaFluor700	Surface
	CD44	IM7	APC	
	CXCR5	L138D7	BV421	
	FR4	12A5	APC/Fire750	
	GITR	DTA-1	PE-Cy7	
BD Biosciences	BCL6	K112-91	PE	Intracellular

Table 2.6 Antibody panel used for investigating T follicular regulatory (Tfr)-like cells within MP Tfh cell population.

Company	Antibody for	Clone	Fluorescent conjugate(s)	Intracellular, Cytokine or Surface Staining
eBiosciences	CD4	RM4-5	PE-Cy5	Surface
	CD44	IM7	APC-eFluor780	
	CD25	PC61.5	APC	
	FOXP3	FJK-16s	PerCP-Cy5.5	Intracellular
Biolegend	CXCR5	L138D7	BV421	Surface
	FR4	12A5	PE	
	BCL6	7D1	PE-Cy7	Intracellular

Table 2.7 Antibody panel used to assess the expression of Egr2, T-bet, and FR4 in memory-phenotype (MP) Tfh cells and the distribution of MP Tfh cells within $Egr2^+$ and $T\text{-}bet^+$ MP $CD4^+$ T-cell subsets.

Company	Antibody for	Clone	Fluorescent conjugate(s)	Intracellular, Cytokine or Surface Staining
eBiosciences	CD4	RM4-5	PE-Cy5	Surface
	CD44	IM7	APC-eFluor780	
	CD25	PC61.5	APC	
	CXCR5	SPRCL5	PerCP-efluor710	
	CD62L	MEL	PE	
Biolegend	FR4	12A5	APC/Fire750	Surface
	BCL6	7D1	PE-Cy7	Intracellular

Table 2.8 Antibody panel used to investigate the role of Egr2 in MP Tfh cell development.

Company	Antibody for	Clone	Fluorescent conjugate(s)	Intracellular, Cytokine or Surface Staining
eBiosciences	CD4	RM4-5	PE-Cy5	Surface
	CD44	IM7	APC-eFluor780	
	CD25	PC61.5	APC	
	CXCR5	SPRCL5	PerCP-efluor710	
	FOXP3	FJK-16s	PerCP-Cy5.5	Intracellular
Biolegend	FR4	12A5	PE	Surface
	BCL6	7D1	PE-Cy7	Intracellular

Table 2.9 Antibody panel used to analyse the expression of Tfh cell-specific and Treg cell-specific markers within MP T cells isolated from GFP-Egr2/AmCyan-T-bet and CD2-Egr2/3$^{-/-}$/AmCyan-T-bet mice.

Egr2+FR4+, Egr2-FR4+, Egr2-FR4- CD44high CD4+ T cell sorting *				
Company	**Antibody for**	**Clone**	**Fluorescent conjugate(s)**	**Intracellular, Cytokine or Surface Staining**
eBiosciences	CD4	RM4-5	PerCP-Cy5.5	Surface
	CD44	IM7	APC-eFluor780	
	CD25	PC61.5	PE-Cy7	
Biolegend	FR4	12A5	PE	
Tfh, Tfr, non-Tfr and naïve T cell sorting				
eBiosciences	CD4	RM4-5	PerCP-Cy5.5	Surface
	CD44	IM7	APC-eFluor780	
	PD1	J43	BV421	
Biolegend	CXCR5	L138D7	BV421	Surface
	GITR	DTA-1	PE-Cy7	
B cell sorting				
eBiosciences	CD4	RM4-5	PE-Cy5	Surface
	B220	RA3-6B2	APC-eFluor780	

Table 2.10 Antibody panels in cell-sorting experiments.

2.4.1 Cell Surface Staining

Isolated white blood cells or $CD4^+$ T cells from both GFP-Egr2/AmCyan-T-bet and CD2-Egr2/3$^{-/-}$ /AmCyan-T-bet mice were stained with 3 µL per cell surface antibody/s (Table 2.2-2.9) and incubated for 30 minutes to 1 hour in ice. The samples were then washed with 1 mL of PBS and centrifuged for 5 minutes at 1300 rpm at room temperature. After which, the cell pellet was resuspended in 600 µL of PBS ready for use on the Cytek Aurora analyser. Single-color compensation and the unstained controls were prepared using the frozen, isolated white blood cells from the Ly5 mouse strains. Single-colour controls were used for spectral unmixing, while unstained controls were used to define the level of autofluorescence and background signal. These control cells were stained using the same procedure outlined above, with a slight modification: the stained cells were resuspended in 300 µL of PBS instead of diluting to 600 µL. This reduced dilution results in a higher concentration of cells to ensure sufficient events are recorded for accurate compensation calculations.

To investigate the expression of the reporter genes, EGR2 and T-bet, in $CD4^+$ T cells from GFP-Egr2/AmCyan-T-bet knockin mice, isolated $CD4^+$ T cells were only stained with anti-mouse CD4-PE-Cy5 (clone RM4-5, eBiosciences).

2.4.2 Intracellular/cytokine staining

The cells to be stained for nuclear transcription factors or intracellular cytokines were first tagged with cell surface antibodies and then pelleted and washed with 1 mL of PBS and then centrifuged. The cells were resuspended in 250 µL of Fixation buffer, prepared following the Intracellular Fixation & Permeabilization Buffer Set guidelines (eBioscience). Briefly, 1 mL of the Perm concentrated was mixed into 3 mL of the diluent. The cell suspension was incubated in the dark at room temperature for 20 minutes to prevent photobleaching of the fluorescent antibodies. After fixing the cells to preserve cellular integrity, they were pelleted by centrifugation and subsequently subjected to permeabilization. The cells were resuspended in 200 µL of Permeabilisation buffer and further incubated for 15 minutes in the same conditions. The cells suspended in the Permeabilisation buffer were centrifuged for 5 minutes and then the supernatant was discarded. The cells were then resuspended in 4 µL of the required intracellular antibodies (Table 2.2, Table 2.4-2.9) diluted in 10 µL of Perm/wash, followed by a 1-hour incubation period at room temperature. The volume of Perm/Wash used was lower than that recommended in the eBioscience Fixation & Permeabilization protocol. However, this modified staining approach was validated in preliminary experiments and produced staining results comparable to those obtained using the manufacturer's recommended protocol. After this, the cells were washed to remove unbound antibodies and finally resuspended in 600 µL of PBS, and ready for analysis using the Cytek Aurora Flow cytometer. The control cells stained for cell surface antibodies were also fixed before resuspending in 300 µL of PBS. The single colour control cells for intracellular staining antibodies were subjected to fixation and permeabilization as described above before being stored in 300 µL of PBS.

2.4.3 Fluorescence-activated Cell Sorting (FACS)

FACS was used to further sort the $CD4^+$ MP T cells into populations of interest for either GFP-Egr2/AmCyan-T-bet or CD2-Egr2/3$^{-/-}$/AmCyan-T-bet mouse models. Sorted cells included $CD4^+$ $CD44^{high}$ $CD25^-$ $FR4^+$ $EGR2^+$, $CD4^+$ $CD44^{high}$ $CD25^-$ $FR4^+$ $EGR2^-$ and $CD4^+$ $CD44^{high}$ $CD25^-$ $FR4^-$ $EGR2^-$ from GFP-Egr2/ AmCyan-T-bet mice for MP Tfh cells functional studies and RNA Seq. And $CD4^+$ $CD44^{high}$ $CD25^-$ $FR4^+$ $EGR2^-$ and $CD4^+$ $CD44^{high}$ $CD25^-$ $FR4^-$ $EGR2^-$ MP T cells from

CD2-Egr2/3$^{-/-}$/AmCyan-T-bet mice for T cell functional studies. The gating strategy is shown in supplementary figure 1A.

And the following cells were also sorted: CD4^{+} CD44^{low} naïve T cells (TN), CXCR5^{+} PD-1^{+} GITR^{-} CD4^{+} CD44^{high} MP Tfh cells, CXCR5^{+} PD-1^{+} GITR^{+} CD4^{+} CD44^{high} MP Tfr cells, and CXCR5^{-} PD-1^{-} GITR^{-} CD4^{+} CD44^{high} non-follicular MP T cells for *in vitro* functional analysis. The cell sorting antibody panel is shown in Table 1.10 and the gating strategy is shown in supplementary figure 1B. This procedure was carried out using the BD FACS Aria III Sorter by the facility manager, Gary Warnes, at the Blizard Institute, Queen Mary University of London.

2.4.4 Flow Cytometry Data Analysis

The data was analysed on the FlowJo software (version 10; Tree Star). The gating employed is indicated in the respective figures. Cells were initially gated on singlets and lymphocytes based on FSC/SSC parameters prior to downstream analysis. Biologically negative populations were used as internal controls to define gating thresholds. For example, naïve CD4^{+} T cells (CD62L^{+} CD44^{low}), which are known not to express CXCR5 or BCL6, were used to set gates for downstream analysis of memory-phenotype CD4^{+} T cells.

2.5 T Cell Functional Studies

2.5.1 T Cell Stimulation and Interferon Gamma Production (IFN-γ)

Sorted EGR2^{+} FR4^{+}, EGR2^{-} FR4^{+}, EGR2^{-} FR4^{-} CD44^{high} CD4^{+} MP T cells from GFP-Egr2/AmCyan-T-bet knockin mice and, CD4^{+} CD44^{high} CD25^{-} EGR2^{-} FR4^{+} and CD4^{+} CD44^{high} CD25^{-} EGR2/3$^{-/-}$FR4^{+} MP T cells from Egr2/3$^{-/-}$/AmCyan-T-bet mice, were stimulated and the IFN-γ production by these cells was measured. The cells were maintained in the RPMI cell culture medium and stimulated in 24-well plate with 50 µL of 5 µg/ml plate-bound anti-CD3 (BD Biosciences) and 2 µg/ml anti-CD28 (BD Biosciences) antibodies in PBS and incubated for 72 hours. This provides TCR-dependent activation of the sorted MP CD4^{+} T cells. Then the cells were stimulated with 100 ng/ml ionomycin (Sigma-Aldrich) and 50 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) with 1:1000 Golgi stop (BD Biosciences) for three hours to enhance TCR signaling in a TCR-independent manner. The addition of Golgi stop prevents the secretion of IFN-γ. After intracellular staining with anti-IFNγ-PE-Cy7 (clone XMG1.2, eBiosciences), stimulated cells were analysed for IFN-γ production by flow cytometry.

2.5.2 CellTrace™ Violet (CTV) Proliferation Assay

Sorted EGR2^{+} FR4^{+}, EGR2^{-} FR4^{+}, EGR2^{-} FR4^{-} CD44^{high} CD4^{+} MP from GFP-Egr2 AmCyan-T-bet knockin mice and CD4^{+} CD44^{high} CD25^{-} EGR2^{+} FR4^{-} and CD4^{+} CD44^{high} CD25^{-} EGR2/3$^{-/-}$ FR4^{+} MP T cells from Egr2/3$^{-/-}$/AmCyan-T-bet mice, were stimulated to measure the rate of proliferation using the CellTrace Violet proliferation assay. These cells were labelled with CellTrace Violet stain, in accordance with the manufacturer's instructions (Invitrogen). Firstly, the stock solution was prepared by adding 20 µL of DMSO to a vial of CellTrace Violet staining solution. After resuspending the sorted cells in PBS containing 0.5% FCS, the CellTrace Violet staining solution was added to the cells in a ratio of 1:1000, which is less toxic to cells, and mixed rapidly to evenly distribute the dye. The cells were incubated at room temperature for 5 minutes after which the cells were washed with 5 µL of PBS and centrifuged to discard the unbound staining solution. Then, 10X medium with 10% FCS was added to the samples and the samples were then centrifuged. The medium was added a second time, and the cells were incubated for 5 minutes at room temperature and then centrifuged. After centrifugation, the cells were resuspended in medium in the well plates. After labelling, the cells were stimulated with anti-CD3 and anti-CD28. After a 72-hour stimulation, these labelled cells were analysed for proliferation by flow cytometry.

2.5.3 *In vitro* Tfh Function Assay

2.5.3.1 B Cell Culture and Stimulation

To investigate the function of MP Tfh and MP Tfr cells, B cell-mediated IgG production was stimulated *in vitro*. This protocol was adapted from previously described methods used to analyse isotype switching following in vivo antigen stimulation (Sage *et al.*, 2013; Ogbe *et al.*, 2015; Sage and Sharpe, 2015; Atala, 2016). The IgG production was then measured by Enzyme-linked immunosorbent assay (ELISA). For this experiment $CD4^+$ $CD44^{low}$ naïve T cells, $CD4^+$ $CD44^{high}$ $CXCR5^+$ $PD\text{-}1^+$ $GITR^-$ MP Tfh cells, and $CD4^+$ $CD44^{high}$ $CXCR5^+$ $PD\text{-}1^+$ $GITR^+$ MP Tfr cells were sorted by FACS, of pure $CD4^+$ T cells from the spleen and lymph nodes of 12-week-old GFP-Egr2/AmCyan-T-bet knockin mice. In addition, $CD4^-$ $B220^+$ B cells were isolated from $CD4^-$ T cells stained with anti-B220 and anti-CD4 (supplementary figure 1C). Then 1×10^5 B cells were cultured with either 3×10^4 naïve T cells, MP Tfh cells, or alone. Another culture included MP Tfh cells in combination with 1.5×10^4 MP Tfr cell, to observe the regulatory effect of MP Tfr cells on MP Tfh function. These cell cultures were then stimulated with 5 µg/mL anti-IgM and 2 µg/mL anti-CD3, the latter required to activate the T cells to provide B cell help.

2.5.3.2 Enzyme-linked immunosorbent assay

ELISA was then used to measure the level of IgG produced from the B cells cultured with either naïve T cells, MP Tfh cells, both MP Tfh cells and MP Tfr cells, or alone. 6 days after stimulation, the supernatants were collected and the total IgG production was analysed by using the Total IgG Mouse ELISA Kit, in line with the manufacturer's guide (Invitrogen). This process relies on the specific binding of capture and detection antibodies to IgG to initiate a colorimetric substrate-enzyme (HRP-TMB) reaction in the presence of IgG. The colour intensity observed is proportional to the concentration of IgG calculated from a standard curve. In brief, the 96-well plate was coated with 100 µL of 250X capture antibody (anti-mouse IgG monoclonal antibody) diluted in 1X Coating buffer (PBS) and incubated at 4^{o}C overnight. After incubation, the wells were washed twice with the wash buffer (0.05% Tween™ 20 + 1X PBS) to discard any unbound capture antibody. To prevent non-specific binding to the plate, 250 µL of the prepared 2X blocking buffer was added to the wells and left for a 2-hour incubation period at room temperature. Then the wells were washed again twice with the wash buffer. After this, 100 µL of the 1X Assay buffer (1% Tween™ 20 + 10% BSA in PBS) was added to the IgG standard and blank control wells and, 90 µL of the assay buffer was added to the sample wells. To form a

standard curve, the IgG isotype standards were serially diluted. Two sets of standard wells (S1-S6) were prepared and 100 µL of the IgG isotype standard was added into the two S1 wells and thoroughly mixed using a pipette before transferring to S2 and so on achieving the concentrations of 100 ng/ml, 50 ng/ml, 25 ng/ml, 12.5 ng/ml, 6.25 ng/ml and 3.123 ng/ml, as advised by the manufacturer's protocol. To the sample wells, 10 µL of the diluted IgG supernatant was added. Finally, 50 µL of the detection antibody (250X HRP-conjugated anti-mouse IgG polyclonal antibody) was then added to each well before incubating the plate for 2 hours at room temperature. Next, the plate was washed 4 times and 100 µL of the Tetramethylbenzidine (TMB) Substrate Solution was added to the wells and incubated for another 15 minutes at room temperature initiating an enzymatic reaction in the presence of IgG. To stop the reaction, 100 µL of the stop solution was added. The absorbance at 450 nm and 570 nm was then read using a CLAIROstar plate reader (BMG Labtech). The absorbance at 570 nm was subtracted from 450 nm, to ensure accurate IgG quantification by removing technical artifacts such as background noise. To ensure the best model was fitted to the standard curve data, 4 and 5 parameter weighted and unweighted models were tested. Unweighted 4 parameter models was confirmed to be the best fit for the data and were used to deduce the concentration of IgG of the samples.

2.6 Quantitative real-time PCR

Much like traditional PCR that was used for genotyping the mice, quantitative real-time PCR (qPCR) also relies on detecting gene expression through the 3-step DNA amplification process involving Denaturation, Annealing, and extension. However, qPCR distinctively involves the quantification of gene expression, of cDNA synthesized from RNA, in real time. The results are then normalised to the expression of housekeeping genes such as actin (Panina *et al.*, 2018). This is because the expression of housekeeping genes remains unchanged regardless of the tissue or the environment. This means experimental factors such as the amount of RNA can be adjusted so the mRNA expressions can be accurately compared between samples.

In this study, qPCR was used to determine the expression of *Egr2*, *Ikzf2*, *Bcl6*, and *Il21* genes in sorted $CD4^+$ $CD44^{low}$ naïve T cells, $CD4^+$ $CD44^{high}$ $CXCR5^+$ $PD\text{-}1^+$ $GITR^-$ MP Tfh cells, $CD4^+$ $CD44^{high}$ $CXCR5^+$ $PD\text{-}1^+$ $GITR^+$ MP Tfr cells and, $CD4^+$ $CD44^{high}$ $CXCR5^-$ $PD\text{-}1^-$ $GITR^-$ non-follicular MP T cells isolated from the spleen and lymph nodes of 12-week-old GFP-Egr2/AmCyan-T-bet knockin mice.

2.6.1 RNA extraction

RNA was extracted from the sorted cells using the RNeasy UCP Micro Kit (Qiagen). This kit's ultra-clean production (UCP) lowers the risk of contamination and selectively purifies large RNA molecules such as mRNA. The cells are first pelleted by centrifugation and then lysed by adding 350 µL of the buffer RULT and vortexed vigorously to facilitate the breakdown of the cells. The RULT lysis buffer contains guanidine thiocyanate which denatures proteins such as RNases and lyses the cells. Further addition of 350 µL of ethanol maintains an environment ideal for RNA to bind to the spin-column membrane. The sample was then vortexed, transferred to the spin-column membrane placed in a collection tube, and centrifuged for 1 minute at 13,000 rpm. After centrifuging, the flow-through was discarded and the RNA-bound to the membrane was washed with 350 µL of RUWT buffer diluted in 30 mL of ethanol. The samples were then centrifuged for 5 minutes at 13,000 rpm and the flow-through was discarded. Then the DNA bound to the spin-membrane was then removed to isolate pure RNA from the sample by adding 80 µL of the DNase in RDD buffer to the spin-column and incubated for 15 minutes. To prepare the DNase in RDD buffer, 10 µL of DNase stock solution was added to 70 µL prepared for every sample and centrifuged for 15 seconds to mix. Then the RNA sample was washed with the

RUWT buffer and centrifuged for 1 minute at 13,000 rpm. The collection tube containing the discarded DNase and DNA impurities was then replaced. 500 µL of RUPE buffer was added, the sample was centrifuged, and the collection tube was emptied. This was then repeated with 500 µL of ethanol. Instead of discarding the flow-through after centrifugation, the collection tube was replaced. The sample was then spun at 13,000 rpm for five minutes with the lid open to dry out the membrane from the ethanol before eluting the RNA. Finally, the pure RNA extracted from the sorted cells was then unbound from the membrane into a clean collection tube by adding 14 µL of RNA-free water, reducing the high salt concentration which releases the RNA bound to the spin membrane into the RNA-free water. The sample was centrifuged for 1 minute with the collection tube lid opened. The extracted RNA was then kept on ice. The extracted RNA was then quantified using the Qubit RNA HS reagent kit (Thermo Scientific).

2.6.2 First strand cDNA synthesis

To conduct qPCR, which relies on DNA amplification, the purified RNA was then used as a single-strand RNA template to synthesise a complementary DNA single strand (cDNA), using an enzyme called the SuperScript IV reverse transcriptase (RT) (Invitrogen) to be then used to synthesise the DNA in the PCR reaction to measure the gene expression. This newly engineered SuperScript IV RT yields higher amounts of cDNA in a shorter time and is comparatively more thermostable complementing an efficient RT reaction. In line with the manufacturer's protocol, the RNA-primer mix was prepared. This mix consisted of the RNA template, 50 µM random hexamers (primer), 10 mM dNTPs, and nuclease-free water. This RNA-primer mix was then incubated for 5 minutes at 65ºC and then left on ice for a minute. Alike to PCR, this first step of reverse transcription involves the denaturation of the secondary structure of the mRNA where the following cooling step allowing annealing of the random hexamers to occur. The annealed RNA was then mixed with the RT reaction mix, which supplies the SuperScript IV RT (200 U/µL), 5X SSIV buffer, 100 mM DTT required for enzyme stability and the RNase Inhibitor to prevent RNA degradation. Then the annealed RNA was further incubated for 10 minutes per temperature change (23ºC, 50-55ºC, and 80ºC). The initial temperature at 23ºC facilitated further annealing of the primer to the RNA strand, then the temperature was increased to 50-55ºC at which the SuperScript IV RT activates and initiate cDNA synthesis. Finally, the SuperScript IV RT was deactivated at 80ºC.

2.6.3 Quantitative real-time PCR and analysis

The cDNA was diluted in 80 µL of distilled water before adding the master mix. The qPCR master mix was prepared using the SYBR green PCR master mix (QIAGEN), PCR-grade water, and the sense and antisense primers listed in Table 2.11. Then 2 µL of cDNA was added to the PCR tubes along with 8 µL of the master mix. Positive and negative controls were also included. The qPCR was then carried out on the Rotor-Gene system (Corbett Robotics).

Locus	Primer
Egr2	Sense- 5'-CTT CAG CCG AAG TGA CCA CC-3' Antisense- 5'-GCT CTT CCG TTC CTT CTG CC-3'
Bcl6	Sense- 5'-CAT GCA GGA AGT TCA TCA AGG-3' Antisense- 5'-CTC AGT GGC ATA TTG TTC TCC-3'
IL21	Sense- 5'-CTC AAG CCA TCA AAC CCT GG-3' Antisense- 5'-CAT ACG AAT CAC AGG AAG GG-3'
Ikzf2	Sense- 5'-AAC GCT GTC ACA ACT ATC TCC-3' Antisense- 5'-CTT TCC CAT ATT TGC CGT GAG-3'
β-actin	Sense- 5'-AAT CGT GCG TGA CAT CAA AG-3' Antisense- 5'-ATG CCA CAG GAT TCC ATA CC-3'

Table 2.11. List of qPCR primer sequences.

The data analysis was carried out on the Rotor-Gene Software. During the three-step PCR cycle, the DNA copies roughly double referred to as the exponential phase, and this increase comes to a gradual plateau with reduced amplification. This is due to the limiting reagents at later time points during the amplification process. The number of PCR cycles until the sample curve reaches the pre-defined threshold value is called the CT value (Figure 2.4). This value is used to calculate the relative mRNA expression normalised to the reference housekeeping gene, β-actin by the equation 2ˆ (CTβ-actin − CT target). The relative mRNA expression is visualised on a bar graph using Microsoft Excel spreadsheet.

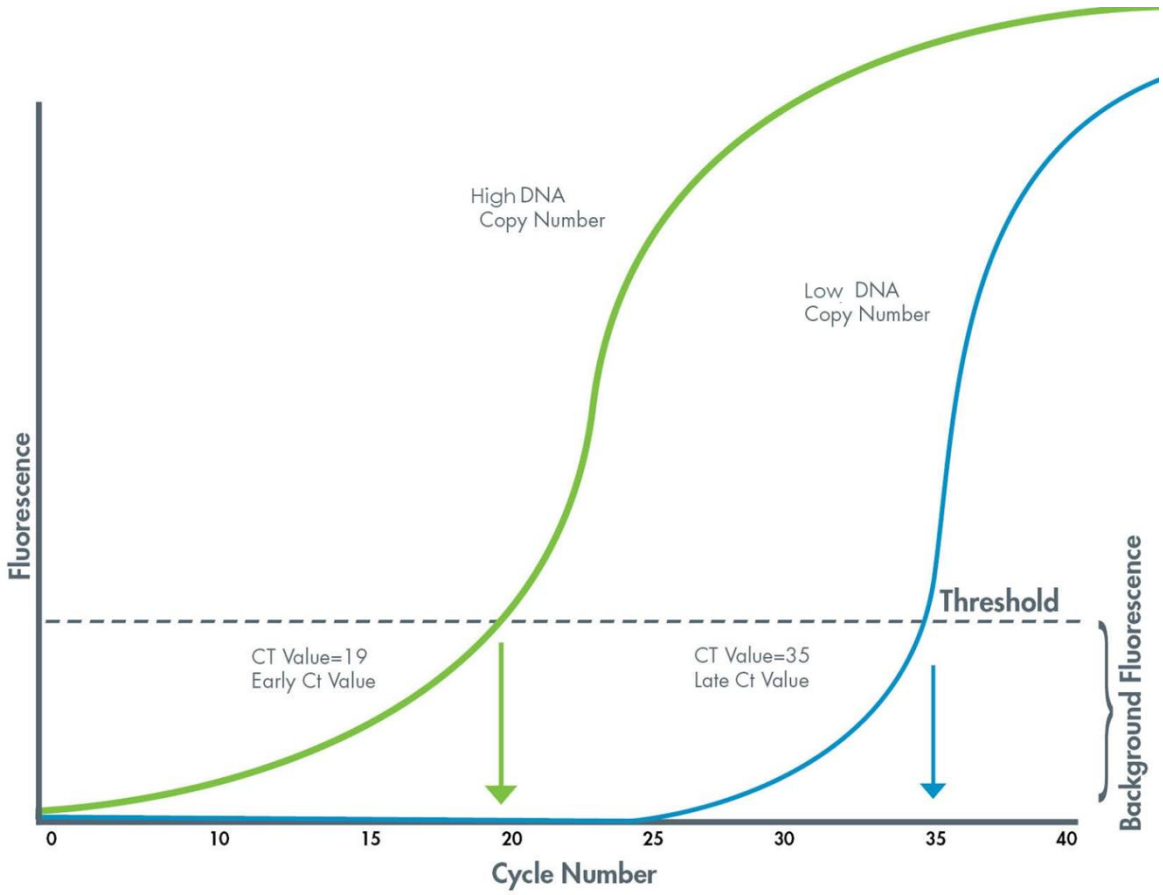


Figure 2.4- PCR amplification curve. The qPCR graph illustrates the fluorescence intensity (Y-axis) against the cycle number (X-axis). The curve shows three main phases during amplification. In phase 1 the baseline phase, at the start of the PCR reaction (below a threshold value), where there is a low fluorescence signal indistinguishable from background noise. In the second exponential phase in which the DNA molecules double. This is followed by the plateau phase by which DNA amplification is restricted due to experimental factors such as limiting reagents. The CT value is the point at which there is a greater PCR cycle and, in turn, a greater fluorescent signal, by which the curve crosses the pre-defined threshold value. The green curve has a lower CT value due to its higher abundance of DNA, whereas the blue line has a higher CT value of 35 due to its low number of DNA copies (LuminUltra Technologies).

2.7 Adoptive Transfer

To investigate MP Tfh cell function *in vivo*, sorted $CD4^+$ $CD44^{high}$ $CD25^-$ $FR4^+$ $EGR2^+$ and $CD4^+$ $CD44^{high}$ $CD25^-$ $FR4^+$ $EGR2^-$ cells from the GFP-Egr2/AmCyan-T-bet mice were transferred into the recipient CD2-Egr2/3$^{-/-}$ mice infected with vaccinia virus. 2 x 10^5 cells, of $CD4^+$ $CD44^{high}$ $CD25^-$ $FR4^+$ $EGR2^+$ and $CD4^+$ $CD44^{high}$ $CD25^-$ $FR4^+$ $EGR2^-$ MP T cells, were isolated from GFP-Egr2/AmCyan-T-bet mice and subsequently suspended in 100 µL physiological saline. The saline mimics the body's environment, maintaining osmotic equilibrium, and thereby protecting the cells from damage and preserving the cell's functionality. The suspension of single cells was then injected intravenously into the dorsal tail vein of 6- to 8-week-old CD2-Egr2/3$^{-/-}$ mice. 24 hours after the adoptive transfer of the cells, during which the cells can migrate throughout the body (including organs such as the spleen), the recipient CD2-Egr2/3$^{-/-}$ mice were infected intraperitoneally with the vaccinia virus.

2.8 Virology studies: Infection and Neutralisation

2.8.1 Cell culture

The thymidine kinase deficient 143 (TK143) cell line was used for rapid viral replication, viral titration test for optimal viral dose to infect the CD2-Egr2/3$^{-/-}$ mice, and to test for the infectiousness of vaccinia virus by studying the plaque formation *in vitro* (known as the neutralisation plaque reduction test). This cell line has been used by our research group multiple times and has been shown effective for viral studies in culture (Ogbe *et al.*, 2015; Miao *et al.*, 2017; Singh *et al.*, 2017). These cells are easily maintained in a culture medium consisting of two mediums GLUTAMAX and Dulbecco's modified Eagle's medium (DMEM) with 2% sodium pyruvate, 10% FBS, 50 µM β-mercaptoethanol (2-ME) and 10% HEPES buffer supplements added. In addition, 50 µg/ml gentamycin antibiotic was added to this medium to prevent bacterial contamination. This list of ingredients was purchased from Invitrogen.

2.8.2 Viruses

The vaccinia virus western strain (VACV-WR), a common laboratory strain of vaccinia virus, was used to infect the CD2-Egr2/3$^{-/-}$/AmCyan-T-bet mice. VACV-WR stocks were grown in TK143 cells in tissue culture T175 flasks, infected with a low multiplicity of infection (MOI) of 0.5 for 72 hours, 37^{O}C, 5% CO_2. At 72 hours, the cells were harvested and suspended in suspended in DMEM medium. A rapid freezing-thawing procedure was repeated three times causing the infected TK143 cells to rupture, releasing the VACV-WR into the DMEM medium. Then the virus containing supernatant was separated from the cellular debris through centrifugation (1300 rpm) for 5 minutes. The VACV-WR containing supernatant was stored in at -80 ^{O}C as virus stocks. The stocks were used for infection of TK143 cells to determine the optimal dosage or viral titre. This process involves serial dilution of the VACV-WR which are then used to infect the TK143 cells to detect the optimal infectious dosage, based on the number of plaques, to use for infecting the CD2-Egr2/3$^{-/-}$/ AmCyan-T-bet mice.

2.8.3 Viral infection

The CD2-Egr2/3$^{-/-}$/AmCyan-T-bet mice were infected with 4X10^{6} plaque forming units (PFU), the measurement unit for the viral titre, of VACV-WR suspended in 100 µL of physiological saline,

using an intraperitoneal injection. The mice were monitored for disease symptoms and weighed daily.

2.8.4 Plaque reduction neutralization test

Plaque reduction neutralization test was used to investigate whether neutralising antibodies were present in the sera of VACV-WR infected CD2-Egr2/3$^{-/-}$mice with either the adoptive transfer of $CD4^+$ $CD44^{high}$ $CD25^-$ $FR4^-$ $EGR2^+$ MP T cells, $CD4^+$ $CD44^{high}$ $CD25^-$ $FR4^+$ $EGR2^+$ MP T cells, or neither. The collected sera were serially diluted in DMEM medium and incubated with an equal volume of $2X10^4$ PFU of VACV-WR per ml for 12 hours at 37^OC, 5% CO_2. After incubation, the viral serum mixture was transferred to pre-prepared TK143 cells. The TK143 cells were prepared by seeding them into 24 well plates (Corning Inc., Corning, NY) and washed with serum-free medium. After that, 100 µL of the virus serum in DMEM medium was added in duplicates per well for 60 minutes, at the same conditions as mentioned before, and periodically swirled to allow even distribution of the viral particles within the wells for absorption of the viral particles by the TK143 cells so these cells can be infected. After 60 minutes, the plates were rinsed again with the serum-free medium to remove unabsorbed viral particles and then the cells were incubated in growth medium for 2 days to allow plaque formation. The plaques formed determine the cytotoxicity of the virus and the comparison of the number of plaques formed by the vial particles from the sera isolated from VACV-WR infected CD2-Egr2/3$^{-/-}$ mice with either the adoptive transfer of $CD4^+$ $CD44^{high}$ $CD25^-$ $FR4^-$ $EGR2^+$ MP T cells, $CD4^+$ $CD44^{high}$ $CD25^-$ $FR4^+$ $EGR2^+$ MP T cells, or transfer of neither cells, will help determine whether or not neutralising antibodies were formed following infection of the CD2-Egr2/3$^{-/-}$ mice. This will help us understand the functionality of MP Tfh cells in response to viral infection.

2.8.5 Light microscopy and analysis

To count plaques using light microscopy, the cells must be stained and fixed. The cells were fixed and stained in a one-step process with 0.1% crystal violet in 20% ethanol. Crystal violet staining allows the plaques to be visualised using a light microscope.

2.9 Immunohistochemistry (IHC)

Much like flow cytometry, IHC involves the addition of fluorescently conjugated antibodies to stain antigens of interest. However, rather than staining cells as carried out in flow cytometry, IHC involves the staining of tissues to be viewed under a fluorescent microscope. This procedure was carried out to analyse spleen tissues for germinal center formation following vaccinia virus infection of CD2-Egr2$^{-/-}$/Egr3$^{-/-}$ mice.

2.9.1 Tissue preparation

Spleens were extracted from the sacrificed CD2-Egr2/3-/- mice infected with VACV-WR. Spleen tissue samples were extracted in PBS and fixed with 4% paraformaldehyde (PFA) in PBS overnight at 4°C. This step ensures that the cellular structure is preserved as the fixative crosslinks proteins through covalent bonds which prevent degradation by proteolytic enzymes and maintain the spleen tissue's architecture. After fixation with 4% PFA, the excess tissue fixative was washed away by rinsing with PBS. The next step involved embedding the tissues in paraffin to allow thin slicing of tissue sections for mounting onto microscope slides for staining and analysis under a fluorescence microscope. Before embedding tissues into paraffin wax, the tissues were passed through ascending concentrations of alcohol in a tissue processor (Leica Biosystems), to dehydrate the tissues. In the processor, the tissues were introduced to xylene to clear the tissue from an alcohol-rich setting to a hydrophobic environment compatible with paraffin wax. Then the tissues were infiltrated with molten paraffin in a Shandon Hypercenter XP Tissue Processor (GMI, USA) and then embedded in a paraffin-filled cassette mold which forms a paraffin block that is manageable on the microtome for tissue sectioning. This is left to solidify on. A microtome was then used to cut the tissue block into 5 µm-thick sections and the sections were placed into a 45°C water bath to allow for the tissue sections to flatten so that the tissue sections could be placed onto the microscope slides. The tissue sections are then transferred onto microscope slides and dried by placing the slides onto a heating block at 65°C for 1 minute.

2.9.2 Dewaxing

The mounted tissue sections were then dewaxed and rehydrated to facilitate antibody penetration, which requires a hydrophilic environment, thereby staining the tissue effectively. To dewax the tissue sections, the slides were first placed on a heating block at 60°C for 15 minutes to soften the paraffin wax and then treated with xylene for 15 minutes at room temperature to

dissolve the wax. The slides were then passed through decreasing concentrations of ethanol (100%, 90%, and 70%) to rehydrate the tissues. In this step, the slides were left in 100% ethanol for 8 minutes followed by passing the slides in 90% and then 70% of ethanol for 4 minutes each. The slides were then rinsed by placing the slides in autoclaved distilled water for 10 minutes.

2.9.3 Tissue staining

List of antibodies

Table 2.12 lists all the antibodies primary and secondary antibodies used for tissue staining.

Type of Antibody	Antibody	Company
Primary	Rat anti-mouse B220	BD Biosciences
	Rabbit anti-CD3	DAKO
	Tritc-conjugated PNA	Sigma
Secondary	Anti-Rat Far-red-labelled IgG	Sigma Chemical Co
	Anti-Rabbit Alexa Fluor 594-labelled IgG	Invitrogen

Table 2.12. Antibody information for IHC staining.

Staining protocol

Prior to staining, the prepared tissue section slides were incubated for an hour with a blocking solution to inhibit non-specific binding of antibodies to the tissue sections. After blocking the tissue sections, primary antibodies were diluted with an appropriate dilution. To the tissue sections, Rat anti-mouse B220, Rabbit anti-CD3 and Tritc-conjugated PNA were added carefully to the slides. The slides were then incubated at 4^{o}C overnight. The following day, the slides were washed for 10 minutes, three times, using PBS. The slides were then introduced to the fluorescently labelled secondary antibodies. The Anti-Rat Far-red-labelled IgG was used to detect the Rat anti-mouse B220 and the Anti-Rabbit Alexa Fluor 594-labelled IgG was used to detect the Rabbit anti-CD3. The slides were incubated for 2 hours at room temperature with Anti-Rat Far-red-labelled IgG and Anti-Rabbit Alexa Fluor 594-labelled IgG. After the incubation period, the slides were rewashed thrice in PBS for 10 minutes per wash.

2.9.4 Fluorescence microscopy and imaging

The slides were then mounted before examining under the fluorescence microscope. This step involved adding drops of Vectasheild medium (Vector Laboratories, Burlingame, CA) to reduce damage to the tissue sections during the imaging process. The slides were then examined using the confocal fluorescence microscope.

2.10 Bulk RNA-seq

In this study, RNA-seq was used to reveal the transcriptome of the newly discovered MP Tfh cell population found within Egr2-GFP/AmCyan-T-bet knockin mice raised under SPF conditions. Due to the well-known heterogeneity of T cells, techniques such as RNA-seq are of great importance to accurately immunophenotype subpopulations of T cells in addition to using flow cytometry for such purposes. A major advantage of RNA-seq over flow cytometry is that flow cytometry limits the number of markers used for phenotyping because of the restriction of fluorophores that can be used in a single run without spectral overlap. Thus, to paint a better picture of the gene expression profile of MP Tfh cells, we used bulk RNA sequencing which measures the average expression of genes across all cells within a the purified cell sample of interest (Valkiers *et al.*, 2022). In addition, the gene expression profiles can be translated to understand the functional characteristics of the cells.

The following sections describe adopted RNA-seq protocol from RNA extraction, library preparation, sequencing and finally the data analysis.

2.10.1 Sample preparation

RNA-seq was used to determine the gene expression profile of MP Tfh cells. We sorted $FR4^+$ $EGR2^+$ $CD4^+$ $CD44^{high}$ $CD25^-$ MP T cells, $FR4^+$ $EGR2^-$ $CD4^+$ $CD44^{high}$ $CD25^-$ MP T cells and $FR4^-$ $EGR2^-$ $CD4^+$ $CD44^{high}$ $CD25^-$ MP T cells from Egr2-GFP/AmCyan-T-bet knockin mice under homeostatic conditions. RNA was isolated and the concentration was quantified using RNeasy UCP Micro Kit (Qiagen) and Qubit with an RNA HS reagent kit (Thermo Scientific), respectively, as described in section 2.6.1.

2.10.2 Library preparation and RNA-seq

The RNA integrity was assessed using the Agilent 4200 Tapestation 2100 Bioanalyzer (Agilent Technologies), of which samples with a median error < ±0.4 RIN units were considered for processing for the library preparation. The RNA samples were processed using the Kapa mRNA HyperPrep Kit (Roche), following the manufacturer's protocol. As mRNA provides the information required for gene expression analysis, the first step involves the isolation of mRNA. The mRNA was isolated from 50 ng-1 g of intact total RNA by two rounds of incubation with poly-T oligo magnetic capture beads, ensuring complete hybridizations of the poly-A tail of the mRNAs to the magnetic capture beads. This ensures a greater level of mRNA enrichment. After

this, the mRNA enriched was resuspended in 1X Fragment, Prime and Elute Buffer and introduced to high temperatures, in the presence of magnesium, to fragment the mRNA. The high temperatures also dehybridise the mRNA from the poly-T oligo beads which is then followed by elution and then priming of the fragmented mRNA to random primers in preparation for cDNA synthesis. The first cDNA strand was synthesized using reverse transcriptase, random primers, and the mRNA template strand. This was followed by the second cDNA strand synthesis using DNA polymerase and then priming the double-stranded cDNA with a 3' adenine overhang in preparation for adaptor ligation. After A-tailing, the IDT xGen UDI-UMI adapters (Integrated DNA Technologies) were ligated to the cDNA and then subjected to amplification by PCR to generate a sequencing library. The UDI-UMI adapters consist of an 8 bp sample index and an 8 bp unique molecular identifiers (UMIs) which allow for multiplexing onto a single flow cell and reduce technical bias from PCR duplicates, respectively. The library was then used for 75 bp single-end sequencing using the Illumina NextSeq 500 platform. The library preparation and sequencing steps were carried out by UCL genomics.

2.10.3 RNA-seq data analysis workflow

The RNA-seq analysis pipeline, as illustrated in Figure 2.5, typically involves pre-processing and aligning the raw sequenced reads to reference genome after which the gene expression is quantified and used for differential expression analysis and downstream analysis to gain more meaningful insights of the results (Kukurba and Montgomery, 2015). RNA-Seq analysis can be quite complex due to the technical artifacts introduced by RNA sequencing technologies during library preparation and sequencing. Thus, computational methods are required to correct for these so that the gene expression levels measured represent true biological variations (Deshpande *et al.*, 2023). The tools and packages used to process and quantify the data for downstream analysis are described in the sections below.

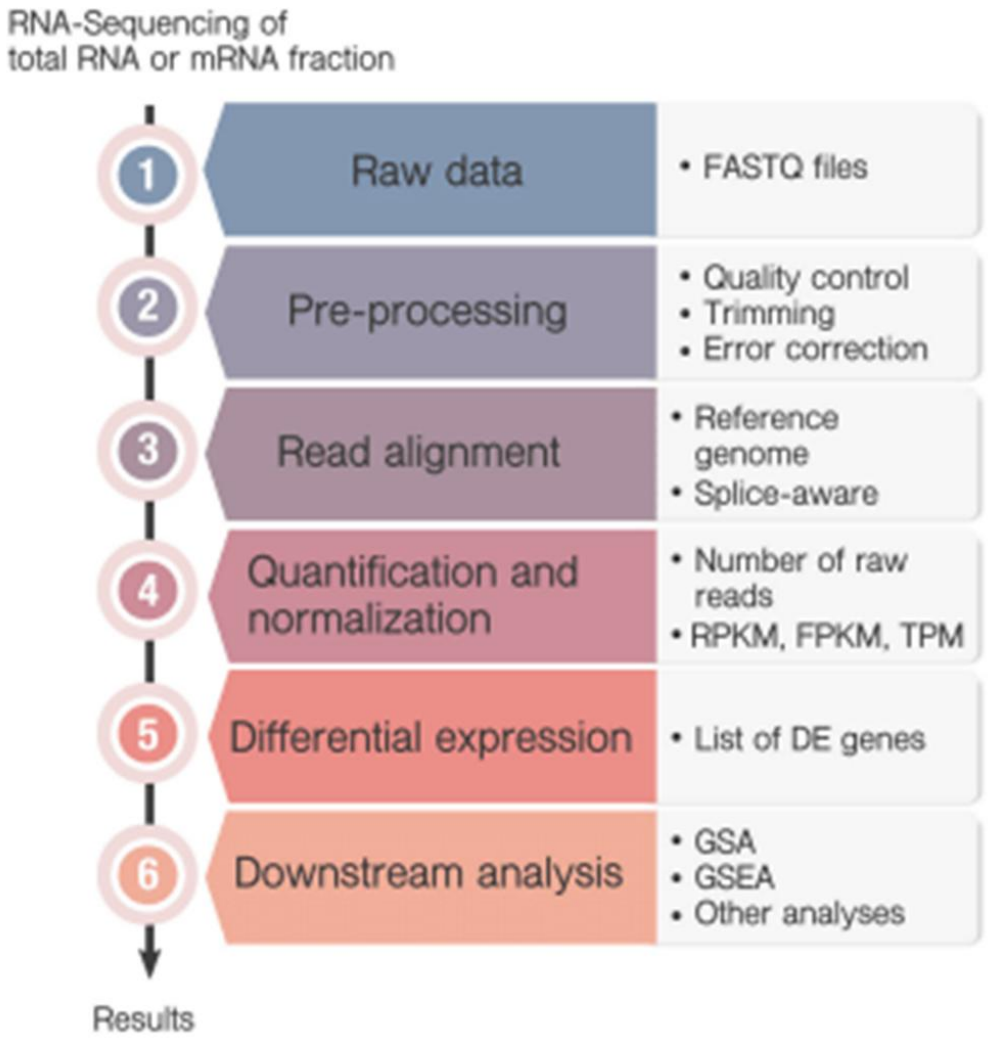


Figure 2.5- RNA Sequencing (RNA-Seq) data analysis workflow. The diagram illustrates the steps followed after sequencing to analyse RNA-Seq data. (1) Firstly, the sequenced raw reads are stored in FASTQ format prior to processing. (2) The preprocessing steps include quality control (QC), trimming and correction for errors. (3) In step 3, the reads are aligned to a reference genome using spliced-aligner tools. (4) After this, the gene expression is quantified based on the amount of reads mapped to per gene and then subjected to normalised using metrics such as Reads Per Kilobase Million (RPKM), Fragments Per Kilobase Million (FPKM) or Transcripts per Kilobase (TPM). (5) Then the differential expression (DE) analysis is carried out to identify the differentially expressed between different sample groups or conditions such as infection versus non-infection groups. (6) Finally, the list of DE genes is interpreted through downstream analysis which includes the Gene Set Enrichment Analysis (GSEA) which aims to find the insights of the biological processes influenced by the results (Martínez-Pérez, Estévez and González-Fernández, 2022).

