Transcriptional and migration regulation of T follicular helper cell differentiation

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A thesis submitted in total fulfilment of degree of Doctor of Philosophy

February 2021

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Abstract

T follicular helper cells (TFH) are specialised $CD4^+$ T cells that promote B cells maturation into antibody secreting plasma and memory cells. Most of the current vaccines generate protection via the induction of long-term antibody responses and circulating TFH are reliable predictors of vaccine response. Conversely, dysfunctional TFH cells are associated with the pathogenesis of immunodeficiency, systemic autoimmunity and allergy. Despite their importance, we have an incomplete understanding of how TFH cells differentiate and function in distinct inflammatory settings. Answering this question has broad health implications to stimulate rational development of vaccines and therapeutics against diverse infections, autoimmune and allergic disease.

In this thesis, I investigated the transcriptional and migration control of TFH differentiation during viral infection. In this setting, TFH cells differentiate in parallel with T helper 1 (TH1) $CD4^+$ T effector cells. I investigated two canonical TH1 factors, T-bet and CXCR3, to understand their roles in TFH cells differentiation. Comparing two viral infections, I demonstrated a context-dependent role for T-bet in TFH differentiation and identified the cytokine and chemokine factors that underlie distinct T cell differentiation. Combined, this work demonstrates that there are multiple paths that direct TFH differentiation. This study has led to further investigations into pathogen-specific TFH programs, which will help us understand how TFH orchestrate tailored B cell responses in diverse infections.

Declaration

This is to certify that:

I. This thesis comprises only my original work towards the degree of Doctor of Philosophy except where indicated in the Preface.

II. Due acknowledgement has been made in the text to all other materials used.

III. The thesis is fewer than 100,000 words in length, exclusive of tables, figures and bibliographies.

Amania Anwar Sheikh

Preface

My contribution to experiments in each of the Chapters was as follows:

Chapter 2: 85%

Chapter 3: 90%

Chapter 4: 90%

The text on the pages 11-14, 24-26 and 113, 114, 116 has been taken from published review (Sheikh and Groom [1], provided in Appendix 1) and my contribution to this publication is 80%.

I acknowledge the important contributions of others to the following experiments presented in this thesis:

Chapter 2: Co-authors on publication included as a chapter have their contributions duly acknowledged in the manuscript

Chapter 3: Light sheet imaging was performed by Brigette Duckworth

Chapter 4: Experiments involving Helminth and T.muris infections were set up with the assistance of Carolina Alvarado, Aidil Zakri and Tabinda Hussain

The publication status of all the chapters is as follows:

Chapter 2: Published by Cell Reports on August 2019

Chapter 3: Unpublished material not submitted for publication

Chapter 4: Unpublished material not submitted for publication

I acknowledge the following sources of funding:

The University of Melbourne

Walter and Eliza Hall Institute of Medical Research

Acknowledgements

First of all, I wish to express my sincere gratitude to my primary supervisor, **Joanna Groom**, for the opportunity to undertake my PhD with her. Thank you for providing me with guidance, unparalleled support and encouragement during my PhD. Thank you for being an amazing mentor, scientist and most importantly, a very loving person. I have learned so much from you and I will always be very grateful because you gave me the greatest gifts of all that is education. I would also like to thank my second supervisor, **Gabrielle Belz**, for her support during my PhD and especially for the feedback on my thesis. I am extremely grateful to my PhD advisory committee, Stephen Nutt, Marc Pellegrini and Katherine Kedzierska for their unwavering support, guidance, encouragement and insightful discussions.

I would like to extend my thanks to colleagues and friends from the Immunology Division, for making me feel like a part division and for being very welcoming, especially Philip Hodgkin, Vanessa Bryant, Susanne Heinzel. I would like to extend my thanks to other people in the division for their insightful discussion and share of protocols and reagents especially Julie Tellier, Yifan Zhan, Fernando S.F. Guimaraes, Miles Horton, Jessica Tempany and Angela D'Amico and many others.

I especially thank collaborators from Monash university Kim Jacobson, Lucy Cooper and Charles Mackay for experiments, reagents and mice. I am very grateful to division coordinators Kim Mcintosh, Jenny Anderson and WEHI Education office Sue Hardy and Keely Bumsted-Obrien for their assistance with my candidature and assistance concerning university requirements.

Special thanks to people from Bioservices, Shannon Oliver, Rhiannan Crawley, Hannah Johnson, Krystal Hughes, Jaclyn Gilbert and Lisa Reid. Grateful to people from Imaging facility Kelly Rogers, Verena Wimmer and Niall Geoghegan for their help. Thanks to people from FACS facility, Simon Monard, Melanie Le Page, Tim McCulloch, Stacey Woodrow and Chayanica Nasa.

I am super grateful to all the past and present members of the groom lab, who made my PhD journey truly wonderful and enjoyable. During my PhD, I cherish all lab coffees, Christmas parties and birthday celebrations with all you young scientists. In particular, I would like to thank Carolina Alvarado for helping me with experiments and for maintaining the lab in the best possible way. Thank you Brigette Duckworth for helping with light sheet imaging. I would

also thank other members of the lab Benjamin Broomfield, Lennard Dalit, Raymond Qin for their support during my PhD. I cannot forget to acknowledge Fanny Lafouresse, a past member of Groom lab for her support and mentoring during early days of my PhD.

Thanks to my awesome friend Suad Abdirahman, you are a gem of a person. I don't know what I would have done without you. I am very grateful that you made sure I was doing okay during the lockdown and thank you for bringing groceries. I would like to thank my other friends, in no particular order; Dawn Lin, Isabella Kong, Angela Huang, Shengbo Zhang and others, Thank you for all the fun during coffee breaks, lunch, drinks, student retreat and conferences.

Finally, special thanks my loving parents, mum, dad and especially my bother Basum Sheikh for his support during my PhD.

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List of Abbreviations

Abbreviation	Full name
AID	Activation-induced cytidine deaminase
AP-1	Activator protein 1
BATF	Basic leucine zipper transcription factor, ATF-like
Bcl6	B-cell lymphoma protein 6
BLIMP-1	B lymphocyte-induced maturation protein-1
c-MAF	c-maf musculoaponeurotic fibrosarcoma oncogene homolog
cTFH	circulating TFH
CCL19	C-C chemokine ligand 19
CCR7	C-C chemokine receptor type 7
CFU	Colony-forming units
CTLA-4	Cytotoxic T-lymphocyte antigen-4
CUT&Tag	Cleavage Under Targets and Tagmentation
CXCR3	Chemokine receptor CXCR3
CXCR5	C-X-C chemokine receptor 5
C. rodentium	*Citrobacter rodentium*
C. albicans	Candida albicans
DC	Dendritic cells
DZ	Dark zone
FOXO1	Forkhead box protein O1
FOXP3	Forkhead box protein P3 (FOXP3)
GATA3	GATA binding protein 3
GC	Germinal centre
GFI1	Growth Factor Independence 1
GM-CSF	granulocyte-macrophage CSF
H. polygyrus	*Heligmosomoides polygyrus*
ICOS	Inducible T cell costimulator
IFN-γ	Interferon-gamma
IL-10	IL-10 Interleukin 10
IRF4	Interferon regulatory factor 4
JAK	Janus kinases
KLF2	Kruppel-like factor 2
LSFM	Light sheet fluorescent microscopy

LCMV	Lymphocytic choriomeningitis virus
LZ	Light zone
NOTCH	Neurogenic locus notch homolog protein
NFAT-1	Nuclear factor of activated T cells-1
PD-1	Programmed cell death protein 1
PFU	Plaque-forming units
PSGL-1	P-selection glycoprotein ligand-1
RNAseq	RNA sequencing
RORγt	Retinoid-related orphan receptor gamma t
RUNX2	Runt-related transcription factor 2
SAP	SLAM-associated protein
SLAM	Signalling lymphocytic activation molecule
STAT3	Signal transducer of activation and transcription 3
T-bet	T-box transcription factor
T:B border	T cell:B cell border
TH cells	T helper cells
TFH	T follicular helper cells
TFR	T follicular regulatory
TGF-β	Transforming growth factor β
TNF-α	Tumour necrosis factor
T. muris	*Trichuris muris*

Chapter 1 Literature Review

1.1 T follicular helper cells

T follicular helper (TFH) cells are a distinct $CD4^+$ T cell subset with an essential function to promote B cell differentiation into high-affinity antibody-secreting plasma and memory cells [2, 3]. The antibodies produced by plasma cells has an indispensable role in the neutralisation and clearance of a range of pathogens including viruses, bacteria, fungi and parasites [2, 3]. Importantly, most current vaccines work by generating neutralising antibodies and memory B cells that are able to mount an immune response upon encountering pathogens [4, 5]. Given that TFH cells have a profound role in the formation of antibody-producing B cells, dysregulated TFH cells have been shown to cause the development of autoantibody-producing B cells that are associated with immunodeficiency, systemic autoimmunity, and allergy [2, 3]. Thus, advances in understanding the factors that determine TFH differentiation and function in the inflammatory setting has broad health implications in the development of vaccines and therapeutics against infections, autoimmune and allergic diseases [2, 3].

1.2 The importance of T follicular helper cells in the germinal centre reactions

TFH cells provide help signals to B cells, which assists in the positive selection of high affinity B cells for an antigen. Subsequently, these high affinity B cells proliferate and differentiate into plasma and memory B cells in response to diverse infections or immunisation [6, 7]. B cell progression through these different differentiation and maturation steps occur in the germinal centres (GCs) which are microstructures formed within the centre of the B cell follicles of the secondary lymphoid tissues such as lymph nodes and spleen, upon infection or immunisation [6, 7, 8].

1.2.1 Germinal centre reactions

B cells undergo multiple steps of development in distinct regions of the GC that is divided into the light zone (LZ) and the dark zone (DZ) [9, 10, 11]. The LZ is composed of TFH cells, GC B cells and follicular dendritic cells. In contrast, DZ mainly consist of highly proliferative B cells (**Figure 1.1**). GC formation begins after resting B cells take different amounts of antigen through their B cell receptor (BCR) [12]. These antigens are then processed and loaded onto MHC complexes and presented to GC TFH cells. Here BCR affinity for an antigen is directly proportional to peptide-MHC density on B cells [13, 14]. *In vitro* studies have shown that T cells

that interact with B cells, display higher surface peptide density [15]. Therefore, GC TFH cells form close interactions with LZ B cells that present higher peptide-MHC density [10, 16, 17]. These interactions are significant because it is through these interactions that GC TFH cells deliver signals to LZ B cells, thus helping to select LZ B cells with high BCR affinity [10]. The selected LZ B cells then migrate to the DZ to proliferate and undergo somatic hypermutation [10]. Somatic hypermutation is a process by which random point mutations are incorporated in the antigen-binding variable (V-) regions of the immunoglobulin gene which encodes BCR [9, 10, 18, 19]. This process results in DZ B cells with antibodies either with high affinity, lower affinity, or no change in affinity for an antigen [10]. The mutated DZ B cells then return to the LZ where B cells with high affinity for an antigen receive additional TFH help signals and are selected to migrate back to the DZ [10]. Again, in the DZ, B cells go through additional rounds of proliferation and somatic hypermutation before they re-enter the LZ for TFH selection [10]. The repetition of GC B cell migration, proliferation and somatic hypermutation in the DZ followed by TFH selection in the LZ results in the expansion of high affinity GC B cells in preference to lower affinity B cells [10, 20]. The lower affinity GC B cell clones present in low numbers are subsequently eliminated by apoptosis, resulting in GC rich in high affinity B cells [10]. The repetition of this process improves BCR affinity for an antigen and drives continuous GC reactions that facilitate the generation of affinity matured, long-lived antibody-secreting plasma and memory B cells [9].

1.2.2 T follicular helper cell help within germinal centres

Within GC LZ, TFH cells provide help to B cells by two known mechanisms, co-stimulatory signals and cytokine signals. Each of these ‘help’ signals has a unique role in driving germinal centre reactions.

1.2.3 T follicular helper cell co-stimulatory signals

TFH and B cells form short, non-stable entangled contacts and here, B cells receive co-stimulatory help signals through CD40L and inducible T cell co-stimulator (ICOS) [6, 7, 21]. The more the surface area of contact between TFH-B cells, the more help is received by B cells. The extent of surface entanglement is augmented by binding of ICOS ligand (ICOSL) expressed by B cells with ICOS on TFH cells. Additionally, ligation of B cell-ICOSL with TFH-ICOS promotes the upregulation of CD40L molecules on TFH cells [21]. A critical component of this T cell help is CD40L, a tumour necrosis factor (TNF) family member that engages with B cell-CD40. CD40 signalling acts as a positive feedback mechanism to promote ICOSL expression by GC B cells [21]. Together, CD40 and ICOS co-stimulation increase the T

cell-B cell entanglement, which leads to more help signals from T cells, facilitating the selection of high-affinity GC B cells [22].

1.2.4 T follicular helper cell-derived soluble signals

The second central mechanism of TFH cell help within GCs is through the production of TFH-derived cytokines. Interleukin (IL)-21 is a canonical TFH cytokine and is produced by TFH cells in most of the pathogen settings [23, 24, 25, 26, 27, 28], while TFH cells secrete other cytokines such as IL-4, IL-17, and interferon-gamma (IFN-γ) under certain circumstances [29]. Each of these cytokines has a unique function in driving GC reactions in infectious diseases and autoimmunity.

1.2.5 Interleukin-21

TFH cells secrete high levels of IL-21 which has a central role in the generation of class-switched B cells, plasma cells and in the formation of GCs [30, 31]. Murine $CD4^+$ T cells cultured with IL-6, IL-21, IL-23 and IL-27 have been shown to activate the signal transducer and activator of transcription 3 (STAT3), to regulate IL-21 production [32]. Although IL-21 signalling activates several Janus kinases (JAK1 and JAK3) and STAT transcription factors (STAT1, STAT3, and STAT5), only downstream signalling through STAT3 is required to regulate multiple roles during humoral immunity [32]. IL-21 exerts a number of key actions in driving antibody production. Firstly, IL-21 promotes immunoglobulin production as naive human B cells cultured with CD40 and IL-21 induced class switching to immunoglobulin G1 (IgG1) and IgG3 [33]. This finding is consistent with a reduction in IgG1 levels in mice deficient in IL-21 receptor [34]. In addition, IL-21, in synergy with transforming growth factor (TGF)-β promotes IgA production [35]. Secondly, IL-21 signalling through STAT3 activates B lymphocyte-induced maturation protein-1 (BLIMP-1), which lead to the differentiation of memory B cells to plasma cells [36]. Thirdly, IL-21 upregulates B-cell lymphoma protein 6 (Bcl6) expression in GC B cells, facilitating in the proliferation of GC B cells and GC formation and maintenance [37]. As discussed above, IL-21 is critical for B cell differentiation, indicating a potential role of IL-21 in promoting B cell-mediated autoimmune diseases. This is supported by studies that find association between mutations within the IL-21 gene and with a number of diseases including systemic lupus erythematosus (SLE), type 1 diabetes, inflammatory bowel disease and psoriasis [37]. Thus, targeting IL-21 may have a role in inhibiting the progression of autoimmune diseases and inflammatory conditions.

1.2.6 Interleukin-4

Cellular sources of IL-4 includes NKT cells [38], basophils [39], eosinophils [40], mast cells [41], TH2 [42, 43] and TFH cells [44]. TFH-derived IL-4 has been shown to a play a multifaceted role in B cells including B cell class switching, proliferation and localisation of B cells in the GCs. Mice that lacked either IL-4 or its high-affinity receptor, IL-4R, failed to generate immunoglobulin IgG1 and IgE in parasite infections [44]. Combined IL-4 and CD40-CD40L signals upregulate the expression of activation-induced cytidine deaminase (AID), in B cells, which is critical for class switch recombination and affinity maturation in B cells [44, 45]. In parasite infections, IL-4 downregulates Epstein-Barr virus-induced G protein-coupled receptor 2 (EBI2) expression, which leads to the retention of B cells in the GCs [46]. Another study found association between IL-4 and the expansion and maturation of GC B cells [47], however it is not yet clear whether IL-4 directly drives GC B cell expansion or if B cell expansion is due to IL-4 mediated retention in the GCs. In addition, IL-4 and IL-21 together direct humoral response; with IL-21 deletion, there was an increase in IL-4-mediated IgE production, while deficiency in both IL-4 and IL-21 led to a complete loss of GC formation that exceeded the defect observed in the loss in either of the cytokines [48, 49].

1.2.7 Interferon-gamma

IFN-γ is predominantly produced by T helper (TH) 1 cells but can be secreted by TFH cells during intracellular pathogen challenge, such as viruses [23, 24]. The induction of IFN-γ in TFH and TH1 cells is upregulated by transcription factors T-box transcription factor (T-bet) and STAT4, acting downstream of IL-12-enriched viral settings [24]. T cell-derived IFN-γ regulates T-bet expression in B cells, which controls IFN-γ-mediated isotype-switching to IgG2a [50, 51, 52]. Similar to murine B cells, IFN-γ promotes switching to IgG2 in humans B cells, whilst reducing IgG1 with no change in IgG3 or IgG4. This shows that IFN-γ in both humans and mice has a similar effect on B cell class switching [53]. A recent study demonstrated that B cell intrinsic T-bet expression was indispensable for the generation of long-lived plasma cells and secondary antibody secreting cells following influenza infection [50]. In addition, IFN-γ is required for the development of IgM^+ memory B cells in bacterial *Salmonella* Typhi infection [53]. Although IFN-γ is essential for the generation of humoral immune response and long-lasting memory, defects in IFN-γ receptor signalling have been implicated in spontaneous autoimmune GC formation and autoantibody production [54].

1.2.8 Interleukin-17

IL-17 is a proinflammatory cytokine associated with inflammation, autoimmune diseases and defence against extracellular bacteria. Although IL-17 is a signature cytokine of T helper 17 cells, it is also expressed by other immune cells, including $CD8^+$ T cells, γδ T cells, natural killer T (NKT) cells, and innate lymphoid cells [55]. In TFH cells, IL-17 encoding genes *Il17a* and *Il17f* are directly suppressed by Bcl6 [56], and these studies could not detect IL-17 production either by human tonsillar TFH or murine TFH cells following immunisation [57, 58]. Of note, TFH cells have been shown to produce excessive IL-17 in an experimental autoimmune encephalomyelitis model [59]. IL-17 is associated with the development of autoantibodies and spontaneous GCs in autoimmune BXD2 mice [60]. BXD2 mice are BXD recombinant inbred (RI) strain that have high titres of autoantibodies against lupus and arthritis autoantigens [61]. In this setting, IL-17 was shown to arrest GC B cell migration between GC LZ and DZ, disrupting affinity maturation of the B cell and selection of B cells within the GCs, resulting in autoreactive B cells [60].

Together, these findings suggest that TFH-derived cytokines direct the generation of humoral immune response against distinct infections, while variation or exacerbation of these signals initiate the development of autoreactive GCs that result in autoimmune diseases and allergies.

1.2.9 T follicular regulatory cell-derived inhibitory signals in germinal centres

GC reactions are the primary site for the development of long-lived antibody-secreting plasma and memory B cells against pathogens [29]. However, autoreactive B cells generated in dysregulated GC reactions lead to autoimmune diseases, chronic inflammation and allergic responses. This highlights that GC reactions need to be tightly regulated [29]. Within GCs, there is a unique type of T regulatory cells (TREGs) known as T follicular regulatory (TFR) cells [62]. TFR cells have been shown to suppress TFH and B cells at multiple levels including (a) restricting IL-21 and IL-4 production by TFH cells, (b) inhibiting antibody production by B cells, and (c) limiting GC size and formation.

One key molecule involved in the suppressive function of TFR is the co-inhibitory molecule cytotoxic T-lymphocyte antigen-4 (CTLA-4). CTLA4 is constitutively expressed by TREGs, and TREGs mediate an immune suppressive function through CTLA-4 [62]. Similar to TREGs, TFR cells express high levels of CTLA-4, and they also use CTLA-4 to control the GC response [62]. The inhibitory function of CTLA-4 was confirmed by studies whereby specific deletion of CTLA-4 in all TREGs, including TFR cells, increased the number of TFH, GC B cells, and IL-

4 production by TFH cells [63, 64]. Briefly, CTLA-4 functions by downregulating CD28 ligands (CD80 and CD86) on GC B cells, which then limit CD28 signalling received by TFH cells [63]. Also, CTLA-4 has been shown to bind to CD80 and CD86 with higher affinity versus CD28, thus, establishing contact between TFR and B cells and limiting CD28 co-stimulation between B and TFH cells, which is required for TFH differentiation [65]. Although CTLA-4 is the central mediator of TFR cell function, another essential TFR-derived inhibitory molecule is TGF-β, which prevents TFH cell accumulation, activation of self-reactive B cells and autoantibody production [62, 66]. In contrast, IL-10 produced by TFR cells was shown to facilitate GC B cell proliferation and GC reactions in acute LCMV infection [67]. Due to the contrasting roles of CTLA-4 and IL-10 in either suppressing or promoting GC B cells, the inhibitory function of TFR might be context dependent. Targeting the mechanism underlying TFR in immunosuppressive function could be a therapeutic strategy for rheumatoid arthritis and other autoimmune diseases.

1.3 Spatial-temporal stages of TFH cell differentiation

Following infection, the fate choice made by $CD4^+$ T cells to differentiate into either TFH or other TH cells is governed by cytokines, interactions with dendritic cells and migration within distinct regions in the secondary lymphoid tissues. There are three spatial-temporal stages of TFH cell differentiation; T cell zone, T cell: B cell border, and GCs (**Figure 1.1**) [6, 7].

1.3.1 T cell zone

Naive $CD4^+$ T cells express C-C chemokine receptor type 7 (CCR7) that engages with C-C chemokine ligand (CCL)19 and CCL21 displayed on high endothelial venules (in the lymph nodes) and reticular cells (in the spleens and lymph nodes) [29]. This allows naive $CD4^+$ T cells to enter the secondary lymphoid tissues from the circulation [29]. CCR7 then directs cells to the T cell zone where CCR7 ligands, CCL19 and CCL21 are expressed by fibroblastic reticular cells [68]. Here, $CD4^+$ T cells differentiate into either TFH cells or other TH cell subsets.

1.3.2 Cytokines

The T cell zone is rich in cytokines that can either promote or inhibit TFH cell program. One of the cytokines that promotes the TFH program is IL-6 [69]. Several immune cells produce IL-6, including DCs, follicular B cells, and non-hematopoietic cells [70]. IL-6 signalling through transcription factor STAT3 rapidly induces TFH lineage defining transcription factor Bcl6 expression in activated T cells [69]. In contrast to IL-6, IL-2 is known to inhibit TFH development by suppressing Bcl6 expression and functions by two mechanisms [1, 71, 72]. Firstly, IL-2-

activated STAT5 competes with STAT3 to bind to *Bcl6* locus, inhibiting Bcl6 expression [1, 71]. Secondly, IL-2 promotes expression of Bcl6 antagonist BLIMP-1, which inhibits Bcl6 transcription and function. Thus, the IL-2-rich cytokine *milieu*-induces BLIMP-1 and STAT5 together to inhibit $CD4^+$ T cell differentiation to TFH cells. Interestingly, TFH cells are one of the cellular sources of IL-2 [71]. A seminal study by DiTora *et al*. directly correlated $CD4^+$ T cells receiving high TCR signal strength with IL-2 production and TFH fate commitment rather than non-TFH cells [73]. Type I interferons (IFNs), (IFN-α and IFN-β) inhibits Bcl6 expression through STAT5 signalling pathway in $CD4^+$ T cells that leads to TH1 cell differentiation while inhibiting TFH [74]. Thus, the level of type I IFNs, IL-6, and IL-2 are essential as they can tip the balance between differentiation of TFH cells and other TH cells (as reviewed in Sheikh and Groom [1], this citation has been included in Appendix 1).

1.3.3 Dendritic cells

DCs are the critical source of antigen, cytokines, and co-stimulatory signals, which are important for initiating TFH program [75, 76, 77, 78, 79]. Two crucial co-stimulatory molecules expressed by DCs include CD80/CD86 and OX40 ligand (OX40L). CD80/CD86 co-stimulatory molecule binds to the CD28 receptor expressed on T cells, which promotes Bcl6, CD40L and CXCR5 expression in T cells [80]. Another DC-derived signal is initiated through OX40L, which ligates to OX40 in T cells, and upregulates expression of TFH molecules such as CXCR5, Bcl6, PD-1, and IL-21 [81]. Programmed cell death protein 1 (PD-1) is a key TFH co-stimulatory molecule required for retaining TFH cells within the GCs [82].

The type of DC subset involved in TFH ontogeny in vivo is dependent on the site of the immunisation and the nature of the antigen. For example, skin-resident type 1 conventional dendritic cells (cDC1-$CD103^+$) expressing C-type lectin Langerin induce TFH cells in the skin draining lymph nodes [83]. In contrast, during intranasal immunisation, migratory type 2 dendritic cells (cDC2) are positioned at the T:B border and are able to initiate TFH priming [79]. Additionally, splenic cDC2s ($CD8\alpha^-$) are more specialised in orchestrating TFH fate as compared with cDC1 ($CD8\alpha^+$) subset following human pathogen challenge [78]. In human tonsils, cDC2 subsets are concentrated in the T cell zone and trigger TFH polarisation [84]. Collectively, this suggests that striking the right combination of cytokines and signals from DCs is a prerequisite to initiate TFH cell differentiation.

1.3.4 T cell: B cell border

Complete polarisation and the maintenance of TFH cell phenotype requires cognate T:B cell interactions at T cell zone-B cell follicle (T:B) border [85]. The T cell zone is rich in CCL19 and CCL21 chemokines that ligate with CCR7 and P-selection glycoprotein ligand-1 (PSGL-1). In T cells, the collective signals from DCs and cytokines in the T cell zone downregulate CCR7 and PSGL-1 in pre-TFH cells (early TFH cells that express initial TFH markers) [29]. As a result, pre-TFH cells anchored in the T cell zone due to the chemotactic influence of CCL19 and CCL21 are released from the T cell zone [29]. The subsequent upregulation of EBI2 helps pre-TFH cells to relocate to the T:B border in response to abundant oxysterol (a receptor for EBI2) expression at the border or outer B cell follicles [29]. Collectively, increased expression of CXCR5 and EBI2 with reciprocal downregulation of CCR7 and PSGL-1 facilitate the relocation of pre-TFH cells to the T:B border.

At the T:B border, antigen-specific B cells establish contact and exchange signals with pre-TFH cells [86, 87]. This contact is stabilised by homotypic engagement between signalling lymphocytic activation molecule (SLAM) family members CD84 and Ly108, expressed by both T and B cells [86, 87]. The adaptor protein SLAM-associated protein (SAP) is recruited to the cytoplasmic tail of these SLAM family receptors [86, 87], initiating an intracellular signalling cascade. SAP-deficiency in TFH cells has been shown to impair extensive T:B interactions which leads to a defect in the development of TFH differentiation, TFH recruitment to GCs [86, 87]. In addition to SAP signals in pre-TFH cells has been shown to provide B cells with CD40L, thus facilitating B cell proliferation, differentiation, and isotype switching [88].

Contact between T and B cells is also established by the ICOSL that binds with ICOS displayed by cognate B and T cells, respectively [89]. ICOS signalling in TFH cells activates the phosphoinositide 3-kinase-Akt pathway [90] and in turn, Akt phosphorylates Forkhead box protein O1 (FOXO1). Phosphorylated FOXO1 stays in the cytoplasm of pre-TFH cells in a functionally inactive state and is unable to promote Krupple-like factor 2 (KLF2) expression [80]. The absence of KLF2 prevents suppression of CXCR5 and activation of CCR7 and PSGL-1 in pre-TFH cells [80]. This suggests that ICOS indirectly suppresses KLF2 *via* FOXO1, which helps to upregulate CXCR5 expression in pre-TFH cells [80]. High levels of CXCR5 expression at the T:B border assist in the migration of pre-TFH cell to the B cell follicles, the region abundant in CXCL13, which is a chemoattractant for CXCR5 [91]. Collectively, cognate T:B cell interaction is essential for the differentiation and further positioning of T and B cells [92, 93].

1.3.5 T follicular helper cells in germinal centres

Following T and B cell interactions at the T:B border, TFH cells migrate into the B cell follicles. Here, TFH cells can either localise in the follicular mantle or in the GCs [91]. The TFH cells that localise in the GCs have shown to express high levels of CXCR5 and PD-1 in comparison to their follicular mantle counterparts [94], facilitating confinement of TFH cells within the GCs versus the follicles [82]. Additionally, GC TFH cells have shown to upregulate the expression of negative regulator of chemotaxis Sphingosine-1-phosphate receptor 2 (S1PR2) [95, 96]. S1PR2 ligates to sphingosine-1-phosphate (SIP)-abundantly present in the subcapsular sinus, which prevents the entry of $S1PR2^{+}$ TFH cells in the subcapsular sinus and maintains TFH cell populations within the GCs [95, 96]. While the expression of CXCR5, PD-1 and S1PR2 is upregulated, the expression of EBI2 is downregulated by TFH cells for localisation in GCs. It has been shown that EBI2 and CXCR5 form heterodimer complexes that impairs the binding of CXCR5 to CXCL13 (abundant in GCs), thus preventing TFH localisation in the GCs. Therefore, EBI2 expression must be reduced at this stage [97]. The localisation of TFH cells within the GCs allows TFH cells to help B cell to differentiation into long-lived plasma and memory B cells.

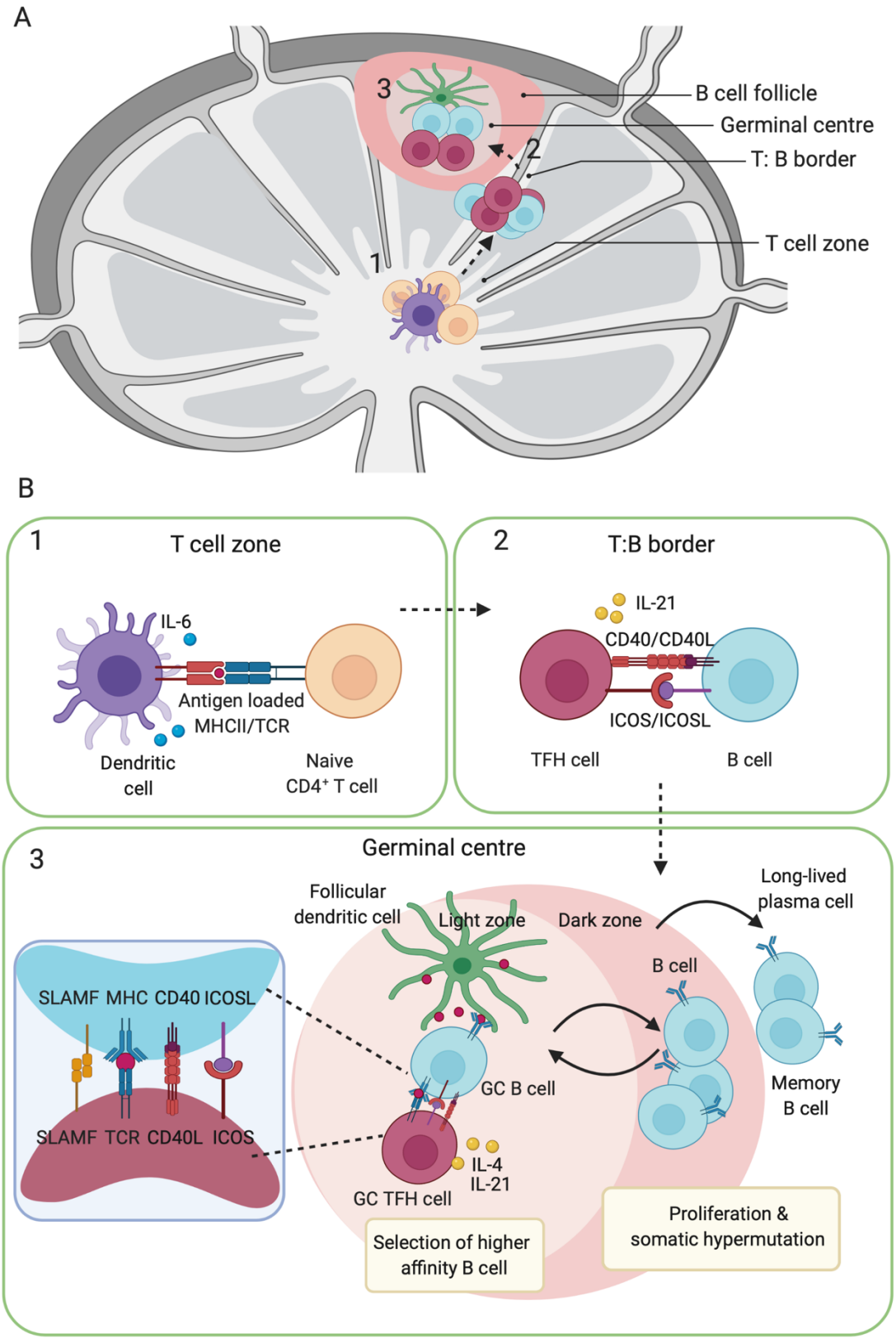

A
3
2
1
B cell follicle
Germinal centre
T: B border
T cell zone
B
1
T cell zone
IL-6
Antigen loaded
MHCII/TCR
Dendritic
cell
Naive
CD4⁺ T cell
2
T:B border
IL-21
CD40/CD40L
ICOS/ICOSL
TFH cell
B cell
3
Germinal centre
Follicular
dendritic cell
Light zone
Dark zone
Long-lived
plasma cell
B cell
SLAMF MHC CD40 ICOSL
SLAMF TCR CD40L ICOS
GC B cell
Memory
B cell
IL-4
IL-21
GC TFH cell
Selection of higher
affinity B cell
Proliferation &
somatic hypermutation

Figure 1.1 Schematic of TFH cell differentiation in the lymph node
(A) TFH differentiation occurs in three steps namely (1) the T cell zone, (2) T:B border and (3) the GCs. (B1) In T cell zone, DCs present antigens and costimulatory molecules to naive CD4$^+$ T cells. These signals together with IL-6 signals drive the TFH program. Following these interactions pre-TFH cells downregulate CCR7, PSGL-1 and upregulate Bcl6, CXCR5 and EBI2 expression. This assist pre-TFH cells to migrate to T:B border (B2) At the T:B border, pre-TFH and B cell establish short contacts and exchange signals through ICOS-ICOSL, CD40L-CD40, TCR-MHC-II and SLAMF-SLAMF. Here, pre-TFH cells downregulate EBI2 and further upregulate CXCR5 expression, promoting migration to the GCs. (B3) GCs contain LZ and DZ. The TFH help in the LZ regulates B cell proliferation and differentiation into long-lived plasma and memory B cells.

1.4 The core transcription factor of TFH differentiation is Bcl6

Bcl6 is a proto-oncogene zinc-finger transcriptional repressor that controls multiple features necessary for TFH differentiation and function [56]. The role of Bcl6 in TFH cell differentiation has been elucidated in studies that showed that CD4$^+$ T cells deficient in Bcl6 failed to develop into TFH cells and were insufficient to support germinal centre reactions *in vivo* [98, 99, 100]. Since the discovery of Bcl6 as the lineage-defining transcription factor, the TFH transcriptional differentiation axis has been focused on the antagonistic relationship between Bcl6 and BLIMP-1 (a transcription factor encoded by *Prdm1*) [98, 99, 100, 101]. Bcl6 and BLIMP-1 antagonise and inversely regulate the expression of each other in TFH cells. In CD4$^+$ T cells, BLIMP-1 is downregulated in TFH, but it is maintained at high levels in non-TFH cells [99]. The overexpression of BLIMP-1 prevents CD4$^+$ T cells to acquires a TFH phenotype by inhibiting expression of canonical markers, including CXCR5, ICOS, and PD-1 [56, 98, 99, 100]. While it was previously proposed that TFH formation was a default differentiation state for CD4$^+$ T cells, this has recently been shown not to be the case, as cells deficient in both Bcl6 and BLIMP-1 fail to form TFH cells *in vivo* following immunisation and viral infection [56]. This study confirmed previous work highlighting that Bcl6 acts as a hub for the transcriptional repression of multiple pathways that inhibit TFH differentiation [56]. Importantly, this and other studies have recently demonstrated that Bcl6 repression of these transcriptional networks occur independent of Blimp-1, further highlighting the indispensable role for Bcl6 in TFH fate commitment [56, 102] (as reviewed in Sheikh and Groom [1], Appendix 1).

Several unique structural features of the Bcl6 protein allow it to interact with diverse transcription factors and chromatin modifiers to form transcriptional complexes. These interactions permit Bcl6 to control gene expression in CD4$^+$ T cell precursors and in mature TFH. The Bcl6 protein consists of an N-terminus POZ (or BTB, broad complex, tramtrack,

bic-a-brac) domain, the middle domain, also known as RDII, and the C-terminal zinc fingers domain. The BTB domain mediates interactions with Bcl6 corepressors, including N-COR, SMRT, and BCOR [103, 104, 105]. These cofactors compete to bind to Bcl6 N-terminus and can recruit the histone deacetylase protein complexes (HDACs) to form a transcriptional repressing complex at the target gene. HDACs are enzymes that alter chromatin structure, and this, in turn prevents the ability of transcription factors to bind to the regulatory regions and activate transcription of target genes [106, 107, 108]. Mutations in the BTB domain inhibit the differentiation of TFH [109]. Further, BCOR-deficient $CD4^+$ T cells fail to differentiate into TFH cells [110]. Combined, this suggests that mutations in the BTB domain prevent BCOR binding. While N-COR and SMRT are also expressed in $CD4^+$ T cells, further research is needed to define their precise role in TFH differentiation. Bcl6 utilises its largest domain, the RDII domain, to associate with the corepressor MTA3. This interaction leads to the repression of BLIMP-1 [111]. The C-terminus of Bcl6 protein harbours six Kruppel-like C2H2-type ZF that can bind to a nine base pair DNA sequence TTCCT(A/C)GAA that shares sequence-binding homology with STAT and AP-1 (Activator Protein 1) DNA binding sites (as reviewed in Sheikh and Groom [1], Appendix 1).

In 2015, a landmark study led by Hatzi *et al.* mapped the cis-acting targets of BCL6 in human GC TFH cells [112]. This research outlined that BCL6 is directly or indirectly recruited to loci of multiple vital genes actively involved in TFH fate [112]. Firstly, BCL6 is indirectly recruited to non-BCL6 DNA binding sites by other transcription factors in TFH [112]. For example, BCL6 was found enriched at AP-1 DNA binding motifs instead of BCL6 DNA binding sites in TFH [112]. This was mediated by the physical association of BCL6 and AP-1 in $CD4^+$ T cells, which enabled BCL6 to be recruited to cis-regulatory regions of many genes in an AP-1-dependent manner [112]. AP-1 is a collective term used for transcription factors, which consist of JUN, FOS, or ATF (Activating Transcription Factor) subunits that form dimers. These transcriptional activators play a vital role in T effector cell differentiation, cell proliferation, and function [113, 114, 115]. It is suggested that recruitment of BCL6 to AP-1 DNA binding motif diverts AP-1-dependent gene activation to repression. Of note, BCL6 and AP-1 colocalise at the *Prdm1* locus, which contains an AP-1 DNA binding motif [112]. It could be that BCL6 uses AP-1 to establish a TFH transcription program through suppression of BLIMP-1.

The second mechanism of Bcl6 action is *via* direct binding to the enhancer and promoter regions of the genes essential in T cell migration. The relocation of TFH precursor cells to the B cell follicle is prerequisite for an effective GC response [22]. Bcl6 regulates multiple T cell

migration factors to establish TFH cell homing to the B cell follicles and to prevent TFH cell egress from secondary lymphoid tissues. Specifically, Bcl6 binds to the promoter and enhancer of *Ccr7* (encodes CCR7) and *Selplg* (encodes PSGL-1 proteins), which are known to regulate the migration of T cells to the T zone of secondary lymphoid tissues [112]. *Selplg* has been shown to be directly repressed by Bcl6 following LCMV infection [56]. Bcl6 binds to the gene, encoding EBI2 and which may lead to repressing its expression [112]. In both B and TFH cells, EBI2 has been shown to play a role in the localisation of cells to the outer B cell follicular regions of the secondary lymphoid tissues [116, 117]. Moreover, Bcl6 promotes the expression of key TFH markers IL-21R and CXCR5 in $CD4^+$ T cell culture, and mutations in Bcl6 zinc finger DNA binding domain restrict Bcl6 mediated upregulation of Bcl6, IL-21R, and CXCR5 in $CD4^+$ T cells [100]. Recently, the Bcl6 repression of GATA binding protein 3 (GATA3), Runt-related transcription factor 2 and Kruppel-like factor (KLF2) was confirmed to increase CXCR5 which promote $CD4^+$ cells migration to B cell follicles *in vivo* [56]. Combined, these actions of Bcl6 on the regulation of T cell migration facilitate the positioning of cells towards the follicle into environmental niches that further promote TFH differentiation (as reviewed in Sheikh and Groom [1], Appendix 1).

One of the most critical roles of Bcl6 in imprinting TFH fate is to block differentiation of alternate TH cells [56, 112]. For example, in human TFH, BCL6 binds to the promoter regions of the genes essential for alternate T helper fates, including *GATA3*, *RORA,* Interferon gamma receptor 1 and the enhancer regions of the *TBX21* gene (that encodes T-BET) [112]. In addition, Bcl6 binding sites in TFH cells are depleted of enhancer histone marks H3K4me1 and H3K27ac in comparison to naive $CD4^+$ T cells, suggesting these regulatory regions are in an inactive state. It is likely that Bcl6, along with its corepressors N-COR, SMRT, and BCOR, recruit HDACs to these sites to dynamically modify histone marks [118]. Further, Bcl6-deficient cells cultured in TH1 conditions demonstrated increased expression of T-bet and RORγt [100], suggesting that this mechanism may be at play even in non-TFH. Collectively, Bcl6 controls TFH fate commitment by direct repression of alternative fates *via* regulating the coercion of cofactors and epigenetic factors and inhibiting alternate TH cell positioning and cytokine signalling. Together, these studies have shown that Bcl6 leaves no stone unturned in establishing the TFH fate (as reviewed in Sheikh and Groom [1], Appendix 1).

1.5 The diversity in CD4$^+$ T helper cells

CD4$^+$ T cells form a bridge between the cell-mediated and humoral arms of the adaptive immune response. This feature is crucial in overcoming a broad range of pathogens that can be intracellular such as viruses and bacteria or extracellular helminth parasites, bacteria, and fungi. Following infection, naive CD4$^+$ T cells can differentiate into distinct TH subsets, including TFH, TH1, T helper 2 (TH2), T helper 17 (TH17) and TREGs [82]. Newly activated CD4$^+$ T cells sense changes in the microenvironment and integrate those signals through the upregulation of lineage-defining transcription factors. The balance of these transcription factors then directs responding CD4$^+$ T cells down a particular development path. CD4$^+$ T cell flexibility to diverge into distinct subsets, guided by pathogen-specific inflammatory cues in secondary lymphoid tissues, enables tailored immune responses against diverse immune challenges (as reviewed in Sheikh and Groom [1], Appendix 1).

Among CD4$^+$ T cell subsets, TH1, TH2 and TH17 cells orchestrate cell-mediated immune responses against intracellular pathogens such as viruses, parasite, and extracellular bacteria respectively [119, 120, 121]. These subsets exit secondary lymphoid organs to infiltrate peripheral tissues and facilitate cell-mediated responses to localised inflammation or infection [119, 120, 121]. Here, owing to their ability to express lineage defining transcription factors, they secrete cytokines that activate other immune cells, promoting the pathogen's clearance. For example, transcription factor T-bet regulates TH1 cell differentiation and induces IFN-γ production to clear viruses. TH2 cells express lineage defining transcription factor GATA3 that induces IL-4, IL-5, and IL-13 production to limit extracellular parasites. TH17 are defined by transcription factor RAR-related orphan receptor gamma (RORγT) which is required to regulate IL-17A and IL-17F expression to eliminate bacterial or fungal infections [121]. The immune response generated by TH1, TH2, TH17 can all be counter regulated by TREGs [122, 123].

TH1, TH2 and TH17 are focused towards a specific type of infection. In comparison, TFH cells co-exist with other TH subsets and share features of other CD4$^+$ T cells in their respective skewed settings [24, 124, 125]. This is exemplified by TFH cells that express a prototypical TH1 transcription factor T-bet and secrete IFN-γ in viral infections, whereas in parasite infections TFH cells produce TH2-associated IL-4 cytokine [47]. These cytokines produced by TFH cells assist in tailoring GC output, which includes B cell class switching, antibody production and GC formation according to the type of infection [23, 24, 25, 26, 27, 28, 29]. This suggests that the pathogen-influenced factors upregulate similar gene signatures in TFH cells and counterpart

$CD4^+$ T cell subsets. The interplay between pathogen-influenced factors and transcription factor is unique for each type of infection. Potentially this allows humoral and cell mediated immune response to work together against diverse infections and this is one of the central hypotheses of this thesis.

1.5.1 T regulatory cells

As mentioned above, TREG cells are a unique $CD4^+$ T cell subset that have a critical role in limiting an immune response by downregulating the activity of other $CD4^+$ TH cells that trigger an immune response [126]. TREG cells are broadly classified into two major subsets, the naturally occurring and adaptive TREG cells on the basis of their site of development. Together they form the peripheral TREG cells pool [127, 128]. Naturally occurring TREG cells are derived from the thymus [129], whereas adaptive TREG cells differentiate from the naive $CD4^+$ T cells following antigen presentation and co-stimulatory signals [128]. These cells are identified by the expression of lineage defining transcription factor Forkhead box P3 (FOXP3) [130]. FOXP3 is critical for the differentiation and function of TREG cells. As mice with mutations in FOXP3 gene showed uncontrolled immune response and elevated levels of inflammation [130]. The peripheral TREG cells can be further divided into central, effector and tissue resident TREG cell populations [127]. Central TREG cells are circulatory in nature like conventional naive $CD4^+$ T cells, whereas effector TREG cells have enhanced functional properties and finally tissue resident TREG cells localise in non-lymphoid organs [127]. In recent years, there has been a growing interest in effector TREG cells that integrate functional properties in response to different microenvironments associated with other $CD4^+$ TH cells [127]. Here, the specific $CD4^+$ TH-associated pathogen-induced cytokine *milieu* upregulates the same transcription factor in both $CD4^+$ T cells and TREGs cells, which influences functional properties and the migratory pattern of TREGs. This allows TREGs to suppress the activity of the $CD4^+$ TH cells in their respective pathogen-induced milieu [131]. For example, in a TH1-skewed viral infection, TREGs express T-bet, the canonical TH1 transcription factor. The expression of T-bet in TREGs cells regulates the transcription of inhibitory molecules that have different suppressive mechanisms such as (a) TREGs produced TGF-β facilitates TREGs suppressive activity by blocking T cell activation and function [132, 133], (b) TREG-derived inhibitory molecule CTLA4 downregulates the expression of CD28 ligands on DCs to inhibit T cell activation by DCs [133], (c) TREGs produced IL-10 reduces IL-6 and IL-12 production by DCs [133, 134]. This suggests that IL-10 and CTLA4 mainly target DC cells to prevent conventional T helper cell differentiation. TREGs orchestrate suppressive activity while in close proximity to TH1 cells in their effector sites.

Thus, TREGs upregulate the expression of chemokine receptor CXCR3 in a T-bet-dependent manner. This facilitates migration of TREG to the sites of TH1 mediated inflammatory responses [135, 136]. The role of T-bet in TREG-mediated TH1 suppression is indispensable as a study demonstrated that loss of T-bet in TREGs led to TH1 autoimmunity [137]. In addition, TREG cells have shown to express STAT3 which is important in the control of TH17 cells [138]. STAT3 expression in TREG cells has also been shown to regulate CCR6 expression which contributes in the migration of TREG cells to the site of inflammation to control TH17-mediated immune responses [138]. TH2 associated transcription factor interferon regulatory factor 4 (IRF4) is required by TREG to inhibit TH2 cell-mediated responses [139]. TREGs cells that lacked IRF4 had reduced expression of IL-10, suggesting TREGs cell mediate TH2 suppression in an IL-10-dependent manner [140]. As introduced previously, TREG cells known as TFR cells are localised in the B cell follicles [65, 141]. Like TFH cells, TFR cells express Bcl6, and CXCR5, which assists in the migration of TFR cells to the B cell follicles and subsequently germinal centres [65]. In the GCs, TFR cells have been shown to counter regulate TFH function, B cell activation and antibody production [65]. Collectively, TREG cells are the most explored example of how coordination between cytokine signals and transcription factors tailors T cell effector function.

1.5.2 T helper 2 cells

TH2 cells regulate cell-mediated immune responses during extracellular helminth parasite infection [120]. In these pathogen settings, TH2 cells have been shown to produce IL-4, IL-5, and IL-13 and through these cytokines TH2 cells activate other immune cells [120]. TH2-derived IL-5 mediates the growth, differentiation, and migration of eosinophils [142]. While TH2-secreted IL-4 promotes mast cell proliferation and degranulation, IL-13 stimulates goblet cells to produce mucus [142].

Helminth-influenced microenvironments have been shown to promote IL-4 in both TH2 and TFH cells [20]. Despite similar patterns of effector cytokine, TFH and TH2 have different effector sites [25, 47, 120]. Specifically, in helminth infection settings, TH2 cells migrate to the mucosal sites to activate innate immune cells, while IL-4 producing TFH cells localise and provide help in the GCs instead. The role of IL-4 in GC output is critical because IL-4 regulates B cell activation and B cell class switching to IgE and IgG1 (mice) in helminth infection [44, 143]. The studies have shown that IgE response is not important in the elimination of helminth infection [144, 145]. Moreover, IL-4 collaborates with IL-21 to augment IgG production [34]. Although, the importance of IL-4 in the GC reaction is clear, the complete mechanistic

understanding of the IL-4 gene regulation in TFH cells is not well defined. Additionally, there is emerging evidence that TFH-derived IL-4 directs IgE class switching that leads to the development of severe asthma [125]. Thus, delineating the pathways that lead to IL-4 production in helminth infection can be used not only to eliminate helminth infection but also to understand the progression of other immune diseases such as asthma.

1.5.2.1 T helper 2 cell differentiation

Following infection, naive $CD4^+$ T cells interact with antigen presenting DCs and IL-4 signals in the local microenvironment and sets the TH2 differentiation program in motion [142]. Several immune cells including natural killer T cells, basophils, inflammatory DCs and previously differentiated TH2 cells are thought to be the source of IL-4 production at the time of T cell priming [120, 146]. In response to IL-4 signals, STAT6 is activated which then translocate to the nucleus and promotes the expression of TH2 lineage defining transcription factor GATA3 [147]. There are two major mechanism through which GATA3 imprints TH2 program and effector function. Firstly, GATA3 makes epigenetic changes in several TH2-specific gene loci which initiates their direct transcription [148]. Studies have shown that GATA3-deficient T cells have impaired TH2 development [148, 149]. Secondly, GATA3 regulates TH2 effector function by directly binding to the promoter regions of the *Il4* and *Il13* gene loci (encode IL-13, IL-15) and enhancer regulatory elements of *Il4* gene loci (which encodes IL-4) [150]. Another important factor in TH2 differentiation and function is STAT5, which is activated downstream of IL-2 signalling [150]. Activated STAT5 regulates chromatin remodelling that leads to de-condensation of *Il4* loci, allowing for transcription. Supporting this data, another study showed that absence of STAT5 dampens IL-4 production in TH2 cells [151].

Other transcription factors such as IRF4, c-maf musculoaponeurotic fibrosarcoma oncogene homolog (c-MAF), and Nuclear factor of activated T cells-1 (NFAT) were shown to promote IL-4 production in TH2 cells [142, 152]. IRF4 belongs to the IRF family of transcription factors and in recent years, IRF4 has gained a lot of attention due to its central role in B and T cell activation and differentiation [153]. This is consistent with the capacity of IRF4 to promote BLIMP-1 and Bcl6 expression to facilitate B cell differentiation into plasma and GC B cells [154]. The expression of IRF4 in T cells is initiated following T cell receptor activation. IRF4 alone is insufficient to bind to the promoter region of *Il4* loci and needs to associate with the NFAT to bind thereby initiating IL-4 transcription. In addition, transcription factor c-MAF is preferentially expressed in TH2 cells but not in TH1 cells [152]. In TH2 cells, c-MAF cooperates with NFAT to further promote IL-4 expression. Collectively, GATA3 and STAT5 are the

primary regulators of IL-4 expression in TH2 cells and other transcription factors IRF4, c-MAF, and NFAT supplement IL-4 production [142].

Neurogenic locus notch homolog protein (NOTCH) is a receptor that has a highly conserved signalling pathway associated with TH2 cell differentiation and IL-4 production. Mechanistically, after activation the intracellular domain (ICD) of the NOTCH receptor in activated $CD4^+$ T cells is detached from the receptor complex and translocated to the nucleus. The main target of ICD is RBPJκ (recombination signal-binding protein for immunoglobulin κ J region, also known as CSL) [142]. CSL is a transcriptional repressor at steady state, but following the binding of ICD with CSL, it switches into a transcriptional activator [142]. The NOTCH/CSL complex directly binds to the promoter regions of *Gata3* and *IL-4* locus to promote gene expression [155]. Moreover, NOTCH has been shown to regulate the expression of the high-affinity IL-2 receptor (also known as CD25) in TH2 cells. Downstream signalling through CD25 activates STAT5, which then regulates GATA3 and IL-4 expression, indicating an indirect role of NOTCH in regulating STAT5 [155]. Together, NOTCH receptor activation regulates GATA3 and STAT5 signalling cascades that orchestrate TH2 differentiation and IL-4 production [155].

1.5.2.2 Transcriptional similarities between TH2 and TFH cells

Following helminth infection, IL-4 rich environmental cues induce similar transcriptional programs in differentiating $CD4^+$ T cell subset TFH and TH2 (**Figure 1.2**). These transcription factors can either positively or negatively regulate TFH cell differentiation.

Transcription factor STAT6 (activated downstream of IL-4 signal) upregulates GATA3 expression in TH2 cells [150]. However, in TFH cells, STAT6 regulates IL-4 expression in a GATA3-independent manner [56]. Firstly, STAT6 bind to the 3′ enhancer region of the *Il4* locus, promoting IL-4 expression [156]. Secondly, STAT6 upregulates the expression of Basic leucine zipper transcription factor, ATF-like (BATF) in TFH cells [150]. BATF forms a complex with IRF4 and directly binds to the 3′ enhancer region of the *Il4* locus, promoting IL-4 production in TFH cells [156]. Thirdly, BATF controls Bcl6 and c-MAF expression in TFH cells [156]. The transcription factor c-MAF is crucial for TFH differentiation in a cell-autonomous manner. This is supported by a previous study which showed that T cells deficient in c-MAF failed to acquire prototypical TFH markers (CXCR5, PD-1) [157]. In addition, c-MAF directly binds to the promoter region of IL-21 and enhancer region IL-4 gene, promoting the expression of these cytokines [156, 158]. The 3′ enhancer region in the *Il4* locus is the main site for IL-4 transcription

in TFH cells and importantly this site is occupied by transcription factors BATF/IRF4, STAT6, and c-MAF. However, it is not clear whether they all form a complex or work independently to transactivate IL-4 expression in TFH cells. Another transcription factor NOTCH associated with TH2 development is also important for TFH ontogeny and IL-4 secretion [155]. The intracellular domain (ICD) of NOTCH receptor in complex with CSL selectively bound to 3′ enhancer region of *Il-4* locus leads to the transcription of IL-4 in TFH cells [155]. NOTCH-deficient mice showed impaired IL-4 and IgE and IgG1 production in TH2-skewed parasite infections, suggesting an important role of NOTCH in TFH-mediated B cell class switching through IL-4 [155]. Another study demonstrated that NOTCH promotes c-MAF and Bcl6 expression, and loss of NOTCH led to a defect in TFH cell differentiation in mice infected with helminths [159]. Collectively, these studies highlight that there are multiple factors that promote IL-4 production in TFH and TH2 cells. While IL-4 has an important role in eliminating helminth infection, exuberated IL-4 response has been implicated in the development of allergies and asthma [160]. Dissecting the factors that result in the production of IL-4 can be targeted to prevent pathogenesis of asthma and allergies.