2.10.3.1 Read mapping

The sequencing reads were then demultiplexed and converted to fastq file format using Illumina's bcl2fastq Conversion Software v2.20. Demultiplexing is a critical step in the bcl2fastq conversion process as it assigns reads, based on the index barcode sequences (UDI adapters),

to their sample generating fastq files per sample. Next, the sequenced reads were aligned to the mm10 assembled mouse reference genome. Spliced junctions are sequences at the exon-intron boundary at the point of RNA splicing. Sequenced reads that span across spliced junctions present a challenge for traditional alignment tools to map the reads against non-spliced reference genomes thereby causing misalignments or failure to align such reads. Thus, several spliced aligner tools such as STAR and TopHat have been designed to overcome this issue and have been tested for their efficiency. In this study, the reads were mapped using the spliced aligner HISAT2 version 2.2.0 which has better performance at a faster rate compared to other alignment tools (Kim *et al.*, 2015; Kukurba and Montgomery, 2015). The output alignment from HISAT2 mapping was produced in a SAM file format and then processed for the removal of secondary alignment using Samtools version 1.1.2 (Li *et al.*, 2009). Further processing was carried out to remove the PCR duplicate reads using UMI barcode information with the UMI-tools package version 1.1.2 (Smith *et al.*, 2017). The gene expression, based on the number of reads mapped per gene, was then quantified using the featureCounts function of the Rsubread package version 2.4.3 (Liao *et al.*, 2019) of the R programming language version 4.0.2 (The R foundation, 2021).

2.10.3.2 Quality control and differential gene expression

The following RNA seq data analysis was carried out using the R programming language version 4.1.0 (The R foundation, 2021) and RStudio version 2022.02.3 (RStudio Team, 2023). The edgeR package was used for quality control and to measure differential gene expression (Robinson *et al.*, 2010). The RNA seq reads were first filtered to remove genes of low quality or null expression that serve no biological importance. Aside from PCR duplicates, technical artifacts such as the variations in library size pose a challenge in detecting true meaningful gene expression differences. Thus, the RNA seq data was normalised using the trimmed mean of M-values (TMM) method after which the MDS plot was generated to ensure the similarity of the replicates and clustering of sample groups. The dispersion was then estimated and the negative binomial generalized linear model was fitted. To determine the differential gene expression, contrasts were designed and the differentially expressed genes between contrasting groups were calculated using the quasi-likelihood F test (Lun *et al.*, 2016). The following thresholds were set for a gene to be considered significantly different in terms of its expression: A false discovery rate (FDR) value of less than or equal to 0.05 and an absolute fold change greater than or equal to 1.5.

2.10.3.3 Downstream differential gene expression analysis

Heatmap.

Using the DESeq2 package version 1.30.1, a variance stabilizing transformation was applied to normalize the dataset required for the visualization of gene expression across samples using techniques such as heatmaps (Love *et al.*, 2014). The raw reads were standardized by calculating the z score for each gene and then genes of similar expression were clustered together using 1- Pearson correlation as a distance metric. The differentially expressed genes across samples were visualised using the ComplexHeatmap package version 2.6.2 (Gu *et al.*, 2016).

Principal component analysis (PCA).

PCA is a dimensionality reduction method that simplifies the dataset by identifying the principal components (PCs) that represent the most variability. PCA was applied to the normalised data using the DeSeq package version 1.30.1 (Love, Huber and Anders, 2014) and the clustering of the data captured by PC1 and PC2 were visualised using the ggplot2 package, version 3.3.6 (Wickham, 2016).

Venn diagram.

Venn diagram was used to determine the shared and unique upregulated and downregulated genes of different contrasting groups. The differentially expressed genes from the contrast groups created in edgeR analysis were used to generate Venn diagrams using the vennDiagram() function in the limma package version 3.50.3 (Ritchie *et al.*, 2015). The gene lists at specific intersections of interest were extracted and exported as Excel files for downstream visualisation on the dot plots.

Dot plot.

The normalised data through the DeSeq package was used to generate dot plots to visualise the expression of selected genes of interest across three sample groups. The gene list was obtained through the Venn diagram intersection points of interest between contrasting groups (supplementary tables 1,2 and 3). Gene annotations, such as the gene symbols and Entrez IDs, were applied to the DESeq2 normalized data using the org.Mm.eg.db package version 3.14.0 (Carlson, 2023). The gene expression patterns for a given sample, including replicates, were represented by a single dot on the dot plot generated using the ggplot2 package, version 3.3.6 (Wickham, 2016).

Gene Set Enrichment Analysis (GSEA).

GSEA plots the differences between a group of genes with a known biological function across the samples, instead of individual genes as visualized using heatmaps and dot plots. For function annotation of the genes, we used the msigdbr package version 7.5.1 (Dolgalev, 2022) to retrieve the immunological and hallmark mouse Molecular Signatures Database (MSigDB) gene sets (Liberzon *et al.*, 2015). To the immunological dataset, we incorporated gene sets generated from memory Tfh cells, memory Th1, and central memory T cells (Tcm) by Kunzli and colleagues (Künzli *et al.*, 2020). From the gene expression omnibus (GEO) database, we downloaded the filtered UMI matrix with accession number of GSE134157. We then applied similar scRNA-seq) analysis methods using R as described by Kunzli and colleagues (Künzli *et al.*, 2020). ScRNA-seq analysis quantifies the gene expression differences between individual cells in a population, in this case among the $CD4^+$ memory T cell compartment. Subsequent analysis steps applied included the normalisation of the dataset using the deconvolution method of the Scran package (Version 1.30.2) in R (version 4.3.2) (Lun, McCarthy and Marioni, 2016). The scran package was also used to remove technical artifacts and select the top 500 highly variable genes. PCA was then performed using the top 500 biologically relevant genes to filter out the identified technical variance whilst retaining biologically meaningful data. The Tfh memory cluster was defined by the highest expression of FR4, PD-1 and CXCR5 encoding by *Izumo1r*, *Pdcd1* and *Cxcr5* genes, respectively. Two clusters of Tcm cells were characterised by the expression of *Il7r* and *Itgb1* but reduced *Izumo1r* levels. The cluster with phenotypic characteristics of both memory Tfh and Tcm cells was excluded from the analysis. The remaining two clusters of Th1 memory cells were identified by the greatest *Cxcr6* and *Ccl15* expressions. The single-cell clusters were used to generate a pseudo bulk samples in which the average gene expression is measured to provide a bulk RNA-seq like gene expression for a specific cell type for comparison across samples. The edgeR package version 4.0.16 was then used to measure the differential gene expression by comparisons of memory Tfh cells gene set against Tcm and memory Th1 cells (Robinson, McCarthy and Smyth, 2010; Lun, Chen and Smyth, 2016). Then for functional annotation, the top 200 differentially expressed genes between the comparison groups stated above, were then included within the immunological signature MSigDB gene set, referred on the GSEA plot in Figure 3.18 as ‘MEMORY TFH VS TCM DOWN’, ‘MEMORY TFH VS TCM UP’, ‘MEMORY TFH VS TH1 DOWN’ and ‘MEMORY TFH VS TH1 UP’.

The clusterProfiler (version 3.18.1) (Yu *et al.*, 2012) and the fgsea (version 1.16.0) (Korotkevich *et al.*, 2021) packages were used to perform pre-ranked GSEA, in which the hallmark and immunological gene sets are tested against a pre-ranked gene list. The ranking metric used is the quasi-likelihood F test statistic, calculated in edgeR, multiplied by the positive or negative sign of the log2 fold change, so that the ranked list consists of strongly upregulated genes at the top end and the downregulated genes further down the ranked list. In addition, to the immunological datasets mentioned previously, Figure 3.18 also includes the 'GSE40068_BCL6_POS_VS_NEG_CXCR5_POS_TFH_UP', denoted as 'Bcl6 pos vs neg UP'. And the 'GSE40068_BCL6_POS_VS_NEG_CXCR5_POS_TFH_DOWN' gene set is abbreviated as 'Bcl6 pos vs neg DOWN'. These gene sets are from Liu and colleagues (Liu *et al.*, 2012).

2.11 Statistics

The statistical analysis was conducted through R (version 4.3.2). The quartiles (upper quartile, median and lower quartile) were calculated for each group. Non-parametric tests were used to determine the statistically significant differences between groups. This approach was considered due to the small size of the samples. For comparisons between two groups, the two-tailed Mann-Whitney U tests were performed using the R package coin (Hothorn *et al.*, 2008). For comparisons between multiple groups, the Kruskal-Wallis tests was employed followed by Conover tests, of the R package PMCMRplus (Pohlert, 2024), for pair-wise comparison to determine the group pairs with significance differences. This was followed by the Benjamini-Hochberg method to correct for multiple comparisons by adjusting p values to control the false discovery rate (FDR). Differences with a p value <0.05 were considered to be significantly significant. All data were included within the analysis. The sample sizes and number of experimental repeats are indicated in the respective figure legends along with statistical methods employed.

Chapter 3

Results

3.1 Validation of GFP-Egr2/AmCyan-T-bet Knockin (Kin) and CD2-Egr2/3$^{-/-}$/AmCyan-T-bet knockout (KO) reporter mice and, the expression of GFP-EGR2 and AmCyan-T-bet in CD4^{+} T cells

MP T cells can be developed from naïve T cell precursors without overt antigen stimulation. Thus, the memory T cell pool consists of memory T cells generated by overt pathogenic antigen stimulation and MP T cells (White, Cross and Kedl, 2017; Kawabe and Sher, 2021). Following aging, MP T cells and pathogen-induced memory T cells accumulate and become a major T cell population with a decline in naïve T cells (Sprent and Surh, 2011; Nikolich-˘ Zugich, 2014). MP T cells have been found to have an important role in both T cell homeostasis and amplifying the immune response (White, Cross and Kedl, 2017; Kawabe and Sher, 2021; Seok *et al.*, 2023). CD4^{+} MP T cells are a heterogeneous population at steady state. However, the mechanisms for the development of these cells and their function in innate and adaptive immune responses against various diseases are largely unknown. Recently, our group found that the transcription factor, EGR2, positively regulates homeostasis and controls the inflammatory response of PD-1^{high} CD4^{+} MP T cells (Symonds *et al.*, 2020). EGR2, and its homolog EGR3, have overlapping regulatory functions towards preventing inflammatory pathology during antigen-specific immune responses and autoimmune disorders (Li *et al.*, 2012). Our group further found that EGR2 suppresses pathogen-induced T helper cell polarisation towards a Th1 phenotype by direct inhibition of the Th1 transcription factor T-bet and, suppression of the STAT1/IFN-γ pathway which mediates the production of Th1 cytokines (Li *et al.*, 2012; Singh *et al.*, 2017). In line with this, PD-1^{high} MP T cells, with defective Egr2 expression exhibited increased levels of T-bet and IFN-γ production to *in vitro* IL-12 stimulation (Symonds *et al.*, 2020). Kawabe and Colleagues have extensively studied the differentiation, at homeostasis, and function of T-bet^{high} CD4^{+} MP T cells which exert antigen non-specific innate-like Th1 response against *Toxoplasma gondii* infection (Kawabe *et al.*, 2017a, 2020). To understand the function of these two transcription factors in MP CD4^{+} T cells in real-time, we established reporter mice of GFP-Egr2/ AmCyan-T-bet mice and CD2-Egr2/3$^{-/-}$/AmCyan-T-bet knockout mice.

The *GFP-Egr2* reporter gene was constructed by the fusion of the *GFP* gene at the N-terminus of the *Egr2* gene, translated as a single GFP-EGR2 fusion protein, illustrated in Figure 3.1A. Through homologous recombination, the *GFP-Egr2* construct was introduced into the *Egr2* locus embryonic stem cells to form knockin mice, as described in our previous publication (Miao *et al.*, 2017). The GFP-Egr2 knock-in mice are crossbred with AmCyan-T-bet reporter mice (Yu

et al., 2015) under the same C57BL background to generate double reporter mice. This allows the visualisation of both T-bet and EGR2 expression in $CD4^+$ MP T cells under flow cytometry.

CD2-Egr2/3$^{-/-}$ /AmCyan-T-bet reporter mice are established by crossbreeding the AmCyan-T-bet with the CD2-Egr2/3$^{-/-}$ mice. CD2-Egr2/3$^{-/-}$ mice were previously established by our group (Li *et al.*, 2012). The Cre-loxP system generated EGR2 deletion in CD2-expressing T cells as whole genome deletion of EGR2 is fatal because EGR2 is required for hindbrain development (Swiatek and Gridley, 1993). Whereas EGR3 was deleted from the whole genome.

The genotypes of the established GFP-Egr2/Amcyan-T-bet and CD2-Egr2/3$^{-/-}$ /AmCyan-T-bet mice are identified by PCR (Figure 3.1, Figure 3.2). This method allows the selection of true GFP-Egr2/Amcyan-T-bet and Amcyan-T-bet/Egr2/3$^{-/-}$ mice. The selected mice are then used for further study.

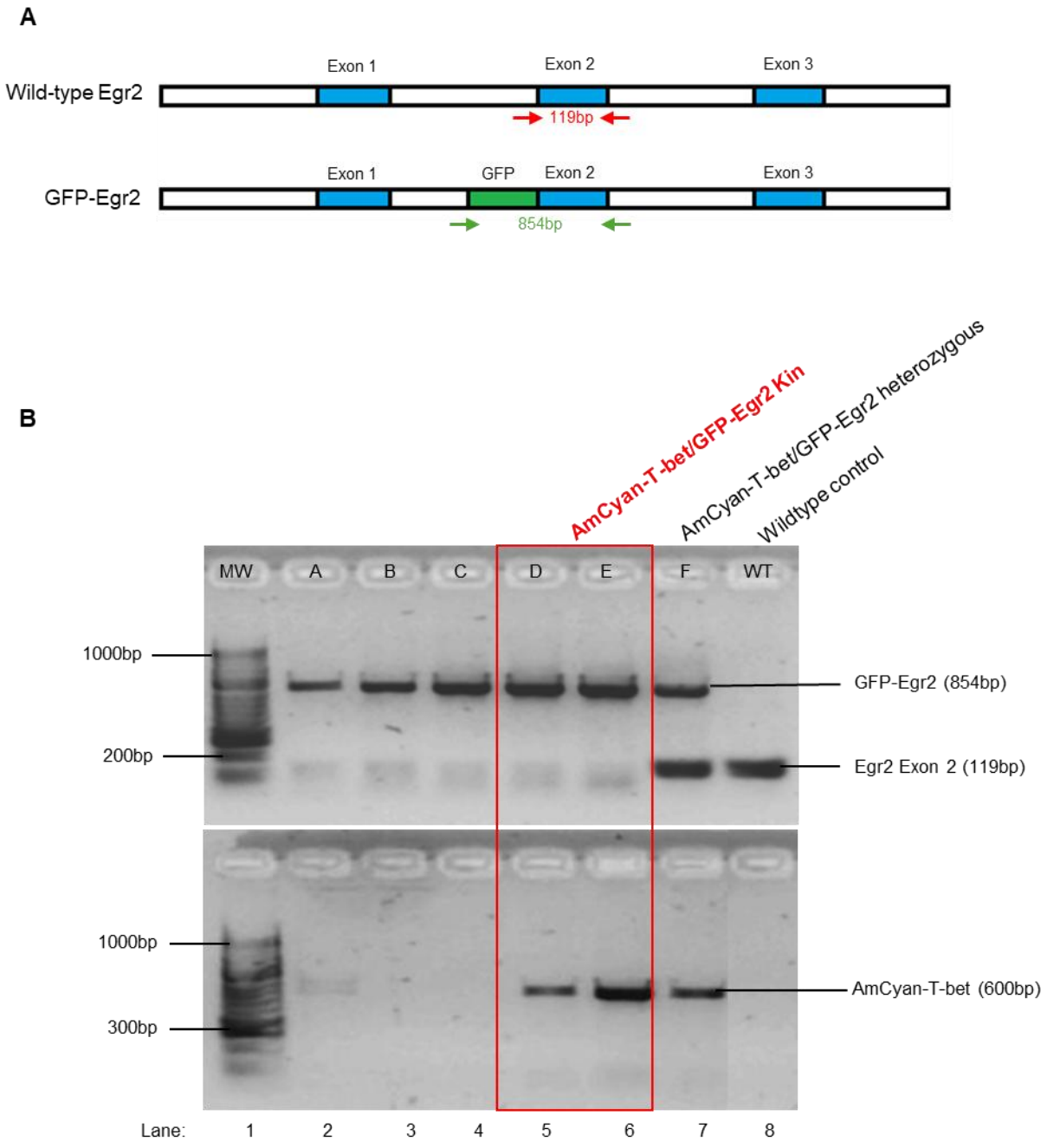


Figure 3.1. Genotyping strategy for GFP-Egr2/AmCyan-T-bet mice. (A) Schematic representation of the exonic structure of the wild-type (WT) and the *GFP-Egr2* constructs used for genotyping. The exons are shown in blue and the GFP insertion into the *Egr2* gene is shown in green. The red arrows indicate the position of the primer (sense/antisense) sequences used to amplify the 199 bp fragment of the *WT* allele whereas, the green arrows represent the primers (sense/antisense) positioning for amplifying an 854 bp fragment of the *GFP-Egr2* allele. **(B)**

PCR gels from genotyping the offspring of AmCyan-T-bet and GFP-Egr2 mice. The GFP-Egr2 Knockin and AmCyan-T-bet transgenic mice were cross-bred, and the ear DNA of the offspring was genotyped by PCR to select AmCyan-T-bet/GFP-Egr2 mice for further study (See Chapter 2- Materials and methods). The representative PCR results are of 6 offspring (Lanes 2-7) and a wildtype (WT) control (Lane 8). The gel image shows distinct bands at 119 bp corresponding to the *Egr2* Exon 2 allele, an 854 bp PCR product indicating the presence of the *GFP-Egr2* allele, and a 600 bp band corresponding to the *AmCyan-T-bet* allele. Samples D and E, highlighted in red, represent true AmCyan-T-bet/GFP-Egr2 Knockin (Kin) mice. Sample F is heterozygous for *GFP-Egr2*, and samples A-C are homozygous for the GFP-Egr2 Knockin. Only true AmCyan-T-bet/GFP-Egr2 Kin mice were selected for further experiments.

As indicated in Figure 3.1B, the PCR products for the *GFP-Egr2* Knockin allele is around 845 bp while the *WT Egr2* allele produces a lower band of around 119 bp in size. Lane 7 shows both GFP-EGR2 and WT-EGR2 PCR products and thus represents an offspring heterozygous for GFP-EGR2. Whereas lanes 2-6 represent mice homozygous for GFP-EGR2, in which only *GFP-Egr2* allele was detected but not the *WT-Egr2* allele. The AmCyan-T-bet mice are a transgenic model. The AmCyan transgene was indicated by a specific product of 600 bp. Therefore, the mice carrying *GFP-Egr2* and *AmCyan-T-bet* showed genotype of lanes 5 and 6. The WT control displaying a single band for *WT-Egr2* allele confirms the specificity of the primers and the validity of the PCR experiments conducted.

Figure 3.2 illustrates the PCR products obtained from genotyping the offspring of breeding the Amcyan-T-bet with the CD2-Egr2/3$^{-/-}$ mice to generate the desired CD2-Egr2/3$^{-/-}$/AmCyan-T-bet reporter mice. The Cre-LoxP system was used for conditional deletion of EGR2 in T cells and B cells. The exon 2 of *Egr2* gene was floxed by LoxP, a 34 bp nucleotide sequence, for excision by Cre-recombinase induced in lymphocytes through CD2 promoter which is expressed in T and B cells. However, this model only completely deletes EGR2 in T cells, but its expression is still observed in B cells (Zhu *et al.*, 2008). The PCR product generated by *CD2-Cre* and *LoxP* genes was 133 bp and 195 bp, respectively. The EGR3 knockout was detected by the *Neomycin resistance cassette (Neo)* gene of around 700 bp which deleted the zinc finger functional domain of exon 2. Egr3 heterozygous knockout mice generated a PCR product of both the *Egr3 WT* allele of 360 bp and the *Neo* gene. The genotype of homozygous Egr3 Knockout, CD2-Cre gene, LoxP floxed Egr2, and AmCyan-T-bet transgene is shown in lanes 2, 4 and 7 representing true CD2-Egr2/3$^{-/-}$/AmCyan-T-bet knockout mice.

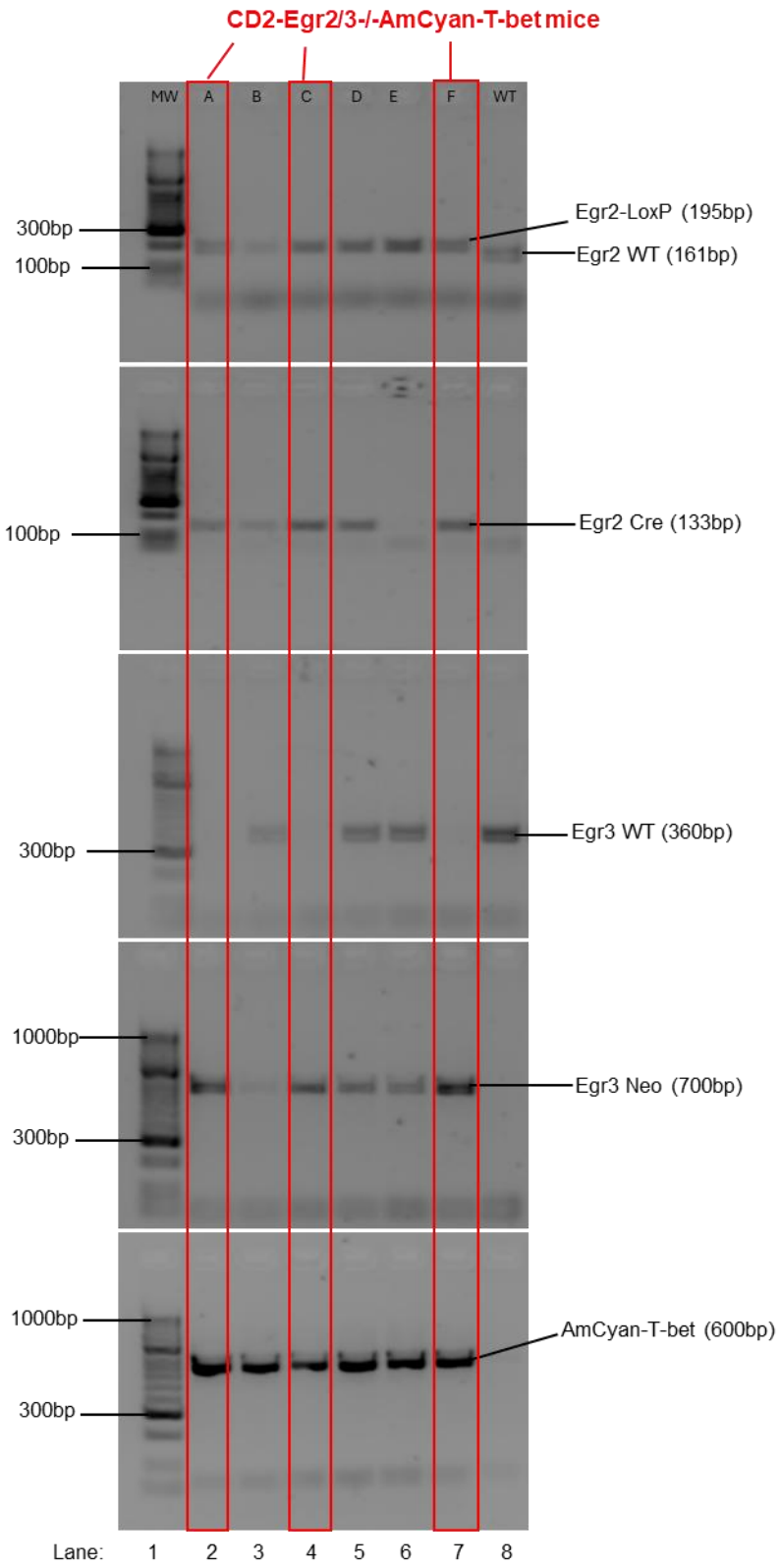

CD2-Egr2/3-/-AmCyan-T-bet mice
MW
A
B
C
D
E
F
WT
300bp
100bp
Egr2-LoxP (195bp)
Egr2 WT (161bp)
100bp
Egr2 Cre (133bp)
300bp
Egr3 WT (360bp)
1000bp
300bp
Egr3 Neo (700bp)
1000bp
300bp
AmCyan-T-bet (600bp)
Lane:
1
2
3
4
5
6
7
8

Figure 3.2. Genotyping of CD2-Egr2/3$^{-/-}$/AmCyan-T-bet knockout mice. CD2-Egr2$^{-/-}$Egr3$^{-/-}$ knockout mice were cross-bred with AmCyan-T-bet transgenic mice. The ear DNA of the offspring were genotyped by PCR (See Chapter 2- Material and methods). The representative PCR results are of 6 offspring (Lanes 2-7) and a wildtype (WT) control (Lane 8), with 5 separate PCRs conducted against specific primers for *Egr2-LoxP*, *Egr2 WT*, *Egr2 Cre*, *Egr3 WT*, *Egr3 Neomycin Cassette (Egr3 Neo)* and *AmCyan* alleles. Highlighted in red (Samples A, C, F) are true CD2-Egr2/3$^{-/-}$ /AmCyan-T-bet mice with distinct bands for *Egr2-LoxP, Egr2 Cre, Egr3 Neo* and *AmCyan* alleles. Samples B and D are Egr3 heterozygous, with a band for *Egr3 WT* and *Egr3 Neo* alleles. Sample E is of Egr2 floxed mice with only *LoxP* alleles. WT control shows bands for *Egr2 WT* and *Egr3 WT* alleles. Only true CD2-Egr2/3$^{-/-}$/AmCyan-T-bet mice were used for further study.

To confirm EGR2 and T-bet expression in CD4$^+$ T cells from the established GFP-Egr2/AmCyan-T-bet knockin mice, GFP-EGR2 and AmCyan-T-bet fluorescence was assessed by flow cytometry. Figure 3.3A shows that, without stimulation, majority (~90%) of the CD4$^+$ T cells do not express GFP-EGR2 nor AmCyan-T-bet. The low expression of EGR2 and T-bet in around 3% or 3.8% of CD4$^+$ T cells, respectively, could represent thymus-derived T regulatory cells or CD4$^+$ MP T cells (Figure 3.3A). This is consistent with previous reports showing that EGR2 is preferentially expressed in regulatory and anergic/memory-like CD4$^+$ T cells (Miao et al., 2017) and a small proportion of Treg or activated CD4$^+$ T cells express T-bet under steady-state conditions (Yu et al., 2015). Upon TCR stimulation, the expression of T-bet and EGR2 increases in activated naïve CD4$^+$ T cells (Anderson et al., 2006). Consistent with this finding, GFP-EGR2 and AmCyan-T-bet expression were highly detectable in stimulated CD4$^+$ T cells with anti-CD3 and anti-CD28 in vitro (Figure 3.3B). Of the CD4$^+$ T cells, 47.3% expressed only GFP-EGR2 and the 42.1% of T-bet expressing CD4$^+$ T cells co-expressed EGR2, with a few CD4$^+$ T cells expressing only AmCyan-T-bet (~1.7%). These results demonstrate that the reporter mice are successfully established with the targeted phenotypes.

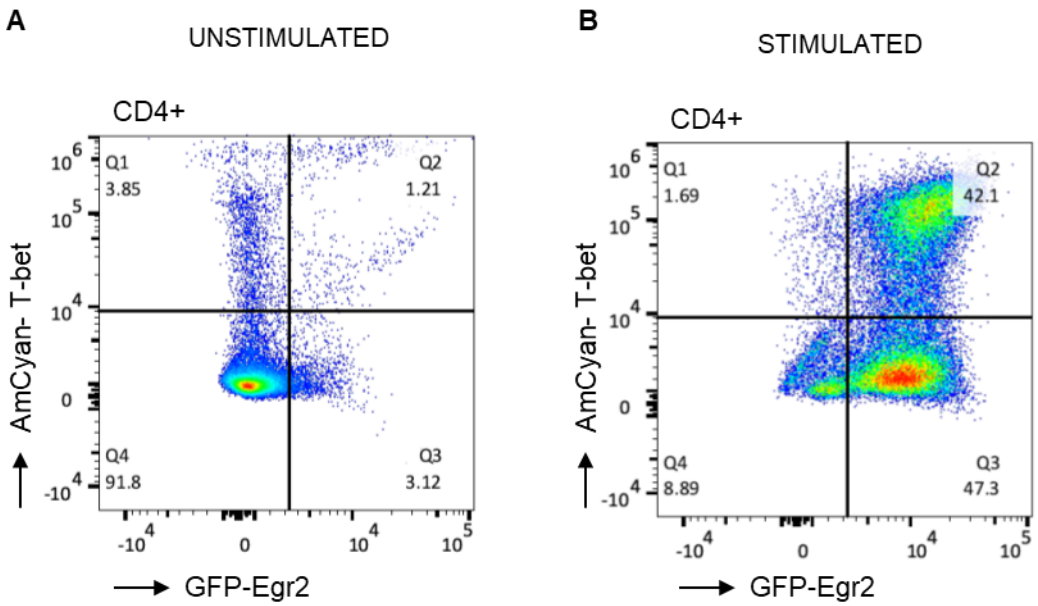


Figure 3.3. The expression of GFP-EGR2 and AmCyan-T-bet in CD4⁺ T cells from GFP-Egr2/ AmCyan-T-bet knockin mice. AmCyan-T-bet and GFP-EGR2 expression from CD4⁺ T cells was analysed by flow cytometry before stimulation (A) and after *in vitro* stimulation with anti-CD3 and anti-CD28 (B). The data were from four mice of three repeated experiments.

3.2 The development of CD4$^+$ MP T cells in specific pathogen-free (SPF) conditions and their expression of GFP-EGR2 and AmCyan-T-bet.

In unimmunised mice raised under GF or SPF or AF conditions, MP T cells can be generated directly from naïve T cells without overt antigen stimulation. These MP T cells can be maintained through homeostatic proliferation (Kawabe and Sher, 2021; Kawabe, 2023). Although CD44^{high} CD4$^+$ MP T cells differ from foreign-antigen-induced memory T cells in terms of their generation and maintenance, they remain similar in their phenotype (Kawabe and Sher, 2021). Pathogen-induced memory CD4$^+$ T cells are typically characterised as CD44^{high} and CD62L$^{high/low}$ whereas naïve CD4$^+$ T cells highly express CD62L but not CD44, an activation marker (Kawabe, Yi and Sprent, 2021). Both MP and pathogen-induced memory CD4$^+$ T cells are defined as CD44^{high} CD62L^{low} (Kawabe, 2023). To analyse the phenotypic characteristics of CD4$^+$ MP T cells against cell surface markers of foreign-antigen induced memory CD4$^+$ T cells, we isolated CD4$^+$ MP T cells from GFP-Egr2/Amcyan-T-bet Kin mice raised and maintained under SPF conditions. Since MP T cells constitute a relatively small proportion of total T cells in young mice (approximately 10–20%) but increase in frequency with age (Sprent and Surh, 2011), older mice (12–22 weeks) were used in this study to ensure sufficient numbers of MP T cells for analysis. In unimmunised GFP-Egr2/ Amcyan-T-bet Kin mice, 11% of CD4$^+$ T cells were CD44^{low} naïve while 15% were CD44^{high} MP T cells (Figure 3.4A). These results are consistent with previous reports where similar numbers of MP T cells were found in both GF and SPF mice (Kawabe *et al.*, 2017). To further characterise CD44^{high} CD25$^-$ CD4$^+$ MP T cells we analysed the expression of CD62L. Within the memory T cell compartment, Tcm cells display high levels of CD62L required for homing to lymphoid tissues whereas Tem cells downregulate CD62L to enter the circulation (Gasper, Tejera and Suresh, 2014). We found that around 30% of CD44^{high} CD25$^-$ CD4$^+$ MP T cells expressed CD62L (Figure 3.4B), a phenotype similar to CD4$^+$ Tcm cells developed after pathogen infection (Gasper, Tejera and Suresh, 2014). The majority (~66%) of CD44^{high} CD25$^-$ CD4$^+$ MP T cells exhibit an effector memory CD4$^+$ T cell phenotype (Figure 3.4B). The percentage and phenotype of central and effector memory of CD4$^+$ MP T cells are similar to those of pathogen-induced memory CD4$^+$ T cells (Crotty, 2014), indicating that CD4$^+$ MP T cells and pathogen-induced memory CD4$^+$ T cells constitute the total memory T cell population with similar phenotypes (Crotty, 2014).

CD4$^+$ MP T cells undergo homeostatic proliferation to maintain the T cell pool (Surh and Sprent, 2000). Using Ki67 as a proliferative marker, we found that CD44^{high} CD25$^-$ CD4$^+$ MP T cells predominantly did not express Ki67 (68.8%) while approximately 30% of the CD44^{high} CD25$^-$

$CD4^+$ MP T cells displayed Ki67 expression at steady state (Figure 3.4B). In addition, only a small number (10.8%) of $CD44^{high}$ $CD25^-$ $CD4^+$ MP T cells with a Tcm phenotype expressed Ki67. Relatively, Ki67 expression was twice as high in $CD44^{high}$ $CD25^-$ $CD4^+$ MP T cells with a Tem phenotype (Figure 3.4B). Thus, in unimmunised GFP-Egr2/Amcyan-T-bet Kin mice in SPF conditions, $CD4^+$ MP T cells pool constitutes of Tcm and predominantly of Tem cells with either proliferative capacity or remain in a quiescent state. Thus, $CD4^+$ MP T cells differ from pathogen-induced memory $CD4^+$ T cells which are found in a quiescent state in absence of pathogenic stimulation (Gasper, Tejera and Suresh, 2014). As Tem phenotype dominates (Kawabe and Sher, 2021), we further looked at the expression of Ki67 within $CD44^{high}$ $CD25^-$ $CD62L^{low}$ $CD4^+$ MP T cell pool. Of the total $CD44^{high}$ $CD25^-$ CD4+ MP T cell population with Tem phenotype, 46.7% did not express Ki67 whereas 20.3% of these cells were positive for Ki67 (Figure 3.4B). This supports the finding of $Ki67^{+/-}$ $CD4^+$ MP T cell subsets described by others which define $CD4^+$ MP T cells as $CD62L^{low}$ (Kawabe *et al.*, 2017; Kawabe, 2022). Although, we have shown that the Tem phenotype of $CD4^+$ MP T cells is dominant, most of our gating will include $CD4^+$ MP T cells of both phenotypes, Tem and Tcm, for subsequent analysis. Any differences in gating to isolate the $CD4^+$ MP T cell population is specified in the text.

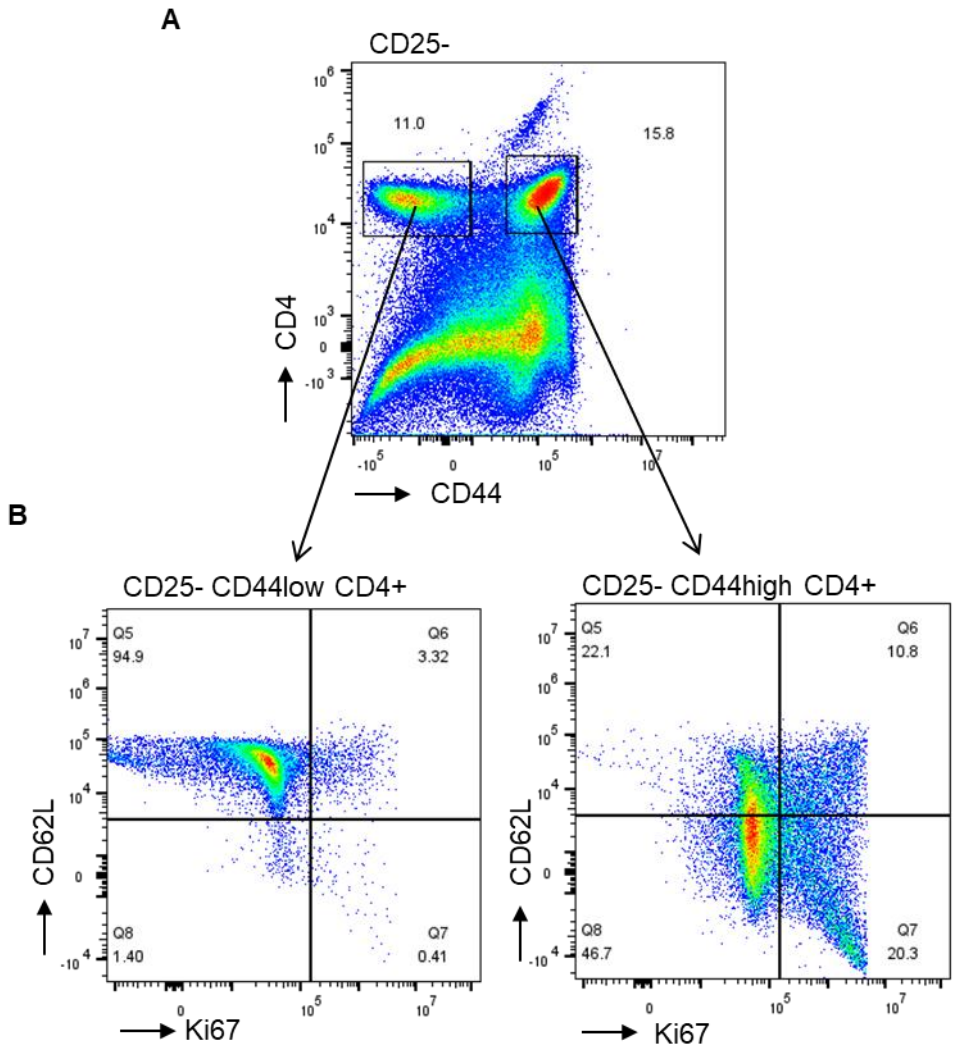


Figure 3.4. Phenotyping of Memory-phenotype (MP) CD4⁺ T cells in steady state. Lymphocytes were isolated from the spleen and lymph nodes of GFP-Egr2/ AmCyan-T-bet mice raised under specific pathogen-free (SPF) conditions and their expression of memory T cell surface markers was analysed by flow cytometry. (A) $CD44^{high}$ $CD4^{+}$ MP T cells were gated from $CD25^{-}$ cells. (B) Then the expression of CD62L and Ki67 was analysed. The data are from four mice and represent three repeated experiments. Data are pre-gated on singlets, lymphocytes, and $CD25^{-}$ cells prior to downstream analysis.

Much like pathogen-induced memory $CD4^{+}$ T cells, MP $CD4^{+}$ T cells have been described as a heterogeneous population by other groups (Charlton *et al.*, 2015; Kawabe *et al.*, 2020; Symonds *et al.*, 2020; Cho *et al.*, 2023). However, there is no clear definitive marker to characterise subsets of MP T cells. We have previously found that Egr2/3 are essential to maintain homeostasis of a subpopulation of $PD1^{high}$ MP T cells similar to conventional $CD4^{+}$ T cells (Symonds *et al.*, 2020). However, we do not know if Egr2 is expressed in all types of $CD4^{+}$ MP

T cells. To analyse the expression of Egr2 in $CD4^+$ MP T cells from GFP-Egr2/ Amcyan-T-bet mice under SPF conditions, we gated for $CD4^+$ T cells then gated $CD44^{low}$ $CD25^-$ naïve T cells and $CD44^{high}$ $CD25^-$ MP T cells (to exclude for Treg-enriched populations) and then analysed the expression of GFP-EGR2 and AmCyan-T-bet. GFP-EGR2 was hardly detected in naïve $CD4^+$ T cells (Figure 3.5). In the MP $CD4^+$ T cell population, around 50% of the cells did not express GFP-EGR2 nor AmCyan-T-bet (Figure 3.5). About 25% only expressed GFP-EGR2. Whereas AmCyan-T-bet was only detected in about 22% of $EGR2^-$ $CD4^+$ MP T cells (Figure 3.5). Very few cells expressed both EGR2 and T-bet. EGR2 and T-bet have been reported to have reciprocal functions in the regulation of homeostasis of conventional $CD4^+$ T cells by counter-regulating the proliferation and inflammation during an immune response. EGR2 is responsible for a suppressive function to control excessive inflammatory and proliferative responses of $CD4^+$ and $CD8^+$ T cells. In the absence of this counter-regulatory control, T-bet becomes dominant promoting elevated inflammatory responses (Li *et al.*, 2012; Singh *et al.*, 2017). The distinct expression of these two transcription factors in MP $CD4^+$ T cells may indicate differences in the regulation of homeostasis and the function of $EGR2^+$ and T-bet^+ MP T cells. Kawabe and colleagues have previously described T-bet^{high} MP T cells as non-specific innate lymphocytes, alike to MP $CD8^+$ T cells, ILCs, and NK cells, during viral infection (Kawabe and Sher, 2021). However, the $EGR2^+$ subset is yet to be defined.

Overall, MP $CD4^+$ T cells have a similar phenotype to pathogen-induced memory T cells and their function may be associated with the roles of Egr2 and T-bet.

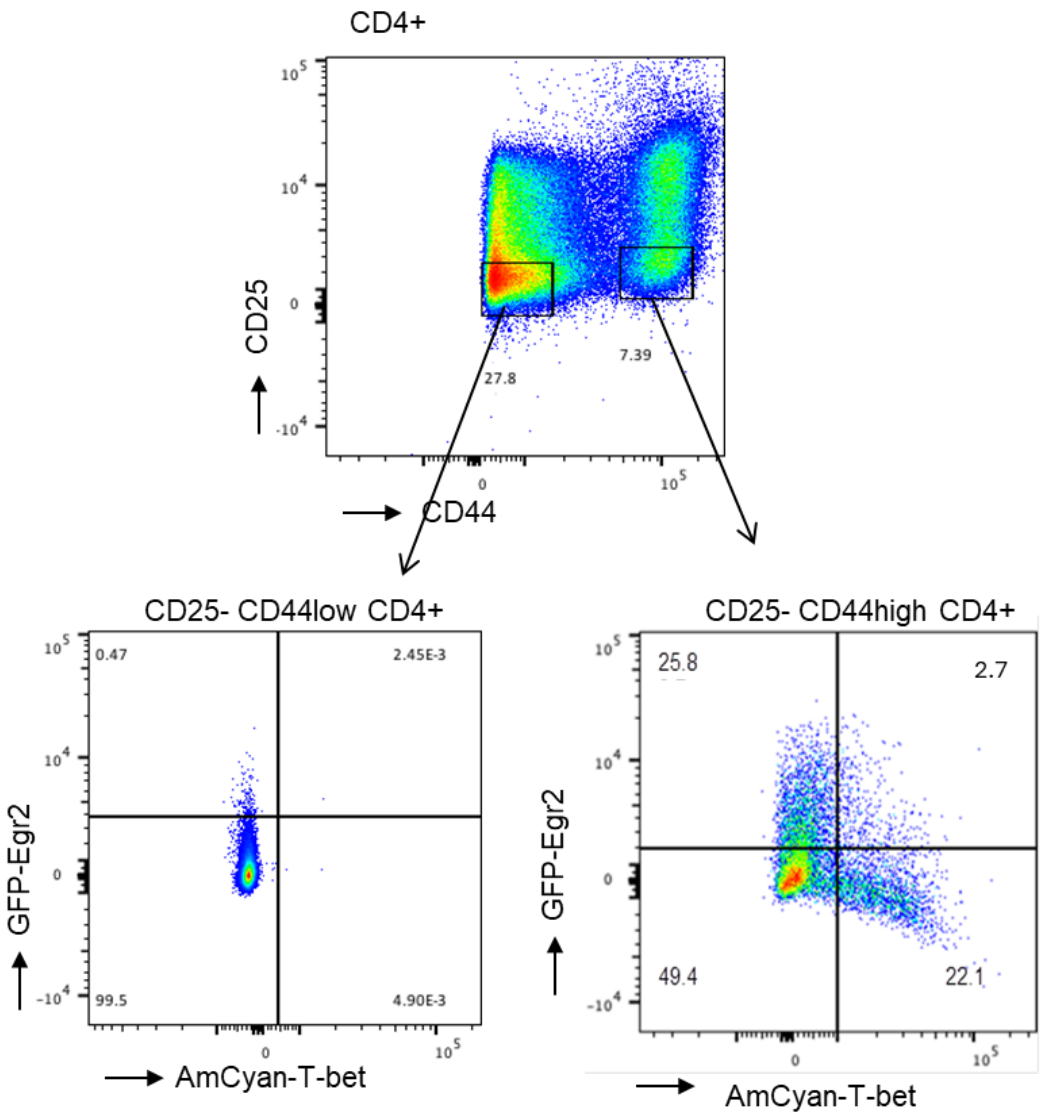
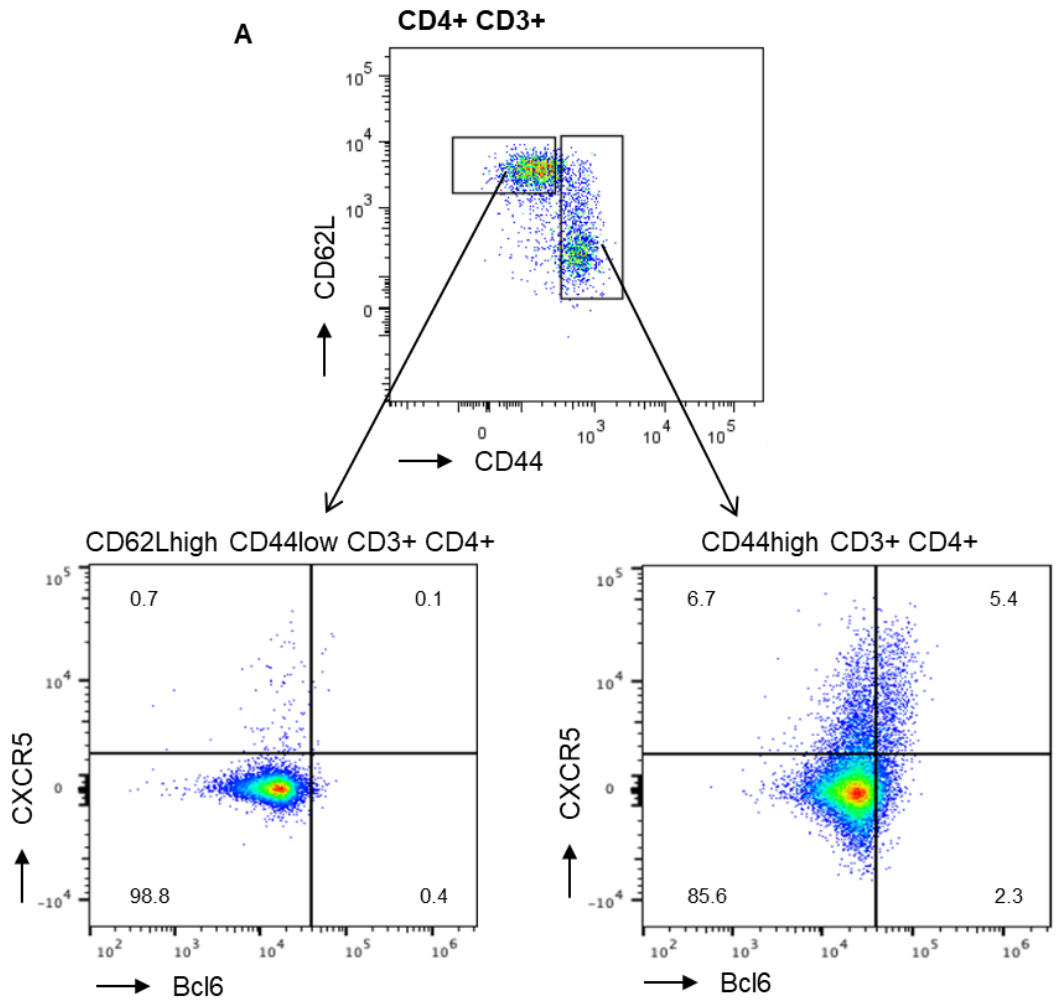


Figure 3.5. GFP-EGR2 and AmCyan-T-bet expression in MP CD4$^+$ T cells in steady conditions. Lymphocytes were isolated from the spleen and lymph nodes of old GFP-Egr2/ AmCyan-T-bet mice raised under specific pathogen-free (SPF) conditions. CD25$^-$ CD44^{low} CD4$^+$ naïve T cells and CD25$^-$ CD44^{high} CD4$^+$ MP T cells were gated and analysed for T-bet and EGR2 positive cells. The data are from four mice and represent three repeated experiments.