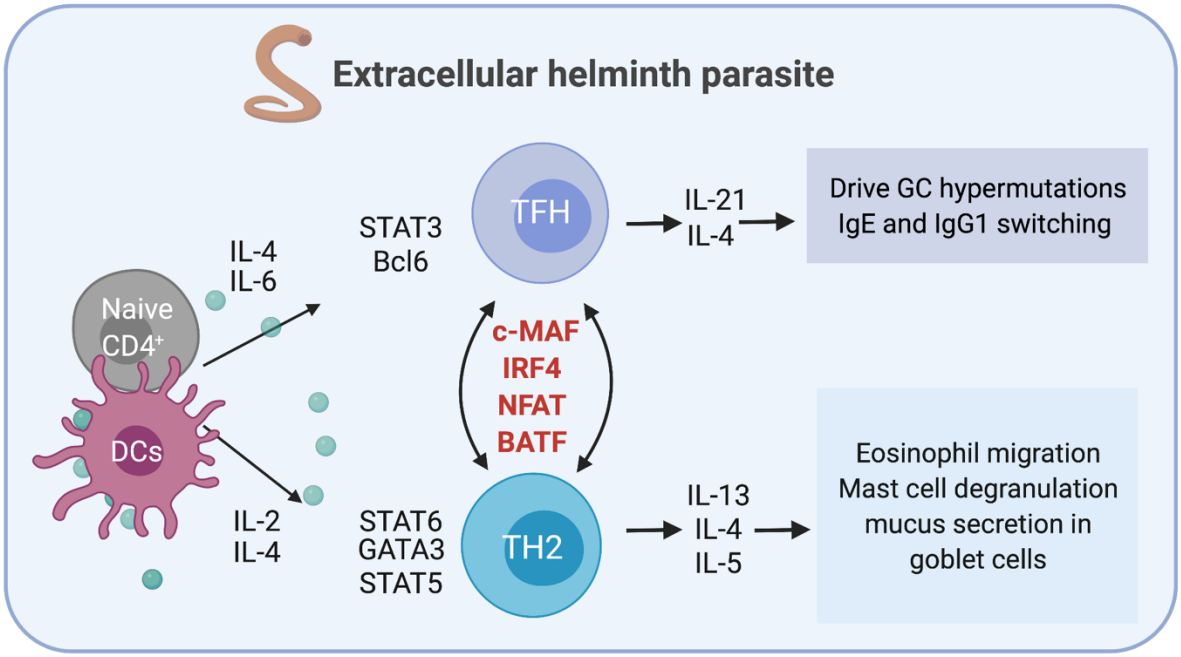


Figure 1.2 TFH and TH2 response in helminth infections

TFH and TH2 cells differentiate in parallel and orchestrate unique functions following extracellular helminth parasite infections. The cytokine milieu regulates expression of transcription factors c-MAF, IRF4, NFAT and BATF in both TFH and TH2 cells. Similar transcription factors in TH2 and TFH regulates IL-4 expression in these cells.

1.5.3 T helper 17 cells

TH17 cells release proinflammatory cytokines that play a pivotal role in the clearance of extracellular bacteria and fungi, especially at mucosal surfaces [121]. One of the primary TH17-derived effector cytokines is IL-17, which targets numerous cells, including epithelial cells, endothelial cells, fibroblasts, osteoclasts, neutrophils, macrophages, and DCs [55, 161, 162]. These cells, in response to IL-17 stimulation release proinflammatory factors such as growth factor granulocyte-macrophage-colony stimulating factor, tumour necrosis factor (TNF)-alpha, and chemokines (CXCL1, CXCL5, and CXCL8) [55]. The resultant microenvironment activates and recruit neutrophils to the tissue site to eliminate invading pathogens [55, 163]. Another cytokine associated with TH17 is IL-22, which collaborates with IL-17 to promote the production of antimicrobial peptides at epithelial surfaces and is key in the elimination of extracellular pathogens [164, 165]. In addition to antimicrobial function, overproduction of IL-17 by TH17 cells is associated with the pathogenesis of autoimmune disorders including multiple sclerosis, psoriasis and, rheumatoid arthritis [166].

1.5.3.1 T helper 17 cell differentiation

The presence of inflammatory cytokines such as IL-6 and TGF-β initiates antigen-activated naive $CD4^+$ T cells to differentiate into non-pathogenic TH17 cells [167, 168]. A number of cells produce IL-6, including monocytes, macrophages, dendritic cells, endothelial cells, and fibroblasts in response to pathogen-associated molecular patterns released by pathogens [169]. In contrast, TGF-β1 production is restricted to stromal, effector, and TREG cells [170]. The combined effect of IL-6 and TGF-β1 stimulation is necessary for the initiation TH17 development. One study demonstrated that in the absence of IL-6, TGF-β1 promotes TREGs program rather than TH17 [171]. Thus, IL-6 signals are required to suppress the upregulation of FOXP3 expression by TGF-β1 [172]. Of note, TGF-β1-deficient mice showed impaired TH17 development [173]. Although TGF-β1 and IL-6 are sufficient to induce TH17 cells, exposure to another cytokine IL-23, leads to the development of pathogenic TH17 cell which is correlated with inflammation in autoimmune diseases [174, 175].

Mechanistically, IL-6 and TGF-β1 promote TH17 differentiation by upregulating the expression of TH17-specific transcription factor retinoid-related orphan receptor gamma t (RORγt) through STAT3 [176]. STAT3 utilizes two main mechanisms to upregulate RORγt expression. Firstly, STAT3 directly binds to the STAT-binding domains into the intronic region of *Rorc* locus (which encodes RORγt), promoting RORγt expression [177]. Secondly, STAT3

introduces the histone modifications H_3K_4me3 at the *Rorc* locus which facilitates binding of other transcription factors to the RORγt gene further promoting RORγt expression [177]. This suggests that STAT3 acting downstream of IL-6 and TGF-β1 upregulates the expression of canonical TH17 transcription factor RORγt which is pivotal for TH17 cell differentiation. In addition, STAT3 binds to the promoter and intergenic regions of *Il17a* gene locus, promoting IL-17 production [178, 179, 180].

The prototypical TH17 transcription factor RORγt belongs to the retinoic acid-related orphan nuclear hormone receptor family including RORα, RORβ, and RORγ, which are encoded by *Rora*, *Rorb,* and *Rorc* genes, respectively [181]. RORγt promotes TH17 phenotype by binding to the regulatory region of several TH17 associated genes, including *Il17a, Il17f,* and *Irf4,* upregulating their expression [182]. RORβ is not expressed in TH17 cells, instead ROR*α* is highly induced in TH17 cells in a STAT3-dependent manner. It has been shown that ROR*α* and RORγ collaborate to promote TH17 development and IL-17 production [183]. However, ROR*α* and RORγ has been shown to be inadequate for the TH17 program and instead requires additional transcriptional factors such as IRF4 and BATF to establish TH17 development. The transcription factor BATF controls the expression of multiple TH17 genes that encode TH17 transcription factor RORγt and cytokines including IL-17, IL-21, and IL-22 [115, 165]. In addition, BATF forms a complex with IRF4 and binds to TH17 related genes that leads to increased chromatin accessibility of these genes. Later these genes are accessed by RORγt that facilitates transcription of these genes [184]. Together these data suggest that multiple transcription factors work in concert to fine-tune non-pathogenic TH17 cell development and cytokines profile.

1.5.3.2 Transcriptional similarities between TH17 and TFH cells

TFH and B cells are efficient in resolving *Citrobacter rodentium* (*C. rodentium*) infection at mucosal surfaces. Previous studies have demonstrated that costimulatory molecules CD28 and CD40L regulate T cell-dependent IgG antibody response against *C. rodentium* [185, 186]. Another recent study correlated TFH-derived IL-21 with IgG1 response to *C. rodentium* infection [27]. In this setting, IL-21-deficient mice showed delayed clearance of pathogen [187]. Collectively, the importance of TFH-derived IL-21 and costimulatory molecules in generating antibody responses in *C. rodentium* is clear. However, the relationship between TFH cells and other CD4 T^+ cell populations that dominate this infection has not been thoroughly investigated. Particularly, TH1 and TH17 cells are often associated with host defence against *C. rodentium* infection by producing their signature cytokines IFN-γ and IL-17 respectively. As mentioned

earlier, TH17 associated transcription factors STAT3, IRF4, and BATF promotes IL-17 production in TH17 cells [115, 165, 178, 179, 180]. Among these transcription factors, STAT3 has a non-redundant role in initiating TFH program [91]. Notably, both IRF4 and BATF are also positively correlated with TFH differentiation [91]. These transcription factors IRF4, BATF and STAT3 are not repressed by Bcl6 in TFH cells. Thus, it is likely that TFH and TH17 cells share expression of transcription factors IRF4, BATF, STAT3 in *C. rodentium*-induced cytokine microenvironment (**Figure 1.3**). The next question is whether these transcription factors can induce IL-17 or IL-4 in TFH cells in *C. rodentium* bacterial infection. Understanding the underlying transcription programs that regulate IL-17 production can be used to target IL-17 in autoimmune diseases since TFH-derived IL-17 contributes to autoreactive B cell responses in BXD2 mice [188].

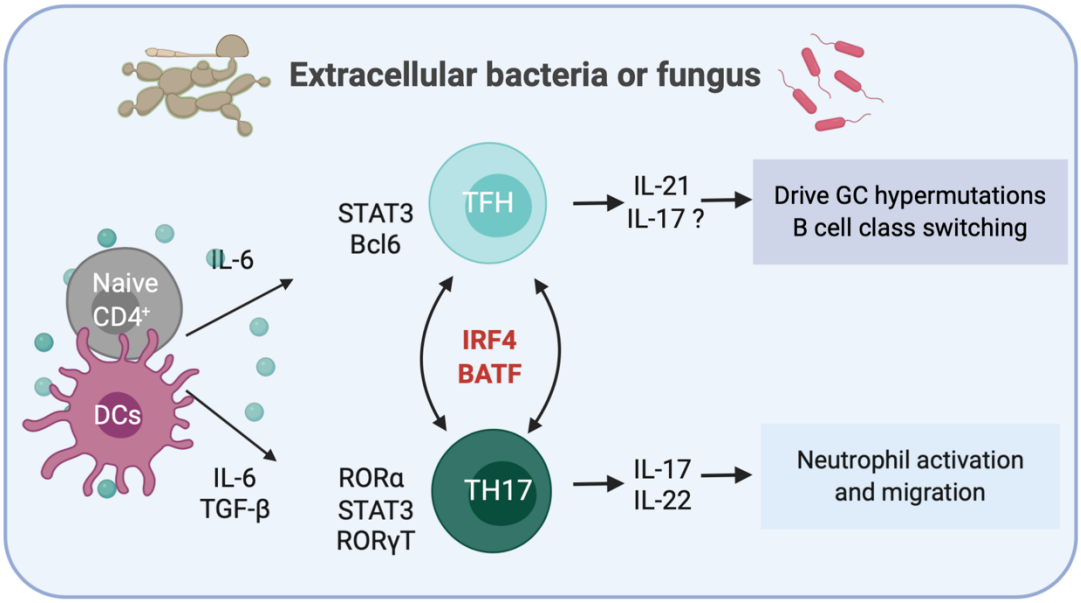


Figure 1.3 TFH and TH17 immune response in C. rodentium bacterial infection

TFH and TH17 cells differentiate in parallel and orchestrate unique functions following C. rodentium bacterial infection. The cytokine milieu regulates expression of transcription factors IRF4 and BATF in both TFH and TH17 cells. Possibly, IRF4 and BATF regulates IL-17 expression in these pathogen settings.

1.5.4 T helper 1 cells

TH1 cells are the key players in eradicating intracellular pathogens, such as influenza A virus and Lymphocytic choriomeningitis virus (LCMV) [119]. TH1 cells execute this effector function by predominantly expressing proinflammatory cytokines interferon (IFN)-γ and tumour necrosis factor (TNF)-α [119]. The most important effects of IFN-γ include (a) activation of macrophages, resulting in enhanced antimicrobial killing ability and phagocytosis, (b) upregulating the antigen processing and presentation by class I and class II major histocompatibility complexes, thereby increasing $CD8^+$ and $CD4^+$ T cell differentiation [189], (c) enhancing leukocyte trafficking to the sites of inflammation by upregulating the expression of adhesion molecules and chemokines, and (d) augmenting IL-12 production in phagocytes while simultaneously inhibiting IL-4 production by TH2 cells [190]. Another TH1-associated cytokine, TNF-α promotes proliferation, apoptosis, and differentiation of macrophages [191]. TNF-α works in synergy with IFN-γ to activate macrophages, thereby enhancing macrophage associated antimicrobial activity. In addition, TNF-α upregulates chemokine expression and inflammatory mediators that act as a chemoattractant for the recruitment of several immune cells such as neutrophils, monocytes, natural killer cells, APC, and DCs to the site of inflammation [192]. Although TH1-derived cytokines IFN-γ and TNF-α act on multiple cells with the same goal of eliminating intracellular pathogens, inappropriate overexpression of these cytokines leads to the development of several diseases including type 1 diabetes, rheumatoid arthritis, and allergy [193].

1.5.4.1 T helper 1 cell differentiation

The differentiation of naive $CD4^+$ T cells into TH1 cells is governed by TCR receptor activation, costimulatory signals by DCs and most importantly IL-12 and IFN-γ cytokine signals in the microenvironment [119]. The proinflammatory cytokines IL-12 is primarily produced by intracellular pathogen-activated DCs and macrophages [194]. Potential cellular source of IFN-γ includes natural killer cells and natural killer T cells [195]. IL-12 signals through STAT4, whereas IFN-γ activates STAT1. The combination of both STAT1 and STAT4 upregulate the transcription of *Tbx21* gene which encodes T-bet, the core TH1 lineage defining transcription factor.

T-bet regulates transcription of multiple genes associated with TH1 differentiation, function and migration [1, 196]. Understanding the mechanism used by T-bet to implement broad TH1 gene programs has been an interesting topic for more than a decade [197]. To regulate TH1 function,

T-bet binding sites exist in the *Ifnγ* locus along with permissive H3K4me3 and H3K36me3 histone modifications in TH1 cells, promoting IFN-γ expression [198]. Additionally, T-bet directly binds to the loci of gene encoding TNF-α. As discussed, TNF-α and IFN-γ are key cytokines regulating TH1 effector function. T-bet also regulates expression of chemokines CCL3 and CCL4 and chemokine receptors CXCR3, which are indispensable for TH1 development and migration of TH1 cells to the site of inflammation [199]. In addition to directly binding to these loci and activating their transcription, T-bet has been shown to bind hundreds of immune regulatory genes across the mouse and human genomes [198, 200, 201, 202]. Like all other T-box proteins, T-bet contains two functional domains [203]. The T-box domain that binds a 24-bp palindromic DNA sequence, consisting of the T-bet recognition sequence TCACACCT. The unique quaternary structure of T-bet enables binding of two distinct DNA sites, potentially allowing T-bet to mediate DNA loop formation and long-range DNA interactions [203]. Meanwhile, the transactivation domain facilitates the binding of T-bet interacting proteins and transcriptional co-factors, such as the recruitment of Mediator and P-TEFb that form the super elongation complex to activate TH1 gene expression [202]. As described below, this domain also allows T-bet to co-opt the function of other transcriptional regulators (as reviewed in Sheikh and Groom [1], Appendix 1).

T-bet implements TH1 fate by preventing alternate T helper gene program by negatively regulating transcription of lineage defining transcription factors and alternate T helper prototypical genes. T-bet can also co-exist with other lineage specifying transcriptional factors in both precursors and committed subsets, such as being co-expressed with RORγt in TH17 cells and with GATA3 in TH2 cells [197]. In these settings, T-bet uses a similar mechanism to sequester these alternative T helper transcription factors during *in vitro* T cell differentiation [204, 205]. TCR signalling *via* tyrosine protein kinase (ITK) phosphorylates motif at the C terminal domain of T-bet. This promotes formation of T-bet-GATA3 complex in $CD4^+$ T cells cultured in TH1 polarized conditions. As a result, GATA3 is sequestered in TH1, which prevents GATA3 from activating TH2 gene program [204]. Furthermore, T-bet directly binds to the sites in *Gata3* locus that are in close proximity to H3K27me3 repressive chromatin modifications, thus, inhibiting GATA3 expression in TH1 cells [200]. Unlike other lineage defining transcription factors, GATA3 is expressed in naive $CD4^+$ T cells [206] and its expression is substantially reduced in TH1 cells [200]. Additionally, T-bet-RUNX3 and T-bet-NFAT1 complexes limits RUNX3 and NFAT1 mediated TH2 signature cytokine expression (IL-4, IL-5, IL-13) [207, 208]. Similarly, T-bet physically interacts with RUNX1 in TH17 polarizing conditions. This blocks

RUNX1 binding to the *Rorc* promoter, thereby inhibiting its transcription. This in turn cripples RORγt-mediated TH17 differentiation [205]. Thus, the constitutive expression of T-bet in T helper cell precursors leads to reduced RORγt blocking TH17 and promoting TH1 differentiation. T-bet in $CD4^+$ T cells can directly bind to its own *Tbx21* locus and this binding site is associated with permissive H3K4me1 histone modifications [201]. However, T-bet induction is unchanged in T-bet-deficient cells when stimulated with IL-12 and IFN-γ and following *Toxoplasma gondii* infection [200]. Conversely, there is a substantial loss in T-bet reporter expression in double T-bet and STAT4-deficient mice to single STAT4-deficient mice during *Toxoplasma gondii* infection, suggesting T-bet may regulate its own expression in certain circumstances [200] (as reviewed in Sheikh and Groom [1], Appendix 1).

1.5.4.2 Overlapping divergent transcription factor of TH1 and TFH cells

One primary attribute of Bcl6 is to control TFH ontogeny by preventing alternate T helper gene circuits by negatively regulating transcription and function of lineage defining transcription factors [56]. However, despite Bcl6 mediated repression of alternative T helper programs, TFH development occurs in parallel with other TH cells. Following viral infections, precursor TFH co-express Bcl6 and T-bet, which is a prototypical TH1 transcription factor (**Figure 1.4**) [99, 198, 209, 210] and as reviewed in Sheikh and Groom [1], Appendix 1. Considering the multiple mechanisms that both Bcl6 and T-bet use to transcriptionally imprint TFH and TH1 differentiation respectively, it may appear counterintuitive for these two factors to not only be co-expressed but work together to direct TFH and TH1 differentiation and function. Indeed, T-bet collaborates with Bcl6 to prolong non-TH1 helper cell program repression even in fully differentiated TH1 cells *in vitro* [210]. While it is possible that T-bet-Bcl6 complexes may bind to Bcl6 DNA binding sites, leading to the TFH fate, it appears that Bcl6 dominates in these interactions, and utilises the transcriptional repressor of Bcl6 to promote TH1 identity. This dominance occurs because the C terminus of T-bet masks the Bcl6 DNA binding site, while leaving T-box DNA binding domain exposed [210]. Among others, the T-bet-Bcl6 complex can be recruited to the *Ifnγ* locus and *Socs1* and *Socs3* promoters in TH1 [210]. As the name suggests, suppressor of cytokine signalling, SOCS1, is involved in blocking the IFN-γ and STAT1 signalling pathways. These signalling pathways are critical for acquisition of the TH1 gene program [211, 212]. However, following the establishment of TH1 cells, T-bet-Bcl6 complexes act to turn these signals down. Indeed, the recruitment of Bcl6 to the *Ifnγ* locus acts to prevent excessive amount of IFN-γ in TH1 culture [210]. Potentially, this action may limit immune pathology and autoimmunity caused by excessive TH1 signals, however further studies are required to determine if this molecular mechanism is relevant *in vivo* and disrupted during immune pathology. In addition, BLIMP-1 is highly expressed in TH1 cells and antagonises *Bcl6* expression [99]. Therefore, BLIMP-1 further prevents expression of Bcl6 and TFH genes in TH1 cells [197]. Together, these findings unravel potential mechanisms implemented by T-bet to block non-TH1, and in particular TFH differentiation. T-bet can be co-expressed with Bcl6 in precursor TFH both in culture and during infection [198, 213] and as reviewed in Sheikh and Groom[1], Appendix 1. However, the molecular mechanisms of T-bet-Bcl6 co-expression are yet to be defined within TFH cells. It is likely that in these cells, they form functional complexes, similar to those described in TH1. It is unclear whether T-bet-Bcl6 complexes regulate TFH cell differentiation or have a role in GC reactions.

1.5.4.3 Overlapping migratory path of TH1 and TFH cells

Following viral infections, T-bet not only regulates TH1 cell differentiation but also shapes TH1 cell migration. Notably, T-bet controls migration by upregulating the expression of CXC-chemokine receptor 3 (CXCR3) [23, 24]. Currently, there are two known mechanisms utilized by T-bet to induce CXCR3 expression. Firstly, T-bet is associated with Jmjd3 and UTX and regulates chromatin remodelling at the promoter region of *Cxcr3* loci, inducing CXCR3 expression [214]. Secondly, T-bet transactivates CXCR3 by increasing positive histone modification at the 5′ enhancer regions of the *Cxcr3* loci [200].

The CXCR3 receptor is activated by three ligands interferon-inducible chemokines CXCL9 (previously known as MIG), CXCL10 (also known as IP-10), and CXCL11 (I-TAC) [215]. Although CXCL11 is highly expressed in humans and most mice strains, it is not encoded in C57BL/6 mice due to mutations in the *Cxcl11* gene [215]. CXCR3 ligands are preferentially expressed at inflamed or infected peripheral tissues and secondary lymphoid tissues. Within the peripheral tissues, these ligands are released by tissue-resident DCs and endothelial cells in response to type I interferons (IFN α/β) and type II (IFN-γ) interferons, resulting in $CXCR3^+$ TH1 cell recruitment [215]. Indeed CXCR3 deficiency result in impaired TH1 cell migration to the site of infection [199]. Furthermore, in secondary lymphoid tissues, CXCR3 ligands are expressed by stromal cells and DCs in the medulla, and interfollicular regions of peripheral draining lymph nodes [216]. This allows $CXCR3^+$ TH1 cells to migrate from the T cell paracortex to the peripheral areas promoting TH1 cell differentiation [216].

In a similar manner to TH1 cells, TFH cells migrate from T-cell paracortex to T:B border or interfollicular areas before migrating deep into the follicles to mediate B cell responses in the GCs [6]. Previous study demonstrated that T cells undergo CXCR3-dependent interactions at interfollicular regions, promoting GC formation and antibody responses. This suggests that CXCR3-dependent interactions outside the B cell follicles are important for TFH differentiation and function following influenza vaccination [217]. Conversely, the inhibitory receptor PD-1 expressed by TFH cells downregulates CXCR3 expression to maintain TFH positioning within the GCs [82]. This potentially implies that TFH cells require CXCR3 to migrate to the T:B border, but also need to block CXCR3-dependent interactions to remain within GCs. CXCR3 is highly expressed in both TH1 and TFH following viral infections, but the exact role of CXCR3 in TFH cell differentiation and function is unclear [23].

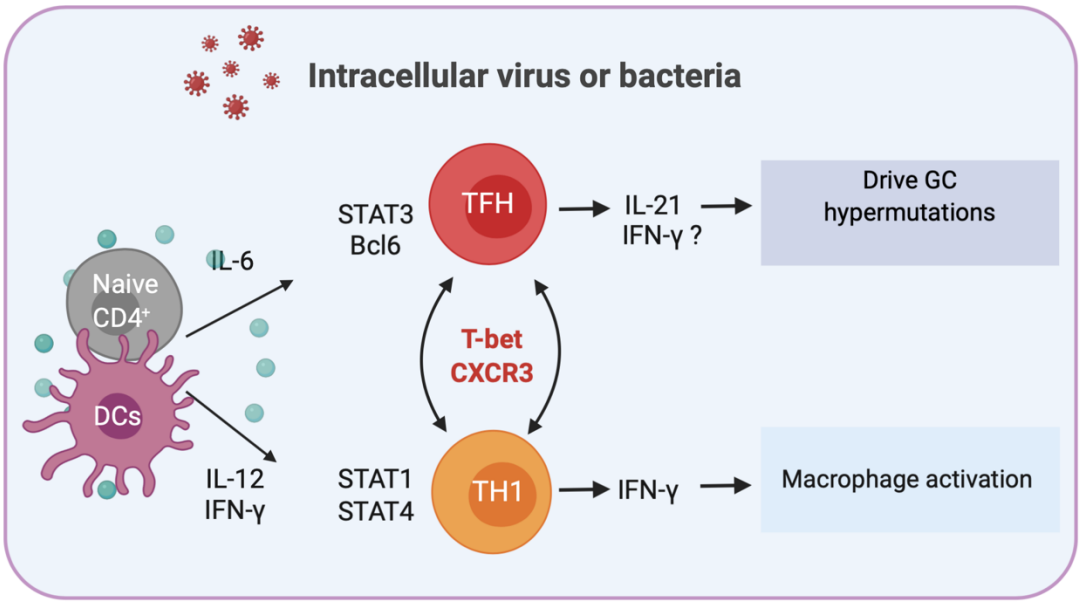


Figure 1.4 TFH and TH1 immune response in viral infections

TFH and TH1 cells differentiate in parallel to limit intracellular viral or bacterial infections. The cytokine milieu regulates expression of transcription factors T-bet and CXCR3 in both TFH and TH1 cells. The role of T-bet and CXCR3 in TFH cell differentiation and function is unclear.

1.6 Hypothesis and Aims

Following infection, naive $CD4^+$ T cells can differentiate into diverse TH subsets, including TFH, TH1, TH2, TH17 and TREGs [82]. Amongst $CD4^+$ T cell subsets, TH1, TH2 and TH17 cells orchestrate cell-mediated immune responses against intracellular pathogens such as viruses, parasite, and extracellular bacteria respectively [119, 120, 121]. In comparison, TFH cells co-exist with counterpart $CD4^+$ T cell subsets and demonstrate similar features as other $CD4^+$ T cells in their respective skewed settings [24, 124, 125]. This implies that the pathogen-influenced factors upregulate similar gene signatures in TFH cells and other TH subsets. Potentially this allows humoral and cell mediated immune response to work in synergy to eliminate diverse infections and this is one of the principal hypotheses of this thesis. The shared gene signatures between TFH and other $CD4^+$ T cell subsets result in transcriptional heterogeneity within TFH cells in diverse infections. However, the role of these shared gene signatures in TFH cell differentiation is unclear. To investigate this, the first aim of the thesis is to establish the role of TH1 associated transcription factor T-bet in TFH cell differentiation and function in viral settings. The second aim of the thesis is to determine whether CXCR3 acts downstream of T-bet and regulates TFH cell differentiation and function in viral infection. Further, the thesis aims to determine shared and pathogen-associated T follicular helper gene signatures in diverse infections including viral, bacterial and parasite infections.

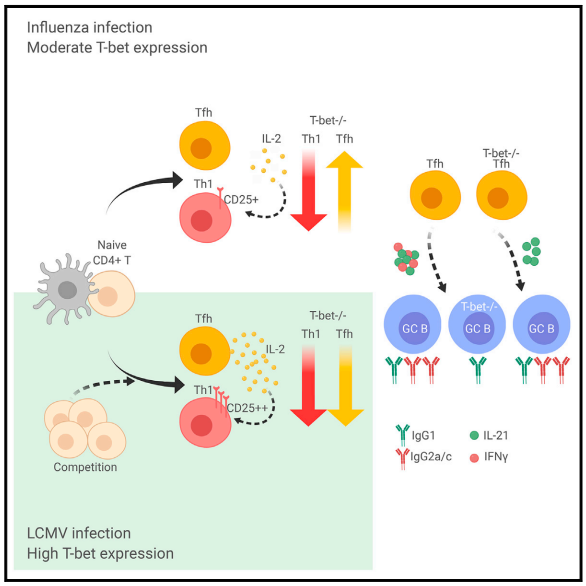
Chapter 2 Context-Dependent Role for T-bet in T Follicular Helper Differentiation and Germinal Centre Function following Viral Infection

Article

Cell Reports

Context-Dependent Role for T-bet in T Follicular Helper Differentiation and Germinal Center Function following Viral Infection

Graphical Abstract



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In Brief

Shiekh et al. show that, in influenza and LCMV infections, the role of the transcription factor T-bet in TFH differentiation is contingent on environmental cues, IL-2 signaling, and T cell competition. Cell-specific T-bet expression independently drives antibody isotype class switching. Therefore T-bet instructs immune protection in a context-dependent manner.