3.3 The analysis of MP Tfh cells

3.3.1 Tfh-like MP T cells develop in the absence of foreign antigen stimulation

Pathogen-induced memory Tfh cells recall their GC Tfh effector functions, providing B cell help in response to reinfection (Hale and Ahmed, 2015). In comparison to GC Tfh cells, pathogen-induced memory Tfh cells are phenotypically characterised with high expression of CXCR5, but reduced BCL6 expression which is elevated once these pathogen-induced memory Tfh cells enter the germinal centres upon secondary infection (Kitano *et al.*, 2011). In healthy humans, memory Tfh like T cells make up 15-25% of the circulatory T cell pool. These peripheral memory Tfh cells are said to be induced by vaccinations. However, the origin of the remaining memory Tfh cells is not known (Schmitt, Bentebibel and Ueno, 2014). Similar to pathogen-induced $CD4^+$ T cells, steady state $CD4^+$ MP T cells can be divided into T helper-like subpopulations, namely Th1- and Th17-like MP T cells (Kawabe *et al.*, 2017, 2020; Cho *et al.*, 2023). In addition, IFN-γ-producing $CD45RO^+$ $CD4^+$ MP T cells are also found in healthy humans which provide a pathogen-specific response to foreign antigens they have not been exposed to before (Su *et al.*, 2013). Since a population of circulating $CXCR5^+$ memory-like $CD4^+$ T cells has been identified in healthy human blood, which cannot be fully classified into conventional T helper lineages and is therefore considered incompletely characterised (Schmitt, Bentebibel and Ueno, 2014), and since there is prior evidence of the existence of $CD4^+$ MP T cells in healthy humans (Su et al., 2013), we postulate that this population of memory Tfh cells may consist of foreign antigen-inexperienced Tfh cells developed in the absence of foreign antigen stimulation. To assess if Tfh cells with a memory-phenotype can develop without pathogen encounter, $CD44^{high}$ $CD62L^{low}$ and $CD62L^{high}$ MP T cells and, $CD62L^+$ $CD44^{low}$ naïve $CD4^+$ T cells, from GFP-Egr2/ AmCyan-T-bet kin mice bred and maintained under SPF conditions, were analysed by flow cytometry. Tfh cells are defined by the expression of the Tfh-lineage specific transcriptional regulator, BCL6, and the chemokine receptor CXCR5 (Yu et al., 2009; Moser, 2015). Of the $CD44^{high}$ $CD62L^{low/high}$ $CD4^+$ MP T cells, 5.4% of the cells were positive for CXCR5 and BCL6, which were absent in $CD62L^{high}$ $CD44^{low}$ $CD4^+$ naïve T cell compartment (Figure 3.6).


A
CD4+ CD3+
CD62L
CD44
CD62Lhigh CD44low CD3+ CD4+
CD44high CD3+ CD4+
CXCR5
Bcl6
0.7
0.1
98.8
0.4
6.7
5.4
85.6
2.3

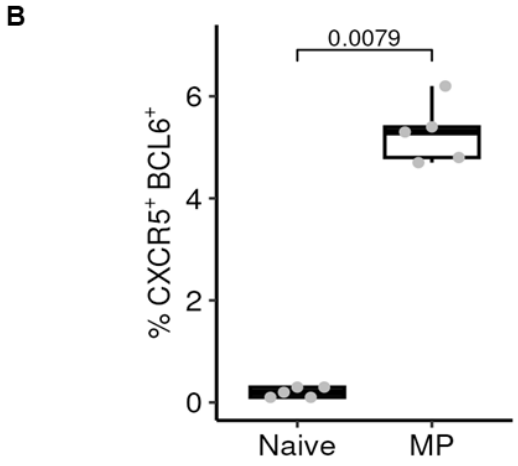

B
0.0079
% CXCR5+ BCL6+
Naive
MP

Figure 3.6. Tfh-like cells in CD4⁺ MP T cell population. Lymphocytes were isolated from the spleen and lymph nodes of old GFP-Egr2/ AmCyan-T-bet mice. (A) $CD4^+$ $CD62L^+$ naïve T cells and $CD4^+$ $CD44^{high}$ MP T cells were gated and the expression of CXCR5 and BCL6 Tfh-cell specific markers was analysed. The data are from five mice in each group and represent three repeated experiments. (B) This graph illustrates the proportion of $CXCR5^+$ $BCL6^+$ Tfh-like cells are within the naïve and $CD44^{high}$ $CD4^+$ MP T cell populations. The box plot represents the lower, median, and upper quartile of five mice analysed. Each data point on the graph is representative of one mouse. The statistical significance was tested for using the two-tailed Mann-Whitney U test. Naïve: CD62Lhigh CD44low CD3+ CD4+ T cells; MP: CD44high CD3+ CD4+ T cells.

Recently, it was shown that although both pathogen-induced Tfh and Tcm cells express CXCR5, only memory Tfh cells retain high levels of the folate receptor FR4, allowing them to be distinguished from $CXCR5^+$ Tcm cells (Künzli *et al.*, 2020). We found that all of the MP Tfh cells expressed FR4 (96.6%) (Figure 3.7). In addition, PD-1 is also used to characterise pathogen-induced Tfh cells in combination with other Tfh cell-specific markers such as CXCR5 and BCL6 (Crotty, 2014). PD-1 controls Tfh cell generation and its expression on Tfh cells is required for regulating B cell responses in the GC (Shi *et al.*, 2018). We detected that PD-1 is also highly expressed in all of the MP Tfh cells (Figure 3.7).

Overall, these results demonstrate that MP Tfh cells share similar phenotypes with pathogen-induced memory Tfh cells.

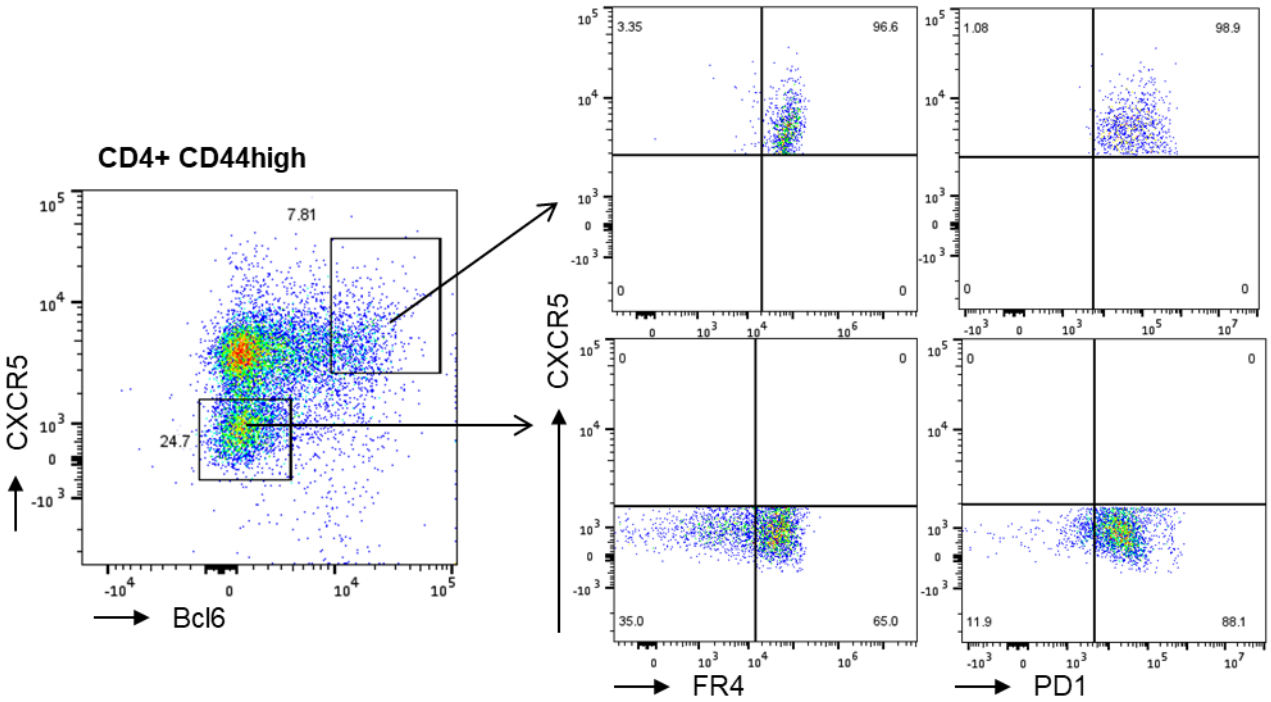


Figure 3.7. Phenotyping of MP Tfh cells. Lymphocytes were isolated from the spleen and lymph nodes of old GFP-Egr2/AmCyan-T-bet mice. CXCR5$^+$ BCL6$^+$ MP Tfh cells and CXCR5$^-$ BCL6$^-$ non-Tfh MP cells were gated for and the expression of other memory Tfh cell markers, FR4 and PD-1, were analysed. The data are from four mice and represent three repeated experiments.

3.3.2 FOXP3$^+$ Tfr cells reside within the Tfh MP cells

Tfr cells are generated from thymic Tregs, expand during infection, and migrate to germinal centres, where they regulate Tfh cell-driven germinal centre responses (Linterman *et al.*, 2011; Chung *et al.*, 2011). These pathogen-induced Tfr cells are defined with the expression of a combination of Tfh- and Treg-specific markers including FOXP3, GITR, CXCR5, BCL6, PD-1 (Linterman *et al.*, 2011). Circulating pathogen-induced Tfr cells adopt similar characteristics to pathogen-induced circulatory memory T cells and reside in the germinal centre to exert a less suppressive effector function than that of effector Tfr cells after reinfection (Sage *et al.*, 2014). To investigate whether or not MP Tfh lineage consists of Tfr cells with a memory-phenotype, we gated for MP Tfh cells and analysed for memory Tfr-like cells. Amongst the CD44^{high} CD4$^+$ T cell population, around 21.3% of CD4$^+$ MP T cells expressed FR4 (Figure 3.8). As expected, MP Tfh cells were found among the CD44^{high} CD4$^+$ FR4$^+$ population but were undetectable without FR4 expression (Figure 3.8 and Figure 3.9). Among these MP Tfh cells, about 10.3% of MP Tfh cells expressed FOXP3 and GITR (Figure 3.9B). GITR was used instead of CD25 to identify regulatory populations, as Tfr cells downregulate CD25 and express higher levels of GITR than conventional Tregs, reflecting their reduced IL-2 responsiveness within the germinal centre environment (Wing *et al.*, 2017). We detected about 15% of Tregs (GITR$^+$ FOXP3$^+$) that did not carry Tfh cell-specific markers (Figure 3.9B), indicating that in steady state conditions, the T regulatory pool consists of thymic-derived Tregs and MP Tfr cells. Consistent with the notion that Tfr cells predominantly originate from thymic-derived FOXP3$^+$ Tregs specialised in controlling self-reactive B cells (Chung *et al.*, 2011), it remains to be determined if these MP Tfr-like cells regulate autoreactive B cell responses.

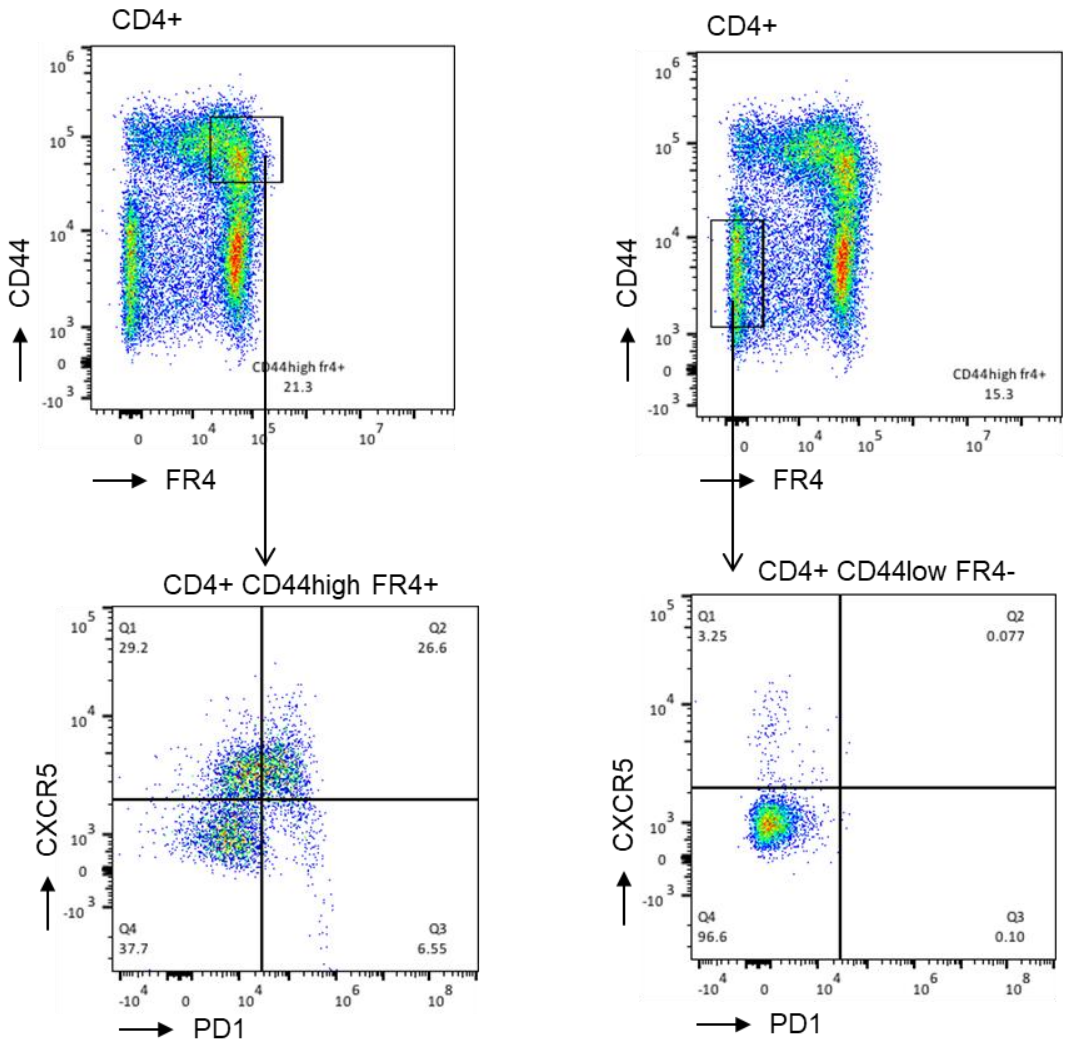


Figure 3.8. The expression of CXCR5 and PD-1 in CD44^{high} FR4^{+} MP T cells and CD44^{low} FR4^{-} naïve T cells under steady conditions. Lymphocytes were isolated from the spleen and lymph nodes of 12-week-old GFP-Egr2 /AmCyan-T-bet mice. CD44^{high} FR4^{+} MP T cells and CD44^{low} FR4^{-} naïve T cells were gated for from CD4^{+} T cells (above). Then the expression of the Tfh cell-specific surface markers, PD-1 and CXCR5, were observed. The data representative of two repeated experiments.

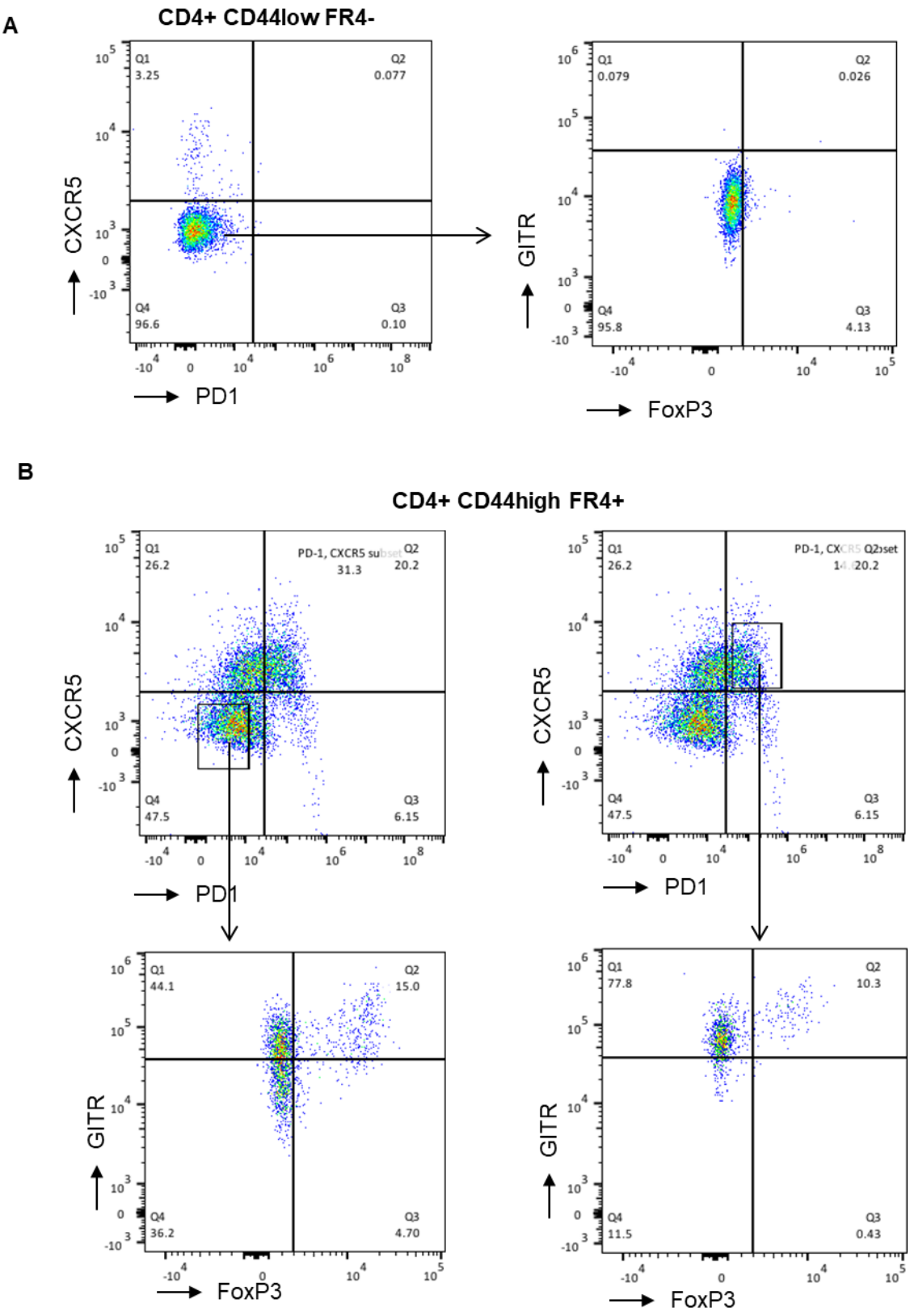

A
CD4+ CD44low FR4-
Q1
3.25
Q2
0.077
Q4
96.6
Q3
0.10
CXCR5
PD1
Q1
0.079
Q2
0.026
Q4
95.8
Q3
4.13
GITR
FoxP3
B
CD4+ CD44high FR4+
Q1
26.2
Q2
20.2
Q4
47.5
Q3
6.15
CXCR5
PD1
Q1
44.1
Q2
15.0
Q4
36.2
Q3
4.70
GITR
FoxP3
Q1
26.2
Q2
20.2
Q4
47.5
Q3
6.15
CXCR5
PD1
Q1
77.8
Q2
10.3
Q4
11.5
Q3
0.43
GITR
FoxP3

Figure 3.9. T follicular regulatory (Tfr)-like cells within MP Tfh cell population. Lymphocytes were isolated from the spleen and lymph nodes of 12-week-old GFP-Egr2/ AmCyan-T-bet mice. (A) CXCR5$^-$ PD-1$^-$ non-Tfh FR4$^-$ CD44^{low} CD4$^+$ naïve T cells were gated for and the expression GITR and FOXP3 were analysed. (B) CXCR5$^+$ PD-1$^+$ MP Tfh cells and CXCR5$^-$ PD-1$^-$ non-Tfh FR4$^+$ CD44^{high} CD4$^+$ MP T cells were gated for and the expression GITR and FOXP3 were analysed. The data representative of two repeated experiments.

3.3.3 MP Tfh cells are distinct from T-bet^{high} CD4$^+$ MP T cells but express EGR2

Pathogen-induced Tfh cells play a central role in promoting antibody production during infection. Unlike other CD4$^+$ T helper subsets, whose differentiation depends on the type of pathogen encountered (e.g., Th1, Th2, Th17), Tfh cells are specialized in providing B cell help for the generation of high-affinity antibodies. For instance, pathogen-induced Th17 cells initiate an immune response against extracellular pathogens. Whereas pathogen-induced Th1 cells play a role in host defence against intracellular pathogens. The differentiation of naïve CD4$^+$ T cells to distinct T helper cell fates is driven by the induction of environmentally influenced lineage-specific transcription factors (T-bet for Th1 cells, GATA-3 for Th2, RORγt for Th17) (Sun *et al.*, 2023). These transcription factors are also co-expressed with BCL6 in pathogen-induced Tfh cells to adapt Tfh cell mediated antibody class switching specific for the type of infection present (Powell *et al.*, 2019; Glatman *et al.*, 2009; Liu *et al.*, 2017; Olatunde, Hale and Lamb, 2021). T-bet mediates IFN-γ production in Th1 cells during an immune response (Szabo *et al.*, 2000). Pathogen-induced Tfh cells express T-bet during the early stages of maturation which initiates IFN-γ production and antibody class switching to IgG2 in a subset of mature Th1-type Tfh cells, during acute viral infection (Wang *et al.*, 2019). In the context of antigen-inexperienced memory T cells, T-bet$^+$ CD4$^+$ MP T cells contribute to an early non-specific Th1-like response to viral infection (Kawabe *et al.*, 2017). Previously, in Figure 3.5, we showed that T-bet$^+$ MP T cells comprised 22.1 % of the total CD44^{high} CD25$^-$ CD4$^+$ MP T cell population (Figure 3.5). To determine whether MP Tfh cells are part of the T-bet$^+$ MP T cell subpopulation, CD44^{high} CD4$^+$ T-bet$^+$ MP T cells were analysed for a Tfh cell phenotype. None of the MP Tfh cells expressed T-bet (Figure 3.10A). Overall, these results indicate that the previously reported innate-like T-bet^{high} CD4$^+$ MP T cells (Kawabe *et al.*, 2017, 2020) are indeed a distinct lineage from MP Tfh cells within the CD4$^+$ MP T cell population. T-bet$^+$ MP CD4$^+$ T cells can mount innate responses (Kawabe *et al.*, 2017). It is unknown how T-bet$^-$ MP CD4$^+$ T cells function during an immune response. Previous studies have identified a minor population of RORγt$^+$ Th17-like MP T cells within the T-bet$^-$ MP T cell subset (Kawabe *et al.*, 2020). In this study, we further demonstrate

that MP Tfh cells are also located within the T-bet^{-} MP T cell subset, suggesting additional heterogeneity among T-bet^{-} MP T cells. These results suggest that MP Tfh cells do not exert an innate-like response driven by T-bet and as pathogen-Tfh cells function in B cell-mediated responses, we propose that MP Tfh cells drive an adaptive function during an immune response.

EGR2/3 are key regulators of MP T cell homeostasis and inflammatory responses to prevent autoimmunity (Symonds *et al.*, 2020). Previously, our group showed that EGR2/3 suppress IFN-γ production in Th1 cells by physically binding to the functional DNA-binding T-box domain of T-bet, upon antigen stimulation (Singh *et al.*, 2017). In contrast to its inhibitory role in Th1 differentiation, EGR2/3 regulates the differentiation and function of pathogen-induced Tfh cells (Ogbe *et al.*, 2015). Earlier we showed that EGR2 and T-bet are differentially expressed in CD4^{+} MP T cells under homeostatic conditions in GFP-Egr2/ AmCyan-T-bet mice raised under SPF conditions (Figure 3.5). As MP Tfh cells are absent in the T-bet^{+} MP T cell population, we determine whether its inhibitor, EGR2, defines MP Tfh cells in unimmunised GFP-Egr2/ AmCyan-T-bet mice. To examine this, we analysed the expression of CXCR5 and BCL6 in EGR2^{+} and EGR2^{-} CD44^{high} CD4^{+} MP T cells. MP Tfh cells are only found within the EGR2^{+} CD44^{high} CD4^{+} MP T cells (~11%), implying a major role of EGR2 in MP Tfh cells (Figure 3.10B). In addition, this indicates that the effector function of MP Tfh cells is driven by EGR2, implying that EGR2 drives an adaptive immune response rather than an innate immune response, distinct from the innate-like T-bet^{+} MP T cells.

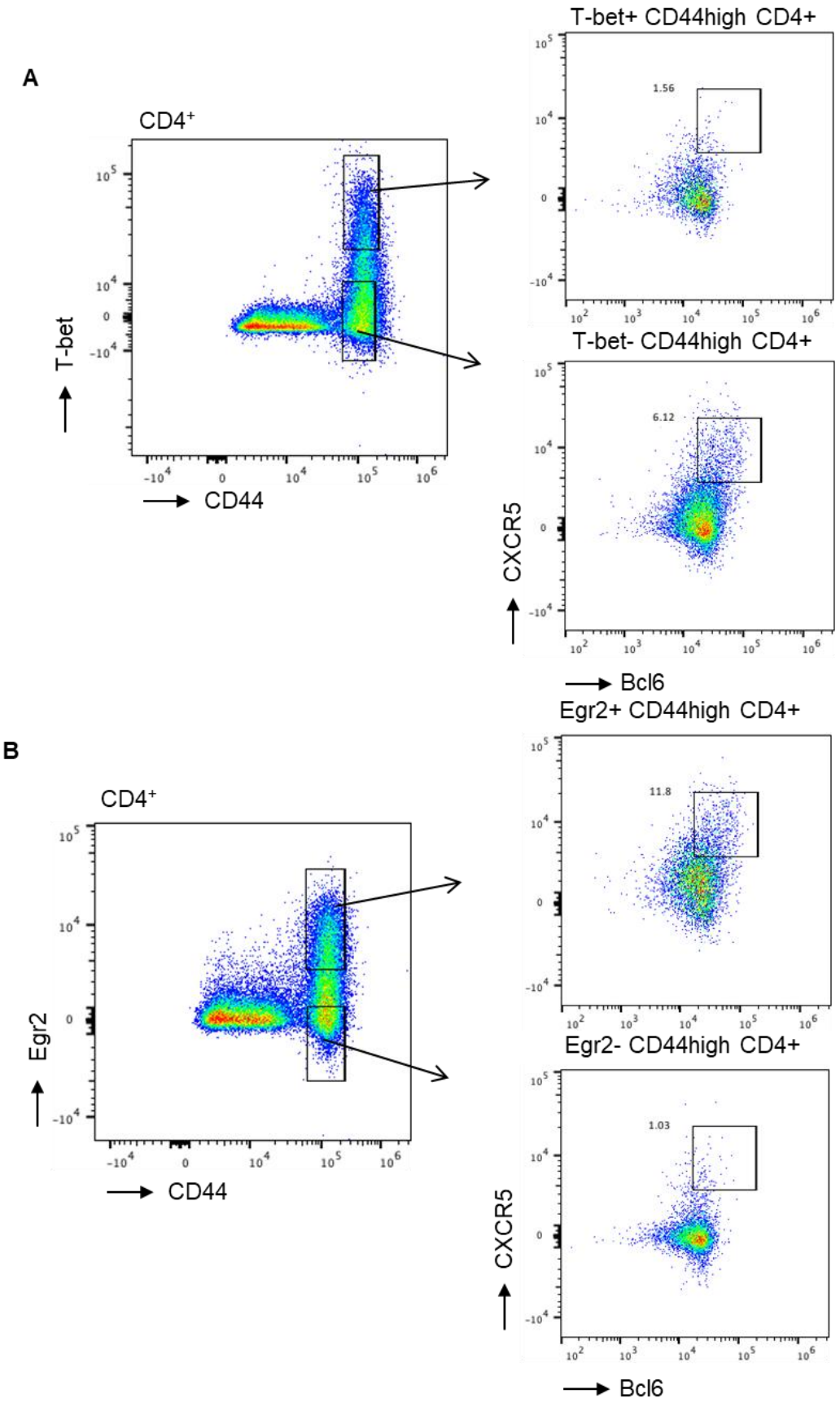

A
CD4+
T-bet
CD44
T-bet+ CD44high CD4+
1.56
T-bet- CD44high CD4+
6.12
CXCR5
Bcl6
B
CD4+
Egr2
CD44
Egr2+ CD44high CD4+
11.8
Egr2- CD44high CD4+
1.03
CXCR5
Bcl6

Figure 3.10. MP Tfh cells are a subset of EGR2^{+} CD44^{high} CD4^{+} MP T cells. Lymphocytes were isolated from the spleen and lymph nodes of 12-week-old GFP-Egr2/ AmCyan-T-bet mice. (A) T-bet^{+} and T-bet^{-} CD44^{high} CD4^{+} MP T cells were gated and the expression of BCL6 and CXCR5 was analysed. (B) EGR2^{+} and EGR2^{-} CD44^{high} CD4^{+} MP T cells were gated and the expression of BCL6 and CXCR5 was analysed. The data are from five mice per group and represent three independent experiments.

3.4 The role of the transcription factor EGR2 in MP Tfh cell generation and the analysis of the gene expression profile of EGR2^{+} FR4^{+} MP Tfh cells

3.4.1 Dual FR4 and EGR2 marker expression defines MP Tfh cells

Alone, CXCR5 is not a good surface marker to identify MP Tfh cells, as 6.7% out of 12.1% of the CXCR5^{+} CD44^{high} CD4^{+} MP T cells did not express BCL6 (Figure 3.6). During Tfh cell differentiation, CXCR5 and BCL6 are expressed at different stages of development, resulting in transient single-positive populations (Chen *et al.*, 2015; Liu *et al.*, 2012). However, mature Tfh cells co-express both CXCR5 and BCL6 (Nurieva *et al.*, 2009), and therefore both markers were used in combination to confidently isolate and define MP Tfh cells in this study**.** In mice, the folic acid nutrient transporter, FR4, is highly expressed in effector Tfh cells and pathogen-induced memory Tfh cells (Iyer *et al.*, 2013; Künzli *et al.*, 2020). We found that FR4 is co-expressed with CXCR5 in MP Tfh cells (Figure 3.7) which localise within the EGR2^{+} MP T cells sub-compartment but do not express T-bet (Figure 3.10). To determine the relationship between FR4 and EGR2 in MP Tfh cells, we gated for MP Tfh and non-Tfh MP T cells and analysed for FR4, GFP-EGR2 and AmCyan-T-bet surface markers. We found that almost all FR4 positive MP Tfh cells co-expressed EGR2 (~80%) (Figure 3.11). However, FR4 was not co-expressed with T-bet in MP Tfh cells. As expected, EGR2 was also not co-expressed with T-bet (Figure 3.11). Although FR4 expression is maintained on about half (52%) of the non-Tfh MP T cells, Egr2 is relatively absent in non-Tfh MP T cells, indicating EGR2 as a distinct marker for MP Tfh cells (Figure 3.11). These findings show that along with Tfh cell markers such as CXCR5, BCL6 and PD-1, a combination of EGR2 and FR4 can isolate MP Tfh cells from the CD4^{+} MP T cell population.

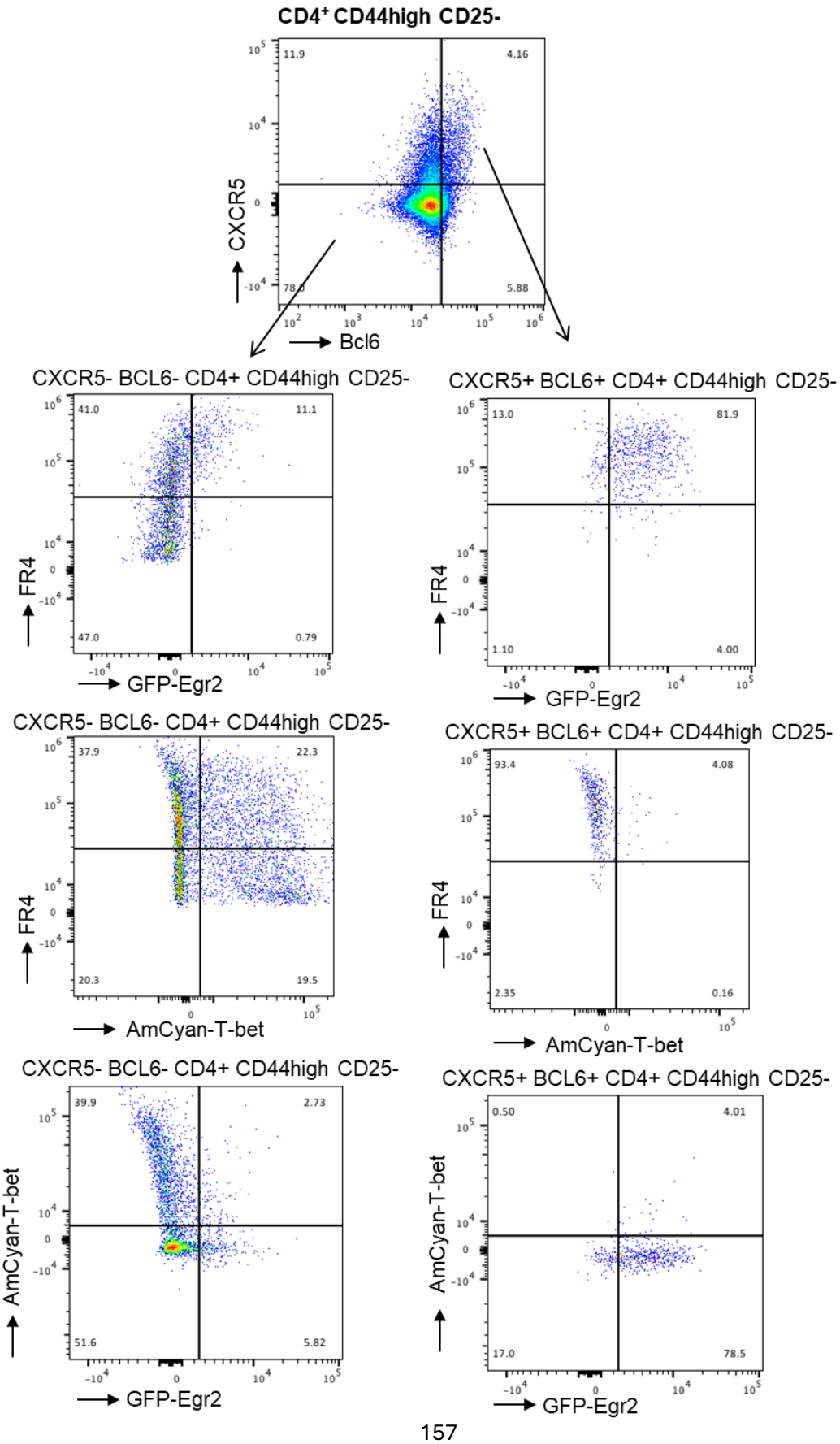

CD4+ CD44high CD25-
CXCR5
Bcl6
11.9
4.16
78.0
5.88
CXCR5- BCL6- CD4+ CD44high CD25-
FR4
GFP-Egr2
41.0
11.1
47.0
0.79
CXCR5+ BCL6+ CD4+ CD44high CD25-
13.0
81.9
1.10
4.00
CXCR5- BCL6- CD4+ CD44high CD25-
AmCyan-T-bet
37.9
22.3
20.3
19.5
CXCR5+ BCL6+ CD4+ CD44high CD25-
93.4
4.08
2.35
0.16
CXCR5- BCL6- CD4+ CD44high CD25-
39.9
2.73
51.6
5.82
CXCR5+ BCL6+ CD4+ CD44high CD25-
0.50
4.01
17.0
78.5

Figure 3.11. FR4 and EGR2 are co-expressed in MP Tfh cells. Lymphocytes were isolated from the spleen and lymph nodes of old GFP-Egr2/ AmCyan-T-bet mice. $CD4^+$ $CD44^{high}$ $CD25^-$ MP T cells were gated and then the expression of CXCR5 and BCL6 was analysed. The $CXCR5^-$ $BCL6^-$ non-Tfh MP T cells and the $CXCR5^+$ $BCL6^+$ MP Tfh cells were analysed for the expression of FR4, EGR2 and T-bet. The data are from four mice and represent three repeated experiments.

3.4.2 The transcription factor EGR2 plays an important role in MP Tfh cell development

EGR2 and EGR3, which play compensatory roles for one another, control BCL6-mediated Tfh cell differentiation and GC responses of pathogen-induced Tfh cells in response to viral infection (Ogbe *et al.*, 2015). We found that along with FR4, EGR2 is highly expressed within MP Tfh cells (Figure 3.11). To elucidate the role of EGR2 in MP Tfh cells, we used both FR4 and EGR2 to isolate MP Tfh cells and MP Tfr cells. FR4 and EGR2 divide $CD4^+$ MP T cells into three distinct subsets: $FR4^+$ $EGR2^+$, $FR4^+$ $EGR2^-$ and $FR4^-$ $EGR2^-$ MP T cell subsets (Figure 3.12). However, within the naïve T cell population, only 15% of cells are $FR4^+$ $EGR2^-$ whereas 80% of naïve T cells do not express FR4 nor EGR2 (Figure 3.12). We found that the $EGR2^+$ $FR4^+$ MP T cell subpopulation consists of both MP Tfh and MP Tfr cells, where the latter shares similar attributes to both MP Tfh and Tregs. Compared to MP Tfh cells, a proportion of $FOXP3^+$ Tregs are found in both $EGR2^+FR4^+$ (15.2%) and $FR4^+$ $EGR2^-$ (18.4%) $CD44^{high}$ $CD4^+$ T cell subsets, indicating $FoxP3^+$ $CD44^{high}$ Tregs do not require Egr2 for their development.

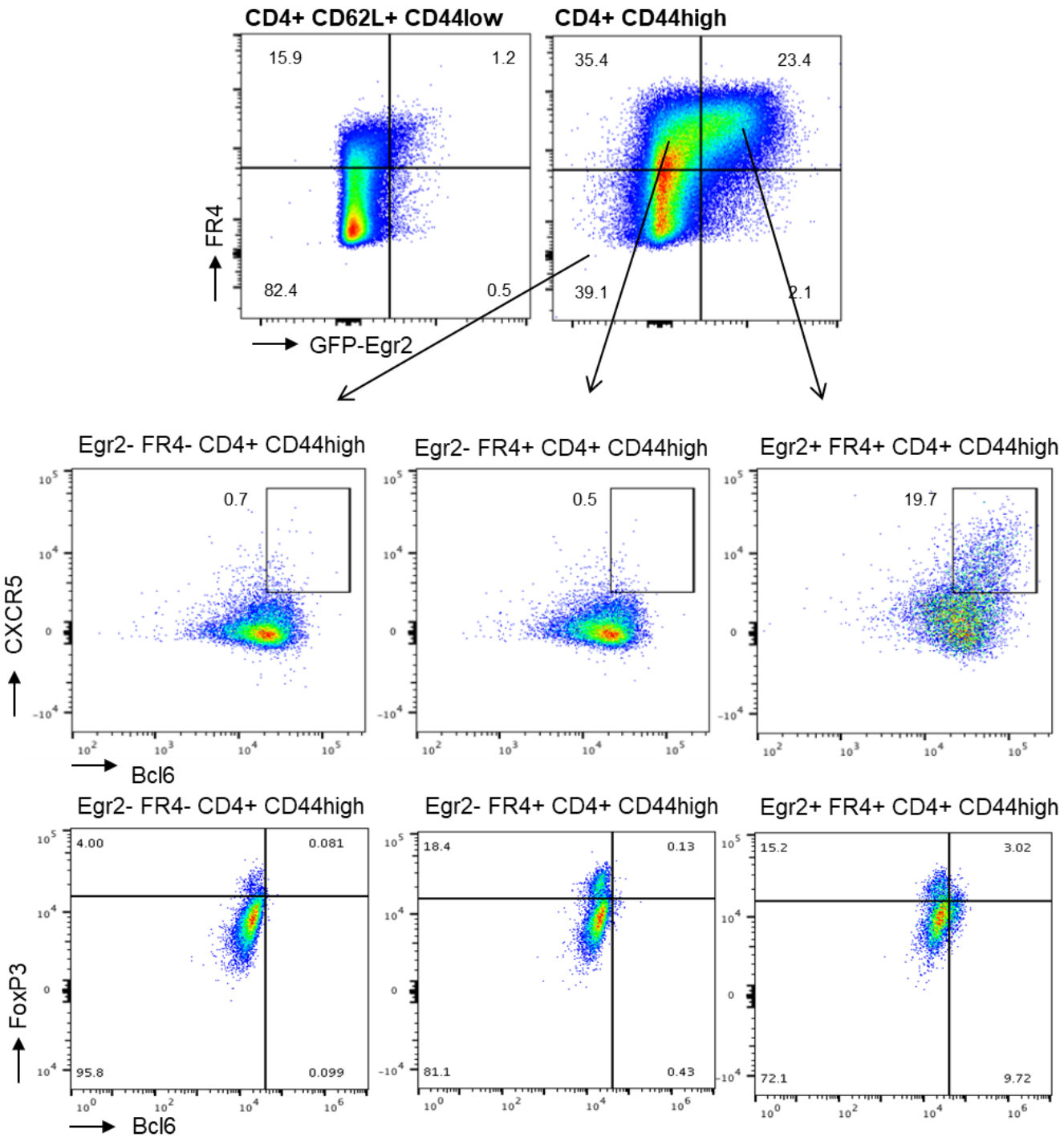


Figure 3.12. EGR2 plays a role in MP Tfh cell development. CD44^{high} CD4^{+} MP T cells from GFP-Egr2/ AmCyan-T-bet mice were gated on the FR4^{+} EGR2^{-}, FR4^{-} EGR2^{-}, FR4^{+} EGR2^{+} subpopulations for the analysis of MP Tfh and MP Tfr cells using FOXP3, BCL6, CXCR5 markers. The data are representative of five mice in each group and are representative of three independent experiments.

Next, to thoroughly determine the impact of EGR2 in MP Tfh cell development, we further analysed MP Tfh cells and $CD44^{high}$ Tregs from GFP-Egr2/ AmCyan-T-bet and CD2-$Egr2/3^{-/-}$ AmCyan-T-bet mice bred and maintained under SPF conditions. We found a similar frequency, of around 50-60%, of FR4 positive MP T cells from both GFP-Egr2/ AmCyan-T-bet and CD2-$Egr2/3^{-/-}$ AmCyan-T-bet mice (Figure 3.13). Over 12% of $CD44^{high}$ Tregs were marked by FR4 expression within MP T cells from both GFP-Egr2/ AmCyan-T-bet and CD2-$Egr2/3^{-/-}$ AmCyan-T-bet mice (Figure 3.13A/C). In contrast, 16.8% of MP Tfh cells were only detected within the $FR4^{+}$ $EGR2^{+/-}$ $CD4^{+}$ MP T cells from GFP-Egr2/ AmCyan-T-bet and severely impaired in CD2-$Egr2/3^{-/-}$ AmCyan-T-bet mice (Figure 3.13A/B). To summarise, EGR2/3 have a role in MP Tfh cell development under homeostatic conditions. However, $CD44^{high}$ Tregs remain unaffected by EGR2 expression.

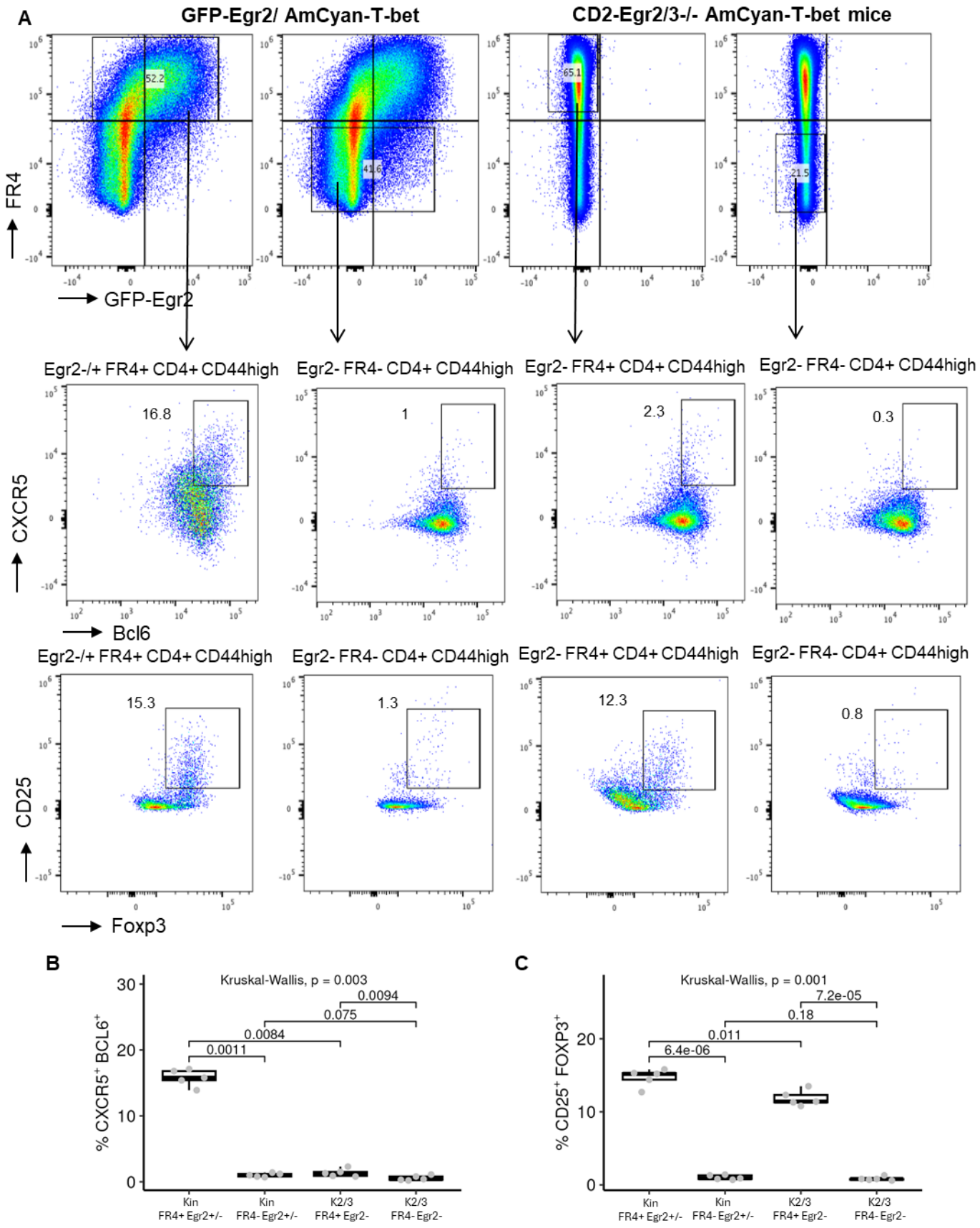

A
GFP-Egr2/ AmCyan-T-bet
CD2-Egr2/3-/- AmCyan-T-bet mice
FR4
GFP-Egr2
52.2
41.6
65.1
21.5
Egr2-/+ FR4+ CD4+ CD44high
Egr2- FR4- CD4+ CD44high
Egr2- FR4+ CD4+ CD44high
Egr2- FR4- CD4+ CD44high
CXCR5
Bcl6
16.8
1
2.3
0.3
Egr2-/+ FR4+ CD4+ CD44high
Egr2- FR4- CD4+ CD44high
Egr2- FR4+ CD4+ CD44high
Egr2- FR4- CD4+ CD44high
CD25
Foxp3
15.3
1.3
12.3
0.8
B
Kruskal-Wallis, p = 0.003
% CXCR5+ BCL6+
0.0094
0.075
0.0084
0.0011
Kin FR4+ Egr2+/-
Kin FR4- Egr2+/-
K2/3 FR4+ Egr2-
K2/3 FR4- Egr2-
C
Kruskal-Wallis, p = 0.001
% CD25+ FOXP3+
7.2e-05
0.18
0.011
6.4e-06
Kin FR4+ Egr2+/-
Kin FR4- Egr2+/-
K2/3 FR4+ Egr2-
K2/3 FR4- Egr2-

Figure 3.13. The analysis of the role of Egr2 in MP Tfh cell development. $FR4^+$ and $FR4^-$ $CD44^{high}$ $CD4^+$ MP T cells without or without EGR2 expression from GFP-Egr2/ AmCyan-T-bet and CD2-Egr2/3$^{-/-}$ AmCyan-T-bet mice were gated on and the co-expression of MP Tfh (A and B) and $CD44^{high}$ Treg (A and C) cell-specific markers was analysed. The data are representative of five mice in each group and are representative of three independent experiments. In (B) and (C) the upper, median and lower quartiles are shown for the five mice, and the significance was tested by Kruskal-Wallis tests, followed by two-tailed Conover tests with Benjamini-Hochberg correction. Kin: GFP-Egr2 / AmCyan-T-bet mice; K2/3: CD2-Egr2/3-/- / AmCyan-T-bet mice.

3.4.3 Gene expression profile of $EGR2^+FR4^+$ MP T cells

3.4.3.1 $CD4^+$ MP T cells with differential expression of EGR2 and FR4 distinctively cluster in Principal Component Analysis (PCA)

MP Tfh cells were enriched in the $EGR2^+FR4^+$ $CD4^+$ MP T cell subset maintained under SPF conditions. The transcription factor, EGR2, regulates the development of $FR4^+$ MP Tfh cells (Figure 3.13). To further investigate the regulatory function of EGR2 in MP Tfh cells, we analysed the transcriptome of $FR4^+$ $EGR2^+$ $CD4^+$ $CD44^{high}$ $CD25^-$ MP T cells from Egr2-GFP/ AmCyan-T-bet kin mice under homeostatic conditions via RNA seq. For comparison, RNA seq was also carried out for the $FR4^+$ $EGR2^-$ and $FR4^-$ $EGR2^-$ $CD4^+$ $CD44^{high}$ $CD25^-$ MP T cells. The PCA plot, in figure 3.14, illustrates the clustering variance of $FR4^+$ $EGR2^+$, $FR4^+$ $EGR2^-$ and $FR4^-$ $EGR2^-$ $CD4^+$ MP T cell subsets with 4 experimental replicates per cell type. Distinct clusters are observed per cell type with a singular outlier evident for both $FR4^+$ $EGR2^+$ and $FR4^+$ $EGR2^-$ $CD4^+$ MP T cells (Figure 3.14). The PCA plot indicates a higher degree of variation when comparing $FR4^-$ $EGR2^-$ $CD4^+$ $CD44^{high}$ $CD25^-$ MP T cells to $FR4^+$ $EGR2^+$ or $FR4^+$ $EGR2^-$ $CD4^+$ T cells. In contrast, there is no significant differences between $FR4^+$ $EGR2^+$ and $FR4^+$ $EGR2^-$ $CD4^+$ MP T cells along the PC 1 axis however factors contributing to PC 2 cause the most variation between both cell types. Overall, this shows that FR4 and EGR2 double expression profiles in $CD4^+$ MP T cells possibly drives the differential expression programmes among the heterogeneous population of MP T cells in terms of their development and function.

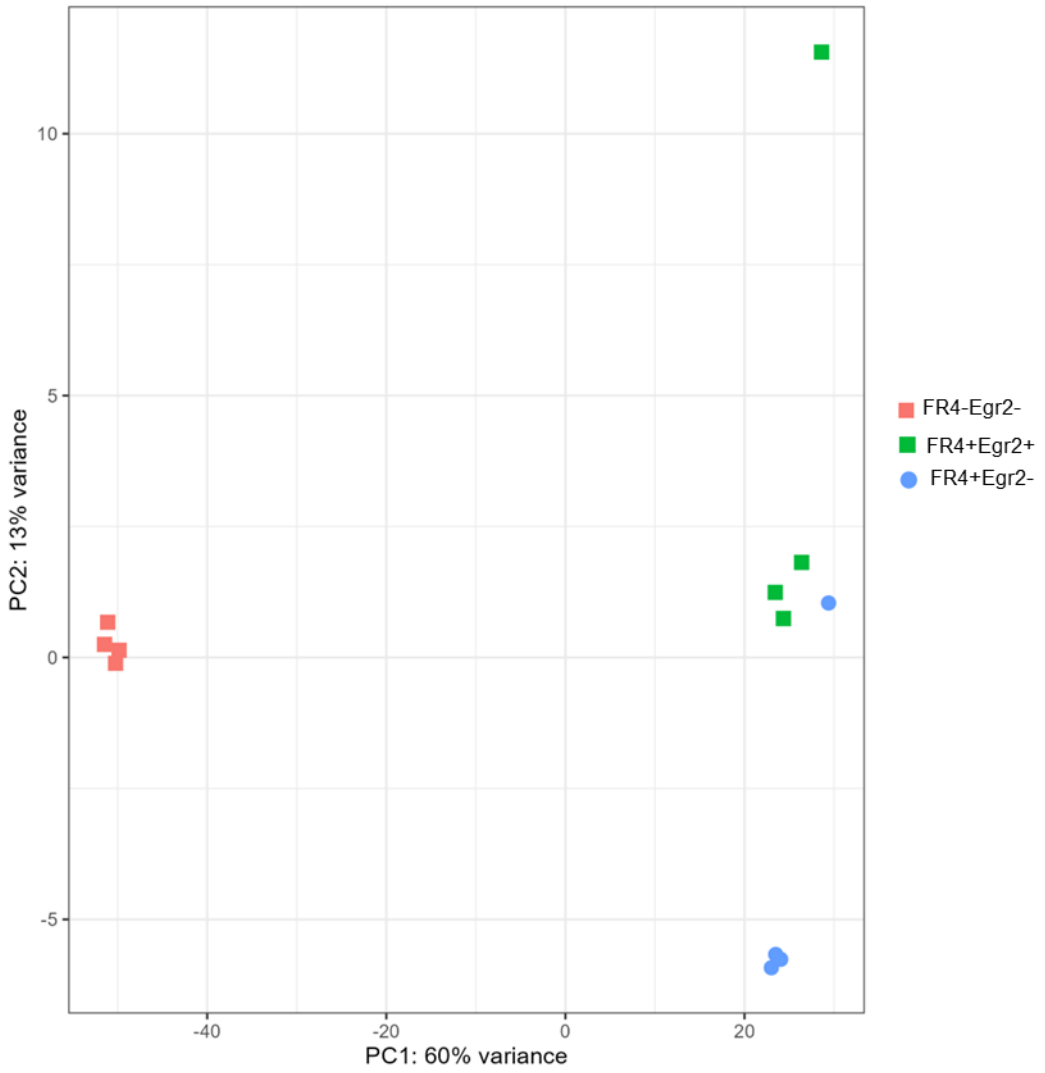


Figure 3.14. Principal component analysis (PCA). FR4^{+} EGR2^{-}, FR4^{+} EGR2^{+}, and FR4^{-} EGR2^{-} CD4^{+} CD44^{high} CD25^{-} MP T cells, from Egr2-GFP/ AmCyan-T-bet mice, were sorted and analysed by RNA sequencing (RNA Seq). The PCA plot is a two-dimensional scatter plot displaying the first two principal components (PCs) of the data. Each point on the plot represents an RNA Seq sample and its identity is color-coded and indicated on the key. Biological replicates for each sample group are clustered together with a one distinctive outlier of the FR4^{+} EGR2^{+} origin. The RNA-Seq data are from four replicates, each with cells pooled from 10 mice for each group.

3.4.3.2 Differential expression profiles of EGR2$^+$ FR4$^+$, EGR2$^-$ FR4$^+$, and EGR2$^-$ FR4$^-$ CD4$^+$ MP T cells

To investigate the influence of EGR2 and FR4 at the gene level, we analysed the differentially expressed genes of FR4$^+$ EGR2$^+$ CD4$^+$ MP T cells when compared to either FR4$^+$ EGR2$^-$ and FR4$^-$ EGR2$^-$ MP T cells using edgeR. Figure 3.15 illustrates the total number of genes upregulated and downregulated upon cell-to-cell type comparison. We focused on comparing the expression profiles, from 4 independent experiments, of FR4$^+$ EGR2$^+$ MP T cells to FR4$^+$ EGR2$^-$ MP T cells, FR4$^+$ EGR2$^+$ MP T cells to FR4$^-$ EGR2$^-$ MP T cells and FR4$^+$ EGR2$^-$ MP T cells to FR4$^-$ EGR2$^-$ MP T cells. The Venn diagram indicates that more than 400 genes were differentially expressed in the FR4$^+$ EGR2$^+$ MP T cells regardless of cell-to-cell type comparison (Figure 3.15). Thus, the expression profile of these genes is consistent within FR4$^+$ EGR2$^+$ MP T cells in comparison to EGR2$^-$ groups regardless of FR4$^+$ or FR4$^-$. Therefore, their expression is dependent on EGR2. Of the EGR2-dependent genes (supplementary table 1), the Tfh cell-associated genes (*Bcl6* and *Il2*), T cell activation and proliferation-associated genes (*Mik67*, *Btla* and *Pdcd1lg2*) are highly expressed in FR4$^+$ EGR2$^+$ MP T cells (Figure 3.16A). In addition, *Ccl2*, which EGR2 regulates, has a role in T-cell migration and is downregulated across all three conditions (Figure 3.16A). Furthermore, over 2500 genes in FR4$^+$ EGR2$^+$ MP T cells are also differentially expressed in FR4$^+$ EGR2$^-$ MP T cells compared to FR4$^-$ EGR2$^-$ MP T cells (Figure 3.15). Thus, these genes represent the number of genes directly regulated by FR4 as they are differentially expressed regardless of EGR2 expression. The genes upregulated by FR4 (listed in supplementary table 2) include Tfh cell-specific markers such as *Icos* and *Cd40l*, and Treg-specific markers such as *Foxp3*, *Ctla4* and *Il2ra* (Figure 3.16B). *Cd5*, which plays a role in T cell biology, is highly expressed in both FR4$^+$ EGR2$^+$ and FR4$^+$ EGR2$^-$ MP T cells (Figure 3.16). The majority of these genes are not expressed by FR4$^-$ EGR2$^-$ MP T cells which supports that FR4 regulates these genes. Apart from the genes regulated solely by EGR2 or FR4, there are at least over 200 genes that may be regulated by the EGR2 and FR4 phenotype as these genes are similarly dysregulated in FR4$^+$ cells with and without EGR2 expression, regardless of the comparisons. These genes include Tfh cell-specific genes (*Cxcr5*, *Il21*) and Treg-associated genes (*Cd200*, *Ascl2* and *Pdcd1*) (supplementary table 3) (Figure 3.16C). Interestingly, both *Cxcr5* and *Bcl6* among other Tfh cell genes are highly expressed in FR4$^+$ EGR2$^+$ MP T cells which is the predominant cell type within a FR4$^+$ EGR2$^+$ MP T cell population (Figure 3.16).

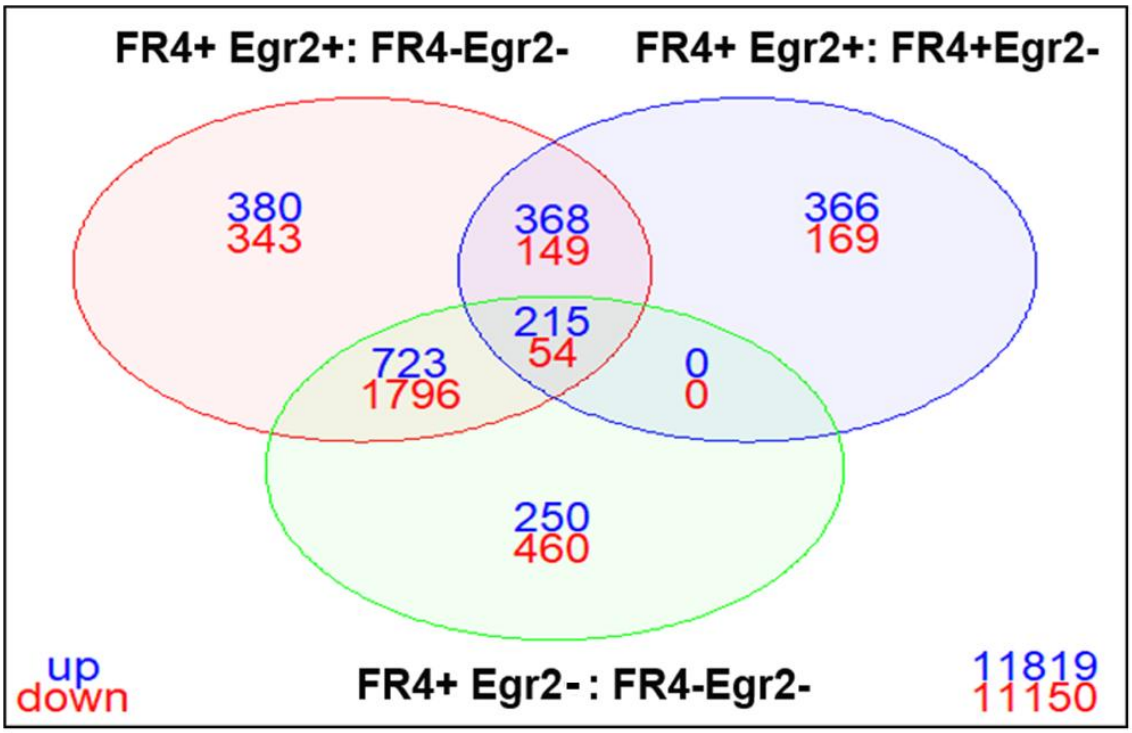


Figure 3.15. Comparison of the differential gene expression of FR4^{+} EGR2^{+}, FR4^{+} EGR2^{-} and FR4^{-} EGR2^{-} MP CD44^{high} CD4^{+} T cells from Egr2-GFP/ AmCyan-T-bet mice by a Venn diagram. The genes were marked differentially expressed with a false discovery rate (FDR) ≤0.05 and a log2 fold difference ≥1.5. The Venn diagram compares the upregulated (in blue) and downregulated (in red) genes of FR4^{+} EGR2^{+}, FR4^{+} EGR2^{-}, and FR4^{-} EGR2^{-} CD44^{high} CD4^{+} MP T cells. The RNA Seq data are from four replicates, each with cells pooled from 10 mice for each group.

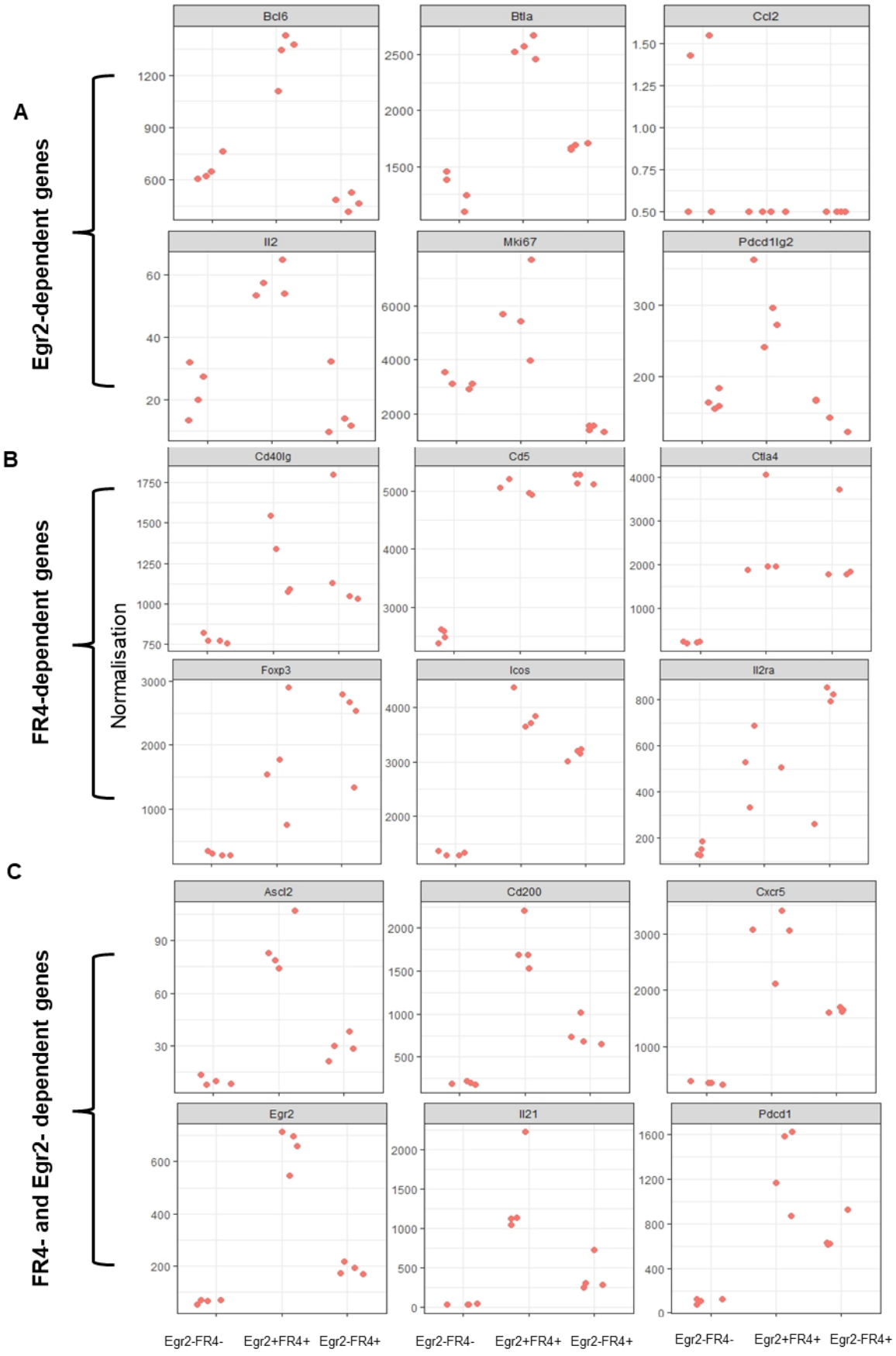

A
Egr2-dependent genes
Bcl6
Btla
Ccl2
Il2
Mki67
Pdcd1lg2
B
FR4-dependent genes
Normalisation
Cd40lg
Cd5
Ctla4
Foxp3
Icos
Il2ra
C
FR4- and Egr2- dependent genes
Ascl2
Cd200
Cxcr5
Egr2
Il21
Pdcd1
Egr2-FR4-
Egr2+FR4+
Egr2-FR4+

Figure 3.16- Differential gene expression analysis of EGR2-, FR4- or Egr2/FR4- dependent genes of FR4$^+$ EGR2$^+$, FR4$^+$ EGR2$^-$ and FR4$^-$ Egr2$^-$ CD44^{high} CD4$^+$ MP T cells. The plot illustrates the comparison of normalized gene expression across each biological replicate and sample group represented by a red dot. The genes were selected from the raw data in supplementary tables 1-3. The RNA-Seq data are from four replicates, each with cells pooled from 10 mice for each group.

Next, we further analysed the transcriptional profile of FR4$^+$ EGR2$^+$, FR4$^-$ EGR2$^-$ and FR4$^+$ EGR2$^-$ CD4$^+$ MP T cells, using a heatmap. FR4$^-$ EGR2$^-$ MP T cells display a unique RNA profile compared to that of FR4$^+$ EGR2$^+$ and FR4$^+$ EGR2$^-$ MP T cells (Figure 3.17). FR4$^-$ EGR2$^-$ MP T cells highly expressed inflammatory genes such as *Il1b*, *Icosl*, *Icam1*, *Ifngr2*, *Il23r* and *Ahr*, as well as the central memory marker, *Ccr7* (Figure 3.17, Supplementary tables 4-6). RNA profiles of FR4$^+$ EGR2$^+$ and FR4$^+$ EGR2$^-$ MP T cells share similar expression of genes associated with T memory cells such as *Tcf7*, *P2rx7*, *Cxcr3* and *Tox2*. However, FR4$^+$ EGR2$^+$ MP T cells uniquely express Tfh cell marker genes such as *Bcl6*, *Cxcr5*, *Pdcd1* and the cytokine, *Il21*. In addition, these cells also highly express proliferation-associated genes such as *Pcna*, *Ccnb1* and *Il2*, under steady conditions (Figure 3.17). Thus, FR4$^+$ EGR2$^+$ MP T cells display a unique profile of MP Tfh-like cells.

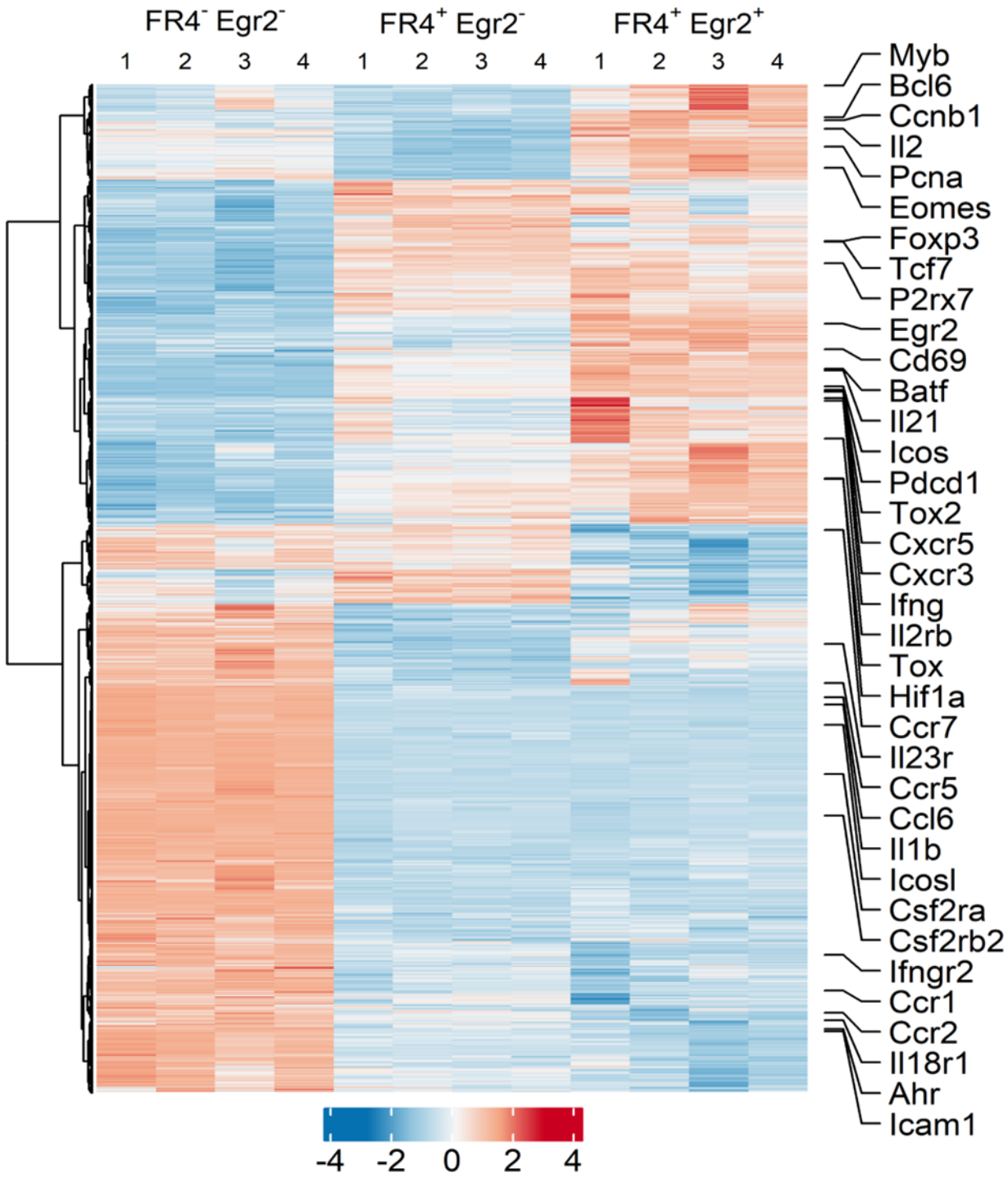


Figure 3.17. Gene expression analysis via Heatmap. The heatmap illustrates the unsupervised hierarchal clustering of differentially expressed genes across FR4^{+} EGR2^{+}, FR4^{+} EGR2^{-} and FR4^{-} EGR2^{-} CD44^{high} CD4^{+} MP T cell sample groups and of the 4 biological replicates per cell type. The gene expressions are standardised on the z-scale with downregulated (in blue) and upregulated (in red) genes displayed with colour patterns and the selected genes are indicated. The RNA-Seq data are from four replicates, each with cells pooled from 10 mice for each group.

3.4.3.3 Unique functional pathways in EGR2$^+$ FR4$^+$ MP Tfh cells

FR4$^+$ EGR2$^+$ MP T cells exhibit a MP Tfh cell-specific profile (Figure 3.17). To assess the impact of Egr2 on the functional pathways of FR4$^+$ EGR2$^+$ MP T cells, we performed GSEA analysis comparing FR4$^+$ EGR2$^+$ and FR4$^+$ EGR2$^-$ MP T cells. The differentially expressed genes between FR4$^+$ EGR2$^+$ MP T cells and FR4$^+$ EGR2$^-$ CD4$^+$ CD44^{high} CD25$^-$ MP T cells were ranked in decreasing order, by the F-statistic. This ranked list was used to assess the distribution of the genes from hallmark gene sets, from the MSigDB, in the ranked gene list. FR4$^+$ EGR2$^+$ MP T cells are highly enriched in genes of BCL6$^+$ Tfh cells and by LCMV-specific memory Tfh cells whereas central memory and Th1 related genes are enriched in FR4$^+$ EGR2$^-$ MP T cells, thus strongly supporting FR4$^+$ EGR2$^+$ MP T cells as MP Tfh cells (Figure 3.18). Further biological differences between FR4$^+$ EGR2$^+$ and FR4$^+$ EGR2$^-$ MP T cells include the high enrichment of genes involved in E2F_Targets and G2M_Checkpoint hallmark gene sets (Figure 3.19). This is represented by the high positive peak of the enrichment score, reflecting that there is a cluster of matched genes from the E2F_Targets and G2M_Checkpoint hallmark gene sets, and BCL6$^+$ Tfh cells, at the top of the ranked gene list. This enrichment score gradually decreases across the ranked list indicating non-significant number of genes associated with E2F_Targets and G2M_Checkpoint hallmark gene sets in FR4$^+$ EGR2$^-$ MP T cells (Figure 3.19). Previously, Kunzli and colleagues illustrated that antigen-induced memory Tfh cells display a metabolic profile of enriched genes of mammalian target of rapamycin (mmTOR) and glycolytic pathways, often connected to TCR activation and T cell expansion, required for memory Tfh cell maintenance (Künzli *et al.*, 2020). Under homeostatic conditions, genes of glycolysis and MTORC1 signalling are highly expressed in FR4$^+$ EGR2$^+$ MP T cells than in FR4$^+$ EGR2$^-$ MP T cells (Figure 3.19), suggesting a similar metabolic profile of MP Tfh cells to antigen-experienced Memory Tfh cells. In contrast, FR4$^+$ EGR2$^-$ CD4$^+$ CD44^{high} CD25$^-$ MP T cells showed an enrichment of genes involved in the inflammatory responses (Figure 3.19). In summary, FR4$^+$ EGR2$^+$ CD4$^+$ CD44^{high} CD25$^-$ MP T cells from GFP-Egr2/ AmCyan-T-bet kin mice, under homeostatic conditions, display a unique RNA profiles of genes associated with proliferation, metabolism and Tfh cell markers.

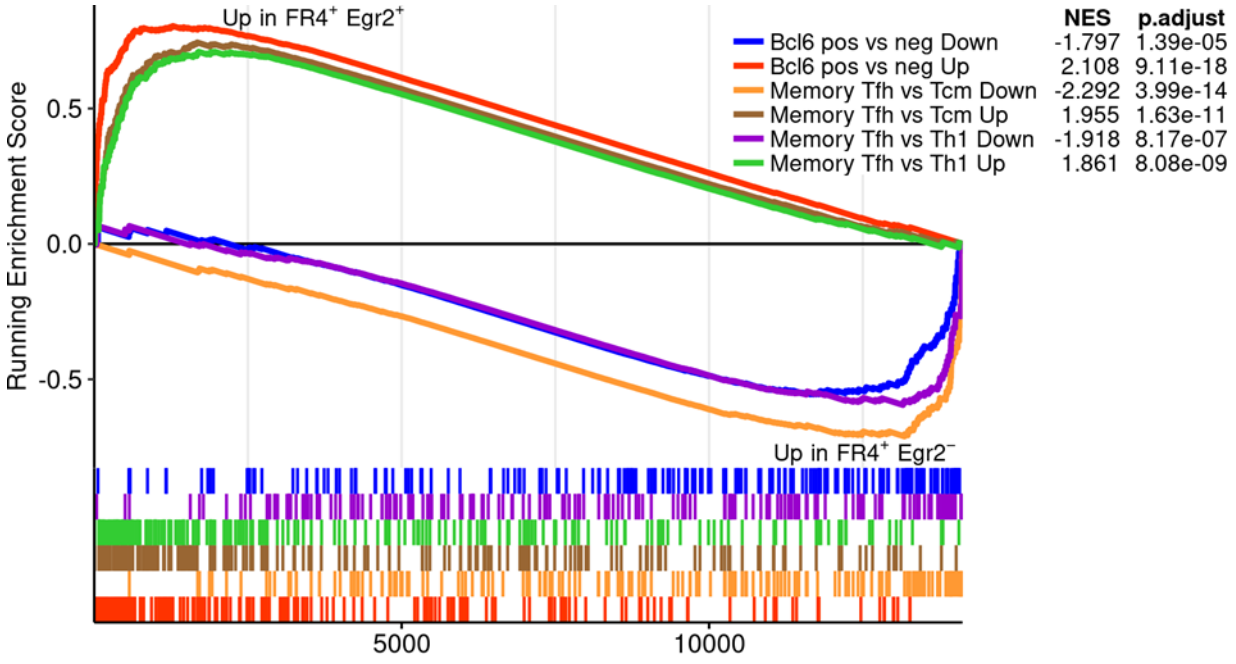


Figure 3.18. Gene Set Enrichment Analysis (GSEA) of FR4^{+} EGR2^{+} and FR4^{+} EGR2^{-} CD4^{+} CD44^{high} CD25^{-} MP T cells from unimmunised GFP-Egr2/ AmCyan-T-bet mice under homeostatic conditions. The enrichment plot shows the distribution of the memory Tfh cell and Bcl6^{+} Tfh gene sets across the ranked genes for FR4^{+} EGR2^{+} and FR4^{+} EGR2^{-} CD4^{+} CD44^{high} CD25^{-} MP T cells. The x-axis corresponds to the individual genes within the ranked gene list and the y-axis represent the degree of enrichment of the gene set across the ranked list. The coloured lines illustrate the enrichment signature associated with the gene sets listed in the key. The gene sets for the BCL6^{+} Tfh and memory Tfh cell are from kunzli's group and, Liu and colleagues, respectively (Liu *et al.*, 2012b; Künzli *et al.*, 2020). The normalised enrichment score (NES) and the p values were adjusted for multiple testing by the Benjamini-Hochberg method.

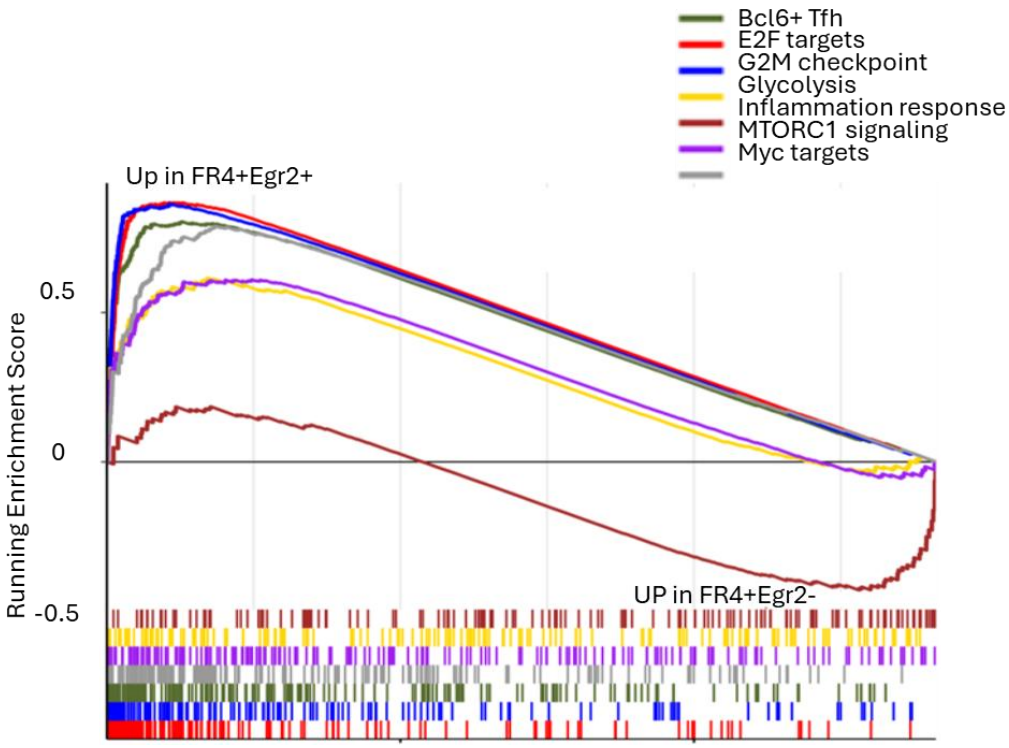


Figure 3.19 Gene set enrichment analysis of FR4$^+$ EGR2$^+$ and FR4$^+$ EGR2$^-$ MP CD4$^+$ T cells. Normalised enrichment scores and Benjamini-Hochberg adjusted p-values are shown. The RNA-seq data are from four biological replicates, each with cells pooled from 10 mice, for each group.

3.5 Functional analysis of MP Tfh cells

3.5.1 Proliferation and interferon gamma production of MP Tfh cells after *in vitro* stimulation

MP Tfh cells highly express proliferation-associated genes under homeostatic conditions, regulated by EGR2 (Figure 3.16, Figure 3.17). Previously, our group showed that EGR2 controls the proliferation of naïve T cells upon TCR activation (Li *et al.*, 2012). To determine the proliferative capacity of MP Tfh cells in homeostasis, we sorted $EGR2^{+}$ $FR4^{+}$, $EGR2^{-}$ $FR4^{+}$ and $EGR2^{-}$ $FR4^{-}$ $CD25^{-}$ $CD44^{high}$ $CD4^{+}$ MP T cells from GFP-Egr2/ AmCyan-T-bet mice and; $EGR2/3^{-/-}$ $FR4^{+}$ and $EGR2/3^{-/-}$ $FR4^{-}$ $CD25^{-}$ $CD44^{high}$ $CD4^{+}$ MP T cells from CD2-$Egr2/3^{-/-}$ /AmCyan-T-bet knockout mice and stimulated, *in vitro*, with anti-CD3 and anti-CD28 antibodies over 72 hours. Before *in vitro* stimulation, the sorted cells were labelled with CellTrace Violet dye which, during cell division, is assumed to distribute evenly between daughter cells visualised as a reduction in the brightness intensity of the cells with several rounds of cell division per cell. Over 80% of $EGR2^{+}$ $FR4^{+}$ MP Tfh cells and more than half of $EGR2^{-}$ $FR4^{-}$ MP T cells proliferated more strongly than $EGR2^{-}$ $FR4^{+}$ MP T cells (Figure 3.20A/C). Without EGR2 expression upon TCR stimulation, no MP T cell proliferation is observed in MP T cells from CD2-$Egr2/3^{-/-}$/AmCyan-T-bet knockout mice (Figure 3.20B/C). Thus, there is a positive correlation between EGR2 upregulation and cellular division upon TCR stimulation in MP T cells, consistent with our previous findings in pathogen-induced T cells (Li *et al.*, 2012).

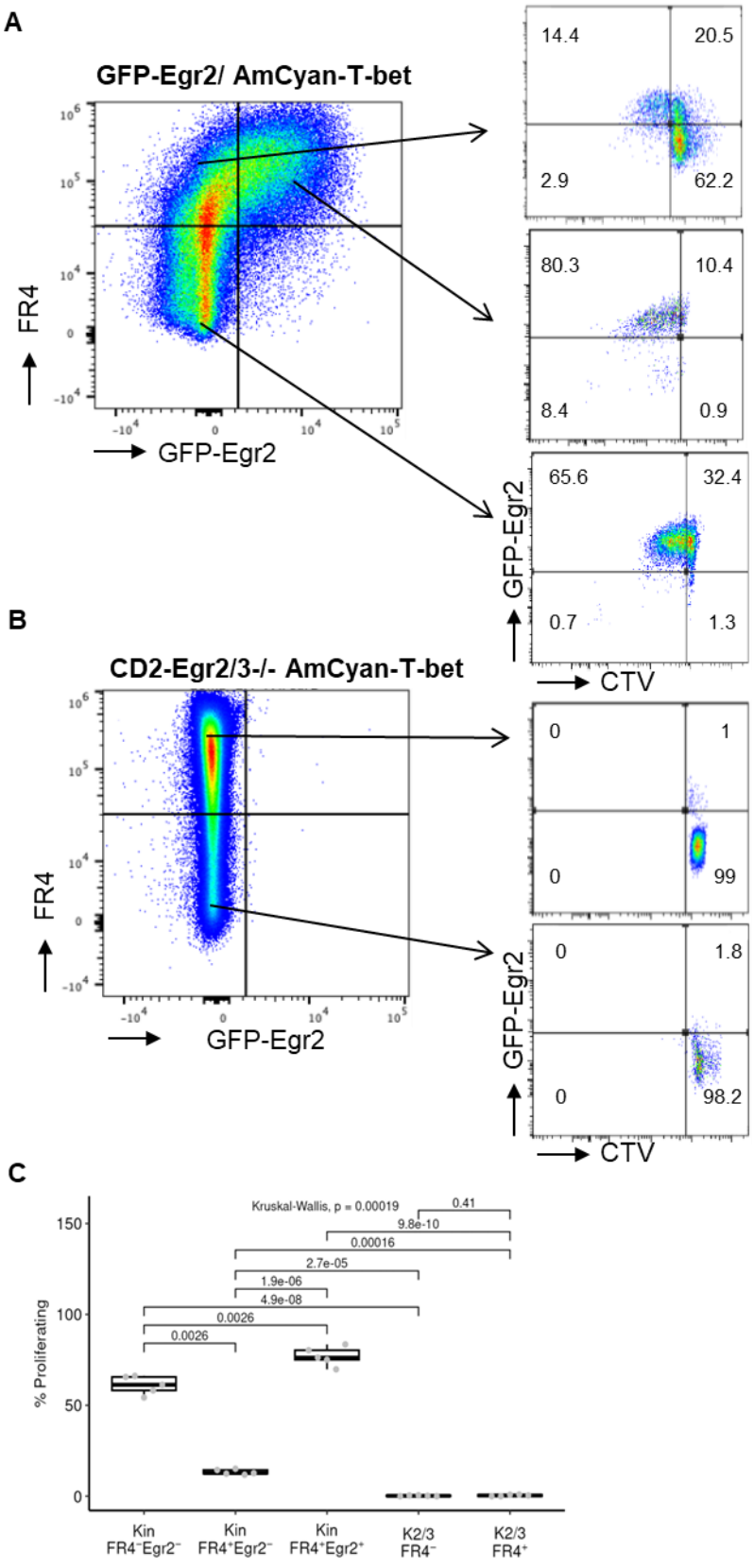

A
GFP-Egr2/ AmCyan-T-bet
FR4
GFP-Egr2
14.4
20.5
2.9
62.2
80.3
10.4
8.4
0.9
65.6
32.4
0.7
1.3
GFP-Egr2
CTV
B
CD2-Egr2/3-/- AmCyan-T-bet
FR4
GFP-Egr2
0
1
0
99
0
1.8
0
98.2
GFP-Egr2
CTV
C
Kruskal-Wallis, p = 0.00019
0.41
9.8e-10
0.00016
2.7e-05
1.9e-06
4.9e-08
0.0026
0.0026
% Proliferating
150
100
50
0
Kin FR4⁻Egr2⁻
Kin FR4⁺Egr2⁻
Kin FR4⁺Egr2⁺
K2/3 FR4⁻
K2/3 FR4⁺

Figure 3.20. Proliferative responses of FR4^{+} EGR2^{+} MP Tfh cells. FR4^{+} EGR2^{+}, FR4^{+} EGR2^{-} and FR4^{-} EGR2^{-} MP T cells from GFP-Egr2/ AmCyan-T-bet mice and FR4^{+} EGR2^{-} and FR4^{-} EGR2^{-} MP T cells from CD2-Egr2/3$^{-/-}$ AmCyan-T-bet mice were isolated and stained for CellTrace Violet dye. The cells were then stimulated with anti-CD3 and anti-CD28 for 72 hours and were analysed for proliferation. The data were from a group of five mice and represent three repeated experiments. In (C) the upper, median and lower quartiles are shown for the five mice and the significance was tested by Kruskal-Wallis tests, followed by two-tailed Conover tests with Benjamini-Hochberg correction. Kin: GFP-Egr2 / AmCyan-T-bet mice; K2/3: CD2-Egr2/3-/- / AmCyan-T-bet mice.