Highlights

- In influenza infection, T-bet represses TFH cells to promote TH1 differentiation
- T-bet is required for differentiation of both TFH and TH1 cells following LCMV
- Distinct IL-2 signaling, T cell competition, and T-bet threshold between infections
- T cell- and B cell-specific T-bet together balance IgG1 and IgG2a/c isotype switching

Sheikh et al., 2019, Cell Reports *28*, 1758–1772
August 13, 2019
https://doi.org/10.1016/j.celrep.2019.07.034

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Context-Dependent Role for T-bet in T Follicular Helper Differentiation and Germinal Center Function following Viral Infection

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https://doi.org/10.1016/j.celrep.2019.07.034

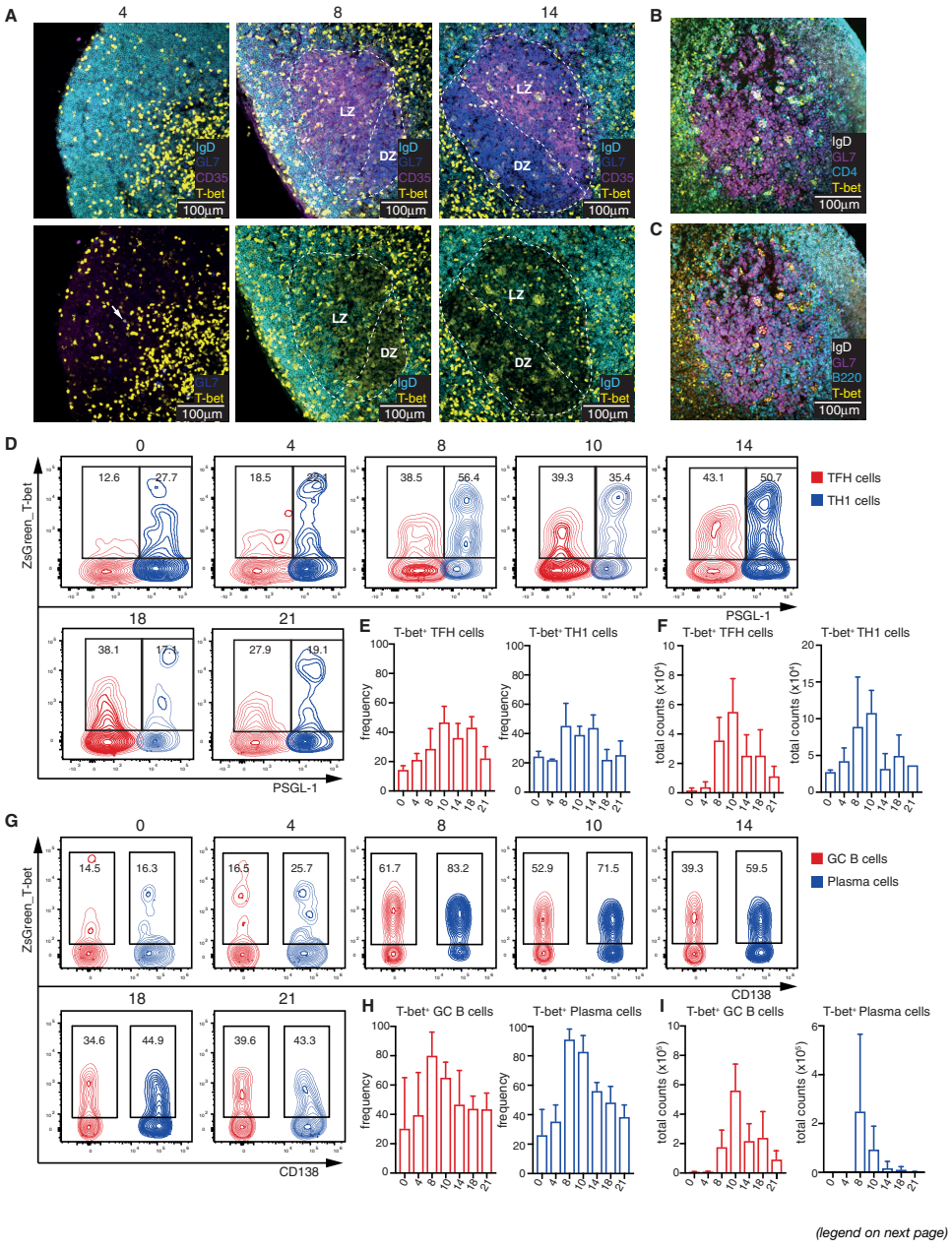
SUMMARY

Following infection, inflammatory cues upregulate core transcriptional programs to establish pathogen-specific protection. In viral infections, T follicular helper (TFH) cells express the prototypical T helper 1 transcription factor T-bet. Several studies have demonstrated essential but conflicting roles for T-bet in TFH biology. Understanding the basis of this controversy is crucial, as modulation of T-bet expression instructs TFH differentiation and ultimately protective antibody responses. Comparing influenza and LCMV viral infections, we demonstrate that the role of T-bet is contingent on the environmental setting of TFH differentiation, IL-2 signaling, and T cell competition. Furthermore, we demonstrate that T-bet expression by either TFH or GC B cells independently drives antibody isotype class switching. Specifically, T cell-specific loss of T-bet promotes IgG1, whereas B cell-specific loss of T-bet inhibits IgG2a/c switching. Combined, this work highlights that the context-dependent induction of T-bet instructs the development of protective, neutralizing antibodies following viral infection or vaccination.

INTRODUCTION

Germinal centers (GCs) are specialized microstructures formed during immune responses and are the cornerstone of protective adaptive immunity. Within GCs, T follicular helper (TFH) cells provide B cells with signals essential for B cell differentiation into isotype-switched antibody-secreting cells. Multiple cytokines and cellular interactions coordinate the expression of a core group of transcription factors that regulate both GC T and B cell differentiation, identity, and function (Good-Jacobson and Groom, 2018). Principally, during both TFH and GC B cell differentiation, Bcl6 upregulation occurs with the reciprocal downregulation of its agonist, B lymphocyte-induced protein-1 (Blimp1) (Crotty et al., 2010; Johnston et al., 2009). In TFH cells, Bcl6 is a transcriptional repressor that acts via multiple mechanisms to functionally activate TFH signature genes and inhibit the different effector T helper (TH) fates (Hatzi et al., 2015; Nurieva et al., 2009; Yu et al., 2009). Despite Bcl6-mediated repression of alternative TH fates, TFH differentiation occurs in parallel with other TH cells. Following viral infection, several prototypical TH1 cell molecules are simultaneously expressed by TFH cells. Notably, this includes co-expression and binding of Bcl6 and the TH1 lineage-specifying transcription factor T-bet (Johnston et al., 2009; Lu et al., 2011; Lüthje et al., 2012; Nakayamada et al., 2011; Oestreich et al., 2011). This interplay is functionally relevant, as T-bet physically recruits Bcl6 to suppress transcription of target genes and blocks the Bcl6 DNA-binding domain, thus establishing appropriate gene expression in TH1 cells (Oestreich et al., 2011, 2012). Similarly, Bcl6 and T-bet can also be co-expressed in B cells following viral infection (Kallies and Good-Jacobson, 2017; Piovesan et al., 2017; Stone et al., 2019). Therefore, the balance in the ratios of different lineage-defining transcription factors may independently alter GC cell function. How extrinsic factors such as distinct infections instruct transcription factor expression and balance is not understood, however, this critically determines cellular differentiation outcomes and ultimately immunological protection.

T-bet is an essential regulator of cellular differentiation and function within multiple lineages. T-bet is the lineage-defining transcription factor for TH1 cells, and it is also highly expressed in $CD8^+$ CTLs, as well as some B and innate lymphoid cell



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subsets (Kallies and Good-Jacobson, 2017). Following viral infection, T cells exhibit graded induction of T-bet expression, which corresponds with their functional capacity. In $CD8^+$ T cells, T-bet functions as a molecular switch between effector and memory differentiation (Intlekofer et al., 2007; Joshi et al., 2007). High expression of T-bet induces and cooperates with Zeb2 to enact a unique transcriptional program that forces effector cell differentiation (Dominguez et al., 2015). In TFH cell differentiation, the role of T-bet is less clear and is an area of active investigation. As T-bet-Bcl6 complexes can inhibit Bcl6 DNA binding, it has been proposed that expression of T-bet during $CD4^+$ T cell activation intrinsically tips the balance of differentiation in favor of TH1 cells (Oestreich et al., 2012). This hypothesis is supported by initial studies in T-bet-deficient animals showing an increased accumulation of TFH cells and reciprocal loss of TH1 cells *in vitro* and following *Toxoplasma gondii* or *Plasmodium berghei* ANKA infections (Nakayamada et al., 2011; Ryg-Cornejo et al., 2016). In contrast, T-bet was shown to promote both TH1 and TFH cell differentiation following lymphocytic choriomeningitis virus (LCMV) infection (Wang et al., 2019; Weinstein et al., 2018). How extrinsic factors underpin these conflicting results in the role of T-bet within differentiating TFH cells and GC biology has not been established.

TFH cytokine signals play an essential role in tailoring effective GC responses (Reinhardt et al., 2009). IL-21 is the cardinal TFH-derived cytokine and along with IFNγ, IL-4, and IL-10 mediates B cell class switching and affinity maturation (Lüthje et al., 2012; Miyauchi et al., 2016; Ramiscal and Vinuesa, 2013). For example, T cell-derived IFNγ induces T-bet in B cells, leading to the upregulation of a broad anti-viral gene expression program (Barnett et al., 2016; Peng et al., 2002; Rubtsova et al., 2013). Together with IL-21, this promotes TH1-skewed antibody-producing cells and drives isotype switching of antibody to IgG2a (named IgG2c in C57BL/6 mice and hereinafter referred to as IgG2a/c) (Lüthje et al., 2012; Miyauchi et al., 2016; Peng et al., 2002; Stone et al., 2019). Switching of antibody to the IgG2a/c isotype mediates clearance of virus and protection against lethal influenza infection (Huber et al., 2006; Miyauchi et al., 2016). In addition, the combined induction of both IgG2a/c and IgG1 is required for optimal vaccine efficacy more than neutralization alone (Huber et al., 2006). The specific external and transcriptional regulators that oversee this balance of antibody class switch following infection are not fully understood.

In this study, we determine the timing and level of expression of T-bet in GC T and B cells following influenza infection using ZsGreen_T-bet reporter mice (Zhu et al., 2012). We show that both $T\text{-}bet^+$ T and B cells are found within GC structures throughout influenza infection, but T-bet levels in the GC do not reach those observed in non-GC effector T cell populations. Given the conflicting literature on the function of T-bet in humoral immunity, we sought to dissect the factors that control these divergent outcomes. We demonstrate that the role of T-bet is contingent on the environmental setting of TFH differentiation, whereby changes in pathogen and T cell competition for antigen alters the kinetics and intensity of T-bet induction, IL-2 production, and consumption and the outcome of T-bet deficiency. Furthermore, to elucidate the function of T-bet individually in GC T and B cells, we use $T\text{-}bet^{fl/fl}$ animals crossed to Cre-expressing animals for each cell type to identify the downstream outcomes of T-bet deficiency. We show that T-bet acts independently in GC T and B cells to enact isotype switching following influenza infection. This revealed that expression in TFH cells directs switching away from IgG1 isotypes, while expression within GC B cells directs switching toward IgG2a/c. Thus, our data demonstrate that T-bet expression within the different cellular compartments of GCs works together to mediate efficient isotype switching that will effectively neutralize virus.

RESULTS

T-bet-Expressing Cells Are Located within GC Structures following Influenza Infection

To characterize the expression of T-bet following influenza infection, we made use of the ZsGreen_T-bet reporter mouse, which faithfully reports the expression of *Tbx21* transcript (Zhu et al., 2012). *Tbx21* transcription has previously been shown to be repressed by Bcl6, but despite this, we found considerable reporter expression within the GC structures of the draining mediastinal lymph node (dLN) at day 8 (d8) following intranasal infection with influenza A HKx31 (H3N2) (Figure 1A). This expression was seen early in infection, with $T\text{-}bet^+GL7^+$ cells detected at d4, prior to established GC structures. T-bet expression was observed following the establishment of GCs d8 and d14 post-infection (p.i). T-bet-expressing cells were present in both the $CD35^+$ FDC-marked light zone (LZ; $GL7^+IgD^-CD35^+$) and to a lesser extent within the dark zone (DZ; $GL7^+IgD^-CD35^-$) (Figure 1A, bottom panels). Furthermore, co-staining with CD4 and B220 showed that both GC T and B cells were positive for *Tbx21* expression (Figures 1B and 1C). We next identified in more detail the T-bet-expressing subsets within GCs using flow cytometry. For this we tracked the upregulation and expression of $T\text{-}bet^+$ cells in dLN during influenza infection. Polyclonal T cell responses were tracked using the gating strategy of Marshall et al. (2011) to identify dLN TH1 cells

Figure 1. T and B GC Cells Express T-bet Following Influenza Infection

ZsGreen_T-bet reporter mice were infected with influenza and dLNs analyzed.

(A–C) Representative confocal micrograph at indicated days p.i. $T\text{-}bet^+$ cells (yellow) in GC LZ ($IgD^-GL7^+CD35^+$) and DZ ($IgD^-GL7^+CD35^+$). Arrow indicates $T\text{-}bet^+GL7^+$ cell at d4 p.i. Dashed lines mark GC regions, bottom panels show $T\text{-}bet^+$ cells without GC markers (A). Day 8 p.i. $T\text{-}bet^+$ GC co-stained with CD4 (cyan) (B) and co-stained with B220 (cyan) (C).

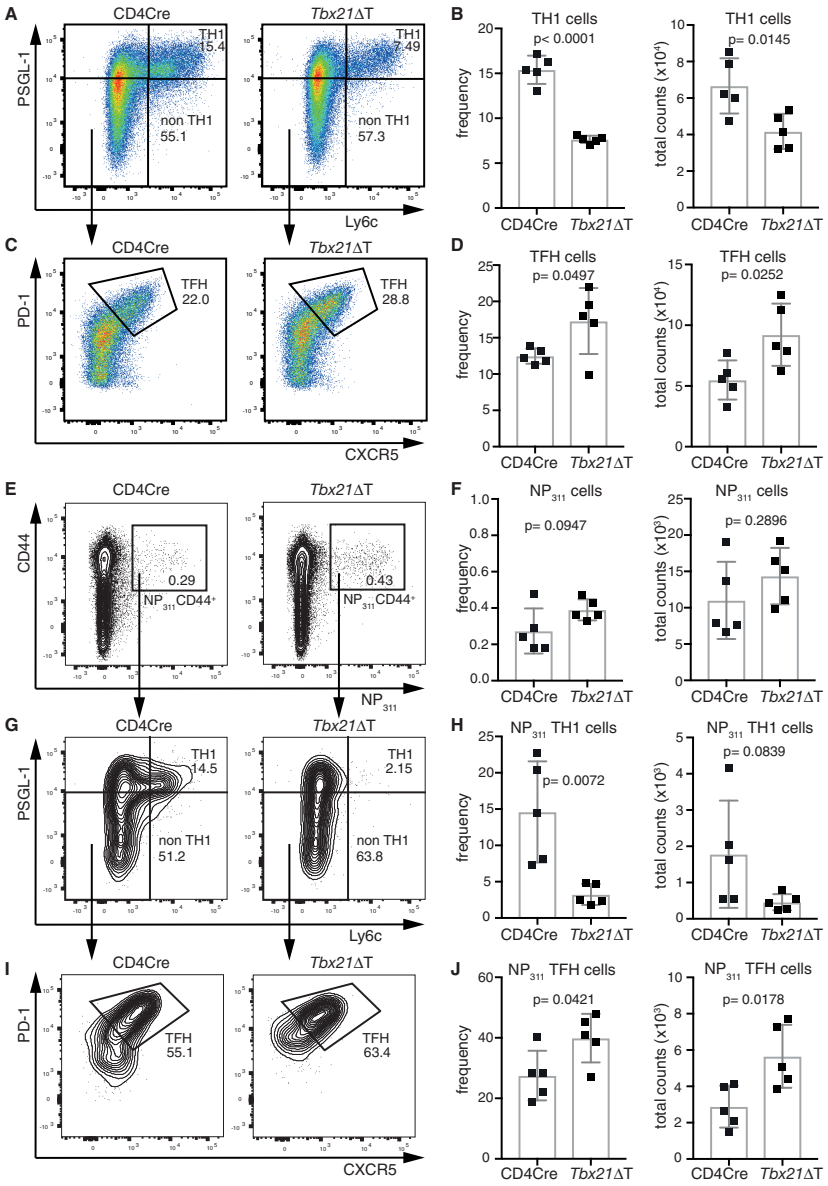
(D) Representative flow cytometry plots showing frequency of $T\text{-}bet^+$ TFH (red, $CD44^+PSGL\text{-}1^-$ $Ly6c^-$ $PD\text{-}1^+$ $CXCR5^+$) and $T\text{-}bet^+$ TH1 (blue, $CD44^+$ $PSGL\text{-}1^+Ly6c^+$) at indicated days p.i.

(E and F) Frequency (E) and total numbers (F) of $T\text{-}bet^+$ TFH and TH1 cells.

(G) Flow cytometry plots showing frequency of $T\text{-}bet^+$ GC B cells (red, $B220^+IgD^-CD95^+CD38^-$) and plasma cells (blue, $B220^{lo}$ $CD138^+$).

(H and I) Frequency (H) and total numbers (I) of $T\text{-}bet^+$ GC B and plasma cells.

In (E), (F), (H), and (I), data are pooled from three independent experiments. Data are mean ± SEM.



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($CD44^+PSGL\text{-}1^+Ly6c^+$) and TFH cells ($CD44^+PSGL\text{-}1^-Ly6c^-PD\text{-}1^+CXCR5^+$) (Marshall et al., 2011). As expected, TH1 cells rapidly upregulated high levels of *Tbx21*, which peaked at d8–d14 of infection (Figures 1D–1F). T-bet expression within the TFH compartment followed a similar kinetic to that of TH1 cells, peaking between d8–d10 of infection. However, TFH cells showed 1–2 logs lower ZsGreen_T-bet reporter expression than the peak MFI of TH1 cells (Figure 1D). This lower expression of T-bet within the TFH compartment is reminiscent of the graded T-bet expression found within the $CD8^+$ T cell compartment, which determines cell fate decisions between effector and memory differentiation (Intlekofer et al., 2007; Joshi et al., 2007). We next identified the kinetics of T-bet induction in GC^+ B cells ($B220^+IgD^-CD95^+$ $CD38^-$) and plasma cells ($B220^{lo}CD138^+$) in dLNs. Within both B cell populations, *Tbx21* transcripts peaked at d8 following infection (Figures 1G–1I). Similar to TFH cells, $T\text{-}bet^+$ B cells expressed a lower level of ZsGreen_T-bet reporter expression than seen in TH1 cells (Figure 1G). Potentially, the lower expression of *Tbx21* within TFH, GC B, and plasma cells may indicate a common mechanism of transcriptional repression that is not present in TH1 and $CD8^+$ T cells (Figures 1D and 1G and data not shown).

T Cell-Specific T-bet Expression Promotes the Accumulation of TH1 Cells with Reciprocal Loss of TFH Cells

As we had observed T-bet expression in GC T and B cells at the peak of the influenza inflammatory response, we next dissected the role of T-bet during TH1 and TFH differentiation *in vivo* at d8 following infection. Initially, we used intact T-bet-deficient ($Tbx21^{-/-}$) animals to confirm previous results that indicated *Tbx21* deficiency promotes TFH cell differentiation *in vivo* (Figure S1) (Nakayamada et al., 2011; Ryg-Cornejo et al., 2016). TFH differentiation *in vivo* is a dynamic, multi-step process that relies on interactions with both dendritic cells and B cells (Groom, 2015; Qi, 2016). To determine whether these differences were T cell intrinsic, we investigated mice lacking T-bet in all T cells ($Tbx21^{fl/fl}$xCD4Cre, *Tbx21*ΔT) following influenza infection. *Tbx21*ΔT and CD4Cre controls were infected, and polyclonal T cell responses were assessed (Figures 2A–2D). This analysis confirmed that T cell-specific *Tbx21* deficiency does indeed promote the accumulation of TFH cells with a reciprocal loss of TH1 cell differentiation. Furthermore, using MHCII $I\text{-}A^b$-restricted tetramers, we identified $CD4^+$ T cells specific for the immunodominant epitope $NP_{311\text{-}325}$ of influenza A virus. We found no differences in the frequency or numbers of NP_{311} $tetramer^+$ cells following infection between CD4Cre controls and *Tbx21*ΔT animals (Figures 2E and 2F). Similar to that observed in the polyclonal population, within the NP_{311} $tetramer^+$ cells, TH1 showed a reduced accumulation, while non-TH1 frequency and TFH numbers were increased (Figures 2G–2J). Combined, these results demonstrate that T-bet acts as a molecular switch that determines $CD4^+$ T cell differentiation between TFH and TH1 effector cells.

The Role of T-bet in $CD4^+$ T Cell Differentiation Is Context and Competition Dependent

In contrast to our data, STAT4-dependent induction of T-bet in $CD4^+$ T cells has been shown to promote the differentiation of both TH1 and TFH cell differentiation following LCMV infection, and in the absence of *Tbx21*, both TH1 and TFH differentiation is limited (Wang et al., 2019; Weinstein et al., 2018). We were intrigued as to how these divergent results may arise and sought to replicate this experimental model. Antigen-specific Smarta TCR Tg cells (STg; specific for the LCMV immunodominant epitope $GP_{66\text{–}77}$) and *Tbx21*ΔT STg cells were transferred into wild-type hosts prior to LCMV (Armstrong) intravenous infection. Splenic STg TH1 and TFH cells were determined *in vivo* at d8 following infection (Figures 3A–3D). Following transfer of cells, the frequency of TH1 cells was significantly decreased in *Tbx21*ΔT STg cells, with a reciprocal increase in the number of non-TH1 cells ($STg^+CD44^+PSGL\text{-}1^-Ly6c^-$) (Figures 3A and 3B). Despite the accumulation of activated, non-TH1 cells, these cells failed to upregulate TFH markers PD-1 and CXCR5, resulting in a deficiency of both TH1 and TFH cells and a complete deficiency in effector $CD4^+$ T cell differentiation. These results are consistent with recent work (Weinstein et al., 2018) but contrast with our findings in intact *Tbx21*ΔT following influenza infection (Figure 2). Thus, T-bet mediates divergent $CD4^+$ T cell differentiation outcomes, which are contingent on the infectious and experimental setting.

Fate decisions between TH1 and TFH can be determined by multiple factors, including antigen affinity and signal strength, in addition to T cell competition for both antigen and environmental cytokines (DiToro et al., 2018; Qi, 2016; Ramiscal and Vinuesa, 2013). These environmental cues lead to the upregulation and balance of multiple transcription factors that regulate $CD4^+$ T cell differentiation (Good-Jacobson and Groom, 2018; Oestreich et al., 2012). It therefore seemed likely that changes in these conditions during an infection could alter the outcome of T-bet deficiency during TFH cell differentiation. To gain insight into the factors that determine the role of T-bet during T cell activation and differentiation, we altered key experimental parameters to understand how viral infection and T cell competition affect TFH differentiation following viral infection. Given that *Tbx21*ΔT STg cells were transferred into a competitive environment with wild-type cells, we reasoned that this competition may significantly alter the outcome of T-bet deficiency following

Figure 2. T Cell-Specific *Tbx21* Deficiency Promotes TFH Differentiation following Influenza Infection

CD4cre and *Tbx21*ΔT mice were infected with influenza, and dLN $CD4^+CD44^+$ polyclonal and antigen-specific cells were analyzed at d8 p.i.

(A–D) Representative flow cytometry plots of polyclonal $CD4^+CD44^+$ (A and C) with frequency and total number (B and D) of natural repertoire of TH1 ($CD44^+PSGL\text{-}1^+Ly6c^+$) (A and B) and TFH ($CD44^+PSGL\text{-}1^-Ly6c^-PD1^+CXCR5^+$) (C and D).

(E and F) Plots (E) and frequency and total number (F) of NP_{311} $CD4^+$ T cells.

(G–J) Plots (G and I) and frequency and total number (H and J) of TH1 ($NP_{311}{}^+CD44^+PSGL\text{-}1^+Ly6c^+$) (G and H) and TFH ($NP_{311}{}^+$ $PSGL\text{-}1^-Ly6c^-PD\text{-}1^+CXCR5^+$) (I and J).

Data are representative of three independent experiments, n = 3–5 mice/group. Data are mean ± SEM.

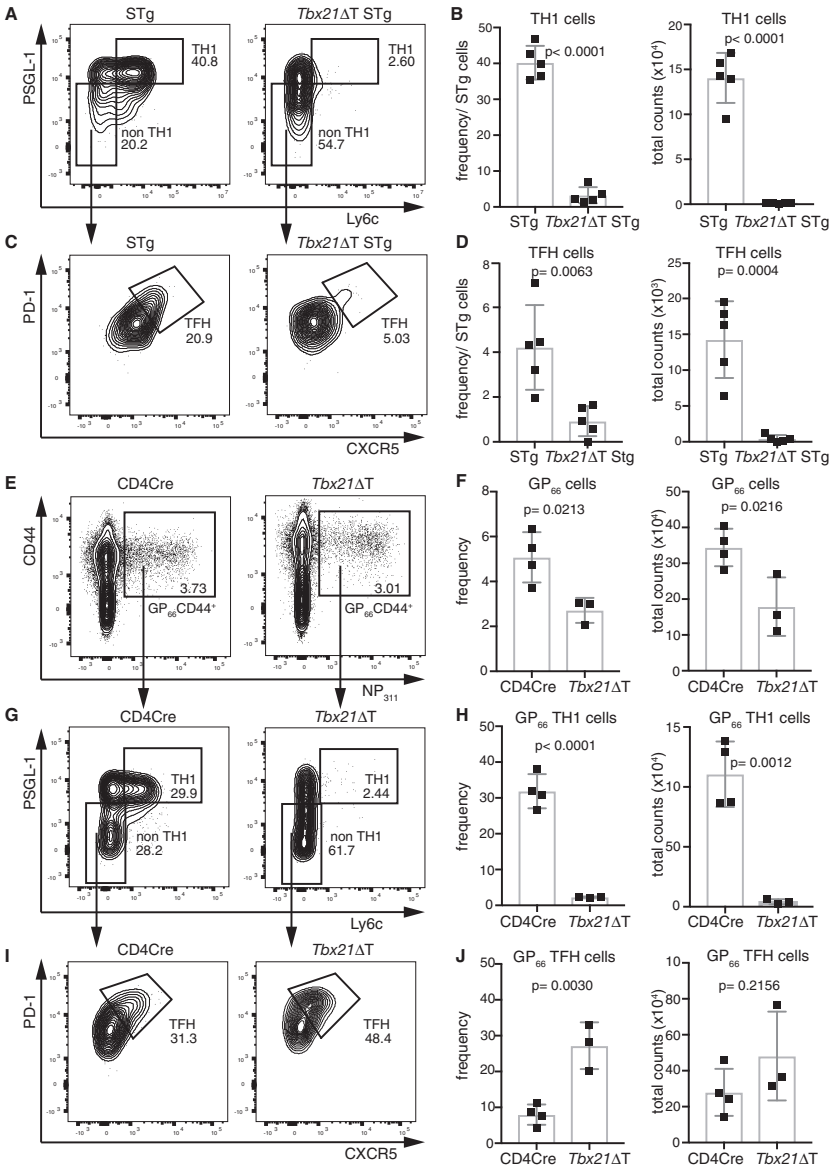

Figure 3. T-bet Regulates TFH Differentiation in a Context-Dependent Manner following LCMV Infection

(A–D) STg and *Tbx21*ΔT STg were transferred into Ly5.1xLy5.2 F1 mice subsequently infected with LCMV, and splenic STg cells were analyzed at d8 p.i. Representative flow cytometry plots (A and C) and frequency and total number (B and D) of TH1 ($CD44^+PSGL\text{-}1^+Ly6c^+$) (A and B) and TFH ($CD44^+PSGL\text{-}1^-Ly6c^-PD\text{-}1^+CXCR5^+$) (C and D) in indicated mice.

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LCMV infection (Figure 3C). To address this question, *Tbx21*ΔT animals were infected with LCMV, and splenic CD4$^+$ T cells specific for the MHCII I-A^b-restricted immunodominant epitope GP$_{66–77}$ of LCMV were assessed at d8 following infection. In these conditions, without competing wild-type T cells, the role of T-bet again resembled what we had observed for influenza infection in both *Tbx21*$^{-/-}$ and *Tbx21*ΔT mice, in which TH1 cells failed to accumulate with a reciprocal increase in the frequency of TFH cells (Figures 3G–3J). We next altered the T cell competition environment of *Tbx21*ΔT mice in influenza infection using a 50:50 BM chimera approach. In this setting, GP$_{66}$ tetramer$^+$ *Tbx21*ΔT TH1 cell frequency was reduced together and was accompanied by diminished accumulation of non-TH1 cells and limited TFH differentiation (Figure S2). Combined, these results suggest that when *Tbx21*ΔT CD4$^+$ T cells are required to compete with wild-type counterparts, the accumulation of effector cells is reduced for both TH1 and TFH cells.

Context-Specific Induction of T-bet Alters T Cell Proliferation, Activation Marker Expression, and Transcription Factor Ratios

To investigate the context-specific basis for the role of T-bet during TFH differentiation, we analyzed in detail the viral infectious settings with the most divergent outcomes: influenza infection in intact animals and LCMV infection with STg transfer (Figure 4). Comparing the ZsGreen_T-bet reporter in these infections showed marked differences in T-bet induction at d4 and d8 during each immune response. Early in LCMV infection, both TFH and TH1 strongly induced T-bet compared with T cells in influenza infection (Figures 4A and 4B). At d8 the frequency of T-bet$^+$ in influenza TH1 cells resembled that of LCMV TFH cells, while T-bet was expressed to a lesser extent in influenza TFH cells. We next investigated how the loss of T-bet expression in these settings influenced proliferation and differentiation readouts during TFH and TH1 differentiation. Using Ki67 as a marker for proliferating cells (Miller et al., 2018), we showed that LCMV induced a higher rate of proliferation at d4 compared with influenza infection (Figures 4C and 4D). Ki67 MFI showed the context-specific role of T-bet whereby at d4 p.i., Ki67 intensity was reduced with T-bet deficiency and increased in influenza. At d8 of infection, the tetramer$^+$ TFH cells in influenza infection showed increased Ki67 expression over that of STg cells in LCMV, but this was not altered further by T-bet deficiency (Figure 4D). We found the kinetics in expression of key regulators of TFH cell differentiation and function was altered in a viral-dependent fashion (Figures 4E–4J). PD-1, Bcl6, and CXCR5 were all more highly expressed in LCMV than influenza d4 of infection, but this was reversed at d8 in TFH cells for PD-1 and Bcl6 (Figures 4E and 4F; Figure S3). Furthermore, at d4 of LCMV and d8 of both LCMV and influenza, T-bet was required for the induction of CXCR3, which may help position cells within GC structures (Figures 4G and 4H; Good-Jacobson and Groom, 2018; Groom, 2015; Kallies and Good-Jacobson, 2017; Shi et al., 2018). Similarly, CD25 also showed context-dependent expression that was regulated by T-bet deficiency at d4 p.i. (Figures 4I and 4J). Combined, this work demonstrates that unique viral infections and T cell competition finely tune TFH differentiation in the context of divergent T-bet induction.

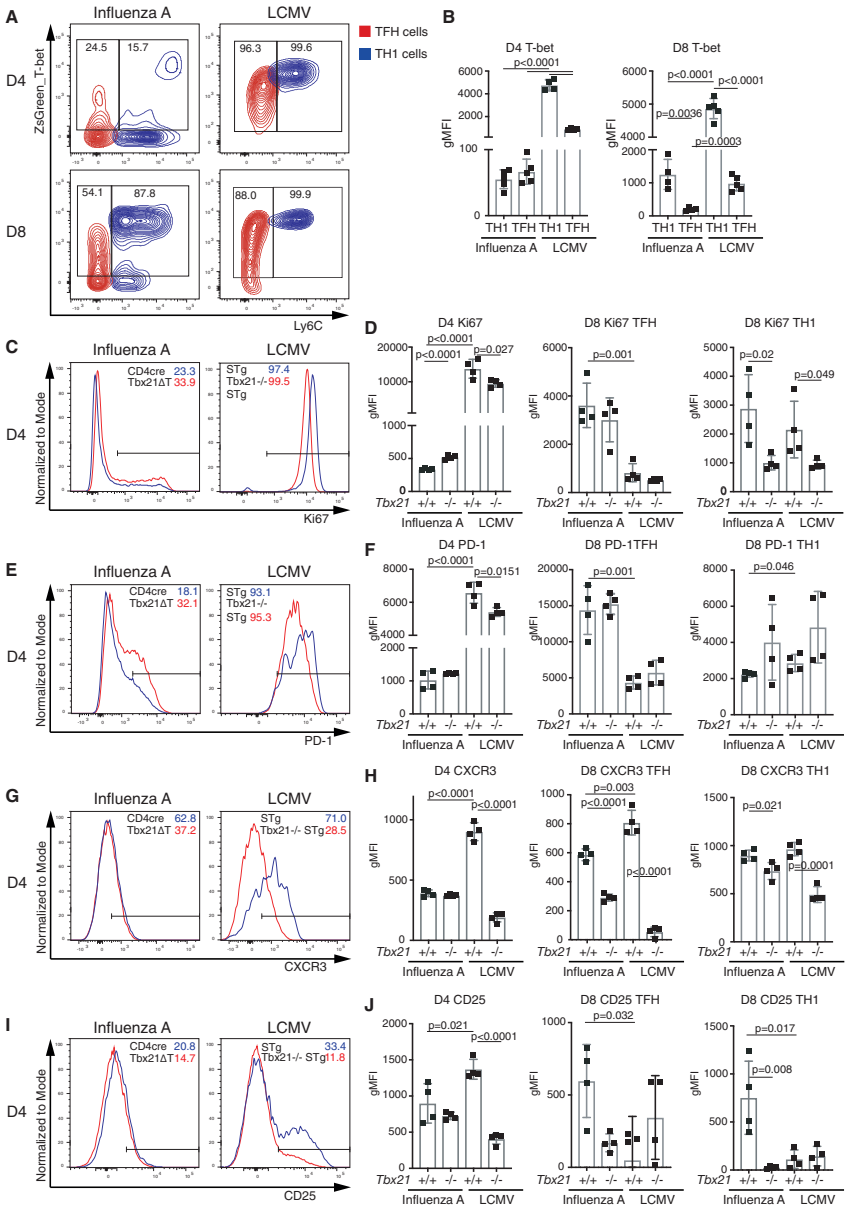
Divergent Induction of T-bet during Viral Infection Alters the Balance of IL-2 Production and Signaling

The different induction of T-bet in two Th1-biased infection models likely reflects unique cytokine milieu between infections. Multiple inflammatory cytokines, including IL-2, TGFβ, IL-7, IL-6, and type I IFNs, alter the balance between TH1 and TFH cells during infection (Ballesteros-Tato et al., 2012; Choi et al., 2011; DiToro et al., 2018; Eto et al., 2011; Harker et al., 2011; Marshall et al., 2015; McDonald et al., 2016; Oestreich et al., 2011; Ray et al., 2014). As we observed that CD25 MFI was altered in a viral- and T-bet-dependent manner (Figures 4I and 4J), we next investigated the role IL-2 production and consumption play in TFH differentiation between influenza and LCMV infections. The prevailing paradigm for IL-2 CD4$^+$ T cell fate decisions dictates that CD25 marks IL-2 consumers that become TH1 cells, while early producers of IL-2 become TFH cells (Ballesteros-Tato et al., 2012; DiToro et al., 2018; Oestreich et al., 2011; Pepper et al., 2011). Fitting this hypothesis, at d4 of infection, CD25 expression was segregated from CXCR5 expression in LCMV infection (Figure 5A). However, the separation of cell fates by CD25 or IL-2 was less well defined in influenza infection (Figures 5A–5C), with low expression of CD25, and the low-affinity IL-2 receptor CD122 was also minimally expressed (Figures S4A and S4B). To demonstrate that the differential expression of CD25 between infections and genotypes was functionally relevant, we determined STAT5 phosphorylation following a brief IL-2 stimulation. Phosphorylated STAT5 (pSTAT5) correlated with the expression of CD25, while no pSTAT5 was detected without IL-2 (Figures 5D and 5E; Figures S4C and S4D). This expression was further dependent on T-bet, whereby CD25$^+$pSTAT5$^+$ frequency in T-bet-deficient T cells was increased in influenza but decreased in LCMV. Furthermore, we investigated whether IL-2 production serves as a marker of TFH differentiation. Similar to described work, at d4, IL-2$^+$ cells were found predominately in the CXCR5$^+$ STg cells following LCMV infection (Figures 5F and 5G). However, in influenza IL-2 production did not indicate CXCR5 cells at this time, suggesting early demarcation of T cell fate is less clear in this infection model.

T Cell-Intrinsic T-bet Deficiency Influences TFH Cytokine Production but Leaves GC B Cell Differentiation Intact

The identification of an experimental model in which *Tbx21* deficiency resulted in the expansion of TFH cells allowed the opportunity to dissect the functional role of T-bet within individual cellular compartments of GCs in intact animals. We first

(E–J) CD4cre and *Tbx21*ΔT mice were infected with LCMV, and splenic CD4$^+$CD44$^+$ antigen-specific cells were analyzed at d8 p.i. Plots (E) and frequency and total number (F) of GP$_{66}$ CD4$^+$ T cells. Plots (G and I) and frequency and number (H and J) of TH1 (GP$_{66}^+$CD44$^+$PSGL-1$^+$Ly6c$^+$) (G and H) and TFH (GP$_{66}$ $^+$CD44$^+$PSGL-1$^-$Ly6c$^-$PD-1$^+$CXCR5$^+$) (I and J).
Data are representative of three independent experiments, n = 3–5 mice/group. Data are mean ± SEM.



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investigated the production of TFH cytokine in *Tbx21*ΔT and control animals following influenza infection by crossing CD4Cre and *Tbx21*ΔT to an IL-21^{GFP} reporter (Lüthje et al., 2012). As T-bet directly controls IFNγ production, we predictably showed a significant decrease in IFNγ production by *Tbx21*ΔT TFH cells following PMA and ionomycin stimulation (Figures 6A and 6B). Interestingly, this drop in IFNγ production appeared to be more significant in influenza compared with published data in LCMV infection (Weinstein et al., 2018) and is in line with fate-mapping data indicating that previous or current T-bet expression is essential for TFH IFNγ production (Fang et al., 2018). Similar to effects on T cell differentiation, the role of T-bet in production of IL-21 may also differ depending on experimental model, with some studies reporting an increase in IL-21 with T-bet-deficiency whereas others show no difference (Lüthje et al., 2012; Nakayamada et al., 2011; Schmitt et al., 2016; Weinstein et al., 2018). Following influenza infection, we did not see an overall change in IL-21 production in *Tbx21*ΔT TFH cells (Figures 6A and 6B). However, combined with the drop in IFNγ, this resulted in a net loss of double-positive IL-21^{+}IFNγ^{+} cells, which may indicate altered functionality of IL-21-expressing TFH population with GCs (Figure 6C). Furthermore, although IL-21 has previously been shown to be produced by CXCR3^{+} TH1 cells, this could not be detected in our experimental system in either control or *Tbx21*ΔT mice (data not shown) (Miyauchi et al., 2016).

We next sought to determine the overall affect that T cell-intrinsic loss of T-bet had on GC B cell responses. We examined GC B cell differentiation by flow cytometry at d8 following influenza infection in *Tbx21*ΔT and CD4Cre mice. We detected an increase in the frequency and total number of GC B cells within dLN, in line with the increased expansion of TFH cells in *Tbx21*ΔT animals (Figures 6D and 6E). However, we did not see any overt effect on the proportion of cells in the LZ (CXCR4^{-}CD86^{+}) or DZ (CXCR4^{+}CD86^{-}) compartments (Figure 6F; Figure S5A). Furthermore, we quantified the number and size of GC structures (GL7^{+}IgD^{-}) in dLNs. We found no difference in the overall size and number of GC structures, suggesting that the expansion of GC B cell numbers was not sufficient to be detected by confocal microscopy (Figure 6G; Figure S5B). Therefore, T cell-intrinsic T-bet deficiency does not result in an overt GC B cell phenotype.

B Cell-Intrinsic T-bet Deficiency Does Not Alter GC Structure

As we and others had observed T-bet expression in GC B cells (Figures 1G–1I), we next investigated the reciprocal question, as to the B cell-intrinsic role of T-bet within GC reactions (Kallies and Good-Jacobson, 2017; Piovesan et al., 2017; Stone et al., 2019). For this we examined *Tbx21*$^{fl/fl}$xCD23Cre (*Tbx21*ΔB) animals at d8 following influenza infection (Piovesan et al., 2017). We found no statistical differences in GC B cell numbers, LZ/DZ accumulation, or GC number and structure between *Tbx21*ΔB and CD23Cre control animals (Figures 6H–6L; Figures S5C and S5D). Therefore, B cell-intrinsic deficiency of T-bet does not result in any overt GC phenotype as examined either by flow cytometry or histologically.

T-bet Acts Independently within TFH and GC B Cells to Influence Isotype Class Switching

Antigen-specific B cells interpret T cell cytokine signals to determine the class switch isoform of high-affinity antibodies. We therefore sought to determine the independent roles of T-bet in T and B cells for antibody class switching. Initially, we identified the level of surface IgG1 and IgG2a/c present on GC B cells at d8 following influenza infection. Comparing complete *Tbx21*$^{-/-}$ animals with controls, we showed not only the expected defect in IgG2a/c but a reciprocal increase in IgG1 (Figures 7A and 7B). GC B cells from *Tbx21*ΔT mice showed no deficiency in IgG2a/c but an increase in IgG1 GC B cell surface staining (Figures 7C and 7D). In contrast, *Tbx21*ΔB GC B cells showed reduced IgG2a/c surface staining as previously described, but no change in IgG1 (Figures 7E and 7F) (Peng et al., 2002; Piovesan et al., 2017). CD138^{+} plasma cells also showed a similar switching bias to GC B cells in each genotype examined (data not shown). We next investigated how differences in isotype switching affect the serum concentration IgG isotypes over the course of influenza infection. Consistent with our IgG isotype surface staining, *Tbx21*$^{-/-}$ animals showed increased IgG1 and decreased IgG2a/c levels over the course of infection (Figures 7G and 7H). Again, T cell-intrinsic T-bet deficiency resulted in increased serum IgG1 (Figures 7I and 7J), while B cell-intrinsic T-bet deficiency resulted in increased serum IgG2a/c (Figures 7K and 7L). Together these cell intrinsic phenotypes observed in individual T and B cell *Tbx21*-deficient animals summed to recapitulate the isotype switching phenotype seen in *Tbx21*$^{-/-}$ animals. Thus, T-bet acts independently in TFH and GC B cells to tailor isotype switching following influenza infection.

DISCUSSION

In this study, we identify T-bet as a molecular switch that determines CD4^{+} T cell differentiation outcomes following different viral infections. In comparison with TH1 cells, TFH and B cells express lower levels of *Tbx21* throughout the immune response to influenza infection. This graded expression between populations is reminiscent of that seen in CD8^{+} cells with different effector

Figure 4. Viral-Specific Induction of T-bet Alters Proliferation, Activation, and Transcription Factor Ratios during T Cell Differentiation

(A and B) ZsGreen_T-bet reporter mice were infected with influenza or naive ZsGreen_T-bet STg cells were transferred prior to LCMV infection. Draining lymph nodes (influenza) and splenocytes (LCMV) were harvested at d4 and d8 p.i. Representative flow cytometry plots (A) and MFI of activated (d4) and NP_{311} (d8) and STg TFH (red) and TH1 (blue) (B) in indicated infection models.

(C–J) CD4cre and *Tbx21*ΔT mice were infected with influenza or naive STg or Tbx21$^{-/-}$ STg cells were transferred prior to LCMV infection and analyzed at d4 and d8 p.i. Activated T cells (CD4^{+}CD44^{+}) and tetramer^{+} (NP_{311}^{+}) were assessed respectively for d4 and d8 p.i. influenza infection. STg cells were assessed for LCMV time points. Viral specific cells were assessed for Ki67 MFI (C and D), PD-1 MFI (E and F), CXCR3 MFI (G and H), and CD25 MFI (I and J). Representative histograms show frequency in positive gate.

Data are representative of two or three independent experiments, n = 3–5 mice/group. Data are mean ± SEM.

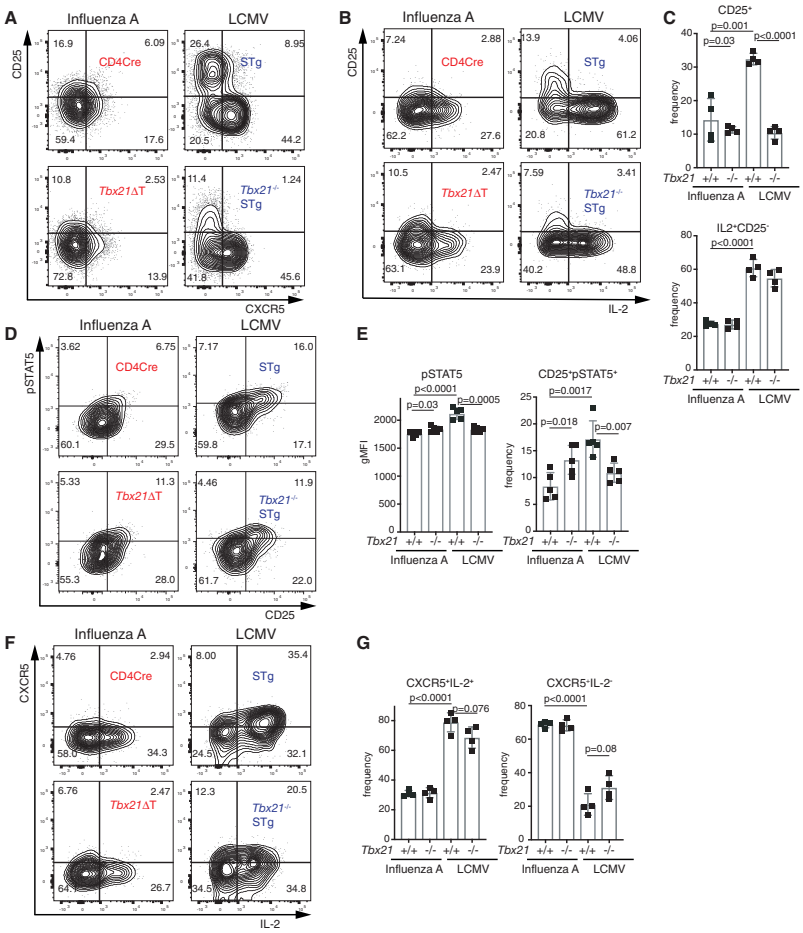

Figure 5. Differential Production and Consumption of IL-2 in Influenza and LCMV Infection Models

CD4cre and *Tbx21*ΔT mice were infected with influenza or naive STg or Tbx21$^{-/-}$ STg cells were transferred prior to LCMV infection and analyzed at d4 p.i. Activated T cells (CD4^{+}CD44^{+}) and STg cells were assessed for influenza and LCMV infections, respectively.

(A–C) Representative flow cytometry plots (A and B) and frequency (C) of CD25^{+} and IL-2^{+} cells in indicated models. Note that re-stimulation for IL-2 detection resulted in lower detection of CD25 (compare A and B), and quantification (C) was performed on samples without re-stimulation.

(D and E) Representative plots (D) and frequency (E) of pSTAT5 and CD25 following IL-2 stimulation.

(F and G) Representative plots (F) and frequency (G) of IL-2^{+} and IL-2^{-} out of CXCR5^{+} gate.

Data are representative of two independent experiments, n = 4 or 5 mice/group. Data are mean ± SEM.

and memory potential (Dominguez et al., 2015; Joshi et al., 2007). T-bet has been shown to be differentially induced following infections with distinct cytokine skewing, for example, TH1 and TH2-skewed infection models (Wang et al., 2019; Zhu et al., 2012). Here, we describe that T-bet expression is graded in cell subsets even between viral models that are thought to evoke similar cytokine skewing. It is interesting to speculate if an overarching rheostat for T-bet expression exists in TFH cells to determine these context-specific differentiation outcomes. We have previously shown that c-Myb can act in this manner in GC B cells to instruct the levels of T-bet depending on the environmental context (Piovesan et al., 2017).

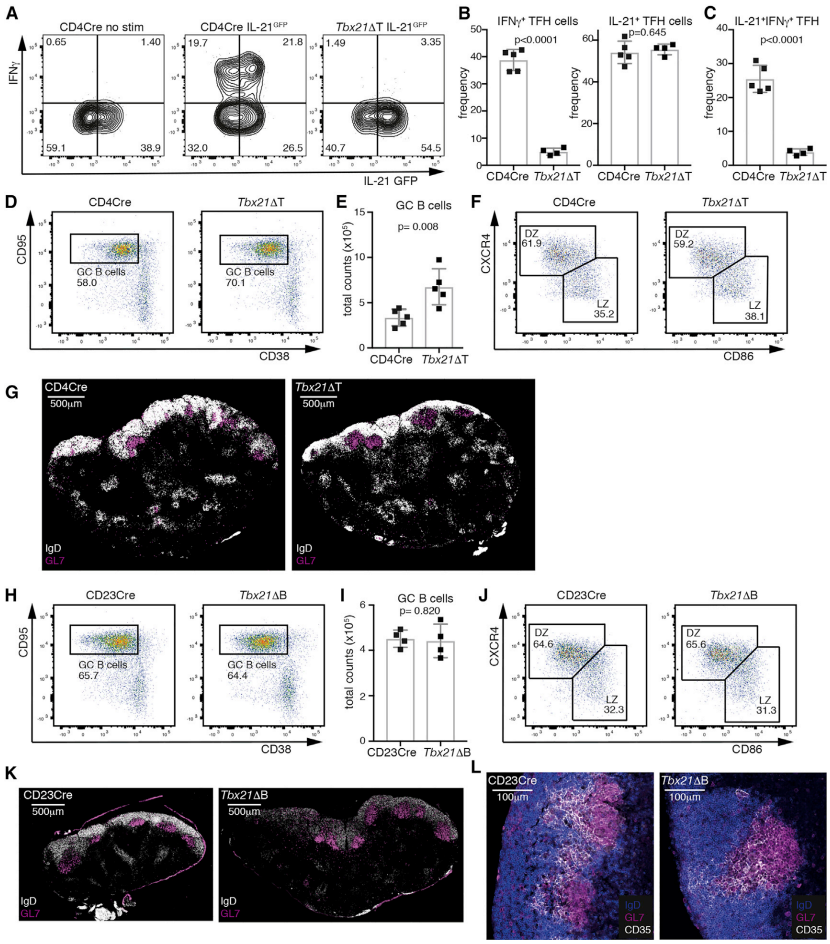

Figure 6. T Cell-Intrinsic T-bet Deficiency Regulates TFH Cytokine Production, While Neither T Cell- or B Cell-Intrinsic T-bet Deficiency Overtly Affects GC Structure

(A–C) CD4Cre_IL-21^{GFP} and *Tbx21*ΔT_IL-21^{GFP} mice were infected with influenza, and dLN TFH (CD44^{+}PSGL-1^{-}Ly6c^{-}PD-1^{+}CXCR5^{+}) cells were re-stimulated and analyzed d8 p.i. Representative flow cytometry plots (A) and frequency (B and C) of IFNγ and IL-21^{GFP} in indicated mice.

(D–G) CD4cre and *Tbx21*ΔT mice were infected with influenza and dLNs analyzed at d8 p.i. Representative flow cytometry plots (D) and total number of GC B cells (B220^{+}IgD^{-}CD95^{+}CD38^{-}) (E). Plots of DZ (CXCR4^{+}CD86^{-}) and LZ (CXCR4^{-}CD86^{+}) GC B cells (F). Confocal micrographs of dLN (white, IgD^{+} B cell follicles; magenta, GL7^{+} GCs) from indicated mice (G).

(H–L) CD23cre and *Tbx21*ΔB mice were infected with influenza, and dLNs were analyzed at d8 p.i. Representative flow cytometry plots (H) and total number of GC B cells (B220^{+}IgD^{-}CD95^{+}CD38^{-}) (I). (J) Plots of DZ (CXCR4^{+}CD86^{-}) and LZ (CXCR4^{-}CD86^{+}) GC B cells. Confocal micrographs of dLNs (white, IgD^{+} B cell follicles; magenta, GL7^{+} GCs) from indicated mice (K). Confocal micrographs of dLN GCs from indicated mice (blue, IgD^{+} B cell follicles; magenta, GL7^{+} GCs; white, CD35^{+} LZ) (L).

In (A)–(F) and (H)–(J), data are representative of three independent experiments, n = 3–5 mice/group. In (G), (K), and (L), data are representative of two independent experiments, n = 3–5 mice/group. Data are mean ± SEM.

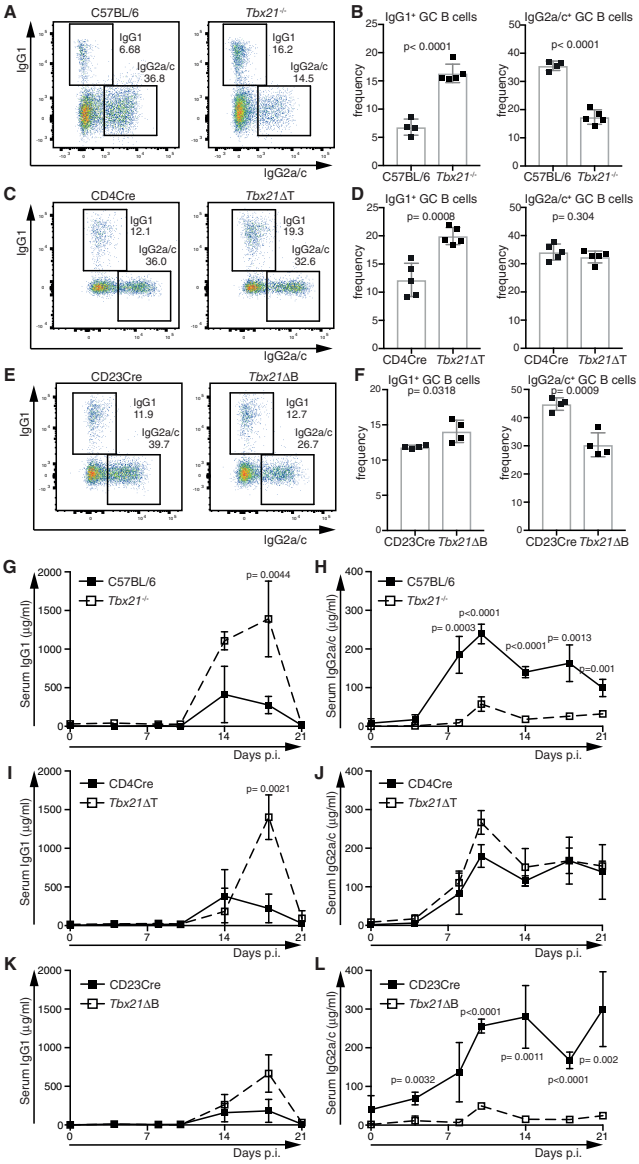

Figure 7. Cell-Intrinsic Role for T-bet in Isotype Class Switching

(A–F) C57BL/6 and *Tbx21*$^{-/-}$ (A and B), CD4Cre and *Tbx21*ΔT (C and D), and CD23Cre and *Tbx21*ΔB (E and F) mice were infected with influenza, and dLNs were analyzed at d8 p.i. (A, C, and E) Representative flow cytometry plots and (B, D, and F) frequency of IgG1 and IgG2a/c surface staining on GC B cells (B220^{+}IgD^{-}CD95^{+}CD38^{-}).

(G–L) C57BL/6 and *Tbx21*$^{-/-}$ (G and H), CD4Cre and *Tbx21*ΔT (I and J), and CD23Cre and *Tbx21*ΔB (K and L) mice were infected with influenza and total serum IgG1 (G, I, and K) and IgG2a/c (H, J, and L) concentrations analyzed at indicated p.i.

In (A)–(F), data are representative of three independent experiments, n = 3–5 mice/group. In (G)–(L), data are representative of two independent experiments, n = 3 or 4 mice/group. Data are mean ± SEM.

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IL-2 signaling plays a central role in the determination of TH1 and TFH bifurcation (Ballesteros-Tato et al., 2012; Choi et al., 2011; DiToro et al., 2018), whereby IL-2 restricts Bcl6 expression and promotes the differentiation of TH1 cells (Oestreich et al., 2012). TGFβ also acts upstream of CD25 expression to inhibit IL-2 signaling (Marshall et al., 2015). Similarly, IL-7 and type I IFNs repress the TFH program (McDonald et al., 2016; Ray et al., 2014), while IL-6 promotes TFH expansion and functional GC reactions (Eto et al., 2011; Harker et al., 2011). We show distinct IL-2 expression between our model infections, but other inflammatory cytokines may also drive altered T-bet induction in these systems. IFNγ plays a critical role in promoting T-bet in TH1 cells, while IL-12 drives T-bet expression in $CD8^+$ T cells (Joshi et al., 2007; Kallies and Good-Jacobson, 2017). Our data suggest that the distinct infection routes (intranasal and intravenous) and level of viral tropism (low and high, respectively) for influenza and LCMV contribute to the differential cytokine production and inflammatory cell recruitment present at the time of T cell priming. Together these viral factors, along with antigen affinity and viral load are intertwined with environmental cytokines to determine the upregulation and overall balance of T-bet and Bcl6 within TFH and GC B cells (DiToro et al., 2018; Fahey et al., 2011; Harker et al., 2011; Snook et al., 2018). That these distinctions alter the role of T-bet during TFH differentiation shifts the dogma of viewing all viral infections in a canonical manner to one in which individual inflammatory and viral factors should be considered when assigning roles for transcriptional regulation of T cell differentiation.