Genes associated with the Inflammatory response were highly expressed in FR4^{-} EGR2^{-} MP T cells (Figure 3.17). In an inflammatory response, T-bet mediates IFN-γ production in pathogen-induced Th1 cells (Szabo *et al.*, 2002) and T-bet^{high} MP T cells during viral infection (Kawabe *et al.*, 2017). As expected, upon *in vitro* stimulation, there is almost no IFN-γ production reported in MP Tfh cells (Figure 3.21A/C). In contrast, more than 60% of EGR2^{-} FR4^{+} and EGR2/3$^{-/-}$ FR4^{+} MP T cells produced IFN-γ (Figure 3.21). These results showed that MP Tfh cell enriched EGR2^{+} FR4^{+} subset is proliferative active and importantly showed limited inflammatory responses.

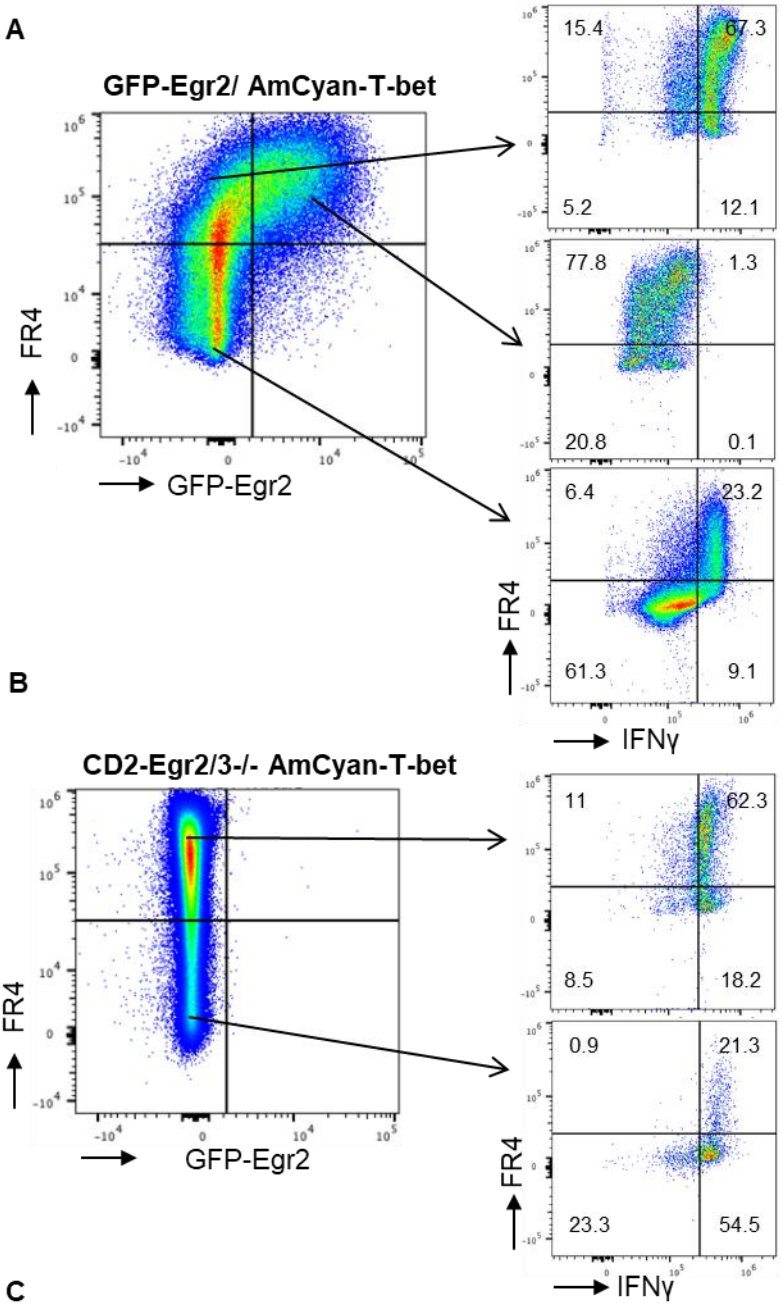

A
GFP-Egr2/ AmCyan-T-bet
FR4
GFP-Egr2
15.4
67.3
5.2
12.1
77.8
1.3
20.8
0.1
6.4
23.2
61.3
9.1
FR4
IFNγ
B
CD2-Egr2/3-/- AmCyan-T-bet
FR4
GFP-Egr2
11
62.3
8.5
18.2
0.9
21.3
23.3
54.5
FR4
IFNγ
C
Kruskal-Wallis, p = 0.00086
0.36
9.4e-06
0.48
0.77
2.6e-05
0.0021
0.068
0.0013
% IFNγ+
150
100
50
0
Kin FR4−Egr2−
Kin FR4+Egr2−
Kin FR4+Egr2+
K2/3 FR4−
K2/3 FR4+

Figure 3.21. Type 1 inflammatory responses of FR4$^+$ EGR2$^+$ MP Tfh cells. FR4$^+$ EGR2$^+$, FR4$^+$ EGR2$^-$ and FR4$^-$ EGR2$^-$ MP T cells from GFP-Egr2/ AmCyan-T-bet mice and FR4$^+$ EGR2$^-$ and FR4$^-$ EGR2$^-$ MP T cells from CD2-Egr2/3$^{-/-}$ AmCyan-T-bet mice were isolated and then stimulated with anti-CD3 and anti-CD28 for 72 hours and analysed for IFN-γ production. The data were from a group of five mice and represent three repeated experiments. In (C) the upper, median and lower quartiles are shown for the five mice and the significance was tested by Kruskal-Wallis tests, followed by two-tailed Conover tests with Benjamini-Hochberg correction. Kin: GFP-Egr2 / AmCyan-T-bet mice; K2/3: CD2-Egr2/3-/- / AmCyan-T-bet mice.

3.5.2 MP Tfh and MP Tfr cell function in B cell-mediated IgG production following *in vitro* stimulation

MP Tfh cells share a similar phenotype to pathogen-induced memory Tfh cells, expressing CD44, CXCR5, BCL6, FR4 and PD-1 (Figure 3.7; Figure 3.8). EGR2 and BCL6 transcriptional regulators are induced upon environmental antigen stimulation driving pathogen-induced Tfh cell differentiation and effector Tfh functions (Ogbe *et al.*, 2015; Yu *et al.*,2009). Upon pathogen encounter, IL-6 and IL-2, in addition to TCR signalling induce early expression of BCL6 initiating the earliest stages of Tfh cell differentiation outside the germinal centres (Choi *et al.*, 2013; Crotty, 2014). EGR2 is also induced upon T cell signalling and plays an important role in Tfh cell development by directly regulating the expression of BCL6 (Ogbe *et al.*, 2015). Once Tfh cells terminally differentiate into GC Tfh cells, GC Tfh cells produce IL-21 and IL-4 which are involved in GC B cell proliferation, differentiation and isotype class switching (Zotos *et al.*, 2010; Weinstein *et al.*, 2016). To assess the functionality of MP Tfh and MP Tfr cells, we first sorted MP Tfh cells, naïve T cells, non-Tfh MP T cells, and MP Tfr cells, and then analysed for the expression of Tfh cell function-associated genes using real-time PCR. The transcription factors, EGR2 and BCL6, were highly expressed in MP Tfh cells and MP Tfr cells but lowly expressed in other non-Tfh MP T cells and naïve T cells (Figure 3.22). In addition, IL-21 was highly expressed in MP Tfh cells compared to MP Tfr cells, indicating MP Tfh cells exert a similar function to pathogen-induced memory Tfh cells whereas MP Tfr cells could possess a Treg-like regulatory function (Figure 3.22). Neither naïve T cells nor non-Tfh MP T cells expressed *Il21*, confirming pure isolation of MP Tfh cells using the Tfh cell markers PD1, CXCR5, and the absence of the Tfr marker, GITR. Helios, encoded by *Ikzf2*, regulates the suppressive function of pathogen-induced Tregs (Kim *et al.*, 2015). We found that GITR$^+$ MP Tfr cells highly express *Ikzf2* (Figure 3.22). In contrast, *Ikzf2* was lowly expressed in MP Tfh cells. These results indicate an adaptive function of MP Tfh cells, similar to that of pathogen-induced memory Tfh cells (Hale and Ahmed, 2015).

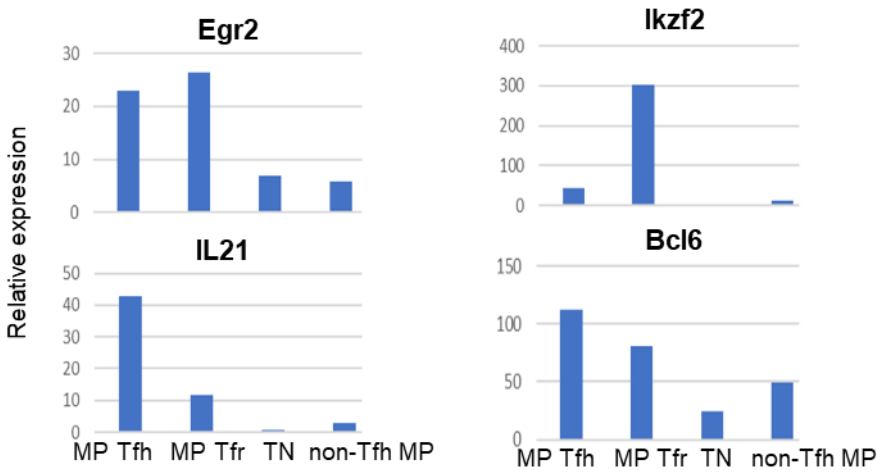


Figure 3.22. Real-time PCR analysis of *Egr2*, *Ikzf2*, *Il21* and *Bcl6* transcripts in memory-phenotype (MP) T cells. $CD4^+$ $CD44^{low}$ naïve T cells (TN), $CXCR5^+$ $PD\text{-}1^+$ $GITR^-$ $CD4^+$ $CD44^{high}$ MP T follicular helper (Tfh) cells, $CXCR5^+$ $PD\text{-}1^+$ $GITR^+$ $CD4^+$ $CD44^{high}$ MP T follicular regulatory (Tfr) cells, and $CXCR5^-$ $PD\text{-}1^-$ $GITR^-$ $CD4^+$ $CD44^{high}$ non-follicular MP T cells (non-Tfh) were isolated by fluorescence activated cell sorting from GFP-Egr2/ AmCyan-T-bet mice. The expression of *Egr2*, *Ikzf2*, *Il21* and *Bcl6* were analysed by real-time PCR. The data represents two independent experiments.

To further investigate the role of these isolated MP Tfh and MP Tfr cells in B cell responses *in vitro*, we cultured isogenic B cells with isolated MP Tfh with and without the addition of MP Tfr cells. The cells were stimulated with anti-CD3 and anti-IgM, and IgG levels were measured using ELISA. As controls, we designed 2 other cultures: B cells alone and, B cells cultured with naïve T cells. Naïve T cells do not support IgG production because they lack Tfh-associated features required to provide B cell help, such as CXCR5, PD-1, and BCL6 (Crotty, 2014). We found that there is a statistically significant difference in IgG levels detected in the culture of the B cell with MP Tfh cells compared to that of the controls, B cell with naïve T cells and B cells alone (P=0.00014 and P=0.00079, respectively) (Figure 3.23). Although there is not a statistically significance difference between the IgG levels detected between the B cell cultures with MP Tfh cells only and the culture with a combination of MP Tfh and MP Tfr cells, the median level of IgG produced with the presence of MP Tfr cells is much lower than without MP Tfr cells in culture but slightly higher than the controls (Figure 3.23). Therefore, this suggests that MP Tfh cells promote B cell-mediated IgG production whereas MP Tfr cells limit IgG production, similarly to conventional Tfr cells (Clement *et al.*, 2019). In addition, there are no significant differences between the baseline controls (Figure 3.23).

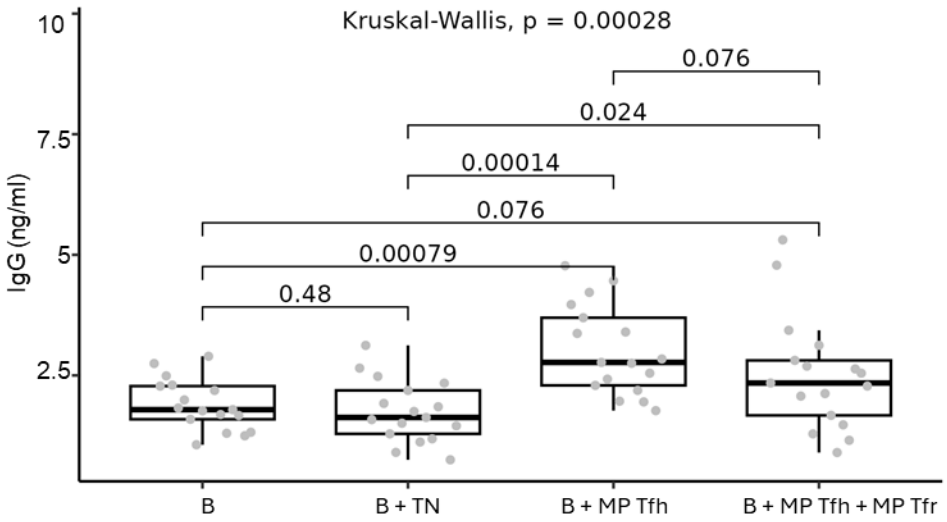


Figure 3.23- The role of memory-phenotype (MP) CD4$^+$ T cells in the regulation of B cell-mediated IgG *in vitro*. The following cells were sorted by fluorescently activated cell sorting: CD4$^+$ CD44^{low} naïve T cells (TN), CXCR5$^+$ PD-1$^+$ GITR$^-$ CD4$^+$ CD44^{high} MP T follicular helper (TF) cells, CXCR5$^+$ PD-1$^+$ GITR$^+$ CD4$^+$ CD44^{high} MP T follicular regulatory (Tfr) cells and B220$^+$ CD4$^-$ B cells. The sorted B cells were cultured with naïve, MP Tfh or MP Tfh and MP Tfr cells and stimulated with anti-CD3 and anti-IgM. The boxplot shows the IgG levels within the different cultures that was measured, on day 6 after stimulation, by enzyme-linked immunosorbent assay (ELISA) IgM (See Materials and Methods). Each dot on the graph represents an individual data point from three independent experiments and the calculated median, lower and upper quartiles for each group are shown by the boxplot. The statistical significance was determined by the Kruskal-Wallis test (p = 0.00028), followed by pairwise comparisons by using Conover tests and corrected for by the Benjamini-Hochberg method. A p value < 0.05 is the cutoff for a statistically significant difference.

3.5.3 MP Tfh cells induce germinal centre development in response to vaccinia virus infection

GCs form within B cell follicles in secondary lymphoid organs such as the lymph nodes and spleen during infection or vaccinations (Young and Brink, 2021). Tfh cell-B cell interactions within the GCs initiate GC B cell expansion and generation of high-affinity antibody-secreting plasma cells and long-lived B cells for protective humoral immunity (Crotty, 2019). Memory Tfh cells are committed to GC Tfh effector function during secondary responses (Hale and Ahmed, 2015). We found that MP Tfh cell development is severely impaired in CD2-Egr2/3$^{-/-}$/AmCyan-T-bet mice (figure 3.13A/B). To examine the functionality of MP Tfh cells in germinal centre development, we sorted FR4^{+} EGR2^{+} and FR4^{+} EGR2^{-} MP T cells, from the Egr2-GFP/ AmCyan-T-bet mice, and adoptively transferred into CD2-Egr2$^{-/-}$ /Egr3$^{-/-}$ mice, following infection with vaccinia virus. To analyse GC formation, spleen tissue was harvested from vaccinia virus infected recipient CD2-Egr2$^{-/-}$ /Egr3$^{-/-}$ mice and stained with B and T cell markers, B220 and CD3, respectively. Peanut agglutinin (PNA) was used to label proliferating GC B cells to visualise GC reactions. In response to vaccinia virus infection, germinal centre formation was defective in CD2-Egr2$^{-/-}$ /Egr3$^{-/-}$ mice, with no donor cells (Figure 3.24A). Upon transfer of FR4^{+} EGR2^{+} MP T cells, but not FR4^{+} EGR2^{-} MP T cells, into infected CD2-Egr2$^{-/-}$ /Egr3$^{-/-}$ recipient mice, GC formation was restored (figure 3.24A). Furthermore, 6.4% of BCL6^{+} CXCR5^{+} MP Tfh cells were only found in FR4^{+} EGR2^{+} MP T cell population in recipient mice, and restored expression of EGR2 (Figure 3.24B/C). However, FR4^{+} EGR2^{-} MP T cells did not restore MP Tfh cells in recipient mice (Figure 3.24B/C). Thus, MP Tfh cells, expressing EGR2, are required for germinal centre reactions during viral infections.

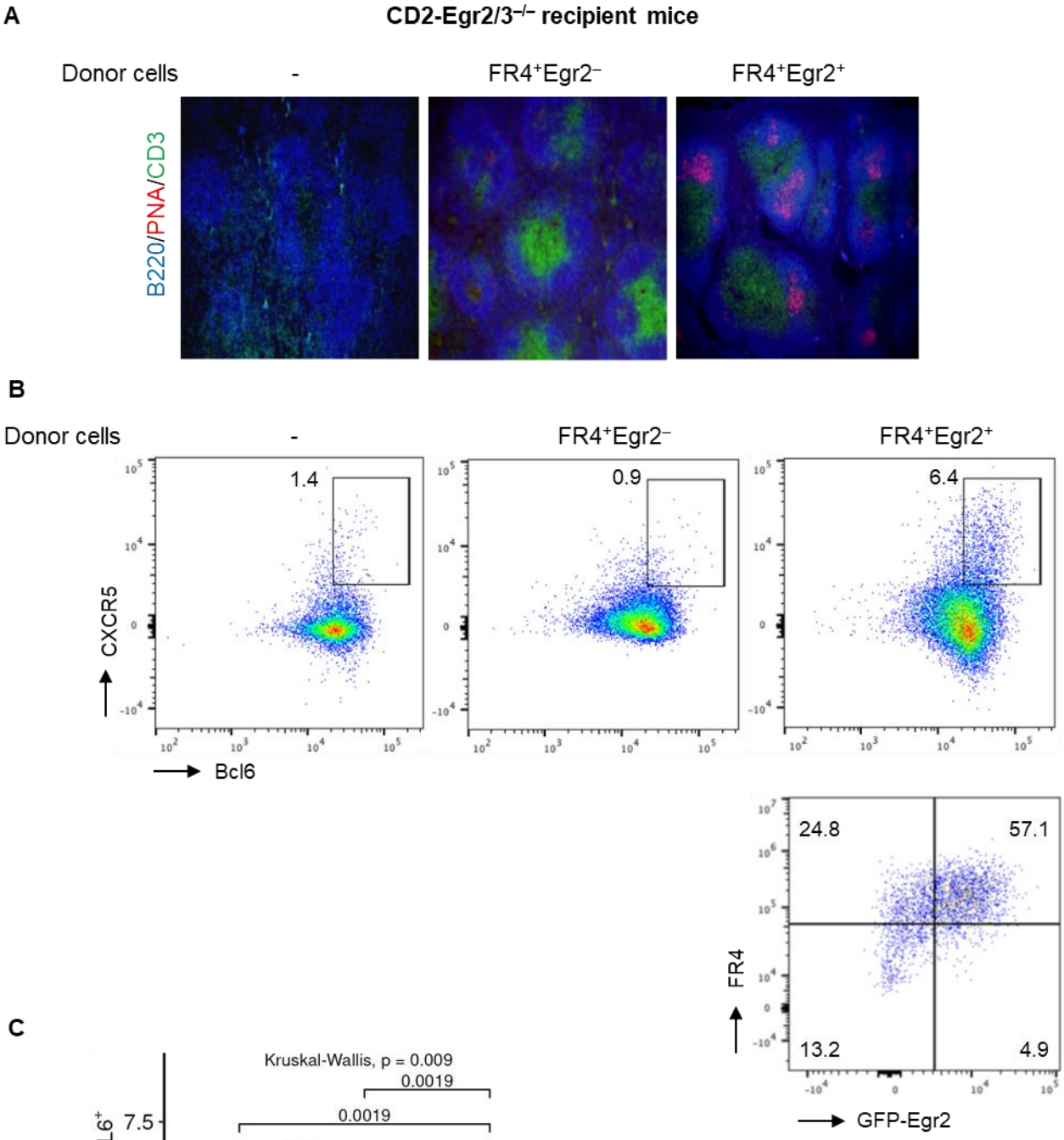

A
CD2-Egr2/3−/− recipient mice
Donor cells
-
FR4+Egr2−
FR4+Egr2+
B220/PNA/CD3
B
Donor cells
-
FR4+Egr2−
FR4+Egr2+
1.4
0.9
6.4
CXCR5
Bcl6
24.8
57.1
13.2
4.9
FR4
GFP-Egr2
C
Kruskal-Wallis, p = 0.009
0.0019
0.0019
0.82
% CXCR5+ BCL6+
7.5
5
2.5
0
K2/3
FR4+Egr2−
FR4+Egr2+

Figure 3.24. Germinal center development in CD2-Egr2/3$^{-/-}$ recipient mice upon transfer of CD4^{+} Memory-phenotype (MP) T cells with differential expression of EGR2 and FR4. Sorted FR4^{-} EGR2^{+} CD25^{-} CD44^{high} CD4^{+} MP T cells and FR4^{+} EGR2^{+} CD25^{-} CD44^{high} CD4^{+} MP T cells from GFP-Egr2/ AmCyan-T-bet mice were adoptively transferred into CD2-Egr2/3$^{-/-}$ recipient mice. 24 hours after transfer, the mice were infected with vaccinia virus infection and then the spleen was extracted for analysis by immunohistochemistry and flow cytometry. (A) The splenic tissues were stained with anti-B220 (in blue), PNA (red) and anti-CD3 (green) to detect B cells, GC B cells and T cells, respectively. (B) CXCR5^{+} BCL6^{+} CD44^{high} CD4^{+} Tfh cells were gated from spleen and lymph nodes from the recipients on day 14 after infection. In (C) the upper, median and lower quartiles are shown, and the significance was tested by Kruskal-Wallis tests, followed by two-tailed Conover tests with Benjamini-Hochberg correction. The data were from a group of five mice and represent three repeated experiments. K2/3: CD2-Egr2/3-/- / AmCyan-T-bet mice.

3.5.4 MP Tfh cells support neutralising antibody production in response to viral infection

MP Tfh cells develop germinal centres in response to viral infection (Figure 25). During pathogen encounters, high-affinity GC B cells produce antibodies through antibody class switching and SHM (Hamel, Liarski and Clark, 2012) . To study for vaccinia virus-neutralising antibody production by MP Tfh cells in CD2-Egr2$^{-/-}$ /Egr3$^{-/-}$ recipient mice, sera from the recipient mice were serially diluted and added to plated TK143 cells and left for infection. After 2 days, plaque formation, indicative of TK143 cell infection by vaccinia virus, was analysed. The plaque reduction neutralisation test showed that serum collected from CD2-Egr2$^{-/-}$ /Egr3$^{-/-}$ mice with transferred FR4^{+} EGR2^{-} MP T cells caused a higher number of plaques in TK143 cells, similar to the plaque levels in the control cells, at each dilution ratio (Figure 3.25). This indicates the serum does not contain neutralising antibodies. In comparison, the number of plaques was significantly reduced in CD2-Egr2$^{-/-}$ /Egr3$^{-/-}$ recipient mice with transferred FR4^{+} EGR2^{+} MP T cells, at each dilution ratio (Figure 3.25). Overall, MP Tfh cells promote GC formation and exert Tfh effector cell functions during effector the humoral responses against pathogens.

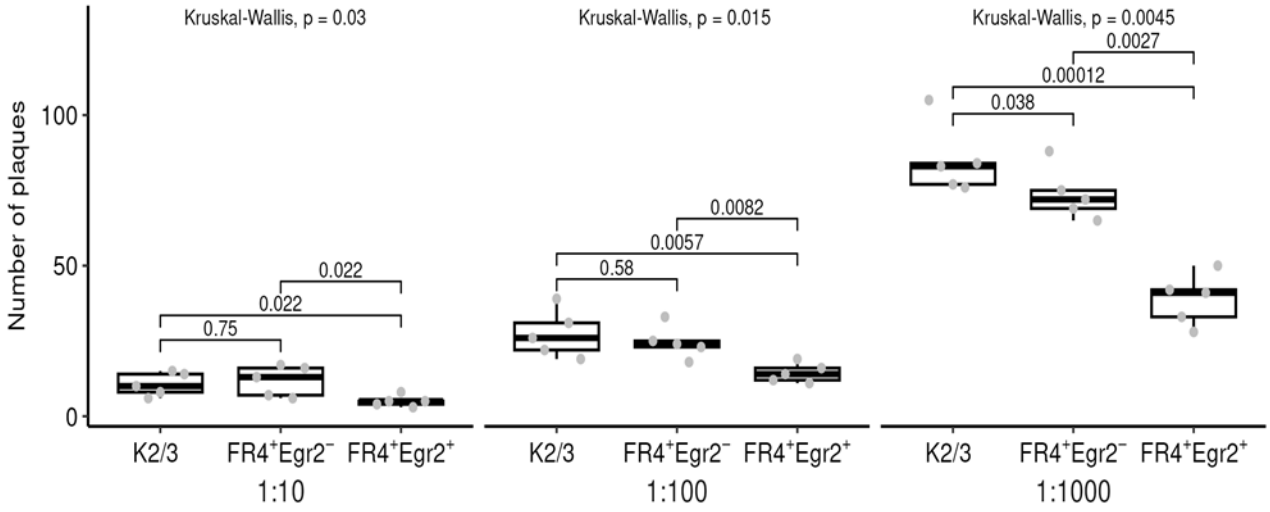


Figure 3.25. Analysis of neutralising antibody production in the presence of CD4⁺ Memory-phenotype (MP) T cells with differential expression of EGR2 and FR4. The sera of VACV-WR infected CD2-Egr2/3$^{-/-}$ recipient mice with either the adoptive transfer of CD4^{+} CD44^{high} CD25^{-}FR4^{-} EGR2^{+} MP T cells, CD4^{+} CD44^{high} CD25^{-} FR4^{+} EGR2^{+} MP T cells, or neither (K2/3) was collected on day 14 after infection. The serum was then tested for neutralising antibodies using the plaque reduction neutralization test. The sera were diluted and the number of viral plaques formed by five mice is shown as individual data points. The calculated upper, median and lower quartiles of the number of viral plaques for the five mice are illustrated on the boxplot. The statistical significance between the three groups was tested by the Kruskal-Wallis tests, followed with comparisons of the viral plaques between two groups using the Conover tests with Benjamini-Hochberg correction applied. The p values are indicated, with significance considered if the p value is less than 0.05. K2/3: CD2-Egr2/3-/- / AmCyan-T-bet mice.

Chapter 4

Discussion

4.1 Summary of findings

$CD4^+$ T cells play a major role in initiating a pathogen-specific immune response by helping other immune cells, such as B cells, exert their effector functions. During an infection, naïve $CD4^+$ T cells with cognate TCR for a specific pathogenic antigen presented on MHC class II become activated and undergo extensive proliferation and differentiation into the classical effector T helper cells, Th1, Th2 or Th17. Although Tfh are activated during infection, unlike classical T helper cells that are specialised to respond to distinct type of infections (e.g. Th1 cells respond to intracellular pathogens whereas Th17 cells respond to extracellular fungi and bacteria), these cells are generated against various infection types including intracellular and extracellular pathogens (Luckheeram *et al.*, 2012). Pathogen-induced Tfh cells are important for high affinity antibody production by cognate B cells (Crotty, 2014). Once an infection clears, majority of the effector T cells undergo apoptotic cell death while ~10% remain as memory T cells, restoring T cell homeostasis (Sallusto *et al.*, 2010). These memory cells remain in a quiescent state in the absence of foreign antigens but provide a faster response upon re-infection (Gasper, Tejera and Suresh, 2014). There is also a population of T regulatory cells, comprising thymic- and peripherally derived subsets, that maintain immune homeostasis by preventing autoimmune responses and controlling excessive inflammation (Workman *et al.*, 2009). Thus, at homeostasis, the T cell pool consists of naïve, memory and regulatory phenotype T cells (Workman *et al.*, 2009; Kawabe, Yi and Sprent, 2021). However, the memory T cell pool not only consists of pathogen-induced memory T cells, but growing evidence suggests that MP T cells develop in pathogen-free conditions through HP of naïve T cell precursors triggered by self-antigen stimulation and homeostatic cytokine signalling (Kawabe and Sher, 2021). This form of HP is found in lymphopenic hosts to restore T cell homeostasis referred to as lymphopenia-induced memory phenotype T cells, but the MP T cell population is also found in similar frequencies within lymphosufficient GF, SPF and AF mice (Min *et al.*, 2003; Kawabe *et al.*, 2017). MP T cells are important for an immune response against infection and in the progression of autoimmune diseases and, are largely present in aged mice where there is a decline of naïve T cells (Sprent *et al.*, 2008; Marusina *et al.*, 2016; Kawabe *et al.*, 2017b; Cho *et al.*, 2023). However, the mechanism of MP T cell development and functions are largely unknown.

In this study, we found the population of $CD44^{high}$ $CD4^+$ MP T cells in unimmunised lymphosufficient GFP-Egr2/ AmCyan-T-bet mice raised and maintained in SPF conditions. Similar to pathogen-induced memory $CD4^+$ T cells, these $CD44^{high}$ $CD4^+$ MP T cells exhibited a

predominant effector $CD4^+$ memory T cell phenotype ($CD62L^-$ cells) and a minor population of $CD44^{high}$ $CD4^+$ MP T cells were phenotypically similar to central memory $CD4^+$ T cells with high expression of CD62L. Unlike pathogen-induced memory T cells, which remain largely quiescent after pathogen clearance, $CD44^{high}$ $CD4^+$ MP T cells, comprising both Tcm and Tem subsets, contain both $Ki67^+$ and $Ki67^-$ populations even at steady state. In addition, we found that $CD44^{high}$ $CD4^+$ MP T cells further display heterogeneity in terms of the differential expression of the transcription factors, EGR2 and T-bet. The following subsets of $CD44^{high}$ $CD4^+$ MP T cells were observed in homeostatic conditions: $EGR2^+$ $T\text{-}bet^-$, $EGR2^-$ $T\text{-}bet^+$ and $EGR2^-$ $T\text{-}bet^-$ $CD44^{high}$ $CD4^+$ MP T cells, with unknown function of EGR2 in $CD44^{high}$ $CD4^+$ MP T cells, and relevance of these distinct populations is yet to be determined.

We found $CD44^{high}$ $CD4^+$ MP T cells with a Tfh-like function developed in the absence of foreign antigen stimulation in GFP-Egr2/ AmCyan-T-bet mice. MP Tfh cells highly express EGR2 and MP Tfh cell development is impaired in CD2-Egr2/3$^{-/-}$ AmCyan-T-bet mice. MP Tfh cells are defined by the expression of pathogen-induced Tfh cell-specific markers, BCL6, CXCR5, FR4, EGR2 and PD-1 and, consisted of $FOXP3^+$ $GITR^+$ MP Tfr subpopulation. *In vitro*, MP Tfh cells promote B cell-mediated IgG production whereas MP Tfr cells exert a suppressive role, similar to conventional Tfr cells. RNA sequencing showed that Tfh cell specific genes are significantly enriched in the $FR4^+$ $EGR2^+$ $CD44^{high}$ $CD4^+$ MP T cells, compared to $FR4^+$ $EGR2^-$ and $FR4^-$ $EGR2^-$ $CD44^{high}$ $CD4^+$ MP T cells. In addition, T cell activation and proliferation genes were also highly upregulated in $FR4^+$ $EGR2^+$ MP T cells, in line with the rapid proliferation of $FR4^+$ $EGR2^+$ MP T cells after *in vitro* stimulation with anti-CD3 and anti-CD28. However, these cells did not produce IFN-γ as expected as inflammatory genes are highly enriched in $FR4^+$ $EGR2^-$ MP T cells. We further showed that $FR4^+$ $EGR2^+$ $CD44^{high}$ $CD4^+$ MP T cells have a metabolic signature similar to that of pathogen-induced memory Tfh cells. Thus, MP Tfh cells can be defined by the dual expression of EGR2 and FR4. *In vivo*, MP Tfh cells restore germinal centre development and anti-viral antibody production upon adoptive transfer of $FR4^+$ $EGR2^+$ $CD44^{high}$ $CD4^+$ MP T cells into CD2-Egr2/3$^{-/-}$ AmCyan-T-bet mice.

Overall, we show that MP Tfh and regulatory MP Tfr cells represent distinct subsets within the broader $CD4^+$ MP T cell compartment, and these subsets display phenotypes and functions similar to pathogen-induced memory T cells. As the T cell pool consists largely of MP T cells with age, these cells remain important to explain the immune responses in elderly people against infections such as COVID (Seok *et al.*, 2023).

4.2 $CD4^+$ MP T cell development

Pathogen-induced memory Tfh cells are generated in response to specific exogenous antigens. During the secondary response, the pathogen-induced memory Tfh cells upregulate BCL6 and re-enter the GCs to regulate the humoral response (Crotty, 2014; Hale and Ahmed, 2015). We have found that MP Tfh cells, developed in pathogen-free conditions, exert similar Tfh effector functions to pathogen-induced Tfh memory cells (Crotty, 2014) in response to viral infection. However, the mechanism for the development of MP Tfh cells in the absence of foreign antigens is unknown.

Pathogen-induced Tfh cell differentiation is a complex mechanism relative to the differentiation of naïve T cells into non-Tfh helper $CD4^+$ T cells. Pathogen-induced Tfh cells require ongoing TCR signalling, first initiated by antigen-presenting DCs, and then by B cells for terminal differentiation. Multiple cytokines, such as IL-6 and IL-2, and costimulatory signalling pathways (e.g. ICOS/ICOSL) are involved in driving the Tfh cell differentiation programme (Crotty, 2014). $CD4^+$ MP T cells require homeostatic cytokine signalling and interactions with antigen-presenting DCs for their development (Kawabe *et al.*, 2017b, 2020; Cho *et al.*, 2023). However, $CD4^+$ MP T cells are generated in the periphery, from naïve T cell precursors, in response to self-antigen stimulation in the absence of foreign antigen stimulation (Min *et al.*, 2005; Kawabe, 2023). Our results demonstrate that CD5, which is a marker of self-reactive T cells (Richards, Kyewski and Feuerer, 2016), is highly expressed in $FR4^+$ $EGR2^+$ $CD44^{high}$ $CD4^+$ MP T cells which are highly enriched in MP Tfh genes. We also found that genes induced upon TCR signalling such as *Egr2* (Anderson *et al.*, 2006; Miao *et al.*, 2017), *Cd69* (Cibrián and Sánchez-Madrid, 2017) and *Icos* (Mahajan *et al.*, 2007) are highly expressed in $FR4^+$ $EGR2^+$ $CD44^{high}$ $CD4^+$ MP T cells. These findings support a possible developmental mechanism in which $CD5^{high}$ naïve T cells, which are highly self-reactive, receive tonic TCR signalling from self-peptide-MHC interactions that induce weaker T cell activation than that elicited by pathogenic antigens and may promote differentiation into $CD5^{high}$ MP Tfh cells under steady-state conditions. Pathway analysis of isolated $FR4^+$ $EGR2^+$ $CD44^{high}$ $CD4^+$ MP T cells showed that glycolytic- and mTORC1- associated genes are significantly enriched in MP Tfh cells. This indicates that MP Tfh cells are in a metabolically activated state as glycolysis is induced in activated T cells, whereas naïve T cells have low metabolic activity (Xu and Powell, 2018). Overall, MP Tfh cells display activated T cell phenotype and metabolic signature which strongly indicates that MP Tfh cells are induced by self-antigens in the absence of pathogen encounters.

DC maturation is induced by PAMPS, however under homeostatic conditions and germ-free conditions, DCs mature and present self-antigen-MHC class II complexes to self-reactive T cells (Bosteels and Janssens, 2024). This suggests that homeostatic DCs, in absence of foreign antigen, could present self-antigens to $CD5^{high}$ naïve T cell precursors inducing MP T cell generation. The differentiation of T-bet^{high} $CXCR3^{+}$ $CD44^{high}$ $CD62L^{low}$ $FOXP3^{-}$ $CD4^{+}$ MP T cell subpopulation has been extensively studied, providing insight into the role of DCs in homeostatic conditions (Kawabe *et al.*, 2020). Conventional type 1 $CD8\alpha^{+}$ DCs (DC1) mediate type 1 immune responses against intracellular pathogens through the secretion of IL-12 (Mashayekhi *et al.*, 2011). In absence of pathogens, TLR-mediated MyD88 signalling primes DC1 for tonic IL-12 production which are further enhanced by costimulatory signalling via CD40L/CD40. Antigen-presenting DC1 located near the T cell zone interact with T cells with relatively high affinity to self-antigens which induces T-bet^{high} $CD4^{+}$ MP T cell differentiation from naïve T cell precursors (Kawabe *et al.*, 2020). During an immune response, pathogen-induced Tfh cells develop from naïve T cells with initial Tfh cell priming by dendritic cells followed by terminal differentiation into GC Tfh cells induced by B cells (Deenick and Ma, 2011; Crotty, 2019). In infectious conditions, initial Tfh cell differentiation is promoted by $CD8\alpha^{-}$ DCs through antigen-presentation, and ICOSL and OX40L signalling which induces the expression of Tfh-cell specific genes such as CXCR5 required for the migration of pre-Tfh cells into the CXCL13-rich B cell follicles for germinal centre development (Moser, 2015; Shin *et al.*, 2015). DCs also produce the cytokines IL-6, IL-27 and IL-12 which are required to induce early Tfh cell differentiation programme (Choi *et al.*, 2013; Gringhuis *et al.*, 2014; Schmitt *et al.*, 2009). Thus, we speculate that a homeostatic DC subset primes naïve T cells by self-antigen-MHC class II complex, in the presence of specific homeostatic cytokines, to drive MP Tfh cell differentiation in unimmunised lymphosufficient mice.

Natural antibodies are found which respond to self-antigens and exhibit a spectrum of affinities towards self-antigens which steers the natural antibodies to either a protective or pathogenic/autoimmune fate (Elkon and Casali, 2008). Natural antibodies include IgM and IgG. The former is produced by B1 B cells independent of T cell-B cell interactions. Whereas the latter is produced by follicular B cells and requires T cell to support SHM responses in the GCs (Elkon and Casali, 2008; Nagele *et al.*, 2013). This indicates a plausible function of MP Tfh cells in helping the follicular B cell-mediated IgG production in steady state conditions.

4.3 CD4$^+$ MP T cell functions

CD4$^+$ MP T cells are a heterogenous population. Although some groups isolate and define CD44^{high} CD4$^+$ MP T cells as CD62L^{low} (Kawabe *et al.*, 2017b, 2020, 2022; Cho *et al.*, 2023; Kawajiri *et al.*, 2024), we and others (Charlton *et al.*, 2015; Marusina *et al.*, 2016) show that CD44^{high} CD4$^+$ MP T cells consist of a central (CD62L^{high}) and effector (CD62L^{low}) memory phenotype compartment. We found that the effector memory phenotype is the predominant population of CD4$^+$ MP T cells in unimmunised mice. By conducting RNA sequencing analysis of FR4 and EGR2 expressing MP cells with an MP Tfh phenotype we found that these cells have reduced expression of Tcm-related genes such as *Ccr7*. Thus, this indicates that MP Tfh cells are unlikely to display a central memory phenotype, which is defined by the expression of CCR7 and CD62L. Instead, MP Tfh cells display an effector memory phenotype, lacking CCR7 expression, and are terminally differentiated with rapid effector function, whereas, central memory T cells retain plasticity, express CCR7 and have higher proliferative potential than effector function during an immune response (Gray, Westerhof and MacLeod, 2018; Künzli and Masopust, 2023).

Pathogen-induced memory T cells with either a Th1, Th2, Th17, Treg or Tfh phenotype remain in the periphery after pathogen clearance (Künzli and Masopust, 2023). Several studies indicate that CD4$^+$ MP T cells found in pathogen-free conditions, differentiate into Th1, Th17, Th2 and Treg MP T cells with a similar phenotype and effector responses to that of their pathogen-induced memory T cells counterparts (Zhu and Paul, 2009; Kawabe *et al.*, 2020; Cho *et al.*, 2023; Kawajiri *et al.*, 2024). CD4$^+$ MP T cells are important part of the immune system and participate in TCR-independent innate responses and pathogen-specific responses (Kawabe *et al.*, 2017b; Cho *et al.*, 2023), even though these cells have not encountered the pathogen before (Su *et al.*, 2013; Marusina *et al.*, 2016; Afroz, Bartolo and Su, 2023).

In vitro, IL-1 family cytokines and STAT-activating cytokines initiate responses of innate immune cells such as NKT cells and stimulate effector responses of T helper cell subsets (Th1, Th2 and Th17) in the absence of TCR-dependent activation (Guo *et al.*, 2009, 2015; Guo, Junttila and Paul, 2012). *In vivo*, upon foreign antigen encounter, pathogen induced T helper cell differentiation is driven by specific cytokine signalling. IL-12 and IL-18 produced by antigen-presenting cells is required to induce T-bet$^+$ Th1 differentiation through activation of STAT4, and GATA3$^+$ Th2 differentiation is promoted by IL-4/STAT6 signalling as well as IL-25 and IL-33 (O'Garra, 2000; Divekar and Kita, 2015; Saravia, Chapman and Chi, 2019). IL-6 and IL-23-mediated STAT3 signalling induced RORγt$^+$ Th17 responses (Saravia, Chapman and Chi,

2019). The Th1, Th2 and Th17 cells then exert their effector functions by producing their signature cytokines IFN-γ, IL-4 and IL-17A, respectively (Saravia, Chapman and Chi, 2019). Single cell RNA sequencing analysis of $CD4^+$ MP T cells cultured in media containing combinations of type 1- (IL-12/IL-18), type 2- (IL-25/IL-33) and type 3- (IL-23/IL-1β) inflammatory cytokines revealed that bystander activation by these cytokine combinations induced the lineage-specific genes required for Th1- (*Tbx21*), Th2- (*Gata3*) and Th17-like (*Roryt*) $CD4^+$ MP T cells, respectively. However, upon TCR stimulation with anti-CD3 and anti-CD28, the cytokine responses are enhanced for certain conditions. For instance, TNF-α, IL-4 and GM-CSF production from IL-12/IL-18-cultured, IL-25/IL-33-cultured and IL-23/IL-1β-cultured $CD4^+$ MP T cells, respectively, required TCR engagement (Cho *et al.*, 2023). Kawabe and colleagues showed that T-bet^{high} $CD44^{high}$ $CD62L^{low}$ MP T cell subset, present in GF, AF and SPF mice, exerts Th1-like non-specific response against early toxoplasma and Mycobacterium tuberculosis infections by rapid production of IFN-γ, similarly, to innate-like lymphocytes such as NK and NKT cells (Kawabe *et al.*, 2017). Cho and colleagues conducted trajectory analysis which illustrates that Th1-like $CD4^+$ MP T cells originate from $CXCR3^{high}$ precursors in response to bystander activation through IL-12/IL-18 cytokines. In contrast, $CCR6^{high}$ MP T cells differentiate into Th17 MP T cells which highly express pathogenic cytokines such as GM-CSF and IL-17A in response to TCR-independent signalling through IL-23 and IL-1β (Cho *et al.*, 2023). Similarly, IL-23 signalling promotes a pathogenic conventional Th17 effector response inducing inflammation in Experimental Autoimmune Encephalomyelitis (EAE), used as a model to study Multiple Sclerosis. Whereas non-pathogenic conventional Th17 differentiation is promoted by IL-6 and TGF-β1 (Lee *et al.*, 2012). Although pathogen-induced Th17 cells play a role in host protection, such a role is not yet known for Th17 MP T cells.