Here we show that T-bet is differentially induced between two important and widely used TH1-biased infection models. Specifically, strong expression in TFH and TH1 precursors in LCMV and TH1 precursors in influenza infection reflects the essential role for T-bet in differentiation of these cell fates. Conversely the lower expression seen in TFH precursors in influenza correlates with the accumulation of these cells in the absence of T-bet. These divergent infections models also uniquely influence the T cell precursor ratios of IL-2 consumers and producers, and this is further augmented with T-bet deficiency. Although T cell differentiation during LCMV infection adheres to the current paradigm that IL-2 production and signaling determine the fate of $CD4^+$ cells in viral infection, this segregation is less clear for influenza, in which T-bet expression and cell fate decisions appear to occur later in infection. This delay potentially allows influenza-specific TFH precursors to pass a survival checkpoint that is not permitted when T-bet is highly induced.

Our findings clarify previous contradictory findings for the role of T-bet in TH1/TFH bifurcation by indicating that transcription factor thresholds are different depending on the environmental context of T-bet and Bcl6 upregulation. Several previous studies have reported that T-bet intrinsically tips the balance of $CD4^+$ T cell differentiation in favor of TH1 cell differentiation in multiple infectious settings (Marshall et al., 2011; Nakayamada et al., 2011; Oestreich et al., 2012; Ryg-Cornejo et al., 2016). Our results in intact animals are also in line with human TFH cells, where T-bet does not inhibit the differentiation of $CXCR5^+$ TFH-like cells (Schmitt et al., 2016). In contrast, we show, along with Weinstein et al. (2018), that the presence of competing T cells, which may influence the availability of the proliferative and differentiation cytokines or antigen, is a key factor in determining the role of T-bet in TH1/TFH fate outcomes (Weinstein et al., 2018). Combined, this work highlights distinct induction of T-bet in specific experimental systems and emphasizes the importance of understanding T-bet modulation when considering vaccination strategies when TFH IFNγ production and/or specific IgG class switching is a major goal.

Within GCs, TFH cells and B cells upregulate a core group of transcription factors (Good-Jacobson and Groom, 2018). Our data suggest that despite shared expression, T-bet acts independently in each cellular compartment to influence class switching following viral infection. Although switching of antibody to the IgG2a/c isotype mediates clearance of virus and protection against lethal influenza infection, IgG1 is required for optimal vaccine efficacy (Huber et al., 2006; Miyauchi et al., 2016). Thus, the overall distribution of antibody isotype is critical for immune protection. T-bet plays a central role in this balance, acting both intrinsically in GC B cells to promote IgG2a/c and indirectly, presumably through TFH cytokine production, to lower IgG1 production. Combined, these results identify T-bet as a context-dependent link between extrinsic inflammatory signals and intrinsic cellular differentiation programs and highlights the importance of understanding the context of T-bet induction for the development of protective, neutralizing antibodies following viral infection and vaccination.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2019.07.034.

ACKNOWLEDGMENTS

We thank members of the Groom lab for technical assistance and Leisa-Rebecca Watson, who performed initial experiments. This work was supported by National Health and Medical Research Council (NHMRC) project grants to J.R.G. and K.L.G.-J. (GNT1137989 and GNT1057707) and to M.P. (GNT1006592, GNT1045549, and GNT1065626) and by NIH grants to J.J.M. (R01 AI107020 and R21 AI124143). A.A.S. and B.C.D. are supported by a University of Melbourne research scholarship. J.R.G. is supported by an Australian Research Council Future Fellowship (FT130100708). K.L.G.-J. is supported by an NHMRC Career Development Fellowship (1108066). G.T.B. and S.L.N. are supported by an NHMRC Senior Principal Research Fellowship. K.L.G.-J. and M.P. are supported by Bellberry-Viertel and Sylvia & Charles

Viertel Senior Medical Research Fellowships. L.C. received a Biomedicine Discovery Institute Scholarship from Monash University. F.L. is supported by a Walter and Eliza Hall Centenary Fellowship sponsored by CSL. This work was made possible through Victorian State Government Operational Infrastructure Support and the Australian Government NHMRC Independent Research Institute Infrastructure Support Scheme (IRIISS).

AUTHOR CONTRIBUTIONS

J.R.G. designed the research. A.A.S. performed the majority of the experiments. L.C., M.F., and F.S.-F.-G. performed additional experiments. K.L.G.-J., G.T.B., S.L.N., M.P., J.J.M., F.L., B.C.D., and N.D.H. provided technical assistance, reagents, and intellectual input. J.R.G. and A.A.S. analyzed the experiments and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no conflicts of interest.

Received: October 9, 2018
Revised: May 29, 2019
Accepted: July 11, 2019
Published: August 13, 2019

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Brilliant Ultraviolet 395-conjugated anti-CD3e (clone 145-2C11)	BD Horizon	Cat#563565; RRID:AB_2738278
Brilliant Ultraviolet 737-conjugated anti-CD4 (clone GK1.5)	BD Horizon	Cat#564298; RRID:AB_2738734
Brilliant Violet 711-conjugated anti-CD162 (clone 2PH1)	BD OptiBuild	Cat#740746; RRID:AB_2740414
APC-Cy7-conjugated anti-CD44 (clone 1M7)	BD PharMingen	Cat#560568; RRID:AB_1727481
Alexa Fluor 488-conjugated anti-Ki-67 (clone B56)	BD PharMingen	Cat#561165; RRID:AB_10611866
Brilliant Violet 711-conjugated anti-CD138 (clone 281-2)	BD Horizon	Cat#563193; RRID:AB_2738060
Brilliant Violet 605-conjugated anti-CD86 (clone GL1)	BD Horizon	Cat#563055; RRID:AB_2737977
Brilliant Violet 510-conjugated anti-B220/CD45R (clone RA3-6B2)	BD Horizon	Cat#563103; RRID:AB_2738007
FITC-conjugated anti-Ig2a/2b (clone R2-40)	BD PharMingen	Cat#553399; RRID:AB_394837
PE-Cy7-conjugated anti-CD95 (clone Jo2)	BD PharMingen	Cat#557653; RRID:AB_396768
Brilliant Ultraviolet 737-conjugated anti-CD127 (clone SB/199)	BD Horizon	Cat#564399; RRID:AB_2738791
Brilliant Ultraviolet 395-conjugated anti-CD8a (clone 53-6.7)	BD Horizon	Cat#563786; RRID:AB_2732919
Brilliant Violet 786-conjugated anti-CD69 (clone H1.2F3)	BD Horizon	Cat#564683; RRID:AB_2738890
Brilliant Violet 786-conjugated anti-CD25 (clone 3C7)	BD Horizon	Cat#564368; RRID:AB_2738771
Brilliant Violet 711-conjugated anti-KLRG1 (clone 2F1)	BD Horizon	Cat#564014; RRID:AB_2738542
PE-Cy7-conjugated anti-IFNγ (clone XMG1.2)	BD PharMingen	Cat#557649; RRID:AB_396766
Brilliant Violet 786-conjugated anti-IgG1 (clone X56)	BD OptiBuild	Cat#742480; RRID:AB_2740814
Brilliant Violet 510-conjugated anti-Ly-6C (clone HK1.4)	Biolegend	Cat#128033; RRID:AB_2562351
PE/Dazzle 594-conjugated anti-CD279 (PD-1) (clone RMP1-30)	Biolegend	Cat#109116; RRID:AB_2566548
Brilliant Violet 421-conjugated anti-CD279 (PD-1) (clone RMP1-30)	Biolegend	Cat#109121; RRID:AB_2687080
Brilliant Violet 421-conjugated anti-CD183 (CXCR3) (clone CXCR3-173)	Biolegend	Cat#126522; RRID:AB_2562205
Brilliant Violet 605-conjugated anti-CD185 (CXCR5) (clone L138D7)	Biolegend	Cat# 145513; RRID:AB_2562208
PerCP-Cy5.5-conjugated anti-CD62L (clone MEL-14)	Biolegend	Cat#104432; RRID:AB_2285839
PE-Cy7-conjugated anti-T-bet (clone 4B10)	eBioscience	Cat#25582582; RRID:AB_11042699
PerCP-Cy5.5-conjugated anti-FOXP3 (clone FJK-16 s)	eBioscience	Cat#45-5773-82; RRID:AB_914351
PerCP-eFluor 710-conjugated anti-CD38 (clone 90)	eBioscience	Cat#46038180; RRID:AB_10852870
PE-conjugated anti-CD184 (CXCR4) (clone 2B11)	eBioscience	Cat#12-9991-81; RRID:AB_891393
PerCP-eFluor 710-conjugated CD122 (clone TM-beta1)	eBioscience	Cat#46-1222-82; RRID:AB_11064442
PE-Cy7-conjugated anti-Phospho- STAT5 (Tyr694) (clone SRBCZX)	eBioscience	Cat#25-9010-41; RRID:AB_2573533
Alexa Fluor 647-conjugated anti-IgD (clone 1126c)	Produced in house	N/A
Alexa Fluor 647-conjugated anti-BCL6 (clone 7D1)	Produced in house	N/A
Alexa Fluor 647-conjugated anti-CD4 (clone GK1.5-7)	Produced in house	N/A
Biotin-conjugated anti-IgD (clone 11-26)	SouthernBiotech	Cat#1120-08; RRID:AB_2631189

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Alexa Fluor 555-conjugated anti-B220 (clone RA3-6B2)	Produced in house	N/A
Alexa Fluor 594-conjugated anti-CD4 (clone GK1.5-7)	Produced in house	N/A
Alexa Fluor 488-conjugated anti-CD4 (clone GK1.5-7)	Produced in house	N/A
Alexa Fluor 647-conjugated anti-GL7 (clone GL7)	Produced in house	N/A
Cy3 conjugated Streptavidin	Jackson Immunoresearch	Cat#016-160-084; RRID:AB_2337244
Dylight 405 conjugated Streptavidin	Jackson Immunoresearch	Cat#016-470-084; RRID:AB_2337248
Brilliant Violet 421-conjugated anti-CD21/CD35 (clone 7G6)	BD Horizon	Cat#562756; RRID:AB_2737772
IgG1-HRP (clone Goat polyclonal)	Southern Biotech	N/A
Unlabeled IgG1 (Goat polyclonal)	Southern Biotech	N/A
IgG1-κ (MOPC 31C)	Sigma-Aldrich	N/A
Unlabeled IgG2a (Goat polyclonal)	Southern Biotech	N/A
IgG2c-HRP (Goat polyclonal)	Southern Biotech	N/A
Bacterial and Virus Strains		
Influenza virus strain HKx31 (H3N2)	Produced in house	N/A
LCMV Armstrong	Produced in house	N/A
Biological Samples		
Mouse tissues	N/A	N/A
Chemicals, Peptides, and Recombinant Proteins		
BD Cytofix/Cytoperm	BD	Cat#554714
BioMag Goat Anti-Rat IgG	QIAGEN	Cat#310107
PHORBOL 12-MYRISTATE 13-ACETATE (PMA)	Sigma-Aldrich	Cat#P1585
Ionomycin	Sigma-Aldrich	Cat#I0634
$NP_{311-325}$:1-A^b-PE (Influenza A $NP_{311-325}$ peptide sequence: QVYSLIRPNENPAHK)	NIH tetramer core	N/A
GP_{66-77}:1-A^b-PE (LCMV GP_{66-77} peptide sequence: DIYKGVYQFKSV)	Moon et al., 2011	N/A
Protein Transport Inhibitor (containing Brefeldin A)	BD	Cat#555029
Protein Transport Inhibitor (containing Monensin)	BD	Cat#554724
FVD eFluor 506	eBioscience	Cat#65-0866
Fixable Viability Stain 700	BD Horizon	Cat#564997
Critical Commercial Assays		
LS columns	Miltenyi Biotec	Cat#130-042-401
Naive $CD4^+$ T Cell Isolation Kit	Miltenyi Biotec	Cat#130-104-453
Brilliant Stain Buffer	BD Horizon	Cat#563794
Foxp3/Transcription Factor Staining Buffer Set	invitrogen	Cat#00-5523
Perm Buffer III	BD Biosciences	Cat# 558050
Lyse/Fix Buffer 5x	BD Biosciences	Cat# 558049
Software and Algorithms		
Prism	Graph Pad	https://www.graphpad.com/scientific-software/prism/
Flowjo (Treestar)	FlowJo, LLC	https://www.flowjo.com/
Zen Black	ZEISS	https://www.zeiss.com/microscopy/int/products/microscope-software/zen.html
ImageJ	National Institutes of Health (NIH)	https://imagej.nih.gov/ij/index.html
Other		
Superfrost Plus Adhesion Microscope Slides	Thermo Scientific	Cat#J1800AMNT
16% Formaldehyde	Thermo Scientific	Cat#28908
O.C.T Compound	Tissue-Tek	Cat#4583

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by Lead Contact, Joanna Groom (groom@wehi.edu.au).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice and BM chimeras

Mice were maintained on a C57BL/6 background in specific-pathogen-free conditions. ZsGreen_T-bet reporter (Zhu et al., 2012), T-bet$^{fl/fl}$ (Wang et al., 2012), T-bet$^{fl/fl}$ CD23Cre (*Tbx21*ΔB) (Piovesan et al., 2017), *Tbx21*$^{-/-}$ (Finotto et al., 2002), Smarta TCR transgenic (STg) (Oxenius et al., 1998) mice have been previously described. T-bet$^{fl/fl}$ were crossed with CD4Cre to generate T-bet$^{fl/fl}$ CD4Cre (*Tbx21*ΔT). *Tbx21*ΔT and CD4Cre were bred with IL21^{GFP} (Lüthje et al., 2012) reporter mice. *Tbx21*ΔT mice were crossed to GFP and STg to generate GFP Stg and *Tbx21*ΔT STg respectively. Both male and female mice at 6-10 weeks of age were used in this study. Mixed chimeras were generated by lethally irradiated Ly5.1xC57BL/6 mice (two doses of 0.55 Cy) and reconstitution with *Tbx21*ΔT and Ly5.1 bone marrow in a 1:1 ratio. Mice were left for 8 weeks before infection. All experiments were performed in accordance with the Walter and Eliza Hall Institute animal ethics committee.

METHOD DETAILS

Viral infections and Adoptive transfer

Mice were inoculated intranasally with 1x10^4 PFU influenza virus strain HKx31 (H3N2) or intravenously with 2x10^5 or 3x10^3 PFU LCMV Armstrong for d4 and d8 experiments respectively. For adoptive transfer, STg CD4$^+$ T cell were isolated by naive CD4$^+$ T cell negative selection kit and 2.0 x10^5 (for d4) or 2.5x10^4 (for d8) naive GFP STg or Tbx21ΔT STg cells were transferred independently to non-irradiated naive recipient mice via intravenous injection. Mice were infected with LCMV the day following cell transfer.

Tetramer staining and Flow Cytometry

Tissues were mechanically dissociated to single cell suspensions and enriched for CD4$^+$ T cells following an incubation with cocktail of antibodies (listed in Key Resource Table) to deplete non-CD4$^+$ T cell lineages. Antibody-bound cells were removed by magnetic bead depletion using BioMag Goat anti-Rat IgG beads. Enriched CD4$^+$ T cells were stained with NP$_{311-325}$:1-A^b or GP$_{66-77}$:1-A^b, conjugated with streptavidin-PE tetramer for 1h at room temperature (Moon et al., 2011). Cells were stained using indicated antibodies (Key Resource Table) and intracellular proteins were detected using Foxp3 staining kit according to manufacturer's protocol. Cytokine intracellular staining experiment was performed as previously described (Lüthje et al., 2012). For pSTAT5 detection, T cells were stimulated in round bottom plates in the presence of 100U/mL murine recombinant IL-2 (DNAX Research Institute) for 25 min at 37 degrees. Intracellular staining of pSTAT5 fixation and permeabilization with Lyse/Fix and Perm III buffers (BD Biosciences) as previously described (Viel et al., 2016). Cytometry data was acquired using a LSR Fortessa x-20 (BD Biosciences).

Immunofluorescence staining

Lymph nodes were fixed in 4% paraformaldehyde and immersed in 30% sucrose before being embedded in OCT compound (Tissue-Tek). Tissues were cut via microtome (Leica) into 12-20 μm sections and mounted on Superfrost Plus slides. Sections were stained as described previously (Rankin et al., 2013) using described antibodies (Key Resource Table). Images were acquired using a LSM780 confocal microscope (Carl Zeiss MicroImaging). The acquisition software was Zen Black 2012 and images were quantified with ImageJ (NIH).

Enzyme-linked immunosorbent assay (ELISA)

Antibodies for detection of total serum are listed (Key Resource Table). 96-well flat-bottom ELISA plates (Sarstedt) were coated with 75 μL per well of respective unlabeled coating antibody (diluted in PBS) 4°C overnight. Plates were blocked with PBS 1% BSA at room temperature for 1h. Plates were washed three times with PBS Tween20 and dH2O. Samples and standards were added to the wells and blank wells were included (PBS 1%BSA). Plates were incubated for 2-4h at 37°C before being washed three times. Secondary antibody, HRP (horseradish peroxidase) diluted in PBS 1%BSA was added and incubated at 37°C for 2h. The plates were then washed five times and 100 μL of OPD (o-phenylenediamine dihydrochloride) substrate solution (ThermoFisher Scientific) was added and plates incubated room temperature in the dark for 10-30 minutes to develop. OD values were read at 405nm using a plate reader (BMG Labtech) and the data was evaluated using MARS (BMG Labtech).

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QUANTIFICATION AND STATISTICAL ANALYSIS

Flow cytometry was analyzed using Flowjo (Treestar) and statistical significance was determined using the paired (for chimeric experiments) or unpaired (two-tailed) Student's t test. For ELISA, differences between the groups were analyzed using the Mann-Whitney non-parametric two-tailed test with a 95% confidence. All experimental data is presented as mean ± standard error of the mean (SEM) with statistical analysis performed using Prism 7 (GraphPad Software).

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Supplemental Information

Context-Dependent Role for T-bet in T Follicular Helper Differentiation and Germinal Center Function following Viral Infection

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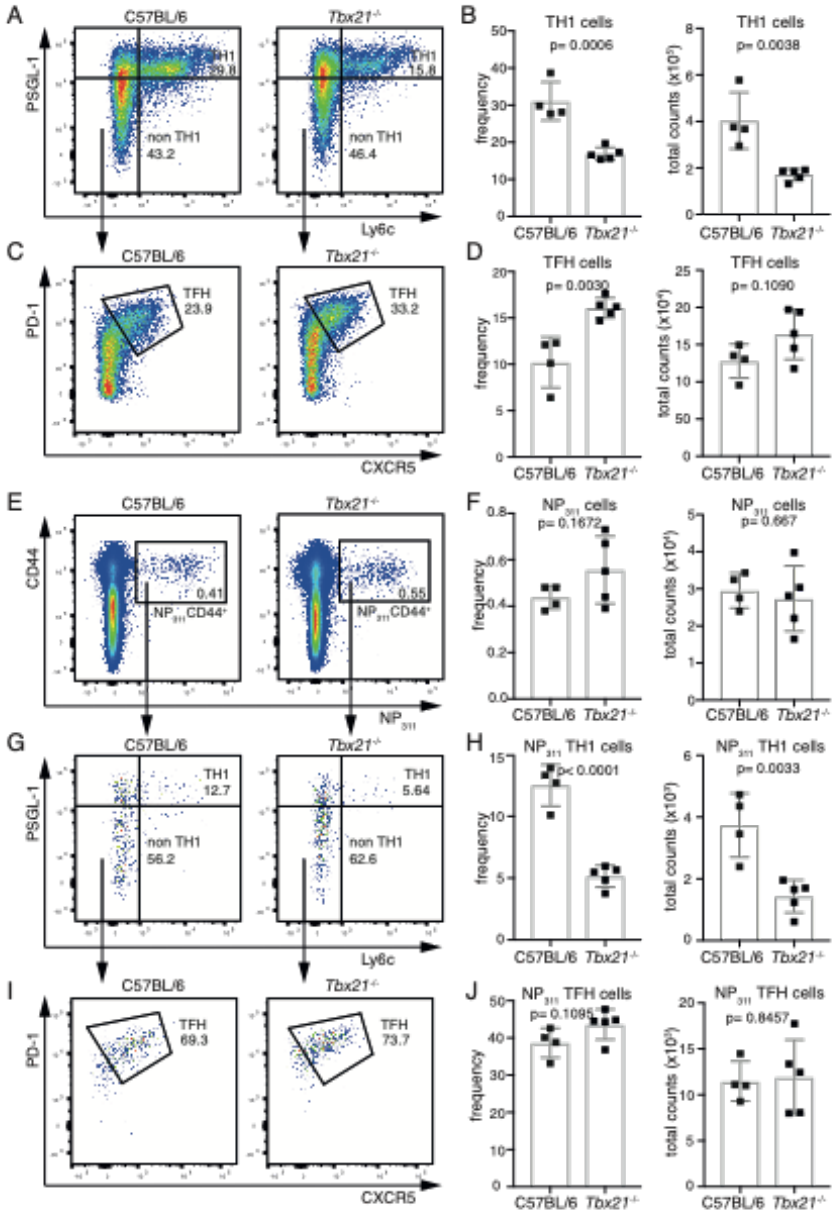

Figure S1. Tbx21-deficiency promotes TFH differentiation following influenza infection. Related to Figure 2. C57BL/6 and Tbx21-/- mice were infected with influenza and dLN CD4+CD44+ polyclonal and antigen-specific cells were analyzed at d8 p.i. (A, C) Representative flow cytometry plots of polyclonal CD4+CD44+ with (B, D) frequency and total number of natural repertoire of (A, B) TH1 (CD44+PSGL-1+Ly6c+) and (C, D) TFH (CD44+PSGL-1-Ly6c-PD1+CXCR5+). (E) Plots and (F) frequency and total number of NP311 CD4+ T cells. (G, I) Plots and (H, J) frequency and total number of (G, H) TH1 (NP311+CD44+PSGL-1+Ly6c+) and (I, J) TFH (NP311+ PSGL-1-Ly6c-PD-1+CXCR5+). Data is representative of 2 independent experiments, n= 3-5 mice/group. Data indicate mean +/- SEM.

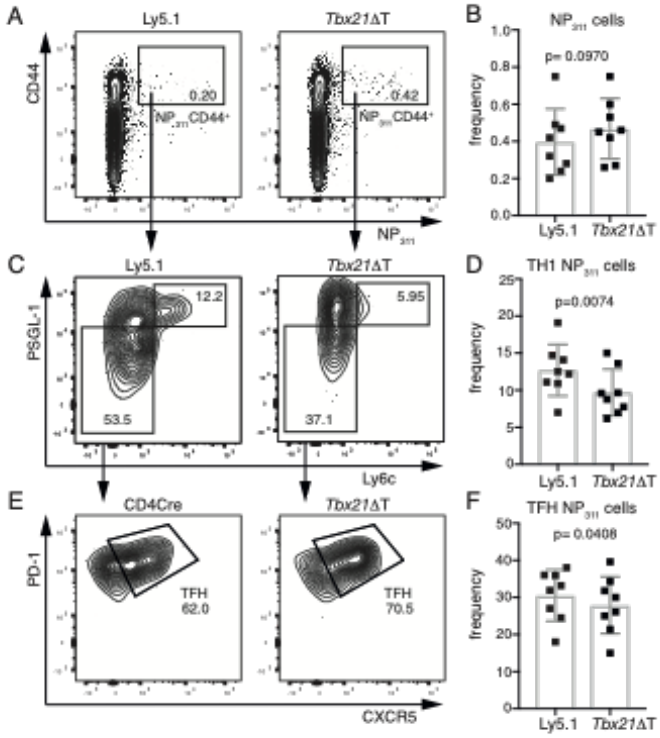

Figure S2. T cell-specific Tbx21-deficiency inhibits TFH differentiation in competitive environment. Related to Figure 3. Lethally irradiated Ly5.1xLy5.2 F1 mice were reconstituted with Ly5.1 and Tbx21ΔT (Ly5.2+) bone marrows at 50:50 ratio. Mice were infected with influenza and dLN CD4+CD44+ antigen-specific cells were analyzed at d8 p.i. (A) Representative flow cytometry plots and (B) frequency of NP311 CD4+ populations from indicated genotypes. (C, E) Plots and (D, F) frequency of (C, D) TH1 (NP311+CD44+PSGL-1+Ly6c+) and (E, F) TFH (NP311+ PSGL-1-Ly6c-PD-1+CXCR5+). Data is representative of 2 independent experiments, n= 4-8 mice/group. Data indicate mean +/- SEM.

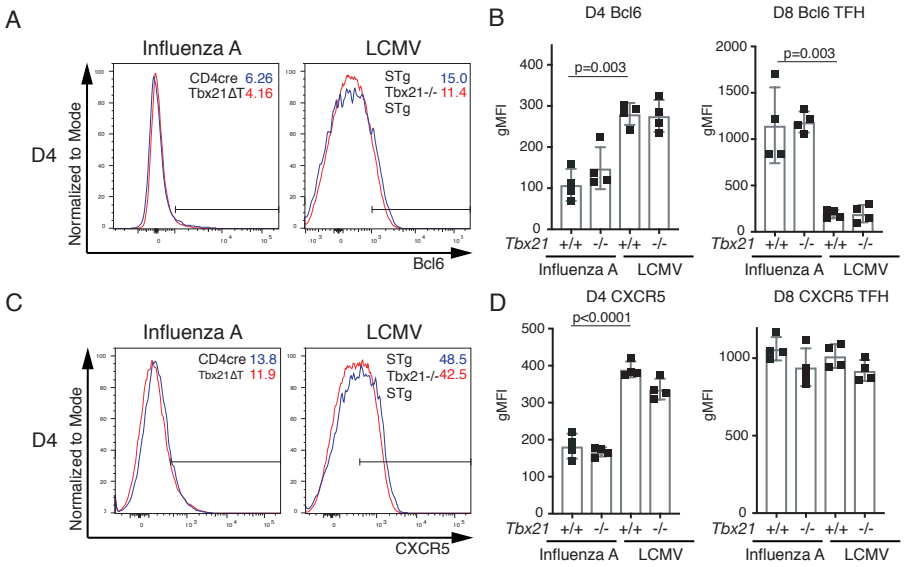

Figure S3. Viral-specific induction of markers of TFH differentiation. Related to Figure 4. CD4cre and Tbx21ΔT mice were infected with influenza or naïve STg or Tbx21-/- STg cells were transferred prior to LCMV infection and analyzed d4 and d8 p.i. Activated T cells (CD4+CD44+) and Tetramer+ (NP311+) were assessed respectively for d4 and d8 p.i. influenza infection. STg cells were assessed for LCMV timepoints. Viral specific cells were assessed for (A, B) Bcl6 MFI and (C, D) CXCR5 MFI. Representative histograms show frequency in positive gate. Data is representative of 2-3 independent experiments, n= 3-5 mice/group. Data indicate mean +/- SEM.