In addition to the function of Th1 and Th17 MP T cells studied in lymphosufficient mice, $CD4^+$ MP T cells play a role in lymphopenia-induced inflammation, suggestive of pathogenic role at homeostasis (Kawajiri *et al.*, 2024). MP T cells were originally studied in lymphopenic settings in which naïve T cells expand to generate MP T cells to restore immune balance. Kawabe and colleagues showed that the transfer of MP T cells in Rag2-deficient mice, resulted in inflammatory responses by IFN-γ and IL-17A in the presence of IL-12 and IL-23, respectively (Kawajiri *et al.*, 2024). These MP T cells induce the development of colitis. However, this response is organ-specific, observed within the colon, but the inflammation in the lungs is independent of these cytokines (Kawajiri *et al.*, 2024). Although scRNAseq of $CD4^+$ MP T cells

stimulated with IL-25 and IL-33 induce Th2 differentiation program, their function in host protection and homeostasis is yet to be determined (Cho *et al.*, 2023).

Although $CD4^+$ MP T cells are considered to be in the same categories as other innate T lymphocytes with innate effector responses which bridge the innate and adaptive immune responses (Kawabe and Sher, 2021), we show that MP Tfh cells, generated in pathogen-free conditions, exert an adaptive response in host protection. Specific T cell-B cell interactions are required for anti-viral antibody production against vaccinia virus infection as germinal centres are defective in absence of MP Tfh cells. As mentioned above, natural antibodies are formed in the steady state in response to self-antigens (Elkon and Casali, 2008) . Autoreactive B cells enter GCs but undergo negative selection to maintain tolerance, thus the production of autoreactive IgG antibodies with high affinity to self-antigens is associated with defective tolerance mechanisms in autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis (Nagele *et al.*, 2013). However, the increased levels of natural IgG in aged healthy individuals suggest that some autoreactive B cells can evade negative selection. Rather than contributing to autoimmunity, moderately autoreactive follicular B cells in secondary lymphoid tissues can undergo the germinal center reaction upon antigenic stimulation. Through somatic hypermutation, these cells refine their BCRs to enhance affinity for foreign antigens while reducing self-reactivity, supporting immune responses without overt autoimmunity. Thus, if this refinement process prevents the generation of high-affinity self-reactive antibodies, the mechanisms underlying the production of natural IgG antibodies remain unclear (Elkon and Casali, 2008; Lee, Ko and Kim, 2020).

As natural IgG antibodies undergo somatic mutations in the germinal centres, it is likely that MP Tfh cells support natural IgG production in the steady state. Natural IgG may function in immune homeostasis by facilitating the clearance of apoptotic debris and damaged cellular components, preventing excessive immune activation. They may also serve as an adaptive response to tissue injury and disease, helping to clear pathology-specific debris and potentially acting as biomarkers for disease detection (Elkon and Casali, 2008). This indicates that MP Tfh cells have a physiological role as well as adaptive function during viral infection (Elkon and Casali, 2008). Conventional MP Tfr cells play a role in suppressing germinal centre responses for optimal B cell-mediated antibody production thereby preventing autoimmunity (Zhu, Zou and Liu, 2015). MP Tfr cells may be important for regulating natural antibody responses at steady state. Thus, MP Tfh and MP Tfr cells may have a physiological role in the production and regulation of

natural IgG, respectively, and MP Tfh cells may promote early B cell-mediated responses against viral infections whilst MP Tfr cells suppress anti-viral antibody production.

4.4 Cross-analysis with pathogen-induced memory Tfh cells

MP Tfh display similar phenotypic and functional characteristics to pathogen-induced memory Tfh cells. In steady state, we found MP Tfh cells with the expression of CXCR5, BCL6, PD-1. This is similar to pathogen-induced Tfh cells which express CXCR5 that is required for B cell homing and is maintained upon circulatory memory Tfh cells. However, once the pathogen-induced Tfh cells exit germinal centres and form memory Tfh cells, they downregulate BCL6 (Crotty, 2014). As the expression of BCL6 on pathogen-induced Tfh cells is specific to localise within the germinal centres (Crotty, 2021), this indicates that $BCL6^{+}$ MP Tfh cells may reside within germinal centres in homeostatic conditions. This is interesting because germinal centre formation is a process associated with immune responses against infections or immunisations (Stebegg *et al.*, 2018). The formation of germinal centres within pathogen-free conditions is not studied. However, as natural antibodies which undergo SHM dependent of T cell interaction suggests that germinal centres formation at steady state is mediated by MP Tfh cells and is required for mediating affinity of natural antibodies which are produced to self-antigens (Nagele *et al.*, 2013). Although we did not study the benefit of MP Tfh cells in homeostasis, we found a role of MP Tfh cells during viral infection similar to pathogen-induced memory Tfh cells. MP Tfh cells express the marker FR4, which is highly expressed on pathogen-induced memory Tfh cells (Künzli *et al.*, 2020). Pathway analysis proved that FR4 and EGR2 expressing $CD44^{high}$ $CD4^{+}$ MP T cells are significantly enriched in memory Tfh genes compared to central memory or Th1 memory genes. In addition, $FR4^{+}$ $EGR2^{+}$ MP T cells have enrichment of BCL6 positive genes which indicates that the genes repressed by BCL6 are enriched in $FR4^{+}$ $EGR2^{-}$ $CD44^{high}$ $CD4^{+}$ MP T cells. This evidence suggests a regulatory role of EGR2 in BCL6 expression which will be discussed in a later section (see section 4.5). Overall, the GSEA pathway analysis shows that FR4 and EGR2 expressing $CD44^{high}$ $CD4^{+}$ MP T cells are significantly enriched in genes found within conventional memory Tfh cells and $BCL6^{+}$ cells. Thus, MP Tfh cells are found within $FR4^{+}$ $EGR2^{+}$ $CD44^{high}$ $CD4^{+}$ MP T cells. By determining the function of MP Tfh cells in host protection, we found that during vaccinia virus infection, the germinal centres are impaired in Egr2/3-deficient mice. However, the adoptive transfer of MP Tfh cells expressing FR4 and EGR2 restores germinal centre development and anti-viral neutralising antibody production. This shows that MP Tfh cells support cognate B cell-mediated antibody production during a pathogen-specific immune response, so have a similar function to pathogen-induced Tfh cells *in vivo*. Although purely MP Tfh cells were not isolated for RNA sequencing, we showed that $CXCR5^{+}$ $PD\text{-}1^{+}$ $GITR^{-}$ $CD4^{+}$ $CD44^{high}$ MP Tfh cells significantly support IgG production by B cells in culture, upon stimulation with anti-CD3 and anti-IgM *in vitro*, whilst $CXCR5^{+}$ $PD\text{-}1^{+}$ $GITR^{+}$

$CD4^+$ $CD44^{high}$ MP Tfr cells repress IgG production by regulating MP Tfh cell responses in germinal centres.

As previously stated, $CD4^+$ MP T cells are generated by rapid homeostatic proliferation of naïve T cell precursors upon self-antigen stimulation in steady condition (Kawabe and Sher, 2021). We showed that MP Tfh cells reside within the $CD4^+$ MP T cell compartment in pathogen-free conditions and the high expression of EGR2 indicates that T cell activation by the recognition of self-peptide-MHC class II by TCR on the surface of naïve T cells (Anderson *et al.*, 2006; Li *et al.*, 2012). Although MP Tfh cell generation differs from pathogen-induced Tfh cells which are generated from naïve T cell activation through exogenous antigens, both perform similar functions in response to viral infections (Crotty, 2014). This indicates that MP Tfh cells may possess cross-reactive TCRs. TCR cross-reactivity is a feature of pathogen-derived T cells, in which a single TCR can recognize homologous pathogen-derived antigens (Petrova, Ferrante and Gorski, 2012). MP Tfh cells, pre-primed by self-antigens, may recognize pathogen-derived antigens during infection and support the ongoing immune response by pathogen-derived Tfh effector cells. However, we do not know if MP Tfh cells are cross-reactive or if their generation is not dependent on self-antigens. Su and colleagues found that memory-phenotype T cells possess cross reactivity to homologous environmental peptides, in unimmunised humans, which determine their development (Su *et al.*, 2013). However, $CD4^+$ MP T cells are found in unimmunised SPF, GF and AF conditions shows that it is likely for $CD4^+$ MP T cells with high CD5 expression to be generated by self-antigens (Kawabe and Sher, 2021), distinct from the findings of memory-phenotype cross reactivity in human hosts.

We found that MP Tfh cells are highly proliferative and exist in a preactivated state, this suggests that they perform an earlier adaptive response to pathogen-induced memory Tfh cells which remain in a quiescent state in absence of pathogen (Tsai and Yu, 2014; Cibrián and Sánchez-Madrid, 2017). MP Tfh cells display similar metabolic and homeostatic proliferation gene signatures to that of pathogen-induced memory Tfh cells. $FR4^+$ $Egr2^+$ $CD44^{high}$ $CD4^+$ MP T cells are highly enriched in genes required for glycolysis, mTOR such as *Hif1α*, which are required by pathogen-induced Tfh memory cells for survival in homeostatic conditions (Künzli *et al.*, 2020; Mayberry *et al.*, 2022). In addition, $FR4^+$ $EGR2^+$ $CD44^{high}$ $CD4^+$ MP T cells are significantly enriched in genes of E2F target, G2M checkpoint and Myc targets, which are associated with cellular proliferation, cell cycle progression and cell growth (Oshi *et al.*, 2021). In addition, *in vitro* stimulation of $FR4^+$ $EGR2^+$ MP T cells stained with CellTrace violet showed that $FR4^+$ $EGR2^+$ MP T cells are highly proliferate compared to $FR4^+$ $EGR2^-$ MP T cells. Overall, this

indicates that MP Tfh cells are found in a metabolically active and proliferative state required for their maintenance in homeostatic conditions.

In addition, we found that MP Tfh cells down regulate the expression of inflammatory genes. Indeed, the nature of Tfh cells, both MP Tfh and pathogen-induced Tfh cells, is to facilitate the inflammatory responses of germinal centre B cells rather than driving an inflammatory response directly (Quinn *et al.*, 2018).

4.5 EGR2 in Tfh cells

EGR2 is a transcription factor which plays a role in T cell responses including tolerance, clonal expansion and effector differentiation (Zhu *et al.*, 2008; Li *et al.*, 2012; Singh *et al.*, 2017; Taefehshokr *et al.*, 2020). Our group previously generated *CD2-Egr2*$^{-/-}$*Egr3*$^{-/-}$ mice to study the regulatory function of EGR2 and 3 in lymphocyte activation and inflammatory responses mediated by B and T cells (Li *et al.*, 2012). By 2 months of age, the mice began to display clinical characteristics of an early-onset of lethal systemic autoimmunity compared to the late-onset of lupus-like autoimmune disease after 15 months of age in mice with conditional Egr2 knockout from T and B lymphocytes (Zhu *et al.*, 2008; Li *et al.*, 2012). In both models, common characteristic features observed included multi-organ infiltration of activated T cells and autoantibodies, and glomerulonephritis, a form of kidney damage (Zhu *et al.*, 2008; Li *et al.*, 2012). The major clinical features of *CD2-Egr2*$^{-/-}$*Egr3*$^{-/-}$ diseased mice included the spleen and lymph node enlargement associated with elevated number of hyperactive T and B cells *in vivo* (Li *et al.*, 2012). Consistently, our newly established Egr2/3$^{-/-}$/AmCyan-T-bet knockout mouse model also displayed splenomegaly in comparison to Egr2-GFP/ AmCyan-T-bet knockin mice, observed during spleen extraction for downstream analysis of the phenotype and functional analysis of MP Tfh cells (data not shown).

EGR2 has been implicated in regulating the expression and/or function of Th lineage-specific transcription factors such as BCL6, BAFT and T-bet required for Tfh, Th17 and Th1 differentiation, respectively (T *et al.*, 2013; Ogbe *et al.*, 2015; Singh *et al.*, 2017). Tfh cell differentiation is an early cell fate determined by factors which promotes Tfh cell differentiation than other T helper cells (Crotty, 2014). BCL6 expression in pathogen-induced Tfh cells is positively regulated by EGR2 (Ogbe *et al.*, 2015). In absence of EGR2/3, BCL6 expression is reduced whereas its antagonist, Blimp-1, is increased. Thus, EGR2 positively regulates Bcl6 expression to induce Tfh cell differentiation, whereas Blimp-1 suppresses Tfh cell differentiation. During viral infection, Tfh cell development and germinal centres formation is restored upon enforced expression of EGR2 and BCL6 in EGR2/3 deficient T cells. Thus, EGR2/3 transcription factors play a major role in Tfh cell differentiation and GC responses in pathogen-induced Tfh cells. In addition, other transcription factors associated with Tfh cells such as TCF7, ASCL2 and LEF1 are also direct targets of EGR2 (Ogbe *et al.*, 2015). As EGR2 is expressed during early stages of T cell activation for clonal expansion but repressed in effector differentiation, this suggests that EGR2 promotes Tfh cell development as an early-stage mechanism dependent on antigen-TCR signalling and cytokine environment. Although EGR2 is required early on T cell

activation to induce Tfh cell fate (Li *et al.*, 2012), whether its expression is sustained in pathogen-induced memory Tfh cells has not been widely studied. It is possible that once pathogen-memory Tfh cells are activated by secondary response EGR2 as well as BCL6 become upregulated to differentiate into GC effector cells to elicit a response. Our group found that EGR2 is expressed in PD-1^{high} CD62L^{-} CD44^{high} CD4^{+} MP T cells. EGR2 is required for the maintenance of PD-1^{high} MP T cells through homeostatic proliferation and in absence of EGR2/3 the TCR repertoire becomes less diverse. *In vitro* stimulation by IL-12 showed greater production of IFN-γ from EGR2/3 deficient T cells than in EGR2^{+} PD1^{high} MP cells. Thus, EGR2 controls the maintenance and inflammatory responses of PD-1^{high} CD4^{+} MP T cells, preventing autoimmune reactions (Symonds *et al.*, 2020). In the present study, we found that EGR2 is highly expressed in MP Tfh cells and MP Tfr cells at steady conditions in unimmunised GFP-Egr2/ AmCyan-T-bet mice raised and maintained in SPF conditions. Because EGR2 is not expressed in all CD4^{+} MP T cells but selectively in MP Tfh and MP Tfr cells, this suggests that EGR2 expression is a stable and defining feature of these cells rather than being induced by self-antigen stimulation. FR4 is highly expressed in pathogen-induced Tfh cells (Künzli *et al.*, 2020), and we found that almost all FR4^{+} MP Tfh cells express EGR2. In absence of EGR2, CD44^{high} Treg generation was not affected but MP Tfh cell development was significantly impaired in CD2-Egr2/3$^{-/-}$/AmCyan-T-bet mice. Thus, similarly to pathogen-induced Tfh cells (Ogbe *et al.*, 2015), EGR2 plays a role in the development of MP Tfh cells in pathogen-free conditions and is required for adaptive response during viral infection. RNA sequencing showed that FR4 and EGR2 expressing MP T cells are enriched in Tfh cell specific genes, T cell activation and proliferation-associated genes, indicating a role for EGR2 in homeostatic proliferation, consistent with our previous finding. *In vitro* analysis also showed that FR4^{+} Egr2^{+} MP T cells were highly proliferative but displayed reduced IFN-γ production compared to FR4^{+} EGR2^{-} MP T cells. Thus, Tfh MP T cells enriched within the FR4^{+} EGR2^{+} MP T cell population require EGR2 for their homeostatic proliferation and limited inflammatory responses in pathogen-free conditions, similarly to pathogen-induced GC Tfh cells, as described above.

4.6 Key transcription factors in CD4$^+$ MP T cells

Transcription factors determine the plasticity and effector responses of T cells during infection and autoimmunity, influenced by cytokine environment during immune responses (Hosokawa and Rothenberg, 2020). T-bet is the transcription factor involved in inducing Th1 response whilst suppressing the expression of other lineage-specific transcription factors such as GATA3, BCL6 and RORγt which are required to induce the Th2, Tfh and Th17 differentiation programmes (Lazarevic *et al.*, 2010; Leavy, 2012; Hertweck *et al.*, 2022). T-bet is one of the main transcription factors involved in MP CD4$^+$ T cell development and innate-like functions. T-bet is differentially expressed in CD4$^+$ MP T cells generating subsets T-bet^{high}, T-bet^{low} and T-bet$^-$ MP T cells. T-bet^{high} MP T cells display Th1 like no-specific response and exists as the dominant subset. T-bet$^-$ Rorγt$^-$ can form into the Th1 like cells, but the Rorγt$^+$ Th17 is a minor population so shows significance of T-bet^{high} MP T cells. In homeostatic conditions, scRNA sequencing of CD4$^+$ MP T cells shows distinct clusters of a predominant Th1-like population, a minor Th17 cells, MP Tregs and a large fraction of undifferentiated T-bet$^-$ Rorγt$^-$ MP T cells with a naïve phenotype. These undifferentiated cells can differentiate into T-bet^{high} MP T cells (Kawajiri *et al.*, 2024).

T-bet and EGR2 have a reciprocal relationship in pathogen-induced CD4$^+$ T cells in whereby EGR2 represses T-bet function, but IFN-γ can regulate EGR2 to get Th1 response (Li *et al.*, 2012; Singh *et al.*, 2017). We have previously shown that in PD-1^{high} MP T cells without EGR2 become inflammatory triggering autoimmunity with increased T-bet expression (Symonds *et al.*, 2020). So EGR2$^+$ MP T cells host protection role but T-bet$^+$ MP T cells in absence of EGR2 there's a pathogenic role. Using our new established mouse model, in which EGR2 and T-bet are fluorescently bound, we found that CD44^{high} CD4$^+$ MP T cells have differential expression of T-bet and EGR2 in steady state in mice raised and maintained in SPF conditions. Three populations exist, T-bet$^+$ EGR2$^-$, T-bet$^-$ EGR2$^+$ and T-bet$^-$ EGR2$^-$. T-bet positive cells have been extensively studied (Kawabe *et al.*, 2017). In this study we show that MP Tfh cells reside in the EGR2$^+$ subset but absent in T-bet$^+$ subset. In the FR4$^+$ EGR2$^-$ CD4$^+$ MP T cell control population in RNA Seq we see that the Th1 genes are highly enriched in FR4$^+$ EGR2$^-$ so EGR2$^-$ population consists of the effector T-bet$^+$ Th1 population. In addition, CD4$^+$ CD44^{high} CD25$^-$ CXCR5$^-$ BCL6$^-$ MP T cells, EGR2 and T-bet not expressed together but only around 20% of the cells co-express FR4 and T-bet so it could be that Th1 MP T cells express FR4 at lower levels than MP Tfh cells. A comparative pathway analysis of FR4^{high} CXCR5$^+$ and FR4^{low} CXCR5$^-$ antigen-specific CD4$^+$ T cells showed that the FR4^{high} CXCR5$^+$ subset is highly enriched in Tfh

cell specific genes whereas the $FR4^{low}$ subset corresponds to pathogen-induced Th1, similar to our observations of FR4 expression in MP Tfh and MP Th1 cells (Iyer *et al.*, 2013). We have conducted further single cell RNA sequencing analysis of $EGR2^{+}$, T-bet^{+} and T-bet^{-}/$EGR2^{-}$ MP T cells which shows that the Th1 like MP T cells in the T-bet^{+} MP T cells and effector memory type cells whereas EGR2 subset has Tfh cells, proliferative cells, and cells with the expression of Eomes/Myb (Manuscript for submission). Thus, EGR2 and T-bet are key transcription factors involved in $CD4^{+}$ MP T cell responses and homeostasis.

4.7 What are the unsolved issues?

Over 3 decades ago, $CD4^+$ MP T cells were found in GF, SPF and conventional mice maintained in clean laboratory conditions but were assumed to be generated in response to the microflora (Dobber *et al.*, 1992). Once $CD4^+$ MP T cells generation in response to self-antigens was discovered (Kim *et al.*, 2016), and since these cells occupy the major T cell population with age (Kawabe *et al.*, 2017), the functional relevance of these cells has become of great interest.

In this study we have found that MP Tfh cells are generated in absence of overt antigen stimulation and mediate germinal centre formation and antiviral antibody production by B cells for host protection. However, several unanswered questions remain. MP Tfh cells respond to viral infections but as EGR2, which is upregulated upon T cell activation (Anderson *et al.*, 2006; Li *et al.*, 2012), is highly induced in MP Tfh cells, this indicates that the MP Tfh cells express self-specific TCRs. This raises the question of whether MP Tfh cells exhibit cross reactivity to self and pathogenic antigens. Su and colleagues have shown that memory-phenotype $CD4^+$ T cells respond to viral pathogens in a primary response as a result of cross reactivity to homologous microbial peptides (Su *et al.*, 2013). This is not an unusual phenomenon described in conventional $CD4^+$ T cells whereby cross-reactive pathogen-induced memory T cells may drive immune responses to novel foreign antigens in conditions in which naïve T cells are depleted such as thymic involution (Petrova, Ferrante and Gorski, 2012).

MP Tfh cells play an important role in early humoral response against viral infections. Although MP Tfh cells exert similar responses to pathogen-induced Tfh cells (Crotty, 2014), their mechanism of development in the steady state is unknown. It is possible that MP Tfh cells develop in the presence of cytokines such as IL-6/IL-12 which drive pathogen-induced Tfh cell differentiation by dendritic cells (Krishnaswamy *et al.*, 2018), in homeostatic conditions. However, the mechanism of development was not explored in this thesis.

In addition, we found a regulatory function of MP Tfh cells *in vitro*. Upon stimulation with anti-CD3 and anti-IgM, MP Tfr cells reduced IgG production by B cells through regulating MP Tfh cells in culture. However, their significance *in vivo* is unknown as well as the mechanism of their development.

Finally, in this thesis, we focused on the adaptive function of MP Tfh cells in response to viral antigens. MP Tfh cells are developed in steady conditions, and we know there are natural antibodies produced in pathogen-free conditions which maintain tissue homeostasis (Nagele *et al.*, 2013). Whether MP Tfh cells exert a physiological function still remains an outstanding

question. In addition, the mechanism of MP Tfr regulation of MP Tfh cells in physiological to maintain tolerance versus repressing excessive antibody production against viral infections would be of interest.

4.8 Limitations of the study

To investigate the transcriptomic profile of MP Tfh cells in unimmunised GFP-Egr2/ AmCyan-T-bet mice raised and maintained in SPF conditions, we isolated $FR4^+$ $EGR2^+$ $CD25^-$ $CD44^{high}$ $CD4^+$ MP T cells. One limitation of this approach is that we did not isolate a pure population of MP Tfh cells defined by the expression of BCL6, CXCR5, PD-1. However, this approach did not compromise the validity of the investigation into the transcriptomic signature of MP Tfh cells as the MP Tfh cells genes were significantly enriched in FR4 and EGR2 expressing cells, presumably a predominant population within this subset of MP $CD4^+$ T cells. In addition, stringent pathway analysis was conducted for MP Tfh cells to determine, for instance, their metabolic profile compared to that of pathogen-induced memory Tfh cells. Thus, the analysis remained focused on MP Tfh cells within FR4 and EGR2 expressing MP T cells.

4.9 Future Studies

In order to address the unanswered questions in this study, future work could focus on the experiments outlined below:

1. To determine whether the **mechanism of MP Tfh cell generation** in pathogen-free conditions is similar to that of pathogen-induced memory Tfh cells, MP Tfh cells generation in SPF mice can be compared to that of IL-6-deficient mice and $CD8\alpha^-$ DC-specific knockout mice. Further experiments can be employed to determine the relevance, of ongoing homeostatic signalling by IL-6, in the maintenance of MP Tfh cells in homeostatic conditions. The proposed methodology was adopted from the study by Kawabe and colleagues which explored the differentiation of T-bet^{high} MP T cell from $CD4^+$ MP T cell precursors in absence of foreign antigen recognition (Kawabe *et al.*, 2020).

2. To examine the **MP Tfh cell cross reactivity of self- and viral- antigens**, the methodology used by Su and colleagues (Su et al., 2013) can be modified by using peptide-MHC tetramer staining with self- and viral-peptides, sort for self-reactive and viral-reactive MP Tfh cells and then apply TCR sequencing to examine for similar or related TCR sequences, determining cross reactivity of MP Tfh cells.

3. To study the **regulatory mechanism of MP Tfr cells *in vivo*** upon viral infection.

4. Investigation of the **impact of MP Tfh cells in autoimmune disease development, and the inflammatory diseases in aging.** MP Tfh cells may serve as biomarkers for disease progression and treatment.

4.10 Conclusion and emerging model of MP T cell differentiation and function

The works in this PhD have demonstrated that novel Tfh-like MP T cells arise without overt antigen stimulation and are important for adaptive immune responses against viral infection. I have formulated an emerging model of the current understanding of $CD4^+$ MP T cell differentiation and function to summarise the work of others as well as the contributions of this PhD to the field of $CD4^+$ MP T cells (Figure 4.1).

$CD4^+$ MP T cells generated by self-antigen stimulation of naïve T cell precursors in the periphery accumulate with age (Kawabe and Sher, 2021). Therefore, $CD4^+$ MP T cells are an important part of the immune system and understanding the innate and adaptive responses of $CD4^+$ MP T cells may explain age-related diseases and the mechanism by which the immune system responds to novel pathogens in aged individuals with reduced naïve T cell repertoire (Yager *et al.*, 2008; Carrasco *et al.*, 2021).

The model in figure 4.1 illustrates the heterogeneity of $CD4^+$ MP T cells regarding their phenotypic characteristics and their effector functions during an immune response against pathogens. Much like pathogen-induced memory T cells, $CD4^+$ MP T cells differentiate into Th1, Th2, Th17, Treg and Tfh-like MP T cells in response to self-antigen stimulation and homeostatic signalling (Kawabe *et al.*, 2017b; Cho *et al.*, 2023; Kawajiri *et al.*, 2024). Th1 and Th17 MP T cells are described as innate responders (Kawabe *et al.*, 2017b; Cho *et al.*, 2023)whereas MP Tfh cells exert an early adaptive response against viral antigens. Thus, $CD4^+$ MP T cells can act as innate lymphocytes such as NK cells and $CD8^+$ MP T cells, involved in bridging the innate and adaptive systems together. And $CD4^+$ MP T cells play a role in specific humoral responses. However, the role of Th2-like MP T cells is yet to be determined. As research has focused on the function of $CD4^+$ MP T cells during host protection against environmental antigens, this indicates that $CD4^+$ MP T cells may possess TCR cross reactivity to self- and environmental antigens that they have not encountered before. For instance, Kawabe and colleagues previously described that T-bet^{high} $CD4^+$ MP T cells exert non-specific IFN-γ production against Toxoplasma gondii and Mycobacterium infections (Kawabe *et al.*, 2017). And in this thesis, we found that MP Tfh cells support B cell- mediated antibody production and germinal centre formation in response to vaccinia virus infection.

$CD4^+$ MP T cells are specific to self-antigens and regulated by MP Tregs and conventional Tregs in the steady state (Kawajiri *et al.*, 2024a; Li *et al.*, 2024). Thus, defects in this system to control $CD4^+$ MP T cells responses may exacerbate autoimmunity. Pathogen-induced Tfh cells are associated with the development of autoimmunity triggered by autoreactive antibodies such

as in rheumatoid arthritis (Crotty, 2019). Therefore, MP Tfh cells, in absence of MP Tfr cells, may have a role in autoimmunity.

To conclude, MP Tfh cells and MP Tfr cells are found in homeostasis and support early humoral response to viral infections. If dysregulated, MP Tfh cells may induce autoreactive B cell responses at homeostasis. Thus, understanding the mechanism of MP Tfh cell development and function in physiological role as well as the previously described role in host protection is of great importance.

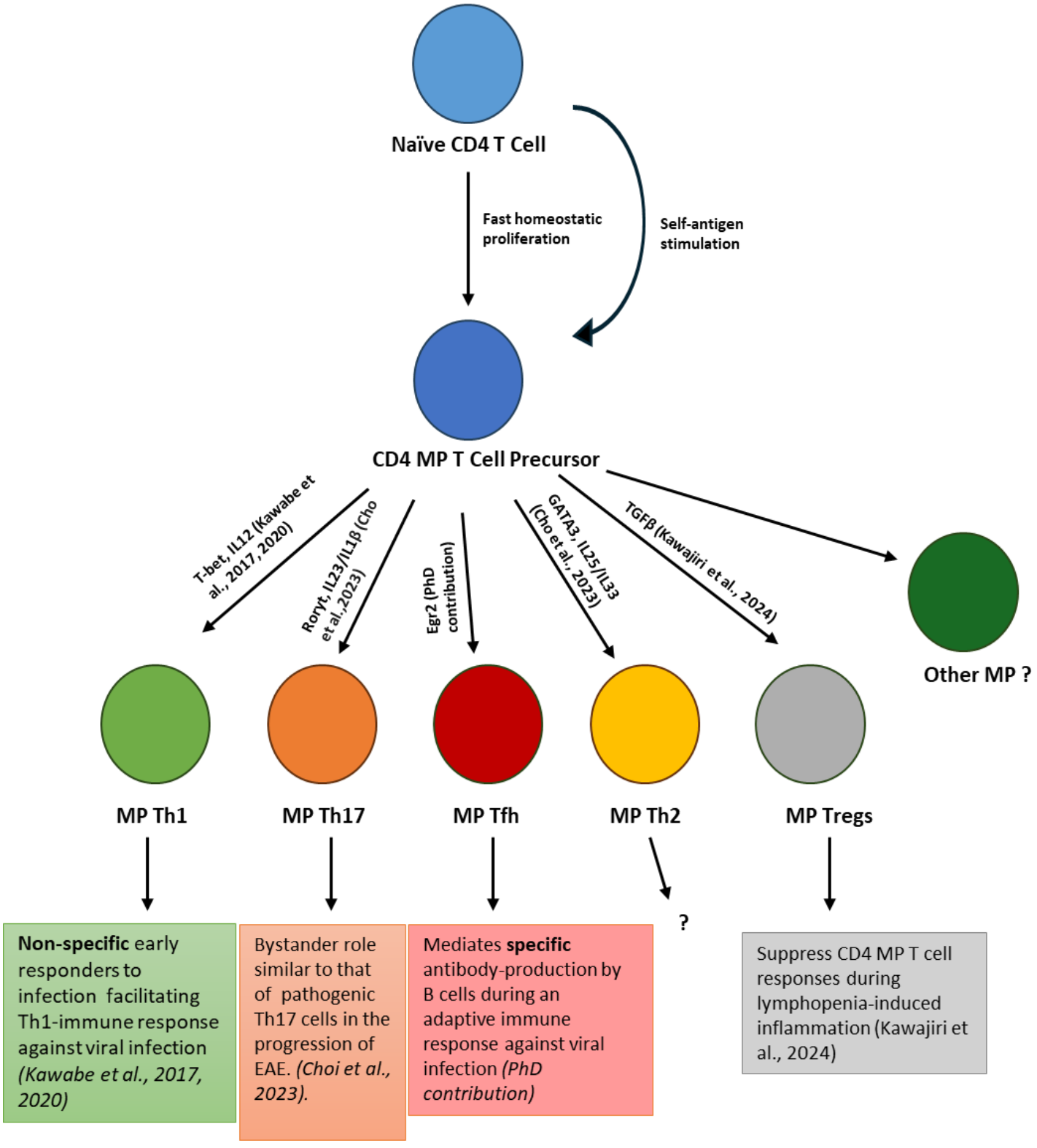

Naïve CD4 T Cell
Fast homeostatic proliferation
Self-antigen stimulation
CD4 MP T Cell Precursor
T-bet, IL12 (Kawabe et al., 2017, 2020)
Rorγt, IL23/IL1β (Cho et al.,2023)
Egr2 (PhD contribution)
GATA3, IL25/IL33 (Cho et al., 2023)
TGFβ (Kawajiri et al., 2024)
Other MP ?
MP Th1
MP Th17
MP Tfh
MP Th2
MP Tregs
?
Non-specific early responders to infection facilitating Th1-immune response against viral infection (Kawabe et al., 2017, 2020)
Bystander role similar to that of pathogenic Th17 cells in the progression of EAE. (Choi et al., 2023).
Mediates specific antibody-production by B cells during an adaptive immune response against viral infection (PhD contribution)
Suppress CD4 MP T cell responses during lymphopenia-induced inflammation (Kawajiri et al., 2024)

Figure 4.1 Emerging model for CD4$^+$ MP T cell differentiation and function. Naïve CD4$^+$ T cell precursor with high expression of CD5, proliferate rapidly in response to self-antigen stimulation. The CD4$^+$ MP T cells proliferate rapidly in pathogen-free conditions and differentiate into distinct subpopulations with similar characteristics to pathogen-induced memory T cells. The production of IL-12 by DCs initiates the differentiation of CD4$^+$ MP T cells into T-bet$^+$ Th1-like MP T cells which provide bystander effector functions during viral infection and enhance the late adaptive immune responses. RORγt$^+$ Th17-like MP T cells are generated by the stimulation of IL-23/IL-1β and produce a pathogenic autoimmune response. RNA sequencing analysis revealed a GATA3$^+$ Th2-like MP T cell population but their role in host protection is unknown. EGR2$^+$ MP Tfh cells are generated and regulate germinal centre responses to support antiviral antibody production by B cells. MP Tregs suppress CD4$^+$ MP T cells and their generation is mediated by TGFβ (Kawabe *et al.*, 2017b, 2020; Kawabe and Sher, 2021; Cho *et al.*, 2023; Kawajiri *et al.*, 2024).

Bibliography

Actor, J. K. (2019) ‘T Lymphocytes: Ringleaders of Adaptive Immune Function’, *Introductory Immunology*. Elsevier, pp. 45–62. doi: 10.1016/B978-0-12-816572-0.00004-8.

Ahmed, R., Bevan, M. J., Reiner, S. L. and Fearon, D. T. (2009) ‘The precursors of memory: models and controversies’, *Nature Reviews Immunology,* 9(9), pp. 662–668. doi: 10.1038/nri2619.

Akkaya, M., Kwak, K. and Pierce, S. K. (2019) ‘B cell memory: building two walls of protection against pathogens’, *Nature Reviews Immunology*, 20(4), pp. 229–238. doi: 10.1038/s41577-019-0244-2.

Andersen, M. H., Schrama, D., Thor Straten, P. and Becker, J. C. (2006) ‘Cytotoxic T Cells’, *Journal of Investigative Dermatology*, 126(1), pp. 32–41. doi: 10.1038/SJ.JID.5700001.

Arulraj, T., Binder, S. C., Robert, P. A. and Meyer-Hermann, M. (2021) ‘Germinal Centre Shutdown’, *Frontiers in Immunology*, 12, p. 705240. doi: 10.3389/fimmu.2021.705240.

Abbas, A. K., Benoist, C., Bluestone, J. A., Campbell, D. J., Ghosh, S., Hori, S., Jiang, S., Kuchroo, V. K., Mathis, D., Roncarolo, M. G., Rudensky, A., Sakaguchi, S., Shevach, E. M., Vignali, D. A. and Ziegler, S. F. (2013) ‘Regulatory T cells: recommendations to simplify the nomenclature’, *Nature Immunology*, 14(4), pp. 307–308. doi: 10.1038/ni.2554.

Alterauge, D., Bagnoli, J. W., Dahlström, F., Bradford, B. M., Mabbott, N. A., Buch, T., Enard, W. and Baumjohann, D. (2020) ‘Continued Bcl6 expression prevents the transdifferentiation of established Tfh cells into Th1 cells during acute viral infection’, *Cell Reports*, 33(1), p. 108232. doi: 10.1016/j.celrep.2020.108232.

Asrir, A., Aloulou, M., Gador, M., Pérals, C. and Fazilleau, N. (2017) ‘Interconnected subsets of memory follicular helper T cells have different effector functions’, *Nature Communications*, 8(1), pp. 1–14. doi: 10.1038/s41467-017-00843-7.

Afroz, S., Bartolo, L. and Su, L.F. (2023) ‘Pre-existing T Cell Memory to Novel Pathogens’, *ImmunoHorizons*, 7(7), pp. 543–553. doi: 10.4049/immunohorizons.2200003.

Anderson, P. O., Manzo, B. A., Sundstedt, A., Minaee, S., Symonds, A., Khalid, S., Rodriguez-Cabezas, M. E., Nicolson, K., Li, S., Wraith, D. C. and Wang, P. (2006) ‘Persistent antigenic stimulation alters the transcription program in T cells, resulting in antigen-specific tolerance’, *European Journal of Immunology*, 36(6), p. 1374. doi: 10.1002/eji.200635883.

Atala, A. (2016) ‘Re: Stable inhibitory activity of regulatory T cells requires the transcription factor Helios’, *Journal of Urology*, 196(2), p. 617. doi: 10.1016/j.juro.2016.05.009.

Basu, A., Ramamoorthi, G., Albert, G., Gallen, C., Beyer, A., Snyder, C., Koski, G., Disis, M. L., Czerniecki, B. J. and Kodumudi, K. (2021) ‘Differentiation and Regulation of TH Cells: A Balancing Act for Cancer Immunotherapy’, *Frontiers in Immunology*, 12, p. 669474. doi: 10.3389/fimmu.2021.669474.

Bednar, K. J., Lee, J. H. and Ort, T. (2022) ‘Tregs in Autoimmunity: Insights Into Intrinsic Brake Mechanism Driving Pathogenesis and Immune Homeostasis’, *Frontiers in Immunology*, 13, p. 932485. doi: 10.3389/fimmu.2022.932485.

Berard, M. and Tough, D. F. (2002) ‘Qualitative differences between naïve and memory T cells’, *Immunology*, 106(2), p. 127. doi: 10.1046/j.1365-2567.2002.01447.x.

Bertschi, N. L., Bazzini, C. and Schlapbach, C. (2021) ‘The Concept of Pathogenic TH2 Cells: Collegium Internationale Allergologicum Update 2021’, *International Archives of Allergy and Immunology*, 182(5), pp. 365–380. doi: 10.1159/000515144.

BIO RAD (2016) T cell receptors overview - Mini-review | Bio-Rad. Available at: https://www.bio-rad-antibodies.com/t-cell-receptor-minireview.html (Accessed: 22 December 2024).

Bonneville, M., O’Brien, R. L. and Born, W. K. (2010) ‘γδ T cell effector functions: a blend of innate programming and acquired plasticity’, *Nature Reviews Immunology*, 10(7), pp. 467–478. doi: 10.1038/nri2781.

Bosteels, V. and Janssens, S. (2024) ‘Striking a balance: new perspectives on homeostatic dendritic cell maturation’, *Nature Reviews Immunology*, 25(2), pp. 125–140. doi: 10.1038/s41577-024-01079-5.

Breitfeld, D., Ohl, L., Kremmer, E., Ellwart, J., Sallusto, F., Lipp, M. and Förster, R. (2000) ‘Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production’, *Journal of Experimental Medicine*, 192(11), pp. 1545–1552. doi: 10.1084/jem.192.11.1545.

Cantrell, D. (1996) ‘T cell antigen receptor signal transduction pathways’, *Annual Review of Immunology*, 14, pp. 259–274. doi: 10.1146/annurev.immunol.14.1.259.

Carleton, M., Haks, M. C., Smeele, S. A. A., Jones, A., Belkowski, S. M., Berger, M. A., Linsley, P., Kruisbeek, A. M. and Wiest, D. L. (2002) ‘Early growth response transcription factors are required for development of CD4(-)CD8(-) thymocytes to the CD4(+)CD8(+) stage’, *The Journal of Immunology*, 168(4), pp. 1649–1658. doi: 10.4049/jimmunol.168.4.1649.

Carlson, M. (2023) *org.Mm.eg.db: Genome wide annotation for Mouse*, Bioconductor.

Carsetti, R. (2000) ‘The development of B cells in the bone marrow is controlled by the balance between cell-autonomous mechanisms and signals from the microenvironment’, *The Journal of Experimental Medicine*, 191(1), p. 5. doi: 10.1084/jem.191.1.5.

Chaplin, D. D. (2010) ‘Overview of the immune response’, *Journal of Allergy and Clinical Immunology*, 125(2), pp. S3–S23. doi: 10.1016/j.jaci.2009.12.980.

Charles A Janeway, J., Travers, P., Walport, M. and Shlomchik, M. J. (2001) ‘The structure of a typical antibody molecule’, Garland Science.

Charlton, J. J., Tsoukatou, D., Mamalaki, C. and Chatzidakis, I. (2015) ‘Programmed Death 1 regulates memory phenotype CD4 T cell accumulation, inhibits expansion of the effector memory phenotype subset and modulates production of effector cytokines’, *PLoS ONE*, 10(3), p. e0119200. doi: 10.1371/journal.pone.0119200.

Chen, J. W., Schickel, J. N., Tsakiris, N., Sng, J., Arbogast, F., Bouis, D., Parisi, D., Gera, R., Boeckers, J. M., Delmotte, F. R., Veselits, M., Schuetz, C., Jacobsen, E. M., Posovszky, C.,

Schulz, A. S., Schwarz, K., Clark, M. R., Menard, L. and Meffre, E. (2022) 'Positive and negative selection shape the human naive B cell repertoire', *The Journal of Clinical Investigation*, 132(2), p. e150985. doi: 10.1172/jci150985.

Chi, X., Li, Y. and Qiu, X. (2020) 'V(D)J recombination, somatic hypermutation and class switch recombination of immunoglobulins: mechanism and regulation', *Immunology*, 160(3), pp. 233–247. doi: 10.1111/imm.13176.

Cho, M. J., Lee, H. G., Yoon, J. W., Kim, G. R., Koo, J. H., Taneja, R., Edelson, B. T., Lee, Y. J. and Choi, J. M. (2023) 'Steady-state memory-phenotype conventional CD4+ T cells exacerbate autoimmune neuroinflammation in a bystander manner via the Bhlhe40/GM-CSF axis', *Experimental & Molecular Medicine*, 55(5), p. 1033. doi: 10.1038/s12276-023-00995-1.

Choi, J. and Crotty, S. (2021) 'Bcl6-mediated transcriptional regulation of follicular helper T cells (TFH)', *Trends in Immunology*, 42(4). doi: 10.1016/j.it.2021.02.002.

Choi, Y. S., Gullicksrud, J. A., Xing, S., Zeng, Z., Shan, Q., Li, F., Love, P. E., Peng, W., Xue, H. H. and Crotty, S. (2015) 'LEF-1 and TCF-1 orchestrate T(FH) differentiation by regulating differentiation circuits upstream of the transcriptional repressor Bcl6', *Nature Immunology*, 16(9), pp. 980–990. doi: 10.1038/ni.3226.

Choi, Y. S., Kageyama, R., Eto, D., Escobar, T. C., Johnston, R. J., Monticelli, L., Lao, C. and Crotty, S. (2011) 'ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6', *Immunity*, 34(6), pp. 932–946. doi: 10.1016/j.immuni.2011.03.023.

Clement, R. L., Daccache, J., Mohammed, M. T., Diallo, A., Blazar, B. R., Kuchroo, V. K., Lovitch, S. B., Sharpe, A. H. and Sage, P. T. (2019) 'Follicular regulatory T cells control humoral and allergic immunity by restraining early B cell responses', *Nature Immunology*, 20(10), p. 1360. doi: 10.1038/s41590-019-0472-4.

Courtney, A. H., Lo, W. L. and Weiss, A. (2017) 'TCR signaling: mechanisms of initiation and propagation', *Trends in Biochemical Sciences*, 43(2), p. 108. doi: 10.1016/j.tibs.2017.11.008.

CreativeDiagnostics (no date) *Flow Cytometry Guide - Creative Diagnostics*. Available at: https://www.creative-diagnostics.com/flow-cytometry-guide.htm (Accessed: 30 November 2024).

Crotty, S. (2014) 'T follicular helper cell differentiation, function, and roles in disease', *Immunity*, 41(4), p. 529. doi: 10.1016/j.immuni.2014.10.004.

Crotty, S. (2019) 'T follicular helper cell biology: a decade of discovery and diseases', *Immunity*, 50(5), p. 1132. doi: 10.1016/j.immuni.2019.04.011.