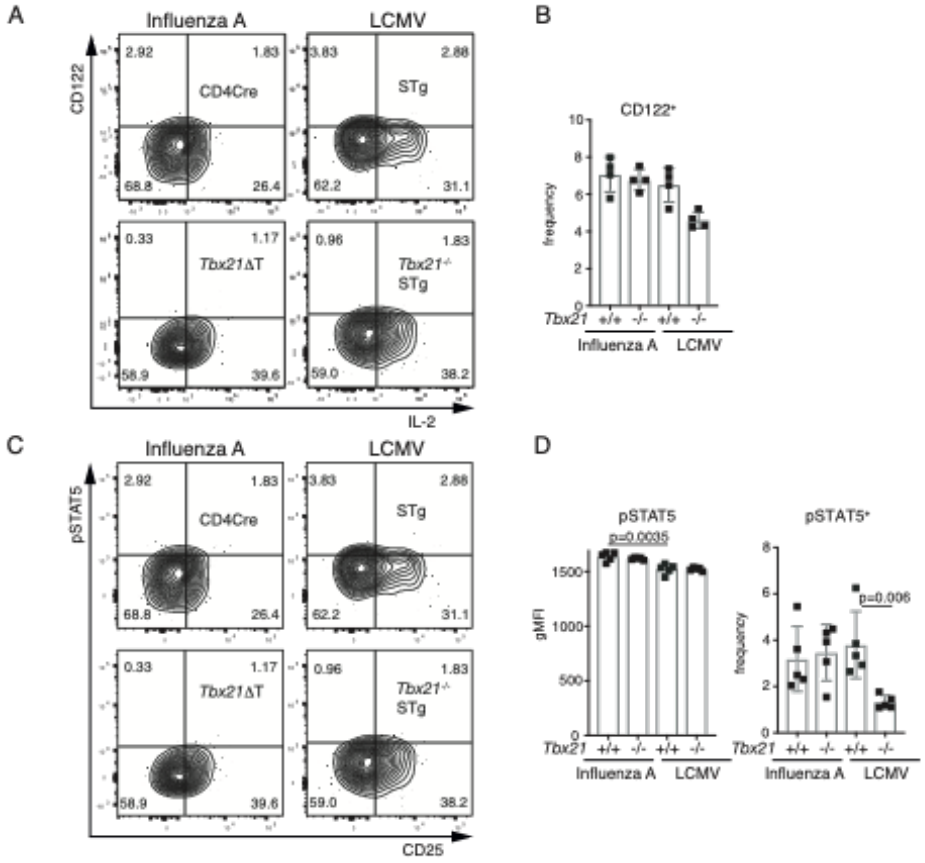

Figure S4. Differential production and consumption of IL-2 in influenza and LCMV infection models. Related to Figure 5. CD4cre and Tbx21ΔT mice were infected with influenza or naïve STg or Tbx21-/- STg cells were transferred prior to LCMV infection and analyzed d4 p.i. Activated T cells (CD4+CD44+) and STg cells were assessed in influenza and LCMV infections respectively. (A) Representative flow cytometry plots and (B) frequency of CD122+ in indicated models. (C) Representative plots and (D) MFI and frequency of pSTAT5 directly ex vivo without IL-2 stimulation. Data is representative of 2 independent experiments, n= 4-5 mice/group. Data indicate mean +/- SEM.

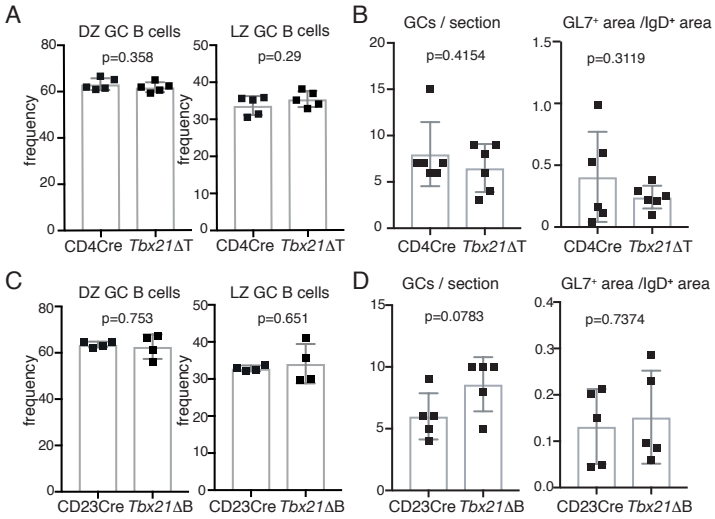

Figure S5. Quantification of GC phenotype in Tbx21ΔT and Tbx21ΔB mice following influenza infection. Related to Figure 6. (A-B) CD4cre and Tbx21ΔT mice were infected with influenza and dLN analyzed d8 p.i. (A) Frequency of DZ (CXCR4+CD86-) and LZ (CXCR4-CD86+) GC B cells. (B) Quantification of micrograph GC number and area from analysis Figure 6G. (C-D) CD23cre and Tbx21ΔB mice were infected with influenza and dLN analyzed d8 p.i. (C) Frequency of DZ (CXCR4+CD86-) and LZ (CXCR4-CD86+) GC B cells. (D) Quantification of micrograph GC number and area from analysis Figure 6K. (A, C) Data is representative of 3 independent experiments, n= 3-5 mice/group. (B, D) Data is representative of 2 independent experiments, n= 3-5 mice/group. Data indicate mean +/- SEM.

Chapter 3 T-bet-dependent TFH differentiation is influenced by chemokine receptor CXCR3

3.1 Introduction

TFH cells are a specialised $CD4^+$ T cell subset that govern B cell differentiation into antibody secreting plasma and memory B cells [6]. TFH cells are characterised by lineage defining transcription factor Bcl6, however, in viral settings TFH cells co-express canonical TH1 transcription factor T-bet [23, 24, 218]. T-bet expression is required for the differentiation of both TH1 and TFH cells in LCMV infection (as shown in **Chapter 2)**. High T-bet expression has been shown in fully differentiated TH1 cells and it is upregulated following $CD4^+$ T cell activation prior to T cell differentiation into TH1 cells [219, 220]. T-bet is induced in two distinct waves whereby initial signalling through the T cell receptor and IFN-γ upregulates T-bet expression in T cells. Later in the polarizing phase, IL-12 secreted by DCs induces and maintains T-bet expression which is required to imprint TH1 phenotype [219, 220, 221, 222]. In addition to T-bet expression in TH1 and TFH cells, $CD8^+$ T cells express T-bet in viral infections [223]. In this setting, T-bet is necessary for the differentiation of effector $CD8^+$ T cells [223, 224, 225]. A recent study showed that T-bet is not required for $CD8^+$ T cell proliferation in influenza infection [226]. T-bet, thus, appears to regulate differentiation in both $CD8^+$ and $CD4^+$ T cells [23, 24, 218, 223]. Potentially, T-bet expression is an important immune checkpoint for $CD4^+$ T cells to undergo proliferation, as it is upregulated following $CD4^+$ T cell activation, but the role of T-bet in $CD4^+$ T cell proliferation remains unknown.

The multifaceted role of T-bet in TH1 cells include differentiation and migration to the site of inflammation and infection [196, 227]. Importantly, T-bet regulates TH1 cell migration by positively regulating the expression of CXCR3 *in vivo* [227]. T-bet upregulates CXCR3 expression by forming a complex with Jmjd3 and UTX at the promoter region of *Cxcr3* loci, which alters chromatin structure and induces CXCR3 expression [214]. In addition, T-bet transactivates CXCR3 by increasing positive histone modification at the 5′ enhancer regions of the *Cxcr3* loci [200]. Supporting these findings, I demonstrated in **Chapter 2** that in the absence of T-bet, CXCR3 expression is severely reduced in viral infections [23]. Similarly, to TH1 cells, CXCR3 is highly expressed in $CD8^+$ T cells in viral infections [226, 228, 229, 230]. In $CD8^+$ T cells, CXCR3 is gradually upregulated and it is dispensable for early $CD8^+$ T cell activation or proliferation [228]. In $CD4^+$ T cells, CXCR3 is rapidly upregulated downstream of T-bet, prior to

$CD4^+$ T cell differentiation into TH1 cells, indicating a potential role of CXCR3 in $CD4^+$ T cell proliferation [216].

T-bet-mediated CXCR3 expression in both TH1 and $CD8^+$ T cells not only contributes to T-cell trafficking to the site of infection or inflammation, but it is required for T-cell positioning in the secondary lymphoid tissues [216, 227, 228]. Within the secondary lymphoid tissues, CXCR3 ligands CXCL9 and CXCL10 are preferentially expressed in the distinct regions allowing $CXCR3^+$ T cells sense these chemokine signals and migrate towards these chemokine rich regions [216]. Particularly, TH1 cells migrate to the interfollicular regions of the LN enriched in CXCR3 ligands produced by stromal and DC cells [216]. This intranodal positioning of TH1 cells to the interfollicular regions is essential for their differentiation and effector function [216]. In another study, $CD8^+$ T cells were shown to relocate to the marginal regions of the spleen in response to CXCL9 chemokine gradient, which facilitated $CD8^+$ T cell differentiation into effector instead of memory T cells [229]. These findings highlight that T cells rely on CXCR3-dependent positioning within distinct regions of the secondary lymphoid tissues which is essential for T cell differentiation.

TFH cells are colocalised with the B cells in the GCs, which are regions present in the centre of the B cell follicles. To reach GCs, early precursors of TFH (pre-TFH) cells migrate from the T cell zone towards the B cell follicles [6]. The coordinated upregulation of CXCR5 and EBI2 by pre-TFH cells assist in their migration from T cell zone to T:B border, which are regions rich in the EBI2 ligand, oxysterol [29]. The relocation of TFH cells within the GCs requires high levels of CXCR5 and PD-1 expression [76, 82, 89]. Following viral infections, the differentiation path of TFH and TH1 is interwoven as exemplified by the expression of prototypical TH1 molecules T-bet and CXCR3 by TFH cells [23, 213]. TFH cells rely on EBI2 and CXCR5-dependent migration to T:B border. However, it remains unresolved whether CXCR3 expression in TFH cells is another mechanism that regulates TFH cell migration to T:B border. It is possible that TFH cells utilise CXCR3-dependent positioning to T:B border as it has been demonstrated that CXCR3-CXCL10-dependent $CD4^+$ T cell interactions in the interfollicular regions promote GC formation and protective antibody responses following vaccination [217]. However, another study demonstrated that PD-1 suppressed CXCR3 expression in TFH cells to maintain their position in the GCs [82]. These two studies are in conflict and the role of CXCR3 in regulating TFH cell migration to T:B border and subsequent TFH differentiation is unclear.

The expression of CXCR3 and another chemokine receptor CCR6 have been used to define three distinct subsets of circulating TFH (cTFH) ($CXCR5^+CD4^+$) cells: TH1 like TFH-1

($CXCR3^+$ $CCR6^-$), TH2 like TFH-2 ($CXCR3^-$ $CCR6^-$) and TH17 like TFH-17 ($CXCR3^-$ $CCR6^+$) and are called T helper- like due to their similarities with conventional $CD4^+$ T helper subsets [231, 232]. Importantly, these cTFH cells exhibit different function for example human $CXCR3^+$ TH1 like cTFH cells were shown to induce memory B cell differentiation into influenza specific plasma cells following seasonal influenza vaccination [232, 233]. On the contrary, human $CXCR3^-$ cTFH cells were correlated with the development of neutralizing antibodies against HIV [234]. Therefore, current studies are insufficient to conclude whether CXCR3 expression in TFH cells regulate B cell help signals which are important for B cell differentiation into long lived plasma and memory B cells.

TFH cell differentiation is regulated by T-bet in LCMV infection (as shown in **Chapter 2**), however the mechanism of this is unknown. In this study, I explored the role of T-bet in $CD4^+$ T cell proliferation and if CXCR3 acts downstream of T-bet to regulate TFH cell differentiation and $CD4^+$ T cell proliferation. Here, I found that T-bet was rapidly induced in $CD4^+$ T cells prior to $CD4^+$ T cells entered the division phase following LCMV infection. High levels of T-bet expressing $CD4^+$ T cells attained maximum number of cell divisions detectable as compared to low T-bet expressing cells. Surprisingly, T-bet-deficient $CD4^+$ T cells demonstrated more cell division as compared to the wildtype (WT), which I speculate is due to expansion of pre-TFH cells. In $CD4^+$ T cells, CXCR3 was upregulated simultaneously with cell division and in the absence of CXCR3 there was no overt change in $CD4^+$ T cell proliferation. This indicates that T-bet regulates $CD4^+$ T cell proliferation in CXCR3-independent manner. At day 4 post LCMV infection, most of the pre-TFH cells were $CXCR3^+$ and CXCR3 expression in pre-TFH cells was upregulated by T-bet. *Cxcr3*$^{-/-}$ TFH cells showed reduced frequency as compared with WT cells in a less competitive environment following LCMV infection. In addition, in a competitive environment there was a higher frequency of *Cxcr3*$^{-/-}$ TFH cells as compared to the WT TFH cells. This suggests that the differentiation of TFH cells is a multifactorial process that could be tuned by antigens, cytokine and nutrients in the microenvironment. Complete CXCR3-deficient mice showed no change in the percentage of TFH cells and TFH-mediated GC B cell differentiation, GC B cells in LZ or DZ and B cell class switching to IgG2c in comparison with WT mice. Together, my current findings imply that CXCR3 has a role in TFH cell differentiation and positioning. Further experiments will determine the role of CXCR3 in TFH-mediated B cell responses in viral settings.

3.2 Results

3.2.1 T-bet induction in T cells occurs prior to cell proliferation

T-bet has been shown to regulate $CD4^+$ T cell differentiation following viral infections [223, 224, 225], while the role of T-bet in $CD4^+$ T cell proliferation in unclear. To understand how $CD4^+$ T cell are regulated, ZsGreen_T-bet reporter mice [200] were crossed to the SMARTA T cell transgenic (STg) mice [235] that recognise the lymphocytic choriomeningitis virus (LCMV)-derived glycoprotein gp61-80 epitope. Cell trace violet dye (CTV) and dsRED-labelled, naive ZsGreen_T-bet STg cells were transferred to individual C57BL/6 host. One day post adoptive cell transfer, mice were infected intravenously with LCMV Armstrong ($3x10^3$ pfu). Spleens were analysed on day 0, 1, 2, 3, 4 post infection to analyse T-bet expression in STg cells in relation to the division of cells marked by CTV dilution (**Figure 3.1**A). Consistent with another study [220], in this setting naive STg cells lacked ZsGreen_T-bet reporter expression (**Figure 3.1**A, **grey**). T-bet expression was induced in STg cells prior to cell division at day 1 post infection (**Figure 3.1**A, **green**). At day 2 post infection, STg cells **(red)** had undergone the first cell division and STg cells showed 8-10-fold higher ZsGreen_T-bet reporter expression than the peak of MFI of day 1 STg cells (**green**) (**Figure 3.1**A). At day 3, activated STg ($CD44^+$) cells demonstrated high, intermediate and low levels of ZsGreen_T-bet reporter expression (**Figure 3.1**B). This graded T-bet expression in STg cells is similar to that in $CD8^+$ T cell compartment, which regulates cell fate decisions between effector and memory differentiation [223, 236].

Given the range of different levels of T-bet expression in STg cells on day 3 post infection, I asked whether T-bet expression was correlated with the proliferation of STg cells (**Figure 3.1**B). To analyse this feature, STg cells were categorised into high, medium, low according to T-bet expression and the frequency of STg cells in each division was determined by CTV dilution. On day 3, higher ZsGreen_T-bet reporter expressing STg cells showed increased frequency of cells that had undergone maximum cell divisions as compared to low ZsGreen_T-bet reporter expressing STg cells (**Figure 3.1**B). Next, I sought to understand the mechanisms by which T-bet regulated cell proliferation. Signalling through IL-2/IL-2rα (high affinity IL-2 receptor subunit, also known as CD25) is associated with cell proliferation [237]. In line with this, high ZsGreen_T-bet reporter expressing STg cells ($CD44^+$) showed increased CD25 expression versus low T-bet expressing cells (**Figure 3.1**C). Together, these findings suggest that STg cells with high levels of T-bet expression have a proliferation advantage and this might be mediated through enhanced CD25 signalling. To formally determine whether T-bet-

expressing cells had a proliferation advantage, CTV-labelled WT or *Tbx21*$^{-/-}$ STg cells were adoptively transferred into a C57BL/6 mice and cell proliferation was analysed in each group on day 3 post infection. In this setting, *Tbx21*$^{-/-}$ STg cells proliferated more compared with WT STg cells, suggesting a correlation of T-bet expression with proliferation (**Figure 3.1**D).

Intriguingly, CD4^{+} *Tbx21*$^{-/-}$ STg cells expressed low levels of CD25, however, *Tbx21*$^{-/-}$ pre-TFH (CXCR5^{+}) proliferated more compared with WT pre-TFH cells (**Figure 3.1**D). These finding suggest that pre-TFH cells proliferate irrespective of T-bet expression (**Figure 3.1**D). Consistent with the increased CTV dilution observed at day 3, *Tbx21*$^{-/-}$ STg cells versus WT accumulated more in the spleens at day 4 post infection (**Figure 3.1**E). These results showed that at day 3, *Tbx21*$^{-/-}$ pre-TFH cells had undergone 1.5-fold more proliferation in comparison to WT pre-TFH cells. As a consequence, more *Tbx21*$^{-/-}$ STg cells are accumulated at day 4 in the spleen. Collectively, these findings suggest that T-bet promotes pre-TH1 cell proliferation, while T-bet is not required for pre-TFH cell proliferation.

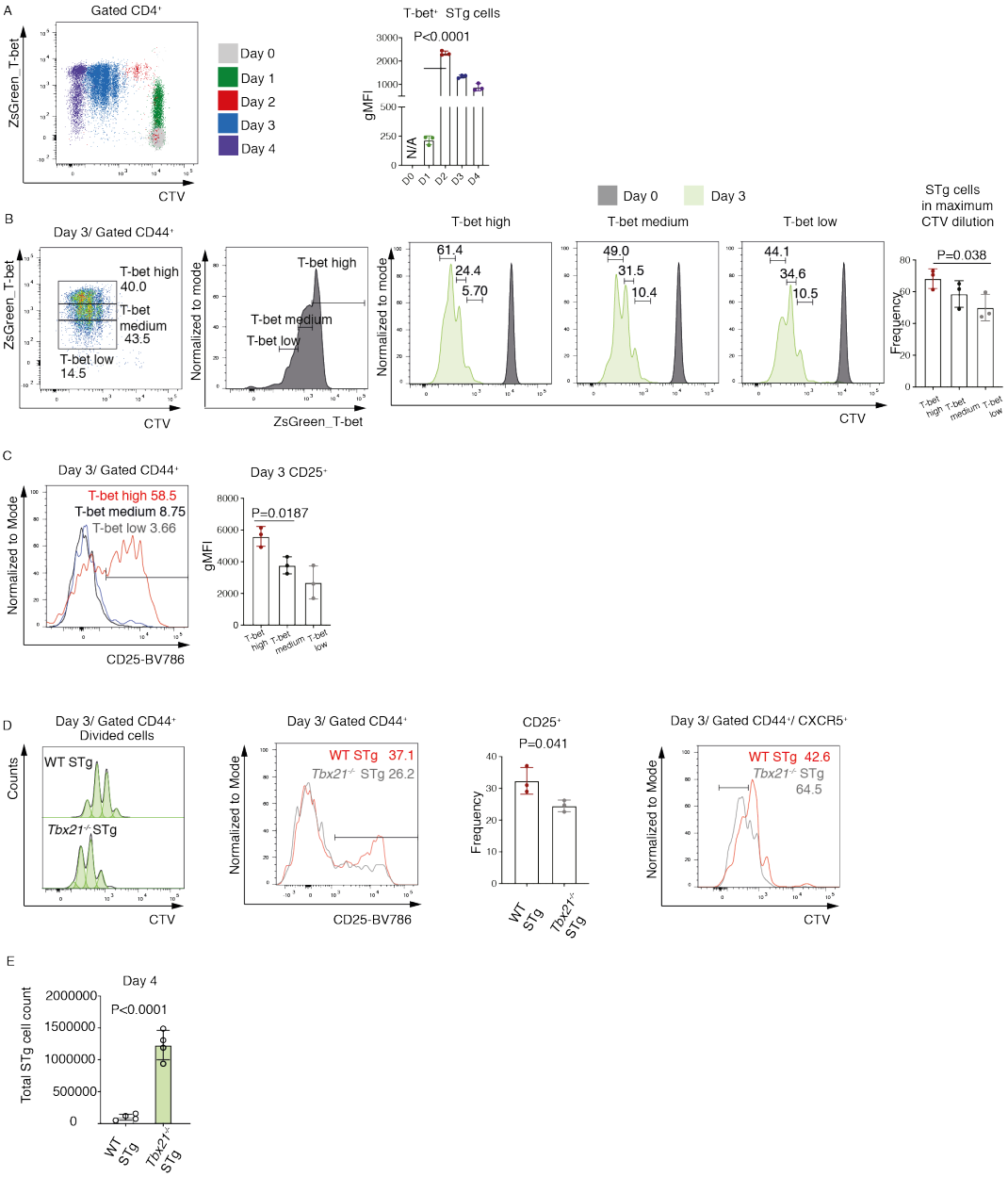
A
Gated CD4+
ZsGreen_T-bet
CTV
Day 0
Day 1
Day 2
Day 3
Day 4
T-bet+ STg cells
P<0.0001
gMFI
N/A
D0 D1 D2 D3 D4
B
Day 3/ Gated CD44+
T-bet high 40.0
T-bet medium 43.5
T-bet low 14.5
Normalized to mode
ZsGreen_T-bet
Day 0
Day 3
T-bet high
T-bet medium
T-bet low
61.4
24.4
5.70
49.0
31.5
10.4
44.1
34.6
10.5
STg cells in maximum CTV dilution
P=0.038
Frequency
C
Day 3/ Gated CD44+
T-bet high 58.5
T-bet medium 8.75
T-bet low 3.66
CD25-BV786
Day 3 CD25+
P=0.0187
D
Day 3/ Gated CD44+ Divided cells
WT STg
Tbx21-/- STg
Counts
Day 3/ Gated CD44+
WT STg 37.1
Tbx21-/- STg 26.2
CD25+
P=0.041
Day 3/ Gated CD44+/ CXCR5+
WT STg 42.6
Tbx21-/- STg 64.5
E
Day 4
P<0.0001
Total STg cell count

Figure 3.1 T-bet induction in T cells occur prior to cell proliferation

*(**A-C**) Naive dsRED_ZsGreen_T-bet reporter STg cells were adoptively transferred into C57BL/6 hosts prior to LCMV Armstrong infection. Spleens were analysed as indicated.*

*(**A**) Flow plot showing T-bet induction versus CTV dilution (indicating proliferation) and bar graph of T-bet MFI in STg cells.*

*(**B**) Flow plot and representative histogram of graded T-bet expression with CTV dilution at day 3 post infection. Representative histograms correlate T-bet expression with the frequency of STg cells in each CTV dilution. Bar graph showing the frequency of STg cells in maximum CTV dilution.*

*(**C**) Histogram of CD25 in high, medium and low ZsGreen_T-bet reporter expressing STg cells ($CD44^+$) at day 3 of infection. Numbers indicate frequency. Bar graph of CD25 MFI in high, medium and low ZsGreen_T-bet reporter expressing STg cells.*

(D-E) CTV-labelled WT STg or $Tbx21^{-/-}$ STg cells were adoptively transferred into individual C57BL/6 mice prior to LCMV Armstrong infection. Spleens were analysed as indicated.

*(**D**) Representative histograms showing WT and $Tbx21^{-/-}$ STg cells to compare CTV dilution. Representative histograms and bar graph of CD25 frequency in WT and $Tbx21^{-/-}$ STg cells. Representative histograms showing $CXCR5^+$ WT and $CXCR5^+Tbx21^{-/-}$ STg cell frequency in maximum CTV dilution.*

*(**E**) Total number of WT and $Tbx21^{-/-}$ STg cells in the spleen at day 4 post infection. Data are representative of two independent experiments, n = 3-4 mice/group. Data show the mean ± SEM.*

3.2.2 CD4$^+$ T cells proliferate in a CXCR3-independent manner

CXCR3 has been shown to mediate CD8$^+$ T cell differentiation following LCMV infection [228]. This led me to ask whether CXCR3-dependent interactions, acting downstream of T-bet might be important for sustained proliferation in CD4$^+$ T cells. To first examine this, CTV and dsRED-labelled, naive ZsGreen_T-bet STg cells were adoptively transferred into individual C57BL/6 host prior to LCMV infection. Spleens were analysed on day 0, 1, 2, 3, 4 post infection to compare CXCR3 expression and the division of cells marked by CTV dilution (**Figure 3.2**A). This showed that CXCR3 was first upregulated on day 2 (**red**) in ZsGreen_T-bet STg cells, one day after T-bet induction which occurred on day 1 post infection (**Figure 3.2**A). CXCR3 expression progressively increased from day 2 (**red**) to 4 (**purple**) with cell division marked by CTV dilution (**Figure 3.2**A), indicating that cell proliferation correlates with CXCR3 expression.

T-bet upregulates CXCR3 expression in CD4$^+$ T cells, under TH1 polarising condition [227]. To determine whether T-bet controls CXCR3 expression in STg cells following LCMV infection, naive WT STg and *Tbx21*$^{-/-}$ STg cells were adoptively transferred into individual C57BL/6 mice prior to LCMV infection. On day 4 post infection, spleens were analysed to determine CXCR3 expression in WT and *Tbx21*$^{-/-}$ STg cells (**Figure 3.2**B). In *Tbx21*$^{-/-}$ STg cells, there was 3-4-fold decrease in CXCR3 expression compared to WT STg cells, indicating that T-bet regulates CXCR3 expression in STg cells in this setting (**Figure 3.2**B).

To determine if CD4$^+$ T cell division occurs independent of CXCR3, naive CTV and GFP-labelled WT or *Cxcr3*$^{-/-}$ STg cells were transferred into C57BL/6 recipient mice prior to LCMV infection. Spleens were analysed on day 3 and 4 post infection to determine the role of CXCR3 in CD4$^+$ T cell proliferation (**Figure 3.2**C). *Cxcr3*$^{-/-}$ and WT STg cells demonstrated similar number of divisions completed on day 3 post infection (**Figure 3.2**C), thus, CXCR3 was not required for the induction of proliferation or subsequent divisions of CD4$^+$ T cells. However, the total number of *Cxcr3*$^{-/-}$ STg cells increased compared with WT cells *in vivo* (**Figure 3.2**C). This increase in cell number maybe be due to the impaired CXCR3-dependent migration to sites other than the spleen, consistent with the known role of CXCR3 in peripheral recruitment [199].

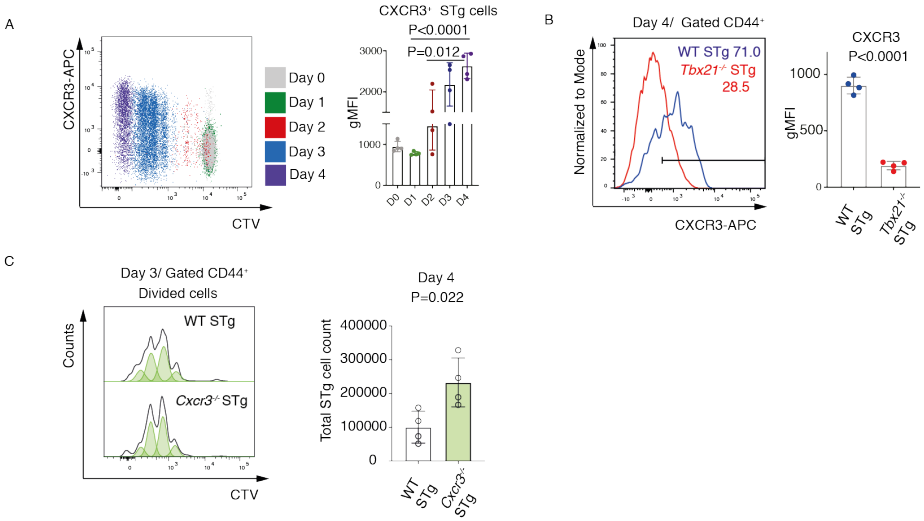

Figure 3.2 T-bet regulates CXCR3 expression in CD4$^+$ T cells and CD4$^+$ T cells proliferate in a CXCR3-independent manner

*(**A**) CTV-labelled naive ZsGreen_T-bet reporter WT STg were transferred into individual C57BL/6 mice prior to LCMV infection. Spleens were analysed on the indicated days. Flow plot of CXCR3 induction versus CTV dilution and bar graph of CXCR3 MFI in STg cells.*

*(**B**) Naive WT or Tbx21$^{-/-}$ STg cells were transferred into individual C57BL/6 mice prior to LCMV. Spleens were analysed at day 4 post infection. Histogram of CXCR3 expression in the WT and Tbx21$^{-/-}$ STg cells. Bar graph of MFI of CXCR3 in WT and Tbx21$^{-/-}$ STg cells.*

*(**C**) CTV-labelled naive ZsGreen_T-bet reporter WT or Cxcr3$^{-/-}$ STg cells were transferred into C57BL/6 mice prior to LCMV infection. Spleens were analysed as indicated. Representative histograms showing WT or Cxcr3$^{-/-}$ STg cells to compare CTV dilution. Total number of WT or Cxcr3$^{-/-}$ STg cells in the spleen at day 4 post infection. Data are representative of two independent experiments, n = 3-4 mice/group. Data show the mean ± SEM.*

3.2.3 T-bet regulates CXCR3 expression in pre-TFH following LCMV infection

TFH cells have been shown to express T-bet, which is required for TFH cell differentiation following LCMV infection (as shown in **Chapter 2**). The mechanism of T-bet in TFH differentiation remains unknown. Given the similarities between the initial migration pathways followed by TH1 and TFH cells [85, 216], T-bet may control TFH cell differentiation through its downstream target CXCR3 whereby CXCR3 regulates pre-TFH cell migration to T:B border. Migration to this niche is known to be an important step in the differentiation of pre-TFH cells to TFH cells [85]. To investigate whether T-bet regulates CXCR3 expression in pre-TFH cells, I first established the time when the $CD4^+$ T cells bifurcate into pre-TFH and pre-TH1 cells. Naive ZsGreen_T-bet STg cells were transferred into C57BL/6 recipient mice prior to LCMV infection. Spleen from these mice were analysed on each consecutive day over the first 4 days of infection (**Figure 3.3**A). Pre-TH1 and pre-TFH were distinguished by CD25 and CXCR5 expression respectively as previously described [76]. Pre-TFH/pre-TH1 cells demonstrated a slight increase in the expression of CD25 and CXCR5 on day 1 relative to day 0 post infection (**Figure 3.3**A). The expression of these markers was further upregulated on day 2 post infection, while at day 3, CD25 and CXCR5 expression became more discrete. On day 4, the expression of CD25 and CXCR5 further increased and pre-TFH ($CXCR5^+$) and pre-TH1 ($CD25^+$) cells were mutually exclusive populations (**Figure 3.3**A). These results demonstrated that $CD4^+$ T cells bifurcate into clearly populations of either pre-TFH and pre-TH1 cells on day 4. Thus, this timepoint was used in this study to analyse T-bet and CXCR3 expression in pre-TFH and pre-TH1.