CytekBiosciences (no date) *Cytek Aurora [Spectral Flow Cytometry] | Cytek Biosciences*. Available at: https://cytekbio.com/pages/aurora (Accessed: 1 December 2024).

Carrasco, E., Gómez de las Heras, M. M., Gabandé-Rodríguez, E., Desdín-Micó, G., Aranda, J. F. and Mittelbrunn, M. (2021) 'The role of T cells in age-related diseases', *Nature Reviews Immunology*, 22(2), pp. 97–111. doi: 10.1038/s41577-021-00557-4.

Cibrián, D. and Sánchez-Madrid, F. (2017) ‘CD69: from activation marker to metabolic gatekeeper’, *European Journal of Immunology*, 47(6), p. 946. doi: 10.1002/eji.201646837.

Crotty, S. (2021) ‘Revealing T follicular helper cells with BCL6’, *Nature Reviews Immunology*, 21(10), pp. 616–617. doi: 10.1038/s41577-021-00591-2.

Choi, Y. S., Eto, D., Yang, J. A., Lao, C. and Crotty, S. (2013) ‘STAT1 is required for IL-6-mediated Bcl6 induction for early follicular helper cell differentiation’, *The Journal of Immunology*, 190(7), pp. 3049–3053. doi: 10.4049/jimmunol.1203032.

Chen, X., Ma, W., Zhang, T., Wu, L. and Qi, H. (2015) ‘Phenotypic Tfh development promoted by CXCR5-controlled re-localization and IL-6 from radiation-resistant cells’, *Protein & Cell*, 6(11), pp. 825–832. doi: 10.1007/s13238-015-0210-0.

Cubas, R. A., Mudd, J. C., Savoye, A. L., Perreau, M., van Grevenynghe, J., Metcalf, T., Connick, E., Meditz, A., Freeman, G. J., Abesada-Terk, G. Jr, Jacobson, J. M., Brooks, A. D., Crotty, S., Estes, J. D., Pantaleo, G., Lederman, M. M. and Haddad, E. K. (2013) ‘Inadequate T follicular cell help impairs B cell immunity during HIV infection’, *Nature Medicine*, 19(4), pp. 494–499. doi: 10.1038/nm.3109.

Chu T, Tyznik AJ, Roepke S, Berkley AM, Woodward-Davis A, Pattacini L, Bevan MJ, Zehn D, Prlic M. Bystander-activated memory CD8 T cells control early pathogen load in an innate-like, NKG2D-dependent manner. Cell Rep. 2013 Mar 28;3(3):701-8. doi: 10.1016/j.celrep.2013.02.020. Epub 2013 Mar 21. PMID: 23523350; PMCID: PMC3628815.

Chung, Y., Tanaka, S., Chu, F., Nurieva, R. I., Martinez, G. J., Rawal, S., Wang, Y. H., Lim, H., Reynolds, J. M., Zhou, X. H., Fan, H. M., Liu, Z. M., Neelapu, S. S. and Dong, C. (2011) ‘Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions’, *Nature Medicine*, 17(8), pp. 983–988. doi: 10.1038/nm.2426.

Deenick, E. K., Chan, A., Ma, C. S., Gatto, D., Schwartzberg, P. L., Brink, R. and Tangye, S. G. (2010) ‘Follicular helper T cell differentiation requires continuous antigen presentation that is independent of unique B cell signaling’, *Immunity*, 33(2), pp. 241–253. doi: 10.1016/j.immuni.2010.07.015.

Deenick, E. K. and Ma, C. S. (2011) ‘The regulation and role of T follicular helper cells in immunity’, *Immunology*, 134(4), p. 361. doi: 10.1111/j.1365-2567.2011.03487.x.

Delaire, S., Huang, Y. H., Chan, S. W. and Robey, E. A. (2004) ‘Dynamic repositioning of CD4 and CD8 genes during T cell development’, *Journal of Experimental Medicine*, 200(11), pp. 1427–1435. doi: 10.1084/jem.20041041.

Delves, P. J. and Roitt, I. M. (2000) ‘The immune system. First of two parts’, *New England Journal of Medicine*, 343(1), pp. 37–49. doi: 10.1056/nejm200007063430107.

Dempsey, P. W., Vaidya, S. A. and Cheng, G. (2003) ‘The art of war: Innate and adaptive immune responses’, *Cellular and Molecular Life Sciences*, 60. doi: 10.1007/s00018-003-3180-y.

Deshpande, D., Chhugani, K., Chang, Y., Karlsberg, A., Loeffler, C., Zhang, J., Muszyńska, A., Munteanu, V., Yang, H., Rotman, J., Tao, L., Balliu, B., Tseng, E., Eskin, E., Zhao, F.,

Mohammadi, P., Łabaj, P. and Mangul, S. (2023) ‘RNA-seq data science: From raw data to effective interpretation’, *Frontiers in Genetics*, 14, p. 997383. doi: 10.3389/fgene.2023.997383.

Dolgalev, I. (2022) *msigdbr: MSigDB Gene Sets for Multiple Organisms in a Tidy Data Format*. Available at: https://cran.r-project.org/package=msigdbr (Accessed: 3 February 2025).

Dong, X., Tu, H., Qin, S., Bai, X., Yang, F. and Li, Z. (2023) ‘Insights into the roles of B cells in patients with sepsis’, *The Journal of Immunology Research*, 2023. doi: 10.1155/2023/7408967.

Dong, Y., Pi, X., Bartels-Burgahn, F., Saltukoglu, D., Liang, Z., Yang, J., Alt, F. W., Reth, M. and Wu, H. (2022) ‘Structural principles of B cell antigen receptor assembly’, *Nature*, 612(7938), pp. 156–161. doi: 10.1038/s41586-022-05412-7.

De Jong, E. C., Smits, H. H. and Kapsenberg, M. L. (2005) ‘Dendritic cell-mediated T cell polarization’, *Springer Seminars in Immunopathology*, 26(3), pp. 289–307. doi: 10.1007/s00281-004-0167-1.

De Silva, N. S. and Klein, U. (2015) ‘Dynamics of B cells in germinal centres’, *Nature Reviews Immunology*, 15(3), p. 137. doi: 10.1038/nri3804.

Divekar, R. and Kita, H. (2015) ‘Recent advances in epithelium-derived cytokines (IL-33, IL-25 and TSLP) and allergic inflammation’, *Current Opinion in Allergy and Clinical Immunology*, 15(1), p. 98. doi: 10.1097/aci.0000000000000133.

Dobber, R., Hertogh-Huijbregts, A., Rozing, J., Nagelkerken, L. and Bottomly, K. (1992) ‘The involvement of the intestinal microflora in the expansion of CD4+ T cells with a-naive phenotype in the periphery’, *Developmental Immunology*, 2(2), p. 141. doi: 10.1155/1992/57057.

Drobek, A., Moudra, A., Mueller, D., Huranova, M., Horkova, V., Pribikova, M., Ivanek, R., Oberle, S., Zehn, D., McCoy, K. D., Draber, P. and Stepanek, O. (2018) ‘Strong homeostatic TCR signals induce formation of self-tolerant virtual memory CD8 T cells’, *EMBO Journal*, 37(14), e98518. doi: 10.15252/embj.201798518.

Eberl, G., Colonna, M., Santo, J. P. D. and McKenzie, A. N. J. (2015) ‘Innate Lymphoid Cells: a new paradigm in immunology’, *Science*, 348(6237), p. aaa6566. doi: 10.1126/science.aaa6566.

Eibel, H., Kraus, H., Sic, H., Kienzler, A. K. and Rizzi, M. (2014) ‘B cell biology: An overview topical collection on basic and applied science’, *Current Allergy and Asthma Reports*, 14(5), pp. 1–10. doi: 10.1007/s11882-014-0434-8.

Esensten, J. H., Helou, Y. A., Chopra, G., Weiss, A. and Bluestone, J. A. (2016) ‘CD28 costimulation: from mechanism to therapy’, *Immunity*, 44(5), p. 973. doi: 10.1016/j.immuni.2016.04.020.

Elkon, K. and Casali, P. (2008) ‘Nature and functions of autoantibodies’, *Nature Clinical Practice Rheumatology*, 4(9), pp. 491–498. doi: 10.1038/ncprheum0895.

Eto, D., Lao, C., DiToro, D., Barnett, B., Escobar, T. C., Kageyama, R., Yusuf, I. and Crotty, S. (2011) ‘IL-21 and IL-6 are critical for different aspects of B cell immunity and redundantly induce optimal follicular helper CD4 T cell (Tfh) differentiation’, *PLoS ONE*, 6(3), p. e17739. doi: 10.1371/journal.pone.0017739.

Gasper, D. J., Tejera, M. M. and Suresh, M. (2014) ‘CD4 T-Cell Memory Generation and Maintenance’, *Critical Reviews in Immunology*, 34(2), p. 121. doi: 10.1615/critrevimmunol.2014010373.

Gatto, D. and Brink, R. (2010) ‘The germinal center reaction’, *Journal of Allergy and Clinical Immunology*, 126(5), pp. 898–907. doi: 10.1016/j.jaci.2010.09.007.

Gol-Ara, M., Jadidi-Niaragh, F., Sadria, R., Azizi, G. and Mirshafiey, A. (2012) ‘The Role of Different Subsets of Regulatory T Cells in Immunopathogenesis of Rheumatoid Arthritis’, *Arthritis*, 2012, p. 805875. doi: 10.1155/2012/805875.

Gray, J. I., Westerhof, L. M. and MacLeod, M. K. L. (2018) ‘The roles of resident, central and effector memory CD4 T-cells in protective immunity following infection or vaccination’, *Immunology*, 154(4), p. 574. doi: 10.1111/imm.12929.

Groom, J. R. and Luster, A. D. (2011) ‘CXCR3 in T cell function’, *Experimental Cell Research*, 317(5), p. 620. doi: 10.1016/j.yexcr.2010.12.017.

Gu, Z., Eils, R. and Schlesner, M. (2016) ‘Complex heatmaps reveal patterns and correlations in multidimensional genomic data’, *Bioinformatics*, 32(18), pp. 2847–2849. doi: 10.1093/bioinformatics/btw313.

Guo, L., Huang, Y., Chen, X., Hu-Li, J., Urban, J. F. and Paul, W. E. (2015) ‘Innate Immune Function of TH2 Cells in vivo’, *Nature Immunology*, 16(10), p. 1051. doi: 10.1038/ni.3244.

Guo, L., Junttila, I. S. and Paul, W. E. (2012) ‘Cytokine-induced cytokine production by conventional and innate lymphoid cells’, *Trends in Immunology*, 33(12), p. 598. doi: 10.1016/j.it.2012.07.006.

Guo, L., Wei, G., Zhu, J., Liao, W., Leonard, W. J., Zhao, K. and Paul, W. (2009) ‘IL-1 family members and STAT activators induce cytokine production by Th2, Th17, and Th1 cells’, *Proceedings of the National Academy of Sciences of the United States of America*, 106(32), p. 13463. doi: 10.1073/pnas.0906988106.

Good-Jacobson, K. L., Szumilas, C. G., Chen, L., Sharpe, A. H., Tomayko, M. M. and Shlomchik, M. J. (2010) ‘PD-1 regulates germinal center B cell survival and the formation and affinity of long-lived plasma cells’, *Nature Immunology*, 11(6), pp. 535–542. doi: 10.1038/ni.1877.

Guo, L., Liu, X. and Su, X. (2023) ‘The role of TEMRA cell-mediated immune senescence in the development and treatment of HIV disease’, *Frontiers in Immunology*, 14, p. 1284293. doi: 10.3389/fimmu.2023.1284293.

Goldrath, A. W., Bogatzki, L. Y. and Bevan, M. J. (2000) ‘Naive T cells transiently acquire a memory-like phenotype during homeostasis-driven proliferation’, *Journal of Experimental Medicine*, 192(4), pp. 557–564. doi: 10.1084/jem.192.4.557.

Goldrath, A. W., Luckey, C. J., Park, R., Benoist, C. and Mathis, D. (2004) ‘The molecular program induced in T cells undergoing homeostatic proliferation’, *Proceedings of the National Academy of Sciences of the United States of America*, 101(48), pp. 16885–16890. doi: 10.1073/pnas.0407417101.

Gatto, D., Paus, D., Basten, A., Mackay, C. R. and Brink, R. (2009) 'Guidance of B cells by the orphan G protein-coupled receptor EBI2 shapes humoral immune responses', *Immunity*, 31(2), pp. 259–269. doi: 10.1016/j.immuni.2009.06.016.

Glatman Zaretsky, A., Taylor, J. J., King, I. L., Marshall, F. A., Mohrs, M. and Pearce, E. J. (2009) 'T follicular helper cells differentiate from Th2 cells in response to helminth antigens', *Journal of Experimental Medicine*, 206(5), pp. 991–999. doi: 10.1084/jem.20090303

Gringhuis, S. I., Kaptein, T. M., Wevers, B. A., van der Vlist, M., Klaver, E. J., van Die, I., Vriend, L. E., de Jong, M. A. and Geijtenbeek, T. B. (2014) 'Fucose-based PAMPs prime dendritic cells for follicular T helper cell polarization via DC-SIGN-dependent IL-27 production', *Nature Communications*, 5, p. 5074. doi: 10.1038/ncomms6074.

Haberman, A. M., Gonzalez, D. G., Wong, P., Zhang, T. ting and Kerfoot, S. M. (2019) 'Germinal Center B cell initiation, GC maturation, and the coevolution of its stromal cell niches', *Immunological Reviews*, 288(1), p. 10. doi: 10.1111/imr.12731.

Hale, J. S., Youngblood, B., Latner, D. R., Mohammed, A. U., Ye, L., Akondy, R. S., Wu, T., Iyer, S. S. and Ahmed, R. (2013) 'Distinct memory CD4+ T cells with commitment to T follicular helper- and T helper 1-cell lineages are generated after acute viral infection', *Immunity*, 38(4), pp. 805–817. doi: 10.1016/j.immuni.2013.02.020.

Hale, J. S. and Ahmed, R. (2015) 'Memory T Follicular Helper CD4 T Cells', *Frontiers in Immunology*, 6, p. 16. doi: 10.3389/fimmu.2015.00016.

Haluszczak, C., Akue, A. D., Hamilton, S. E., Johnson, L. D. S., Pujanauski, L., Teodorovic, L., Jameson, S. C. and Kedl, R. M. (2009) 'The antigen-specific CD8+ T cell repertoire in unimmunized mice includes memory phenotype cells bearing markers of homeostatic expansion', *Journal of Experimental Medicine*, 206(2), p. 435. doi: 10.1084/jem.20081829.

Hamel, K. M., Liarski, V. M. and Clark, M. R. (2012) 'Germinal Center B-cells', *Autoimmunity*, 45(5), pp. 333–347. doi: 10.3109/08916934.2012.665524.

He, J., Tsai, L. M., Leong, Y. A., Hu, X., Ma, C. S., Chevalier, N., Sun, X., Vandenberg, K., Rockman, S., Ding, Y., Zhu, L., Wei, W., Wang, C., Karnowski, A., Belz, G. T., Ghali, J. R., Cook, M. C., Riminton, D. S., Veillette, A., Schwartzberg, P. L., Mackay, F., Brink, R., Tangye, S. G., Vinuesa, C. G., Mackay, C. R., Li, Z. and Yu, D. (2013) 'Circulating precursor CCR7loPD-1hi CXCR5+ CD4+ T cells indicate Tfh cell activity and promote antibody responses upon antigen reexposure', *Immunity*, 39(4), pp. 770–781. doi: 10.1016/j.immuni.2013.09.007.

Heath, W. R., Kato, Y., Steiner, T. M. and Caminschi, I. (2019) 'Antigen presentation by dendritic cells for B cell activation', *Current Opinion in Immunology*, 58, pp. 44–52. doi: 10.1016/j.coi.2019.04.003.

Hegenbarth, J.-C., Lezzoche, G., De Windt, L. J. and Stoll, M. (2022) 'Perspectives on Bulk-Tissue RNA Sequencing and Single-Cell RNA Sequencing for Cardiac Transcriptomics', *Frontiers in Molecular Medicine*, 2, p. 839338. doi: 10.3389/fmmed.2022.839338.

Hernandez, J. B., Newton, R. H. and Walsh, C. M. (2010) 'Life and death in the thymus—cell death signaling during T cell development', *Current Opinion in Cell Biology*, pp. 865–871. doi: 10.1016/j.ceb.2010.08.003.

Hirayama, D., Iida, T. and Nakase, H. (2017) ‘The Phagocytic Function of Macrophage-Enforcing Innate Immunity and Tissue Homeostasis’, *International Journal of Molecular Sciences*, 19(1), p. 92. doi: 10.3390/ijms19010092.

Ho, I. C., Tai, T. S. and Pai, S. Y. (2009) ‘GATA3 and the T-cell lineage: essential functions before and after T-helper-2-cell differentiation’, *Nature Reviews Immunology*, 9(2), p. 125. doi: 10.1038/nri2476.

Hothorn, T., Van De Wiel, M. A., Hornik, K. and Zeileis, A. (2008) ‘Implementing a Class of Permutation Tests: The coin Package’, *Journal of Statistical Software*, 28(8), pp. 1–23. doi: 10.18637/jss.v028.i08.

Hussain, T., Nguyen, A., Daunt, C., Thiele, D., Pang, E. S., Li, J., Zaini, A., O’Keeffe, M., Zaph, C., Harris, N. L., Quinn, K. M. and La Gruta, N. L. (2023) ‘Helminth Infection–Induced Increase in Virtual Memory CD8 T Cells Is Transient, Driven by IL-15, and Absent in Aged Mice’, *The Journal of Immunology*, 210(3), pp. 297–309. doi: 10.4049/jimmunol.2200316.

Hussain, T. and Quinn, K. M. (2019) ‘Similar but different: virtual memory CD8 T cells as a memory-like cell population’, *Immunology and Cell Biology*, 97(7), pp. 675–684. doi: 10.1111/imcb.12277.

Hertweck, A., Vila De Mucha, M., Barber, P. R., Dagil, R., Porter, H., Ramos, A., Lord, G. M. and Jenner, R. G. (2022) ‘The TH1 cell lineage-determining transcription factor T-bet suppresses TH2 gene expression by redistributing GATA3 away from TH2 genes’, *Nucleic Acids Research*, 50(8), p. 4557. doi: 10.1093/nar/gkac258.

Hosokawa, H. and Rothenberg, E. V. (2020) ‘How transcription factors drive choice of T cell fate’, *Nature Reviews Immunology*, 21(3), p. 162. doi: 10.1038/S41577-020-00426-6.

Hams, E., McCarron, M. J., Amu, S., Yagita, H., Azuma, M., Chen, L. and Fallon, P. G. (2011) ‘Blockade of B7-H1 (programmed death ligand 1) enhances humoral immunity by positively regulating the generation of T follicular helper cells’, *The Journal of Immunology*, 186(10), pp. 5648–5655. doi: 10.4049/jimmunol.1003161.

Hardtke, S., Ohl, L. and Förster, R. (2005) ‘Balanced expression of CXCR5 and CCR7 on follicular T helper cells determines their transient positioning to lymph node follicles and is essential for efficient B-cell help’, *Blood*, 106(6), pp. 1924–1931. doi: 10.1182/blood-2004-11-4494.

Hatzi, K., Nance, J. P., Kroenke, M. A., Bothwell, M., Haddad, E. K., Melnick, A. and Crotty, S. (2015) ‘BCL6 orchestrates Tfh cell differentiation via multiple distinct mechanisms’, *Journal of Experimental Medicine*, 212(4), pp. 539–553. doi: 10.1084/jem.20141380.

Iyer, S. S., Latner, D. R., Zilliox, M. J., McCausland, M., Akondy, R. S., Peñaloza-Macmaster, P., Hale, J. S., Ye, L., Mohammed, A. U. R., Yamaguchi, T., Sakaguchi, S., Amara, R. R. and Ahmed, R. (2013) ‘Identification of novel markers for mouse CD4 T follicular helper cells’, *European Journal of Immunology*, 43(12). doi: 10.1002/eji.201343469.

Ise, W., Inoue, T., McLachlan, J. B., Kometani, K., Kubo, M., Okada, T. and Kurosaki, T. (2014) ‘Memory B cells contribute to rapid Bcl6 expression by memory follicular helper T cells’, *Proceedings of the National Academy of Sciences of the United States of America*, 111(32), pp. 11792–11797. doi: 10.1073/pnas.1404671111.

Janda, A., Bowen, A., Greenspan, N. S. and Casadevall, A. (2016) 'Ig constant region effects on variable region structure and function', *Frontiers in Microbiology*, 7, p. 22. doi: 10.3389/fmicb.2016.00022.

Jogdand, G. M., Mohanty, S. and Devadas, S. (2016) 'Regulators of Tfh cell differentiation', *Frontiers in Immunology*, 7, p. 226877. doi: 10.3389/fimmu.2016.00520.

Joyce, S. (2022) 'Life In-Between: Bridging Innate and Adaptive Immunity', *The Journal of Immunology*, 209(2), pp. 193–195. doi: 10.4049/jimmunol.2290012.

Johnston, R. J., Poholek, A. C., DiToro, D., Yusuf, I., Eto, D., Barnett, B., Dent, A. L., Craft, J. and Crotty, S. (2009) 'Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation', *Science*, 325(5943), pp. 1006–1010. doi: 10.1126/science.1175870.

Johnston, R. J., Choi, Y. S., Diamond, J. A., Yang, J. A. and Crotty, S. (2012) 'STAT5 is a potent negative regulator of TFH cell differentiation', *Journal of Experimental Medicine*, 209(2), pp. 243–250. doi: 10.1084/jem.20111174.

Jacomet, F., Cayssials, E., Basbous, S., Levescot, A., Piccirilli, N., Desmier, D., Robin, A., Barra, A., Giraud, C., Guilhot, F., Roy, L., Herbelin, A. and Gombert, J. M. (2015) 'Evidence for eomesodermin-expressing innate-like CD8(+) KIR/NKG2A(+) T cells in human adults and cord blood samples', *European Journal of Immunology*, 45(7), pp. 1926–1933. doi: 10.1002/eji.201545539.

Kaech, S. M., Wherry, E. J. and Ahmed, R. (2002) 'Effector and memory T-cell differentiation: implications for vaccine development', *Nature Reviews Immunology*, 2(4), pp. 251–262. doi: 10.1038/nri778.

Kawabe, T. (2022) 'Memory-phenotype CD4+ T Lymphocytes: A Novel Therapeutic Target in Infectious or Autoimmune Diseases?', *Japan Medical Association Journal*, 5(3), p. 298. doi: 10.31662/jmaj.2022-0048.

Kawabe, T. (2023) 'Homeostasis and immunological function of self-driven memory-phenotype CD4+ T lymphocytes', *Immunological Medicine*, 46(1), pp. 1–8. doi: 10.1080/25785826.2022.2129370.

Kawabe, T., Ciucci, T., Kim, K. S., Tayama, S., Kawajiri, A., Suzuki, T., Tanaka, R., Ishii, N., Jankovic, D., Zhu, J., Sprent, J., Bosselut, R. and Sher, A. (2022) 'Redefining the Foreign Antigen and Self-Driven Memory CD4+ T-Cell Compartments via Transcriptomic, Phenotypic, and Functional Analyses', *Frontiers in Immunology*, 13. doi: 10.3389/fimmu.2022.870542.

Kawabe, T., Jankovic, D., Kawabe, S., Huang, Y., Lee, P. H., Yamane, H., Zhu, J., Sher, A., Germain, R. N. and Paul, W. E. (2017) 'Memory-phenotype CD4+ T cells spontaneously generated under steady state conditions exert innate Th1-like effector function', *Science Immunology*, 2(12). doi: 10.1126/sciimmunol.aam9304.

Kawabe, T. and Sher, A. (2021) 'Memory-phenotype CD4+ T cells: a naturally arising T lymphocyte population possessing innate immune function', *International Immunology*, 34(4), p. 189. doi: 10.1093/intimm/dxab108.

Kawabe, T., Yi, J., Kawajiri, A., Hilligan, K., Fang, D., Ishii, N., Yamane, H., Zhu, J., Jankovic, D., Kim, K. S., Trinchieri, G. and Sher, A. (2020) 'Requirements for the differentiation of innate

T-bethigh memory-phenotype CD4+ T lymphocytes under steady state', *Nature Communications*, 11(1), p. 3366. doi: 10.1038/s41467-020-17136-1.

Kawabe, T., Yi, J. and Sprent, J. (2021) 'Homeostasis of Naive and Memory T Lymphocytes', *Cold Spring Harbor Perspectives in Biology*, 13(9), p. a037879. doi: 10.1101/cshperspect.a037879.

Khantakova, J. N., Bulygin, A. S. and Sennikov, S. V. (2022) 'The Regulatory-T-Cell Memory Phenotype: What We Know', *Cells*, 11(10), p. 1687. doi: 10.3390/cells11101687.

Khatoon, Z., Figler, B., Zhang, H. and Cheng, F. (2014) 'Introduction to RNA-Seq and its applications to drug discovery and development', *Drug Development Research*, 75(5), pp. 324–330. doi: 10.1002/ddr.21215.

Kim, D., Langmead, B. and Salzberg, S. L. (2015) 'HISAT: a fast spliced aligner with low memory requirements', *Nature Methods*, 12(4), p. 357. doi: 10.1038/nmeth.3317.

Kim, H. J., Barnitz, R. A., Kreslavsky, T., Brown, F. D., Moffett, H., Lemieux, M. E., Kaygusuz, Y., Meissner, T., Holderried, T. A. W., Chan, S., Kastner, P., Haining, W. N. and Cantor, H. (2015) 'Stable inhibitory activity of regulatory T cells requires the transcription factor Helios', *Science*, 350(6258), p. 334. doi: 10.1126/science.aad0616.

Kobayashi, S. D., Voyich, J. M., Burlak, C. and DeLeo, F. R. (2005) 'Neutrophils in the innate immune response', *Archives of Immunology and Therapeutic Experimentation (Warsaw)*, 53(6), pp. 505–517. PMID: 16407783.

Koch, M. A., Tucker-Heard, G., Perdue, N. R., Killebrew, J. R., Urdahl, K. B. and Campbell, D. J. (2009) 'T-bet controls regulatory T cell homeostasis and function during type-1 inflammation', *Nature Immunology*, 10(6), p. 595. doi: 10.1038/ni.1731.

Koh, C. H., Lee, S., Kwak, M., Kim, B. S. and Chung, Y. (2023) 'CD8 T-cell subsets: heterogeneity, functions, and therapeutic potential', *Experimental & Molecular Medicine*, 55(11), pp. 2287–2299. doi: 10.1038/s12276-023-01105-x.

Kokubo, K., Onodera, A., Kiuchi, M., Tsuji, K., Hirahara, K. and Nakayama, T. (2022) 'Conventional and pathogenic Th2 cells in inflammation, tissue repair, and fibrosis', *Frontiers in Immunology*, 13, p. 945063. doi: 10.3389/fimmu.2022.945063.

Korotkevich, G., Sukhov, V., Budin, N., Shpak, B., Artyomov, M. N. and Sergushichev, A. (2021) 'Fast gene set enrichment analysis', *bioRxiv*, p. 060012. doi: 10.1101/060012.

Kotnis, A., Du, L., Liu, C., Popov, S. W. and Pan-Hammarström, Q. (2008) 'Non-homologous end joining in class switch recombination: the beginning of the end', *Philosophical Transactions of the Royal Society B: Biological Sciences*, 364(1517), p. 653. doi: 10.1098/rstb.2008.0196.

Krangel, M. S. (2009) 'Mechanics of T cell receptor gene rearrangement', *Current Opinion in Immunology*, 21(2), p. 133. doi: 10.1016/j.coi.2009.03.009.

Kreslavsky, T., Gleimer, M., Garbe, A. I. and von Boehmer, H. (2010) 'αβ versus γδ fate choice: Counting the T-cell lineages at the branch point', *Immunological Reviews*, 238(1), pp. 169–181. doi: 10.1111/j.1600-065X.2010.00947.x.

Krishnaswamy, J. K., Alsén, S., Yrlid, U., Eisenbarth, S. C. and Williams, A. (2018) 'Determination of T Follicular Helper Cell Fate by Dendritic Cells', *Frontiers in Immunology*, 9, p. 408585. doi: 10.3389/fimmu.2018.02169/pdf.

Krystel-Whittemore, M., Dileepan, K. N. and Wood, J. G. (2015) 'Mast Cell: A Multi-Functional Master Cell', *Frontiers in Immunology*, 6, p. 620. doi: 10.3389/fimmu.2015.00620.

Kukurba, K. R. and Montgomery, S. B. (2015) 'RNA Sequencing and Analysis', *Cold Spring Harbor Protocols*, 2015(11), p. 951. doi: 10.1101/pdb.top084970.

Kumar, B. V., Connors, T. J. and Farber, D. L. (2018) 'Human T Cell Development, Localization, and Function throughout Life', *Immunity*, doi: 10.1016/j.immuni.2018.01.007.

Künzli, M. and Masopust, D. (2023) 'CD4+ T cell memory', *Nature Immunology*, 24(6), pp. 903–914. doi: 10.1038/s41590-023-01510-4.

Künzli, M., Schreiner, D., Pereboom, T. C., Swarnalekha, N., Litzler, L. C., Lötscher, J., Ertuna, Y. I., Roux, J., Geier, F., Jakob, R. P., Maier, T., Hess, C., Taylor, J. J. and King, C. G. (2020) 'Long-lived T follicular helper cells retain plasticity and help sustain humoral immunity', *Science Immunology*, 5(45), pp. eaay5552. doi: 10.1126/sciimmunol.aay5552.

Kawajiri, A., Li, J., Koinuma, K., Yang, Z., Yoon, H. J., Yi, J., Nagashima, H., Ishii, M., Gao, F., Sato, K., Tayama, S., Harigae, H., Iwakura, Y., Ishii, N., Sher, A., Ishigaki, K., Zhu, J., Kim, K. S. and Kawabe, T. (2024) 'Naturally arising memory-phenotype CD4+ T lymphocytes contain an undifferentiated population that can generate TH1, TH17, and Treg cells', *Science Advances*, 10(49), p. eadq6618. doi: 10.1126/sciadv.adq6618.

Kim, K. S., Hong, S. W., Han, D., Yi, J., Jung, J., Yang, B. G., Lee, J. Y., Lee, M. and Surh, C. D. (2016) 'Dietary antigens limit mucosal immunity by inducing regulatory T cells in the small intestine', *Science*, 351(6275), pp. 858–863. doi: 10.1126/science.aac5560.

Kusam, S., Toney, L. M., Sato, H. and Dent, A. L. (2003) 'Inhibition of Th2 differentiation and GATA-3 expression by BCL-6', *Journal of Immunology*, 170(5), pp. 2435–2441. doi: 10.4049/jimmunol.170.5.2435.

Karnowski, A., Chevrier, S., Belz, G. T., Mount, A., Emslie, D., D'Costa, K., Tarlinton, D. M., Kallies, A. and Corcoran, L. M. (2012) 'B and T cells collaborate in antiviral responses via IL-6, IL-21, and transcriptional activator and coactivator, Oct2 and OBF-1', *Journal of Experimental Medicine*, 209(11), pp. 2049–2064. doi: 10.1084/jem.20111504.

Kitano, M., Moriyama, S., Ando, Y., Hikida, M., Mori, Y., Kurosaki, T. and Okada, T. (2011) 'Bcl6 protein expression shapes pre-germinal center B cell dynamics and follicular helper T cell heterogeneity', *Immunity*, 34(6), pp. 961–972. doi: 10.1016/j.immuni.2011.03.025.

La Gruta, N. L., Gras, S., Daley, S. R., Thomas, P. G. and Rossjohn, J. (2018) 'Understanding the drivers of MHC restriction of T cell receptors', *Nature Reviews Immunology*, 18(7), pp. 467–478. doi: 10.1038/s41577-018-0007-5.

Lam, N., Lee, Y. S. and Farber, D. L. (2024) 'A guide to adaptive immune memory', *Nature Reviews Immunology*, 24(11), pp. 810–829. doi: 10.1038/s41577-024-01040-6.

Lebien, T. W. and Tedder, T. F. (2008) 'B lymphocytes: how they develop and function', *Blood*, 112(5), pp. 1570–1580. doi: 10.1182/blood-2008-02-078071.

Lee, J. Y., Hamilton, S. E., Akue, A. D., Hogquist, K. A. and Jameson, S. C. (2013) 'Virtual memory CD8 T cells display unique functional properties', *Proceedings of the National Academy of Sciences of the United States of America*, 110(33), pp. 13498–13503. doi: 10.1073/pnas.1307572110

Lehtimäki, S. and Lahesmaa, R. (2013) 'Regulatory T cells control immune responses through their non-redundant tissue-specific features', *Frontiers in Immunology*, 4, p. 294. doi: 10.3389/fimmu.2013.00294.

Lendeckel, U., Venz, S. and Wolke, C. (2022) 'Macrophages: shapes and functions', *ChemTexts*, 8(2), pp. 1–12. doi: 10.1007/s40828-022-00163-4.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G. and Durbin, R. (2009) 'The Sequence Alignment/Map format and SAMtools', *Bioinformatics*, 25(16), p. 2078. doi: 10.1093/bioinformatics/btp352.

Li, S., Miao, T., Sebastian, M., Bhullar, P., Ghaffari, E., Liu, M., Symonds, A. L. J. L. J. and Wang, P. (2012) 'The transcription factors Egr2 and Egr3 are essential for the control of inflammation and antigen-induced proliferation of B and T cells', *Immunity*, 37, pp. 685–696. doi: 10.1016/j.immuni.2012.08.001.

Li, X. and Wang, C. Y. (2021) 'From bulk, single-cell to spatial RNA sequencing', *International Journal of Oral Science*, 13(1). doi: 10.1038/S41368-021-00146-0.

Liao, Y., Smyth, G. K. and Shi, W. (2019) 'The R package Rsubread is easier, faster, cheaper, and better for alignment and quantification of RNA sequencing reads', *Nucleic Acids Research*, 47(8), p. e47. doi: 10.1093/nar/gkz114.

Liberzon, A., Birger, C., Thorvaldsdóttir, H., Ghandi, M., Mesirov, J. P. and Tamayo, P. (2015) 'The Molecular Signatures Database (MSigDB) hallmark gene set collection', *Cell Systems*, 1(6), p. 417. doi: 10.1016/j.cels.2015.12.004.

Lin, C. C., Bradstreet, T. R., Schwarzkopf, E. A., Sim, J., Carrero, J. A., Chou, C., Cook, L. E., Egawa, T., Taneja, R., Murphy, T. L., Russell, J. H. and Edelson, B. T. (2014) 'Bhlhe40 controls cytokine production by T cells and is essential for pathogenicity in autoimmune neuroinflammation', *Nature Communications*, 5(1), pp. 1–13. doi: 10.1038/ncomms4551.

Linterman, M. A., Pierson, W., Lee, S. K., Kallies, A., Kawamoto, S., Rayner, T. F., Srivastava, M., Divekar, D. P., Beaton, L., Hogan, J. J., Fagarasan, S., Liston, A., Smith, K. G. C. and Vinuesa, C. G. (2011) 'Foxp3+ follicular regulatory T cells control T follicular helper cells and the germinal center response', *Nature Medicine*, 17(8), p. 975. doi: 10.1038/nm.2425.

Liu, X., Yan, X., Zhong, B., Nurieva, R. I., Wang, A., Wang, X., Martin-Orozco, N., Wang, Y., Chang, S. H., Esplugues, E., Flavell, R. A., Tian, Q. and Dong, C. (2012) 'Bcl6 expression specifies the T follicular helper cell program in vivo', *Journal of Experimental Medicine*, 209(10), pp. 1841–1852. doi: 10.1084/jem.20120219.

Liu, D., Liu, J., Wang, J., Guo, L., Liu, C., Jiang, Y., Wang, H. and Yang, S. (2017) 'Distribution of circulating T follicular helper cell subsets is altered in immunoglobulin A vasculitis in children', *PLoS One*, 12(12), e0189133. doi: 10.1371/journal.pone.0189133.

Lo, J. W., de Mucha, M. V., Henderson, S., Roberts, L. B., Constable, L. E., Garrido-Mesa, N., Hertweck, A., Stolarczyk, E., Houlder, E. L., Jackson, I., MacDonald, A. S., Powell, N., Neves, J. F., Howard, J. K., Jenner, R. G. and Lord, G. M. (2022) 'A population of naive-like CD4+ T cells stably polarized to the TH1 lineage', *European Journal of Immunology*, 52(4), p. 566. doi: 10.1002/eji.202149228.

Locci, M., Havenar-Daughton, C., Landais, E., Wu, J., Kroenke, M. A., Arlehamn, C. L., Su, L. F., Cubas, R., Davis, M. M., Sette, A., Haddad, E. K., Poignard, P. and Crotty, S. (2013) 'Human Circulating PD-1+CXCR3−CXCR5+ Memory Tfh Cells Are Highly Functional and Correlate with Broadly Neutralizing HIV Antibody Responses', *Immunity*, 39(4), p. 758. doi: 10.1016/j.immuni.2013.08.031.

Lopes-Carvalho, T. and Kearney, J. F. (2004) 'Development and selection of marginal zone B cells', *Immunological Reviews*, 197, pp. 192–205. doi: 10.1111/j.0105-2896.2004.0112.x.

Love, M. I., Huber, W. and Anders, S. (2014) 'Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2', *Genome Biology*, 15(12), pp. 1–21. doi: 10.1186/s13059-014-0550-8.

Luckheeram, R. V., Zhou, R., Verma, A. D. and Xia, B. (2012) 'CD4+T Cells: Differentiation and Functions', *Clinical and Developmental Immunology*, 2012, p. 925135. doi: 10.1155/2012/925135.

LuminUltra Technologies (no date) 'How do I interpret my qPCR results?', LuminUltra Technologies.

Lun, A. T. L., Chen, Y. and Smyth, G. K. (2016) 'It's DE-licious: A Recipe for Differential Expression Analyses of RNA-seq Experiments Using Quasi-Likelihood Methods in edgeR', *Methods in Molecular Biology*, 1418, pp. 391–416. doi: 10.1007/978-1-4939-3578-9_19

Lun, A. T. L., McCarthy, D. J. and Marioni, J. C. (2016) 'A step-by-step workflow for low-level analysis of single-cell RNA-seq data with Bioconductor', *F1000Research*, 5. doi: 10.12688/f1000research.9501.2/doi.

Lazarevic, V., Chen, X., Shim, J.H., Hwang, E.S., Jang, E., Bolm, A.N., Oukka, M., Kuchroo, V.K. and Glimcher, L.H. (2010) 'Transcription factor T-bet represses TH17 differentiation by preventing Runx1-mediated activation of the RORγt gene', *Nature Immunology*, 12(1), p. 96. doi: 10.1038/ni.1969.

Leavy, O. (2012) 'The TFH-like transition of TH1 cells', *Nature Reviews Immunology*, 12(2), pp. 75–75. doi: 10.1038/nri3161.

Lee, S., Ko, Y. and Kim, T.J. (2020) 'Homeostasis and regulation of autoreactive B cells', *Cellular and Molecular Immunology*, 17(6), p. 561. doi: 10.1038/s41423-020-0445-4.

Lee, Y., Awasthi, A., Yosef, N., Quintana, F.J., Xiao, S., Peters, A., Wu, C., Kleinewietfeld, M., Kunder, S., Hafler, D.A., Sobel, R.A., Regev, A. and Kuchroo, V.K. (2012) 'Induction and molecular signature of pathogenic TH17 cells', *Nature Immunology*, 13(10), pp. 991–999. doi: 10.1038/ni.2416.

Li, J., Yang, Z., Kawajiri, A., Sato, K., Tayama, S., Ishii, N., Zhu, J. and Kawabe, T. (2024) 'Excess generation and activation of naturally arising memory-phenotype CD4+ T lymphocytes are inhibited by regulatory T cells in steady state', *Frontiers in Immunology*, 15, p. 1429954. doi: 10.3389/fimmu.2024.1429954.

Lee, J. Y., Skon, C. N., Lee, Y. J., Oh, S., Taylor, J. J., Malhotra, D., Jenkins, M. K., Rosenfeld, M. G., Hogquist, K. A. and Jameson, S. C. (2015) 'The transcription factor KLF2 restrains $CD4^+$ T follicular helper cell differentiation', *Immunity*, 42(2), pp. 252–264. doi: 10.1016/j.immuni.2015.01.013.

Liu, X., Yan, X., Zhong, B., Nurieva, R. I., Wang, A., Wang, X., Martin-Orozco, N., Wang, Y., Chang, S. H., Esplugues, E., Flavell, R. A., Tian, Q. and Dong, C. (2012) 'Bcl6 expression specifies the T follicular helper cell program in vivo', *Journal of Experimental Medicine*, 209(10), pp. 1841–1852. doi: 10.1084/jem.20120219.

Ma, X. and Nakayamada, S. (2021) 'Multi-Source Pathways of T Follicular Helper Cell Differentiation', *Frontiers in Immunology*, 12, p. 621105. doi: 10.3389/fimmu.2021.621105.

Maddur, M. S., Miossec, P., Kaveri, S. V. and Bayry, J. (2012) 'Th17 Cells: Biology, Pathogenesis of Autoimmune and Inflammatory Diseases, and Therapeutic Strategies', *The American Journal of Pathology*, 181(1), pp. 8–18. doi: 10.1016/j.ajpath.2012.03.044.

Mariuzza, R. A., Agnihotri, P. and Orban, J. (2019) 'The structural basis of T-cell receptor (TCR) activation: An enduring enigma', *The Journal of Biological Chemistry*, 295(4), p. 914. doi: 10.1074/jbc.rev119.009411.

Marshall, A., Celentano, A., Cirillo, N., McCullough, M. and Porter, S. (2017) 'Tissue-specific regulation of CXCL9/10/11 chemokines in keratinocytes: Implications for oral inflammatory disease', *PLOS ONE*, 12(3), p. e0172821. doi: 10.1371/journal.pone.0172821.

Martínez-Pérez, A., Estévez, O. and González-Fernández, Á. (2022) 'Contribution and Future of High-Throughput Transcriptomics in Battling Tuberculosis', *Frontiers in Microbiology*, 13. doi: 10.3389/fmicb.2022.835620.

Marusina, A. I., Ono, Y., Merleev, A. A., Shimoda, M., Ogawa, H., Wang, E. A., Kondo, K., Olney, L., Luxardi, G., Miyamura, Y., Yilma, T. D., Villalobos, I. B., Bergstrom, J. W., Kronenberg, D. G., Soulika, A. M., Adamopoulos, I. E. and Maverakis, E. (2016) 'CD4+ virtual memory: Antigen-inexperienced T cells reside in the naïve, regulatory, and memory T cell compartments at similar frequencies, implications for autoimmunity', *Journal of Autoimmunity*, 77. doi: 10.1016/j.jaut.2016.11.001.

McAleer, J. P. and Kolls, J. K. (2011) 'Mechanisms controlling Th17 cytokine expression and host defense', *Journal of Leukocyte Biology*, 90(2), p. 263. doi: 10.1189/jlb.0211099.

McKinnon, K. M. (2018) ‘Flow Cytometry: An Overview’, *Current Protocols in Immunology*, 120, p. 5.1.1. doi: 10.1002/cpim.40.

Mellman, I. (2013) ‘Dendritic Cells: Master Regulators of the Immune Response’, *Cancer Immunology Research*, 1(3), pp. 145–149. doi: 10.1158/2326-6066.cir-13-0102.

Mesin, L., Ersching, J. and Victora, G. D. (2016) ‘Germinal center B cell dynamics’, *Immunity*, 45(3), p. 471. doi: 10.1016/j.immuni.2016.09.001.

Miao, T., Symonds, A. L. J., Singh, R., Symonds, J. D., Ogbe, A., Omodho, B., Zhu, B., Li, S. and Wang, P. (2017) ‘Egr2 and 3 control adaptive immune responses by temporally uncoupling expansion from T cell differentiation’, *Journal of Experimental Medicine*, 214(6), pp. 1787–1808. doi: 10.1084/jem.20160553.

Miller, C. H., Klawon, D. E. J., Zeng, S., Lee, V., Socci, N. D. and Savage, P. A. (2020) ‘Eomes identifies thymic precursors of self-specific memory-phenotype CD8+ T cells’, *Nature Immunology*, 21(5), pp. 567–577. doi: 10.1038/s41590-020-0653-1.

Miao, T., Ramachandran, M., Bhullar, P., Ghaffari, E., Sebastian, A. L. J., Miao, U. C., Ghaffari, G., Li, S. and Wang, P. (2013) ‘Early growth response gene-2 controls IL-17 expression and Th17 differentiation by negatively regulating Batf’, *The Journal of Immunology*, 190(1), pp. 58–65. doi: 10.4049/jimmunol.1200868.

Min, B., Yamane, H., Hu-Li, J. and Paul, W. E. (2005) ‘Spontaneous and Homeostatic Proliferation of CD4 T Cells Are Regulated by Different Mechanisms’, *The Journal of Immunology*, 174(10), pp. 6039–6044. doi: 10.4049/jimmunol.174.10.6039.

Morita, R., Schmitt, N., Bentebibel, S. E., Ranganathan, R., Bourdery, L., Zurawski, G., Foucat, E., Dullaers, M., Oh, S. K., Sabzghabaei, N., Lavecchio, E. M., Punaro, M., Pascual, V., Banchereau, J. and Ueno, H. (2011) ‘Human Blood CXCR5+CD4+ T Cells Are Counterparts of T Follicular Cells and Contain Specific Subsets that Differentially Support Antibody Secretion’, *Immunity*, 34(1), p. 108. doi: 10.1016/j.immuni.2010.12.012.

Mahajan, S., Cervera, A., MacLeod, M., Fillatreau, S., Perona-Wright, G., Meek, S., Smith, A., MacDonald, A. and Gray, D. (2007) ‘The role of ICOS in the development of CD4 T cell help and the reactivation of memory T cells’, *European Journal of Immunology*, 37(7), p. 1796. doi: 10.1002/eji.200636661.

Mayberry, C. L., Logan, N. A., Wilson, J. J. and Chang, C. H. (2022) ‘Providing a Helping Hand: Metabolic Regulation of T Follicular Helper Cells and Their Association With Disease’, *Frontiers in Immunology*, 13, p. 864949. doi: 10.3389/fimmu.2022.864949.

Min, B., McHugh, R., Sempowski, G. D., Mackall, C., Foucras, G. and Paul, W. E. (2003) ‘Neonates support lymphopenia-induced proliferation’, *Immunity*, 18(1), pp. 131–140. doi: 10.1016/s1074-7613(02)00508-3.

Moser, B. (2015) ‘CXCR5, the Defining Marker for Follicular B Helper T (TFH) Cells’, *Frontiers in Immunology*, 6, p. 296. doi: 10.3389/fimmu.2015.00296.

Mashayekhi, M., Sandau, M. M., Dunay, I. R., Frickel, E. M., Khan, A., Goldszmid, R. S., Sher, A., Ploegh, H. L., Murphy, T. L., Sibley, L. D. and Murphy, K. M. (2011) 'CD8α(+) dendritic cells are the critical source of interleukin-12 that controls acute infection by *Toxoplasma gondii* tachyzoites', *Immunity*, 35(2), pp. 249–259. doi: 10.1016/j.immuni.2011.08.008.

Nikolich-Žugich, J. (2014) 'Aging of the T Cell Compartment in Mice and Humans: From No Naive Expectations to Foggy Memories', *The Journal of Immunology*, 193(6), pp. 2622–2629. doi: 10.4049/jimmunol.1401174.

Nitta, T., Nitta, S., Yu, L., Lipp, M. and Takahama, Y. (2009) 'CCR7-mediated migration of developing thymocytes to the medulla is essential for negative selection to tissue-restricted antigens', *Proceedings of the National Academy of Sciences of the United States of America*, 106(40), pp. 17129–17133. doi: 10.1073/pnas.0906956106.

Nolan, J. P. (2022) 'The evolution of spectral flow cytometry', *Cytometry Part A*, 101(10), pp. 812–817. doi: 10.1002/cyto.a.24566.

Nagele, E. P., Han, M., Acharya, N. K., DeMarshall, C., Kosciuk, M. C. and Nagele, R. G. (2013) 'Natural IgG Autoantibodies Are Abundant and Ubiquitous in Human Sera, and Their Number Is Influenced By Age, Gender, and Disease', *PLOS ONE*, 8(4), p. e60726. doi: 10.1371/journal.pone.0060726.

Nurieva, R. I., Chung, Y., Martinez, G. J., Yang, X. O., Tanaka, S., Matskevitch, T. D., Wang, Y. H. and Dong, C. (2009) 'Bcl6 mediates the development of T follicular helper cells', *Science*, 325(5943), pp. 1001–1005. doi: 10.1126/science.1176676.

Ogbe, A., Miao, T., Symonds, A. L. J., Omodho, B., Singh, R., Bhullar, P., Li, S. and Wang, P. (2015) 'Early Growth Response Genes 2 and 3 Regulate the Expression of Bcl6 and Differentiation of T Follicular Helper Cells', *The Journal of Biological Chemistry*, 290(33), p. 20455. doi: 10.1074/jbc.m114.634816.

Olatunde, A. C., Hale, J. S. and Lamb, T. J. (2021) 'Cytokine-skewed Tfh cells: functional consequences for B cell help', *Trends in Immunology*, 42(6), pp. 536–550. doi: 10.1016/j.it.2021.04.006.

Osińska, I., Popko, K. and Demkow, U. (2014) 'Perforin: an important player in immune response', *Central-European Journal of Immunology*, 39(1), p. 109. doi: 10.5114/ceji.2014.42135.

O'Garra, A. (2000) 'T-cell differentiation: Commitment factors for T helper cells', *Current Biology*, 10(13), pp. R492–R494. doi: 10.1016/s0960-9822(00)00556-x.

Oshi, M., Patel, A., Le, L., Tokumaru, Y., Yan, L., Matsuyama, R., Endo, I. and Takabe, K. (2021) 'G2M checkpoint pathway alone is associated with drug response and survival among cell proliferation-related pathways in pancreatic cancer', *American Journal of Cancer Research*, 11(6), p. 3070. PMID: 34249445.

Ozaki, K., Spolski, R., Ettinger, R., Kim, H. P., Wang, G., Qi, C. F., Hwu, P., Shaffer, D. J., Akilesh, S., Roopenian, D. C., Morse, H. C. III, Lipsky, P. E. and Leonard, W. J. (2004)

‘Regulation of B cell differentiation and plasma cell generation by IL-21, a novel inducer of Blimp-1 and Bcl-6’, *The Journal of Immunology*, 173(9), pp. 5361–5371. doi: 10.4049/jimmunol.173.9.5361.

Palm, A. K. E. and Henry, C. (2019) ‘Remembrance of Things Past: Long-Term B Cell Memory After Infection and Vaccination’, *Frontiers in Immunology*, 10, p. 1787. doi: 10.3389/fimmu.2019.01787.

Panina, Y., Germond, A., Masui, S. and Watanabe, T. M. (2018) ‘Validation of Common Housekeeping Genes as Reference for qPCR Gene Expression Analysis During iPS Reprogramming Process’, *Scientific Reports*, 8(1), pp. 1–8. doi: 10.1038/s41598-018-26707-8.

Papayannopoulos, V. (2017) ‘Neutrophil extracellular traps in immunity and disease’, *Nature Reviews Immunology*, 18(2), pp. 134–147. doi: 10.1038/nri.2017.105.

Parker, M. E. and Ciofani, M. (2020) ‘Regulation of γδ T Cell Effector Diversification in the Thymus’, *Frontiers in Immunology*, 11, p. 42. doi: 10.3389/fimmu.2020.00042.

Parkin, J. and Cohen, B. (2001) ‘An overview of the immune system’, *The Lancet*. doi: 10.1016/s0140-6736(00)04904-7.

Pepper, M. and Jenkins, M. K. (2011) ‘Origins of CD4+ effector and central memory T cells’, *Nature Immunology*, 12(6), p. 467. doi: 10.1038/ni.2038.

Pohlert, T. (2024) ‘Calculate Pairwise Multiple Comparisons of Mean Rank Sums Extended (R package PMCMRplus version 1.9.12)’, *CRAN: Contributed Packages*. doi: 10.32614/cran.package.pmcmrplus.

Porciello, N. and Tuosto, L. (2016) ‘CD28 costimulatory signals in T lymphocyte activation: Emerging functions beyond a qualitative and quantitative support to TCR signalling’, *Cytokine & Growth Factor Reviews*, 28, pp. 11–19. doi: 10.1016/j.cytogfr.2016.02.004.

Pribikova, M., Moudra, A. and Stepanek, O. (2018) ‘Opinion: Virtual memory CD8 T cells and lymphopenia-induced memory CD8 T cells represent a single subset: Homeostatic memory T cells’, *Immunology Letters*, 203, pp. 57–61. doi: 10.1016/j.imlet.2018.09.003.

Petrova, G., Ferrante, A. and Gorski, J. (2012) ‘Cross-Reactivity of T Cells and Its Role in the Immune System’, *Critical Reviews in Immunology*, 32(4), p. 349. doi: 10.1615/critrevimmunol.v32.i4.50.

Poholek, A. C., Hansen, K., Hernandez, S. G., Eto, D., Chandele, A., Weinstein, J. S., Dong, X., Odegard, J. M., Kaech, S. M., Dent, A. L., Crotty, S. and Craft, J. (2010) ‘In vivo regulation of Bcl6 and T follicular helper cell development’, *Journal of Immunology*, 185(1), pp. 313–326. doi: 10.4049/jimmunol.090402.

Powell, M. D., Read, K. A., Sreekumar, B. K., Jones, D. M. and Oestreich, K. J. (2019) ‘IL-12 signaling drives the differentiation and function of a TH1-derived TFH1-like cell population’, *Scientific Reports*, 9(1), p. 13991. doi: 10.1038/s41598-019-50614-1.

Qi, Q., Liu, Y., Cheng, Y., Glanville, J., Zhang, D., Lee, J. Y., Olshen, R. A., Weyand, C. M., Boyd, S. D. and Goronzy, J. J. (2014) 'Diversity and clonal selection in the human T-cell repertoire', *Proceedings of the National Academy of Sciences of the United States of America*, 111(36), pp. 13139–13144. doi: 10.1073/pnas.1409155111.

Quinn, K. M., Fox, A., Harland, K. L., Russ, B. E., Li, J., Nguyen, T. H. O., Loh, L., Olshansky, M., Naeem, H., Tsyganov, K., Wiede, F., Webster, R., Blyth, C., Sng, X. Y. X., Tiganis, T., Powell, D., Doherty, P. C., Turner, S. J., Kedzierska, K. and La Gruta, N. L. (2018) 'Age-Related Decline in Primary CD8+ T Cell Responses Is Associated with the Development of Senescence in Virtual Memory CD8+ T Cells', *Cell Reports*, 23(12), pp. 3512–3524. doi: 10.1016/j.celrep.2018.05.057.

Quinn, J. L., Kumar, G., Agasing, A., Ko, R. M. and Axtell, R. C. (2018) 'Role of TFH cells in promoting T Helper 17-induced neuroinflammation', *Frontiers in Immunology*, 9, p. 382. doi: 10.3389/fimmu.2018.00382.

Ramponi, G., Brunetta, E. and Folci, M. (2022) 'Role of Th1 and Th2 in autoimmunity', *Translational Autoimmunity: Etiology of Autoimmune Diseases*, pp. 61–92. doi: 10.1016/b978-0-12-822564-6.00020-3.

Ratajczak, W., Niedźwiedzka-Rystwej, P., Tokarz-Deptuła, B. and Deptuła, W. (2018) 'Immunological memory cells', *Central-European Journal of Immunology*, 43(2), p. 194. doi: 10.5114/ceji.2018.77390.

Recaldin, T. and Fear, D. J. (2016) 'Transcription factors regulating B cell fate in the germinal centre', *Clinical and Experimental Immunology*, 183(1), pp. 65–75. doi: 10.1111/cei.12702.

Ritchie, M. E., Phipson, B., Wu, D., Hu, Y., Law, C. W., Shi, W. and Smyth, G. K. (2015) 'limma powers differential expression analyses for RNA-sequencing and microarray studies', *Nucleic Acids Research*, 43(7), p. e47. doi: 10.1093/nar/gkv007.

Robinson, M. D., McCarthy, D. J. and Smyth, G. K. (2010) 'edgeR: a Bioconductor package for differential expression analysis of digital gene expression data', *Bioinformatics*, 26(1), pp. 139–140. doi: 10.1093/bioinformatics/btp616.

Rolot, M., Dougall, A. M., Chetty, A., Javaux, J., Chen, T., Xiao, X., Machiels, B., Selkirk, M. E., Maizels, R. M., Hokke, C., Denis, O., Brombacher, F., Vanderplasschen, A., Gillet, L., Horsnell, W. G. C. and Dewals, B. G. (2018) 'Helminth-induced IL-4 expands bystander memory CD8+ T cells for early control of viral infection', *Nature Communications*, 9(1), p. 4516. doi: 10.1038/s41467-018-06978-5.

Romagnani, S. (1999) 'Th1/Th2 Cells', *Inflammatory Bowel Diseases*, 5(4), pp. 285–294. doi: 10.1097/00054725-199911000-00009.

Rothenberg, E. V., Moore, J. E. and Yui, M. A. (2008) 'Launching the T-Lineage Developmental Programme', *Nature Reviews Immunology*, 8(1), p. 9. doi: 10.1038/nri2232.

RStudio Team (2023) *Posit | The Open-Source Data Science Company*. Available at: https://posit.co/ (Accessed: 19 December 2024).

Ryu, S., Lim, M. Y., Kim, J. and Kim, H. Y. (2023) ‘Versatile roles of innate lymphoid cells at the mucosal barrier: from homeostasis to pathological inflammation’, *Experimental & Molecular Medicine*, 55(9), pp. 1845–1857. doi: 10.1038/s12276-023-01022-z.

Richards, D. M., Kyewski, B. and Feuerer, M. (2016) ‘Re-examining the nature and function of self-reactive T cells’, *Trends in Immunology*, 37(2), p. 114. doi: 10.1016/j.it.2015.12.005.

Rudd, C. E., Taylor, A. and Schneider, H. (2009) ‘CD28 and CTLA-4 coreceptor expression and signal transduction’, *Immunological Reviews*, 229(1), pp. 12–26. doi: 10.1111/j.1600-065x.2009.00770.x.

Saadh, M. J., Rasulova, I., Khalil, M., Farahim, F., Sârbu, I., Ciongradi, C. I., Omar, T. M., Alhili, A., Jawad, M. J., Hani, T., Ali, T. and Lakshmaiya, N. (2024) ‘Natural killer cell-mediated immune surveillance in cancer: Role of tumor microenvironment’, *Pathology – Research and Practice*, 254, p. 155120. doi: 10.1016/j.prp.2024.155120.

Sage, P. T., Alvarez, D., Godec, J., Von Andrian, U. H. and Sharpe, A. H. (2014) ‘Circulating T follicular regulatory and helper cells have memory-like properties’, *The Journal of Clinical Investigation*, 124(12), p. 5191. doi: 10.1172/jci76861.

Sallusto, F., Lanzavecchia, A., Araki, K. and Ahmed, R. (2010) ‘From vaccines to memory and back’, *Immunity*, 33(4), pp. 451–463. doi: 10.1016/j.immuni.2010.10.008.

Savid-Frontera, C., Viano, M. E., Baez, N. S., Lidon, N. L., Fontaine, Q., Young, H. A., Vimeux, L., Donnadieu, E. and Rodriguez-Galan, M. C. (2022) ‘Exploring the immunomodulatory role of virtual memory CD8+ T cells: Role of IFN gamma in tumor growth control’, *Frontiers in Immunology*, 13, p. 971001. doi: 10.3389/fimmu.2022.971001.

Schmitt, N., Morita, R., Bourdery, L., Bentebibel, S. E., Zurawski, S. M., Banchereau, J. and Ueno, H. (2009) ‘Human dendritic cells induce the differentiation of interleukin-21-producing T follicular helper-like cells through interleukin-12’, *Immunity*, 31(1), pp. 158–169. doi: 10.1016/j.immuni.2009.04.016.

Schmitt, N., Bentebibel, S. E. and Ueno, H. (2014) ‘Phenotype and functions of memory Tfh cells in human blood’, *Trends in Immunology*, 35(9), p. 436. doi: 10.1016/j.it.2014.06.002.

Schmitt, N., Liu, Y., Bentebibel, S. E., Munagala, I., Bourdery, L., Venuprasad, K., Banchereau, J. and Ueno, H. (2014) ‘The cytokine TGF-β co-opts signaling via STAT3–STAT4 to promote the differentiation of human TFH cells’, *Nature Immunology*, 15(9), pp. 856–865. doi: 10.1038/ni.2947.

Seok, J., Cho, S. D., Seo, S. J. and Park, S. H. (2023) ‘Roles of virtual memory T cells in diseases’, *Immune Network*, 23(1), p. e11. doi: 10.4110/in.2023.23.e11.

Shah, K., Al-Haidari, A., Sun, J. and Kazi, J. U. (2021) ‘T cell receptor (TCR) signaling in health and disease’, *Signal Transduction and Targeted Therapy*, 6(1), pp. 1–26. doi: 10.1038/s41392-021-00823-w.

Shamri, R., Xenakis, J. J. and Spencer, L. A. (2011) 'Eosinophils in innate immunity: an evolving story', *Cell and Tissue Research*, 343(1), p. 57. doi: 10.1007/s00441-010-1049-6.

Shi, J., Hou, S., Fang, Q., Liu, X., Liu, X. and Qi, H. (2018) 'PD-1 controls follicular T helper cell positioning and function', *Immunity*, 49(2), p. 264. doi: 10.1016/j.immuni.2018.06.012.

Singh, R., Miao, T., Symonds, A. L. J., Omodho, B., Li, S. and Wang, P. (2017) 'Egr2 and 3 inhibit T-bet–mediated IFN-γ production in T cells', *The Journal of Immunology*, 198(11), pp. 4394–4402. doi: 10.4049/jimmunol.1601723.

Sjaastad, L. E., Owen, D. L., Tracy, S. I. and Farrar, M. A. (2021) 'Phenotypic and functional diversity in regulatory T cells', *Frontiers in Cell and Developmental Biology*, 9, p. 715901. doi: 10.3389/fcell.2021.715901.

Smith, F. L. and Baumgarth, N. (2019) 'B-1 cell responses to infections', *Current Opinion in Immunology*, 57, p. 23. doi: 10.1016/j.coi.2018.12.001.

Smith, T., Heger, A. and Sudbery, I. (2017) 'UMI-tools: Modeling sequencing errors in unique molecular identifiers to improve quantification accuracy', *Genome Research*, 27(3), pp. 491–499. doi: 10.1101/gr.209601.116.

Sobecki, M., Mrouj, K., Colinge, J., Gerbe, F., Jay, P., Krasinska, L., Dulic, V. and Fisher, D. (2017) 'Cell-cycle regulation accounts for variability in Ki-67 expression levels', *Cancer Research*, 77(10), pp. 2722–2734. doi: 10.1158/0008-5472.can-16-0707.

Sosinowski, T., White, J. T., Cross, E. W., Haluszczak, C., Marrack, P., Gapin, L. and Kedl, R. M. (2013) 'CD8α+ DC trans-presentation of IL-15 to naïve CD8+ T cells produces antigen inexperienced T cells in the periphery with memory phenotype and function', *The Journal of Immunology*, 190(5), p. 1936. doi: 10.4049/jimmunol.1203149.

Spellberg, B. and Edwards, J. E. (2001) 'Type 1/Type 2 immunity in infectious diseases', *Clinical Infectious Diseases*, 32(1), pp. 76–102. doi: 10.1086/317537.

Spinner, C. A. and Lazarevic, V. (2021) 'Transcriptional regulation of adaptive and innate lymphoid lineage specification', *Immunological Reviews*, 300(1), pp. 65–81. doi: 10.1111/imr.12935.

Sprent, J. and Surh, C. D. (2011) 'Normal T cell homeostasis: the conversion of naive cells into memory-phenotype cells', *Nature Immunology*, 12(6), pp. 478–484. doi: 10.1038/ni.2018.

Stark, J. M., Tibbitt, C. A. and Coquet, J. M. (2019) 'The metabolic requirements of Th2 cell differentiation', *Frontiers in Immunology*, 10, p. 2318. doi: 10.3389/fimmu.2019.02318.

Su, J., Berg, R. E., Murray, S. and Forman, J. (2005) 'Thymus-dependent memory phenotype CD8 T cells in naive B6.H-2Kb−/−Db−/− animals mediate an antigen-specific response against *Listeria monocytogenes*', *The Journal of Immunology*, 175(10), pp. 6450–6457. doi: 10.4049/jimmunol.175.10.6450.

Su, L. F., Kidd, B. A., Han, A., Kotzin, J. J. and Davis, M. M. (2013) 'Virus-specific CD4+ memory-phenotype T cells are abundant in unexposed adults', *Immunity*, 38(2), pp. 373–383. doi: 10.1016/j.immuni.2012.10.021.

Sun, L., Su, Y., Jiao, A., Wang, X. and Zhang, B. (2023) 'T cells in health and disease', *Signal Transduction and Targeted Therapy*, 8(1), pp. 1–50. doi: 10.1038/s41392-023-01471-y.

Surh, C. D. and Sprent, J. (2000) 'Homeostatic T cell proliferation: How far can T cells be activated to self-ligands?', *The Journal of Experimental Medicine*, 192(4), p. F9. doi: 10.1084/jem.192.4.f9.

Swiatek, P. J. and Gridley, T. (1993) 'Perinatal lethality and defects in hindbrain development in mice homozygous for a targeted mutation of the zinc finger gene Krox20', *Genes & Development*, 7(11), pp. 2071–2084. doi: 10.1101/gad.7.11.2071.

Symonds, A. L. J., Zheng, W., Miao, T., Wang, H., Wang, T. S., Kiome, R., Hou, X., Li, S. and Wang, P. (2020) 'Egr2 and 3 control inflammation, but maintain homeostasis, of PD-1high memory phenotype CD4 T cells', *Life Science Alliance*, 3(9), p. e202000766. doi: 10.26508/lsa.202000766.

Szabo, S. J., Kim, S. T., Costa, G. L., Zhang, X., Fathman, C. G. and Glimcher, L. H. (2000) 'A novel transcription factor, T-bet, directs Th1 lineage commitment', *Cell*, 100(6), pp. 655–669. doi: 10.1016/s0092-8674(00)80702-3.

Szabo, S. J., Sullivan, B. M., Stemmann, C., Satoskar, A. R., Sleckman, B. P. and Glimcher, L. H. (2002) 'Distinct effects of T-bet in TH1 lineage commitment and IFN-gamma production in CD4 and CD8 T cells', *Science*, 295(5553), pp. 338–342. doi: 10.1126/science.1065543.

Saravia, J., Chapman, N. M. and Chi, H. (2019) 'Helper T cell differentiation', *Cellular & Molecular Immunology*, 16(7), pp. 634–643. doi: 10.1038/s41423-019-0220-6.

Seif, F., Khoshmirsafa, M., Aazami, H., Mohsenzadegan, M., Sedighi, G. and Bahar, M. (2017) 'The role of JAK-STAT signaling pathway and its regulators in the fate of T helper cells', *Cell Communication and Signaling*, 15(1), pp. 1–13. doi: 10.1186/s12964-017-0177-y.

Shin, C., Han, J. A., Koh, H., Choi, B., Cho, Y., Jeong, H., Ra, J. S., Sung, P. S., Shin, E. C., Ryu, S. and Do, Y. (2015) 'CD8α− dendritic cells induce antigen-specific T follicular helper cells generating efficient humoral immune responses', *Cell Reports*, 11(12), pp. 1929–1940. doi: 10.1016/j.celrep.2015.05.042.

Silva, S. L., Albuquerque, A. S., Serra-Caetano, A., Foxall, R. B., Pires, A. R., Matoso, P., Fernandes, S. M., Ferreira, J., Cheynier, R., Victorino, R. M. M., Caramalho, I., Barata, J. T. and Sousa, A. E. (2016) 'Human naïve regulatory T-cells feature high steady-state turnover and are maintained by IL-7', *Oncotarget*, 7(11), p. 12163. doi: 10.18632/oncotarget.7512.

Sprent, J., Cho, J. H., Boyman, O. and Surh, C. D. (2008) 'T cell homeostasis', *Immunology and Cell Biology*, 86(4), pp. 312–319. doi: 10.1038/icb.2008.12.

Stebegg, M., Kumar, S. D., Silva-Cayetano, A., Fonseca, V. R., Linterman, M. A. and Graca, L. (2018) 'Regulation of the germinal center response', *Frontiers in Immunology*, 9, p. 2469. doi: 10.3389/fimmu.2018.02469.

Su, L. F., Kidd, B. A., Han, A., Kotzin, J. J. and Davis, M. M. (2013) 'Virus-specific CD4+ memory-phenotype T cells are abundant in unexposed adults', *Immunity*, 38(2), pp. 373–383. doi: 10.1016/j.immuni.2012.10.021.

Symonds, A. L. J., Zheng, W., Miao, T., Wang, H., Wang, T. S., Kiome, R., Hou, X., Li, S. and Wang, P. (2020) 'Egr2 and 3 control inflammation, but maintain homeostasis, of PD-1high memory phenotype CD4 T cells', *Life Science Alliance*, 3(9), p. e202000766. doi: 10.26508/lsa.202000766.

Sage, P. T. and Sharpe, A. H. (2015) 'T follicular regulatory cells in the regulation of B cell responses', *Trends in Immunology*, 36(7), pp. 410–418. doi: 10.1016/j.it.2015.05.005.

Suan, D., Nguyen, A., Moran, I., Bourne, K., Hermes, J. R., Arshi, M., Hampton, H. R., Tomura, M., Miwa, Y., Kelleher, A. D., Kaplan, W., Deenick, E. K., Tangye, S. G., Brink, R., Chtanova, T. and Phan, T. G. (2015) 'T follicular helper cells have distinct modes of migration and molecular signatures in naive and memory immune responses', *Immunity*, 42(4), pp. 704–718. doi: 10.1016/j.immuni.2015.03.002.

Sage, P. T. and Sharpe, A. H. (2015) 'In vitro assay to sensitively measure T(FR) suppressive capacity and T(FH) stimulation of B cell responses', *Methods in Molecular Biology*, 1291, pp. 151–160. doi: 10.1007/978-1-4939-2498-1_13.

Sage, P. T., Francisco, L. M., Carman, C. V. and Sharpe, A. H. (2013) 'The receptor PD-1 controls follicular regulatory T cells in the lymph nodes and blood', *Nature Immunology*, 14(2), pp. 152–161. doi: 10.1038/ni.2496.

Thapa, P. and Farber, D. L. (2019) 'The role of the thymus in the immune response', *Thoracic Surgery Clinics*, pp. 123–131. doi: 10.1016/j.thorsurg.2018.12.001.

The R foundation (2021) *R: The R Project for Statistical Computing*. Available at: https://www.r-project.org/ (Accessed: 18 December 2024).

ThermoFisher (no date) *How a Flow Cytometer Works - UK*. Available at: https://www.thermofisher.com/uk/en/home/life-science/cell-analysis/cell-analysis-learning-center/molecular-probes-school-of-fluorescence/flow-cytometry-basics/flow-cytometry-fundamentals/how-flow-cytometer-works.html (Accessed: 30 November 2024).

Thiele, D., La Gruta, N. L., Nguyen, A. and Hussain, T. (2020) 'Hiding in plain sight: virtually unrecognizable memory phenotype CD8+ T cells', *International Journal of Molecular Sciences*, 21(22), p. 8626. doi: 10.3390/ijms21228626.

Taefehshokr, N., Miao, T., Symonds, A. L. J., Wang, P. and Li, S. (2020) 'Egr2 regulation in T cells is mediated through IFNγ/STAT1 and IL-6/STAT3 signalling pathway', *Pathology Research and Practice*, 216(12), p. 153259. doi: 10.1016/j.prp.2020.153259.

Tsai, L. M. and Yu, D. (2014) ‘Follicular helper T-cell memory: establishing new frontiers during antibody response’, *Immunology and Cell Biology*, 92(1), pp. 57–63. doi: 10.1038/icb.2013.68.

Van Kaer, L. (2015) ‘Innate and virtual memory T cells in man’, *European Journal of Immunology*, 45(7), p. 1916. doi: 10.1002/eji.201545761.

Valeri, M. and Raffatellu, M. (2016) ‘Cytokines IL-17 and IL-22 in the host response to infection’, *Pathogens and Disease*, 74(9), p. ftw111. doi: 10.1093/femspd/ftw111.

Valkiers, S., de Vrij, N., Gielis, S., Verbandt, S., Ogunjimi, B., Laukens, K. and Meysman, P. (2022) ‘Recent advances in T-cell receptor repertoire analysis: bridging the gap with multimodal single-cell RNA sequencing’, *ImmunoInformatics*, 5, p. 100009. doi: 10.1016/j.immuno.2022.100009.

van der Veeken, J., Gonzalez, A. J., Cho, H., Arvey, A., Hemmers, S., Leslie, C. S. and Rudensky, A. Y. (2016) ‘Memory of inflammation in regulatory T cells’, *Cell*, 166(4), p. 977. doi: 10.1016/j.cell.2016.07.006.

Viano, M. E., Baez, N. S., Savid-Frontera, C., Lidon, N. L., Hodge, D. L., Herbelin, A., Gombert, J. M., Barbarin, A. and Rodriguez-Galan, M. C. (2022) ‘Virtual memory CD8+ T cells: origin and beyond’, *Journal of Interferon & Cytokine Research*, 42(12), p. 624. doi: 10.1089/jir.2022.0053.

Vinuesa, C. G. and Chang, P. P. (2013) ‘Innate B cell helpers reveal novel types of antibody responses’, *Nature Reviews Immunology*, 14(2), pp. 119–126. doi: 10.1038/ni.2511.

Vivier, E., Tomasello, E., Baratin, M., Walzer, T. and Ugolini, S. (2008) ‘Functions of natural killer cells’, *Nature Immunology*, 9(5), pp. 503–510. doi: 10.1038/ni1582.

Verykokakis, M., Boos, M. D., Bendelac, A. and Kee, B. L. (2010) ‘SAP protein-dependent natural killer T-like cells regulate the development of CD8(+) T cells with innate lymphocyte characteristics’, *Immunity*, 33(2), pp. 203–215. doi: 10.1016/j.immuni.2010.07.013.

Walker, J. A. and McKenzie, A. N. J. (2017) ‘TH2 cell development and function’, *Nature Reviews Immunology*, 18(2), pp. 121–133. doi: 10.1038/nri.2017.118.

Wang, P., Wang, Y., Xie, L., Xiao, M., Wu, J., Xu, L., Bai, Q., Hao, Y., Huang, Q., Chen, X., He, R., Li, B., Yang, S., Chen, Y., Wu, Y. and Ye, L. (2019) ‘The transcription factor T-bet is required for optimal type I follicular helper T cell maintenance during acute viral infection’, *Frontiers in Immunology*, 10, p. 606. doi: 10.3389/fimmu.2019.00606.

Wellford, S. A. and Schwartzberg, P. L. (2024) ‘Help me help you: emerging concepts in T follicular helper cell differentiation, identity, and function’, *Current Opinion in Immunology*, 87, p. 102421. doi: 10.1016/j.coi.2024.102421.

Wen, Y., Jing, Y., Yang, L., Kang, D., Jiang, P., Li, N., Cheng, J., Li, J., Li, X., Peng, Z., Sun, X., Miller, H., Sui, Z., Gong, Q., Ren, B., Yin, W. and Liu, C. (2019) ‘The regulators of BCR signaling during B cell activation’, *Blood Science*, 1(2), p. 119. doi: 10.1097/bs9.0000000000000026.

White, J. T., Cross, E. W. and Kedl, R. M. (2017) ‘Antigen-inexperienced memory CD8+ T cells: where they come from and why we need them’, *Nature Reviews Immunology*, 17(6), p. 391. doi: 10.1038/nri.2017.34.

Wickham, H. (2016) *ggplot2: elegant graphics for data analysis*. Cham: Springer International Publishing. doi: 10.1007/978-3-319-24277-4.

Wieczorek, M., Abualrous, E. T., Sticht, J., Álvaro-Benito, M., Stolzenberg, S., Noé, F. and Freund, C. (2017) ‘Major histocompatibility complex (MHC) class I and MHC class II proteins: conformational plasticity in antigen presentation’, *Frontiers in Immunology*, 8, p. 292. doi: 10.3389/fimmu.2017.00292.

Workman, C. J., Szymczak-Workman, A. L., Collison, L. W., Pillai, M. R. and Vignali, D. A. A. (2009) ‘The development and function of regulatory T cells’, *Cellular and Molecular Life Sciences*, 66(16), p. 2603. doi: 10.1007/s00018-009-0026-2.

Wu, Y., Zhang, N., Hashimoto, K., Xia, C. and Dijkstra, J. M. (2021) ‘Structural comparison between MHC classes I and II; in evolution, a class-II-like molecule probably came first’, *Frontiers in Immunology*, 12, p. 621153. doi: 10.3389/fimmu.2021.621153.

Weinstein, J. S., Herman, E. I., Lainez, B., Licona-Limón, P., Esplugues, E., Flavell, R. and Craft, J. (2016) ‘TFH cells progressively differentiate to regulate the germinal center response’, *Nature Immunology*, 17(10), pp. 1197–1205. doi: 10.1038/ni.3554.

Xu, W. and Powell, J. D. (2018) ‘Peeking under the hood of naive T cells’, *Cell Metabolism*, 28(6), pp. 801–802. doi: 10.1016/j.cmet.2018.11.008.

Weber, J. P., Fuhrmann, F., Feist, R. K., Lahmann, A., Al Baz, M. S., Gentz, L. J., Vu Van, D., Mages, H. W., Haftmann, C., Riedel, R., Grün, J. R., Schuh, W., Kroczek, R. A., Radbruch, A., Mashreghi, M. F. and Hutloff, A. (2015) ‘ICOS maintains the T follicular helper cell phenotype by down-regulating Krüppel-like factor 2’, *The Journal of Experimental Medicine*, 212(2), pp. 217–233. doi: 10.1084/jem.20141432.

Weinreich, M. A., Odumade, O. A., Jameson, S. C. and Hogquist, K. A. (2010) ‘T cells expressing the transcription factor PLZF regulate the development of memory-like CD8+ T cells’, *Nature Immunology*, 11(8), pp. 709–716. doi: 10.1038/ni.1898.

White, J. T., Cross, E. W., Burchill, M. A., Danhorn, T., McCarter, M. D., Rosen, H. R., O’Connor, B. and Kedl, R. M. (2016) ‘Virtual memory T cells develop and mediate bystander protective immunity in an IL-15-dependent manner’, *Nature Communications*, 7, p. 11291. doi: 10.1038/ncomms11291.

Wing, J. B., Kitagawa, Y., Locci, M., Hume, H., Tay, C., Morita, T., Kidani, Y., Matsuda, K., Inoue, T., Kurosaki, T., Crotty, S., Coban, C., Ohkura, N. and Sakaguchi, S. (2017) ‘A distinct subpopulation of CD25- T-follicular regulatory cells localizes in the germinal centers’, *Proceedings of the National Academy of Sciences of the United States of America*, 114(31), pp. E6400–E6409. doi: 10.1073/pnas.1705551114.

Yamaguchi, T., Hirota, K., Nagahama, K., Ohkawa, K., Takahashi, T., Nomura, T. and Sakaguchi, S. (2007) 'Control of immune responses by antigen-specific regulatory T cells expressing the folate receptor', *Immunity*, 27(1), pp. 145–159. doi: 10.1016/j.immuni.2007.04.017.

Yokoyama, W. M. (2005) 'Natural killer cell immune responses', *Immunologic Research*, 32(1–3), pp. 317–325. doi: 10.1385/ir:32:1-3:317.

Younes, S. A., Punkosdy, G., Caucheteux, S., Chen, T., Grossman, Z. and Paul, W. E. (2011) 'Memory phenotype CD4 T cells undergoing rapid, nonburst-like, cytokine-driven proliferation can be distinguished from antigen-experienced memory cells', *PLoS Biology*, 9(10), p. e1001171. doi: 10.1371/journal.pbio.1001171.

Young, C. and Brink, R. (2021) 'The unique biology of germinal center B cells', *Immunity*, 54(8), pp. 1652–1664. doi: 10.1016/j.immuni.2021.07.015.

Yu, D., Rao, S., Tsai, L. M., Lee, S. K., He, Y., Sutcliffe, E. L., Srivastava, M., Linterman, M., Zheng, L., Simpson, N., Ellyard, J. I., Parish, I. A., Ma, C. S., Li, Q. J., Parish, C. R., Mackay, C. R. and Vinuesa, C. G. (2009) 'The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment', *Immunity*, 31(3), pp. 457–468. doi: 10.1016/j.immuni.2009.07.002.

Yu, F., Sharma, S., Edwards, J., Feigenbaum, L. and Zhu, J. (2015) 'Dynamic expression of T-bet and GATA3 by regulatory T cells maintains immune tolerance', *Nature Immunology*, 16(2), p. 197. doi: 10.1038/ni.3053.

Yu, G., Wang, L. G., Han, Y. and He, Q. Y. (2012) 'clusterProfiler: an R package for comparing biological themes among gene clusters', *OMICS: A Journal of Integrative Biology*, 16(5), p. 284. doi: 10.1089/omi.2011.0118.

Yager, E. J., Ahmed, M., Lanzer, K., Randall, T. D., Woodland, D. L. and Blackman, M. A. (2008) 'Age-associated decline in T cell repertoire diversity leads to holes in the repertoire and impaired immunity to influenza virus', *The Journal of Experimental Medicine*, 205(3), pp. 711–723. doi: 10.1084/jem.20071140.

Zhu, J. and Paul, W. E. (2009) 'Heterogeneity and plasticity of T helper cells', *Cell Research*, 20(1), pp. 4–12. doi: 10.1038/cr.2009.138.

Zhu, Y., Zou, L. and Liu, Y. C. (2016) 'T follicular helper cells, T follicular regulatory cells and autoimmunity', *International Immunology*, 28(4), p. 173. doi: 10.1093/intimm/dxv079.

Zhang, N. and Bevan, M. J. (2011) 'CD8+ T cells: foot soldiers of the immune system', *Immunity*, 35(2), p. 161. doi: 10.1016/j.immuni.2011.07.010.

Zhang, N., Zhang, Z. M. and Wang, X. F. (2021) 'The roles of basophils in mediating the immune responses', *European Journal of Inflammation*, 19. doi: 10.1177/20587392211047644.

Zhang, X., Ge, R., Chen, H., Ahiafor, M., Liu, B., Chen, J. and Fan, X. (2021) 'Follicular helper CD4+ T cells, follicular regulatory CD4+ T cells, and inducible costimulator and their roles in

multiple sclerosis and experimental autoimmune encephalomyelitis', *Mediators of Inflammation*, 2021, p. 2058964. doi: 10.1155/2021/2058964.

Zhang, X., Mozeleski, B., Lemoine, S., Deŕiaud, E., Lim, A., Zhivaki, D., Azria, E., Le Ray, C., Roguet, G., Launay, O., Vanet, A., Leclerc, C. and Lo-Man, R. (2014) 'CD4 T cells with effector memory phenotype and function develop in the sterile environment of the fetus', *Science Translational Medicine*, 6(238). doi: 10.1126/scitranslmed.3008748.

Zhu, B., Symonds, A. L. J., Martin, J. E., Kioussis, D., Wraith, D. C., Li, S. and Wang, P. (2008) 'Early growth response gene 2 (Egr-2) controls the self-tolerance of T cells and prevents the development of lupus-like autoimmune disease', *The Journal of Experimental Medicine*, 205(10), pp. 2295–2308. doi: 10.1084/jem.20080187.

Zhu, J., Jankovic, D., Oler, A. J., Wei, G., Sharma, S., Hu, G., Guo, L., Yagi, R., Yamane, H., Punkosdy, G., Feigenbaum, L., Zhao, K. and Paul, W. E. (2012) 'The transcription factor T-bet is induced by multiple pathways and prevents an endogenous T helper-2 program during T helper-1 responses', *Immunity*, 37(4), p. 660. doi: 10.1016/j.immuni.2012.09.007.

Zhu, Y., Zou, L. and Liu, Y. C. (2015) 'T follicular helper cells, T follicular regulatory cells and autoimmunity', *International Immunology*, 28(4), p. 173. doi: 10.1093/intimm/dxv079.

Zotos, D., Coquet, J. M., Zhang, Y., Light, A., D'Costa, K., Kallies, A., Corcoran, L. M., Godfrey, D. I., Toellner, K. M., Smyth, M. J., Nutt, S. L. and Tarlinton, D. M. (2010) 'IL-21 regulates germinal center B cell differentiation and proliferation through a B cell-intrinsic mechanism', *Journal of Experimental Medicine*, 207(2), pp. 365–378. doi: 10.1084/jem.20091777.

Supplementary information

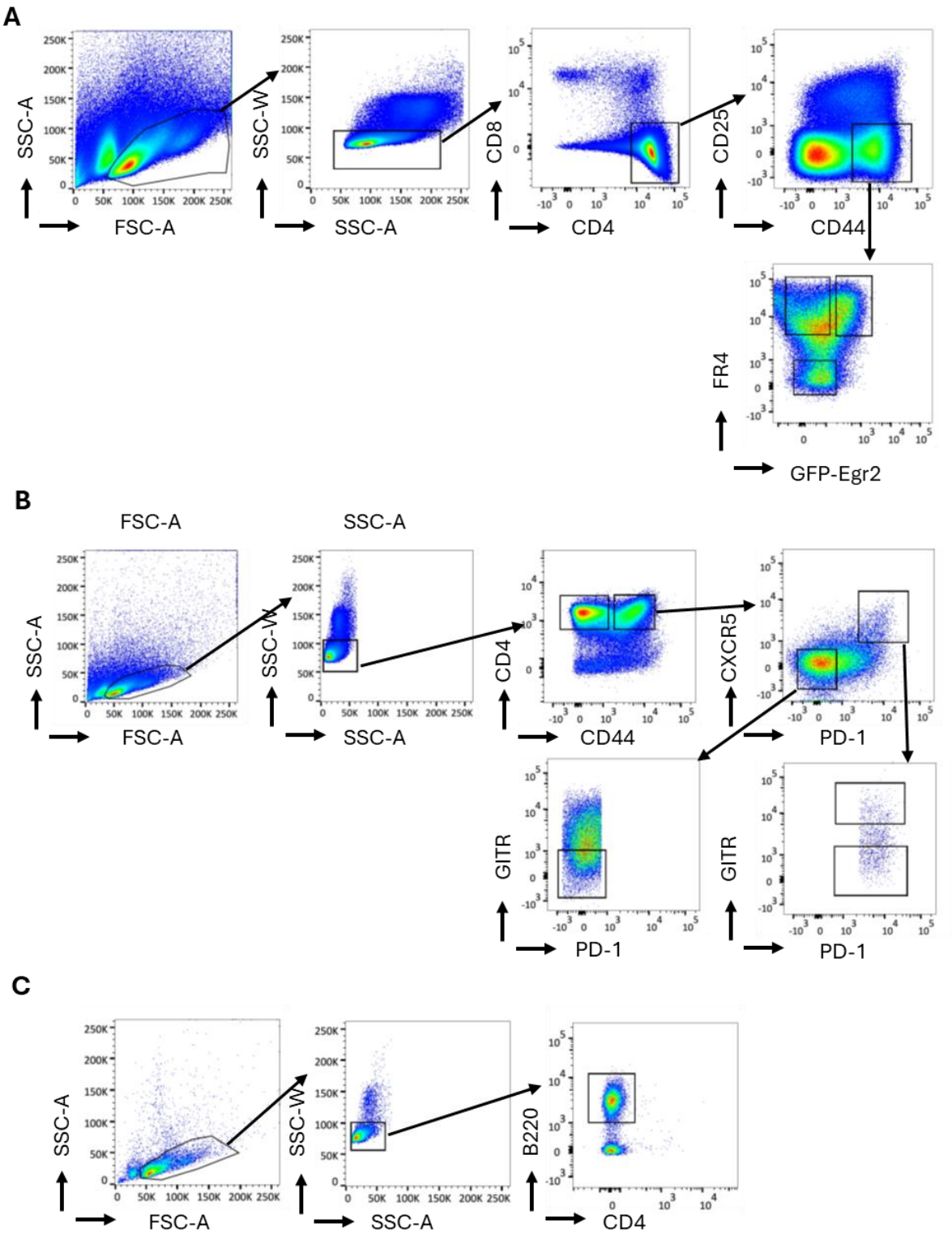


Figure S1. Cell sorting. Gating strategies for cell sorting. (A) FR4^{+}Egr2^{+}, FR4^{+}Egr2^{-} and FR4^{-}Egr2^{-} cells were isolated by FACS. (B) Tfh, Tfr, non-Tf and naïve T cell sorting strategy. CD4^{+} cells were isolated by MACS and then Tfh, Tfr, non-Tf and naïve T cells were isolated by FACS. (C) B cell sorting strategy. CD4^{-} cells were isolated by MACS and then B cells were isolated by FACS.

Publications

- Symonds, A. L. J., Miao, T., Busharat, Z., Li, S., & Wang, P. (2023). **Egr2 and 3 maintain anti-tumour responses of exhausted tumour infiltrating CD8+ T cells.** Cancer Immunology, Immunotherapy, 72(5), 1139–1151. https://doi.org/10.1007/S00262-022-03319-W/FIGURES/7
- Symonds ALJ, Busharat Z, Du M, Miao T, Li S, Hou X, Wang P. **Memory Phenotype Tfh Cells Develop Without Overt Infection and Support Germinal Center Formation and B Cell Responses to Viral Infection**. Eur J Immunol. 2025 Jan;55(1):e202451291. doi: 10.1002/eji.202451291. Epub 2024 Nov 20. PMID: 39568245; PMCID: PMC11739680.
- Alistair L. J. Symonds, Zabreen Busharat, Tizong Miao, Suling Li and Ping Wang. **Function of memory phenotype CD4 T cells developed without overt pathogen infection are balanced between innate and adaptive responses**. (manuscript for submission)
- Zabreen Busharat, Alistair L. J. Symonds, Tizong Miao, Suling Li and Ping Wang. **Characterisation of the GFP-Egr2/ AmCyan-T-bet mouse model.** (manuscript in preparation)