To determine whether T-bet controls TFH cell differentiation through its downstream target CXCR3, I next examined T-bet expression in pre-TFH cells. Naive ZsGreen_T-bet STg cells were transferred into individual C57BL/6 recipient mice prior to LCMV infection. Spleen were analysed on day 4 post infection (**Figure 3.3**B). T-bet was highly expressed in pre-TH1 cells, while pre-TFH cells expressed intermediate to low level of T-bet (**Figure 3.3**B), suggesting moderate T-bet expression in pre-TFH cells might be sufficient to upregulate CXCR3. To test this hypothesis, I next determined CXCR3 expression in pre-TFH and pre-TH1 cells in the same experimental settings. Here, about 45% of STg cells were pre-TFH ($CXCR5^+$, $CXCR3^+$, **blue**). Importantly, the vast majority of these pre-TFH cells lacked expression of other TH1 markers (PSGL-1 and CD25, **blue**) (**Figure 3.3**C). In contrast to pre-TFH cells, about 25% of STg cells were pre-TH1 ($CXCR5^-$, $CXCR3^+$, **red**) and co-expressed TH1 markers (PSGL-1 and CD25, **red**) (**Figure 3.3**C). Interestingly, 20% of STg cells were $CXCR3^-$ (**black**) but

expressed high levels of TH1 markers (PSGL-1 and CD25, **black**), indicating that not all pre-TH1 cells are CXCR3^{+} (**Figure 3.3**C). These data suggest that the majority of the moderate T-bet expressing pre-TFH cells were CXCR3^{+}. Next, to investigate whether T-bet specifically regulates CXCR3 expression in pre-TFH, naive WT or *Tbx21*$^{-/-}$, STg cells were transferred into individual C57BL/6 mice prior to LCMV infection. Spleens were analysed on day 4 post infection. In *Tbx21*$^{-/-}$ STg cells, there was a significant decrease in CXCR3 expression compared with WT, indicating that T-bet directly regulates CXCR3 expression in pre-TFH cells (**Figure 3.3**D). Collectively, these findings indicate that pre-TFH cells express CXCR3 in a T-bet-dependent manner.

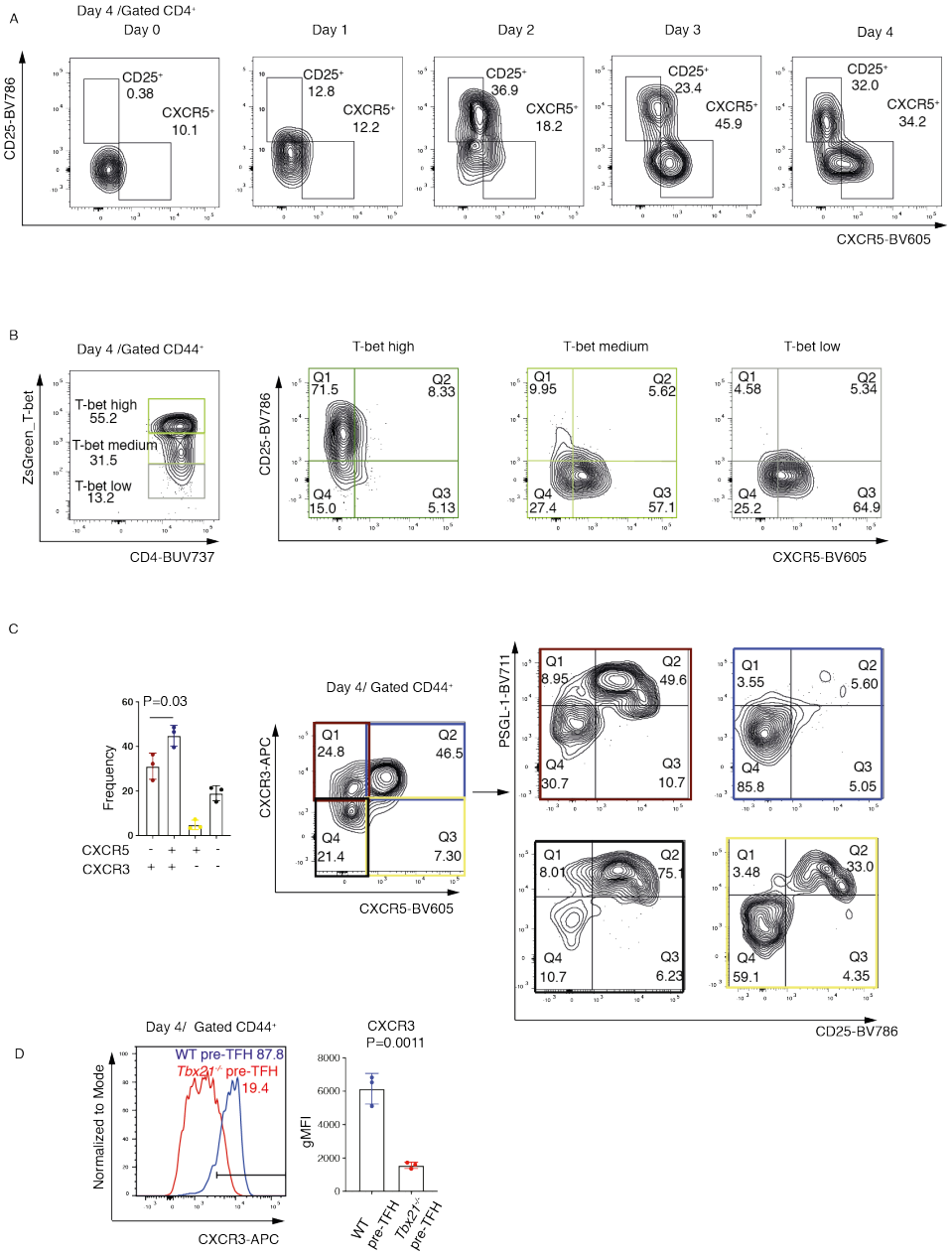
A
Day 4 /Gated CD4+
Day 0
Day 1
Day 2
Day 3
Day 4
CD25+ 0.38
CXCR5+ 10.1
CD25+ 12.8
CXCR5+ 12.2
CD25+ 36.9
CXCR5+ 18.2
CD25+ 23.4
CXCR5+ 45.9
CD25+ 32.0
CXCR5+ 34.2
CD25-BV786
CXCR5-BV605
B
Day 4 /Gated CD44+
T-bet high 55.2
T-bet medium 31.5
T-bet low 13.2
ZsGreen_T-bet
CD4-BUV737
T-bet high
T-bet medium
T-bet low
Q1 71.5
Q2 8.33
Q4 15.0
Q3 5.13
Q1 9.95
Q2 5.62
Q4 27.4
Q3 57.1
Q1 4.58
Q2 5.34
Q4 25.2
Q3 64.9
CD25-BV786
CXCR5-BV605
C
P=0.03
Frequency
CXCR5
CXCR3
Day 4/ Gated CD44+
Q1 24.8
Q2 46.5
Q4 21.4
Q3 7.30
CXCR3-APC
CXCR5-BV605
PSGL-1-BV711
Q1 8.95
Q2 49.6
Q4 30.7
Q3 10.7
Q1 3.55
Q2 5.60
Q4 85.8
Q3 5.05
Q1 8.01
Q2 75.1
Q4 10.7
Q3 6.23
Q1 3.48
Q2 33.0
Q4 59.1
Q3 4.35
CD25-BV786
D
Day 4/ Gated CD44+
WT pre-TFH 87.8
Tbx21-/- pre-TFH 19.4
Normalized to Mode
CXCR3-APC
CXCR3
P=0.0011
gMFI
WT pre-TFH
Tbx21-/- pre-TFH

Figure 3.3 T-bet controls CXCR3 expression in pre-TFH

*(**A-C**) Naive ZsGreen_T-bet STg cells were adoptively transferred into C57BL/6 mice prior to LCMV infection. Spleens were analysed on the indicated days. Representative flow plots showing bifurcation of $CD4^+$ STg cells into pre-TFH ($CD25^-$ $CXCR5^+$) and pre-TH1 ($CXCR5^-$ $CD25^+$).*

*(**B**) Representative plots of T-bet expression in pre-TH1 ($CXCR5^-$ $CD25^+$) and pre-TFH ($CD25^-$ $CXCR5^+$) cells.*

*(**C**) Frequency and representative flow plots showing pre-TFH ($CXCR5^+$ $CXCR3^+$) and pre-TH1 ($CXCR3^+$ $CXCR5^-$) in WT STg cells.*

*(**D**) Naive WT or $Tbx21^{-/-}$ STg cells were transferred into C57BL/6 mice prior to LCMV infection. Histogram of CXCR3 in WT and $Tbx21^{-/-}$ pre-TFH cells. Bar graph of MFI of CXCR3 in WT and $Tbx21^{-/-}$ pre-TFH cells.*

Data are representative of two independent experiments, n = 3-4 mice/group. Data show the mean ± SEM.

3.2.4 CXCR3-dependent intranodal migration of $CD4^+$ T cells

CXCR3 regulates intranodal positioning of $CD4^+$ T cells to the interfollicular and T:B border in the LNs following immunisation [216]. Earlier studies have shown that the positioning of TFH and TH1 cells at the interfollicular regions is required for their differentiation [85, 216]. To this point, I showed that CXCR3 was upregulated in both pre-TFH and pre-TH1 cells following LCMV infection (**Figure 3.3**C). Thus, to understand whether CXCR3 orchestrates $CD4^+$ T cell location following LCMV infection, naive GFP-labelled WT, *Tbx21*$^{-/-}$ or *Cxcr3*$^{-/-}$ STg cells were adoptively transferred into C57BL/6 mice prior to LCMV infection. On day 4 post infection, the inguinal LNs were harvested, cleared, stained with antibodies and imaged using light sheet fluorescent microscopy (LSFM) (**Figure 3.4)**. B cells were stained with B220 to outline follicles (**yellow**), endothelial cells are shown as $CD31^+$ (**magenta**) and STg cells (**cyan)** (**Figure 3.4**A). WT STg cells were visually observed throughout the LNs (**Figure 3.4**B). In contrast, *Tbx21*$^{-/-}$ STg cells and vast majority of the *Cxcr3*$^{-/-}$ STg cells were confined to the centre of the node (**Figure 3.4**C-D). This initial experiment suggests that CXCR3 orchestrate the location of $CD4^+$ T cells in the LNs following LCMV infection. This is an interesting result; however, this still requires confirmation and quantification of T cell location in 3D. Additionally, it would be interesting to stain STg cells to determine the location of pre-TH1 and pre-TFH within the LN.

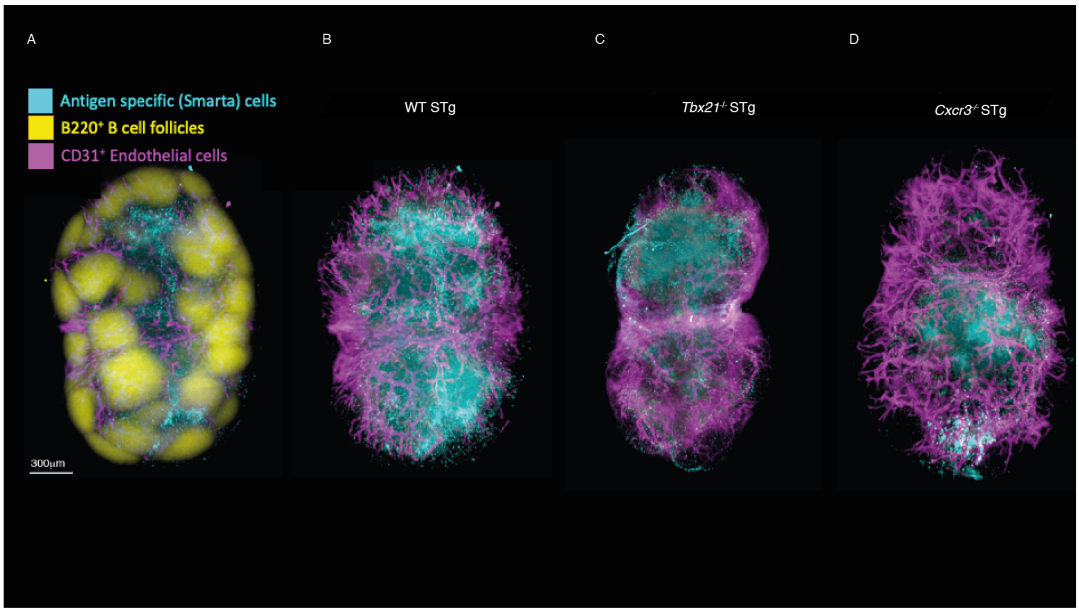

Figure 3.4 CXCR3 orchestrates CD4$^+$ T cell location in vivo following LCMV infection

Naive WT, Tbx21$^{-/-}$ or Cxcr3$^{-/-}$ STg cells were adoptively transferred into C57BL/6 mice prior to LCMV infection. Inguinal LNs were harvest at day 4 post infection then cleared using the Ce3D method and imaged by LSFM.

*(**A**) Representative LSFM micrograph showing (yellow, B220$^+$ B cell follicles; magenta, CD31$^+$ endothelial cells; green, STg cells).*

*(**B-D**) Representative LSFM micrographs showing the location of WT, Tbx21$^{-/-}$ or Cxcr3$^{-/-}$ STg cells.*

Data are representative of individual LNs from a single experiment, n = 3-4 mice/group.

3.2.5 T-bet and CXCR3-deficiency in CD4$^+$ T cells promote pre-TFH differentiation

The migration to T:B border is an important step in the differentiation of both TFH and TH1 cells [85, 216]. Given that the location of *Cxcr3*$^{-/-}$ STg cells differed from that of WT STg cells (**Figure 3.4**), I sought to understand whether the loss of CXCR3 affects pre-TFH/TH1 cell development. To investigate this, naive GFP-labelled WT, *Tbx21*$^{-/-}$ or *Cxcr3*$^{-/-}$ STg cells were adoptively transferred into C57BL/6 mice prior to LCMV infection. Spleens were harvested on day 4 post infection (**Figure 3.5**). The results showed that a higher frequency of WT STg cells (**grey**) differentiated into pre-TH1 cells as compared to *Tbx21*$^{-/-}$ STg (**blue**) and *Cxcr3*$^{-/-}$ STg (**red**) cells (**Figure 3.5**B). There was a decrease in the frequency of WT STg cells (**grey**) with reciprocal increase in the *Cxcr3*$^{-/-}$ STg (**red**), *Tbx21*$^{-/-}$ STg (**blue**) cells that differentiated into pre-TFH cells (**Figure 3.5**B). Importantly, the outcome of T-bet and CXCR3-deficiency in pre-TFH cell differentiation was similar. The localisation of CXCR3-deficient STg cells differed as compared to WT STg cells (**Figure 3.4**), herein, at early differentiation stage data suggest that deficiency in T-bet or CXCR3 results in expansion of pre-TFH cells at the expense of pre-TH1 cells.

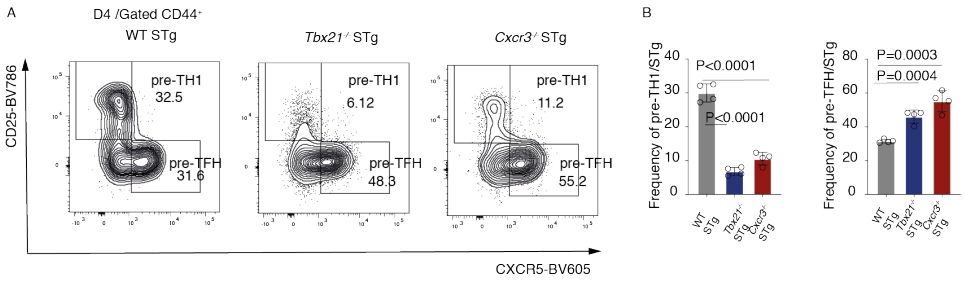

Figure 3.5 T-bet and CXCR3-deficiency in T cells promote pre-TFH differentiation

*(**A-B**) Naive GFP-labelled WT, Tbx21*$^{-/-}$ *or Cxcr3*$^{-/-}$ *STg cells were transferred into C57BL/6 mice prior to LCMV infection. Splenic STg cells were analysed at day 4 post infection.*

*Representative flow plots (**A**) and frequency (**B**) of pre-TH1 (CD25*$^+$ *CXCR5*$^-$*) and pre-TFH (CD25*$^-$ *CXCR5*$^+$*).*

Data are representative of two independent experiments, n = 3-4 mice/group. Data show the mean ± SEM.

3.2.6 T-bet regulates TFH cell differentiation in CXCR3-dependent manner

The observation that loss of T-bet and CXCR3 promotes early expansion of TFH cells (**Figure 3.5**) was surprising, given that T-bet-deficiency is known to inhibit TFH cell differentiation during acute LCMV infection (Chapter 2 and by others [24, 218]). Therefore, I next investigated whether the initially expanded pre-TFH cells can differentiate into fully polarized TFH cells. To test this, naive GFP-labelled WT, *Tbx21*$^{-/-}$ or *Cxcr3*$^{-/-}$ STg cells were transferred into C57BL/6 host prior to LCMV infection. Spleens were harvested from infected mice on day 8 post infection (**Figure 3.6**). There was a significant decrease in the number and frequency of *Tbx21*$^{-/-}$ STg cells (**blue**) in comparison to WT STg cells (**grey**) (**Figure 3.6**A-B). In contrast, there was an increase in the total number of *Cxcr3*$^{-/-}$ (**red**) versus WT STg cells (**grey**) (**Figure 3.6**A-B). Consistent with previous studies, there was a significant reduction in the frequency and number of *Tbx21*$^{-/-}$ TH1 (**blue**) compared to WT cells (**Figure 3.6**C-D) [23, 24]. However, there was no significant change in the frequency and number of *Cxcr3*$^{-/-}$ TH1 cells (**red**) as compared to WT TH1 cells (**grey**) (**Figure 3.6**C-D). As demonstrated in **Chapter 2**, there was a decrease in the frequency and number of *Tbx21*$^{-/-}$ TFH cells (**blue**) versus WT TFH cells (**grey**) (**Figure 3.6**E-F). Similar to *Tbx21*$^{-/-}$ cells, there was a reduction in the frequency of *Cxcr3*$^{-/-}$ TFH cells (**red**). However, given the increase in the total number of *Cxcr3*$^{-/-}$ STg cells in the spleen (**Figure 3.6**A-B), the total number of *Cxcr3*$^{-/-}$ TFH cells remained comparable to WT (**Figure 3.6**E-F).

TFH cells colocalise with the B cells in the GCs, which are present at the centre of the B cell follicles [6]. To investigate whether CXCR3-deficient STg cells were able to position themselves in the B cell follicles, WT and *Cxcr3*$^{-/-}$ STg cells were transferred into C57BL/6 mice prior to LCMV infection. Inguinal LNs were harvested from the infected mice on day 14 post infection and were cleared by Ce3D clearing method. The intact LNs were stained with IgD (**magenta**), which marked the B cell follicles excluding the GCs, thus GCs were observed as non-stained regions in the centre of the B cell follicles in **magenta** (**Figure 3.6**G). By visual observation of the LSFM micrographs, more WT TFH cells were positioned in the GCs in comparison to *Cxcr3*$^{-/-}$ TFH cells (**Figure 3.6**G), however, confirmation and quantification of T cell location in 3D is required as this experiment was done once.

T-bet regulates multiple genes associated with cytokine production, cell division, metabolic processes and cell migration in T cells [218, 238]. CXCR3 likely exerts its role downstream of T-bet induction to regulate T cell migration within a particular niche of the LNs [239]. This CXCR3-dependent positioning allows T cells to access cytokines signals and cellular interactions at that

particular niche which are important for their differentiation [216, 239]. In **Chapter 2**, T-bet has been shown to be required for TFH cell during LCMV infection. While some roles of T-bet maybe mediated via the induction of CXCR3, the results here suggest that loss of CXCR3 promotes early TFH cell differentiation but impairs TFH cell differentiation and positioning within the GCs at later time points.

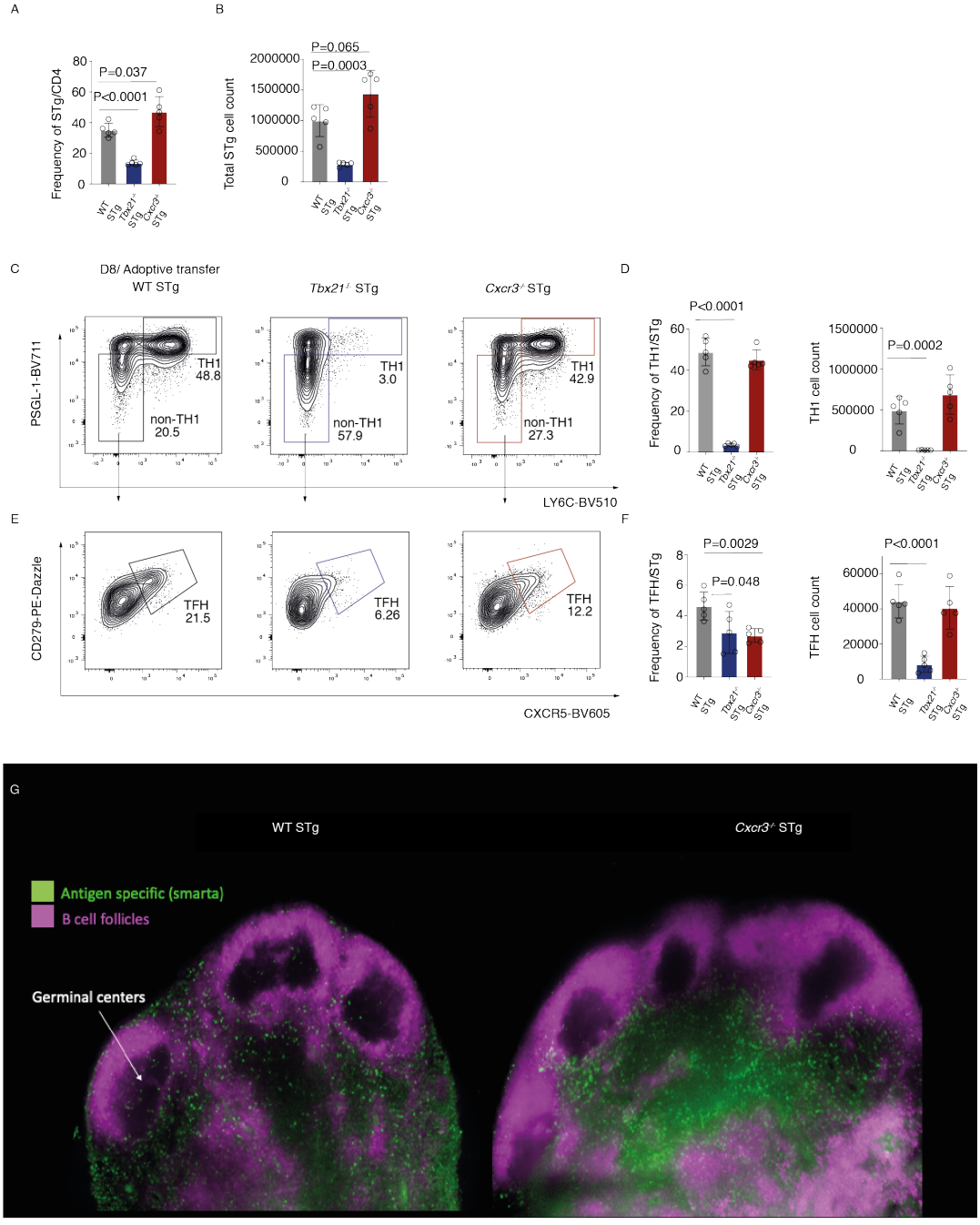

Figure 3.6 CXCR3 regulates TFH cell differentiation and positioning in GCs

*(**A-F**) Naive WT, Tbx21$^{-/-}$ or Cxcr3$^{-/-}$ STg cells were transferred into C57BL/6 mice prior to LCMV infection. Splenic STg cells were analysed at day 8 post infection.*

*(**A-B**) Frequency and total cell number of splenic STg cells. Representative flow plots (C-E) and frequency and total number (**D-F**) of TH1 (CD162^{+} Ly6C^{+}) and TFH (CD162^{-} Ly6C^{-} CD279^{+} CXCR5^{+}) cells. Data are representative of two independent experiments, n = 3-5 mice/group. Data show the mean ± SEM.*

*(**G**) Naive WT or Cxcr3$^{-/-}$ STg cells were transferred into C57BL/6 mice prior to LCMV infection. Representative LSFM micrographs of inguinal LNs (magenta, IgD^{+}, B cell follicles; green, STg cells) at day 14 post infection.*

Data are representative of one independent experiment, n = 3-5 mice/group.

3.2.7 The microenvironment influences the proportion of WT versus CXCR3-deficient TFH cells

The role of T-bet in TFH cell differentiation is contingent on T cell competition as shown in **Chapter 2**. Next, to investigate whether altered T cell competitive environment regulates the defect in TFH cell differentiation due to loss of CXCR3. To setup a competing environment, 2.5×10^4 of each WT and *Cxcr3*$^{-/-}$ STg were co-transferred into individual C57BL/6 recipient mice prior to LCMV infection. Spleens from these mice were harvested on day 8 post infection (**Figure 3.7**). In this setting, the frequency of *Cxcr3*$^{-/-}$ TH1 cells (**red**) was reduced as compared to WT TH1 cells (**grey**) (**Figure 3.7**A-C), whereas there was an increase in the percentage of *Cxcr3*$^{-/-}$ TFH (**red**) versus WT TFH cells (**grey**) (**Figure 3.7**D-F). These data indicate that the ratio of CXCR3-deficient versus WT TFH cells is influenced by the microenvironment following LCMV infection. To confirm this result using an alternate approach, T cell competitive environment was established between the WT and *Cxcr3*$^{-/-}$ cells by using 50:50 BM chimeras. Mice were allowed 8 weeks to reconstitute and then infected with LCMV. Spleens were harvested on day 8 post infection. The analysis showed an increase in GP_{66} tetramer^{+} *Cxcr3*$^{-/-}$ TFH cells (**red**) versus WT TFH cells (**grey**) (**Figure 3.7**G-K). Combined, in a more competitive environment, CXCR3-deficiency TFH cells outcompete WT TFH cells. In this setting, it is likely that CXCR3-deficient T cells compete with T cells for nutrients, cytokines or interactions generated by the expression of CXCR3 ligands in the secondary lymphoid tissues [240].

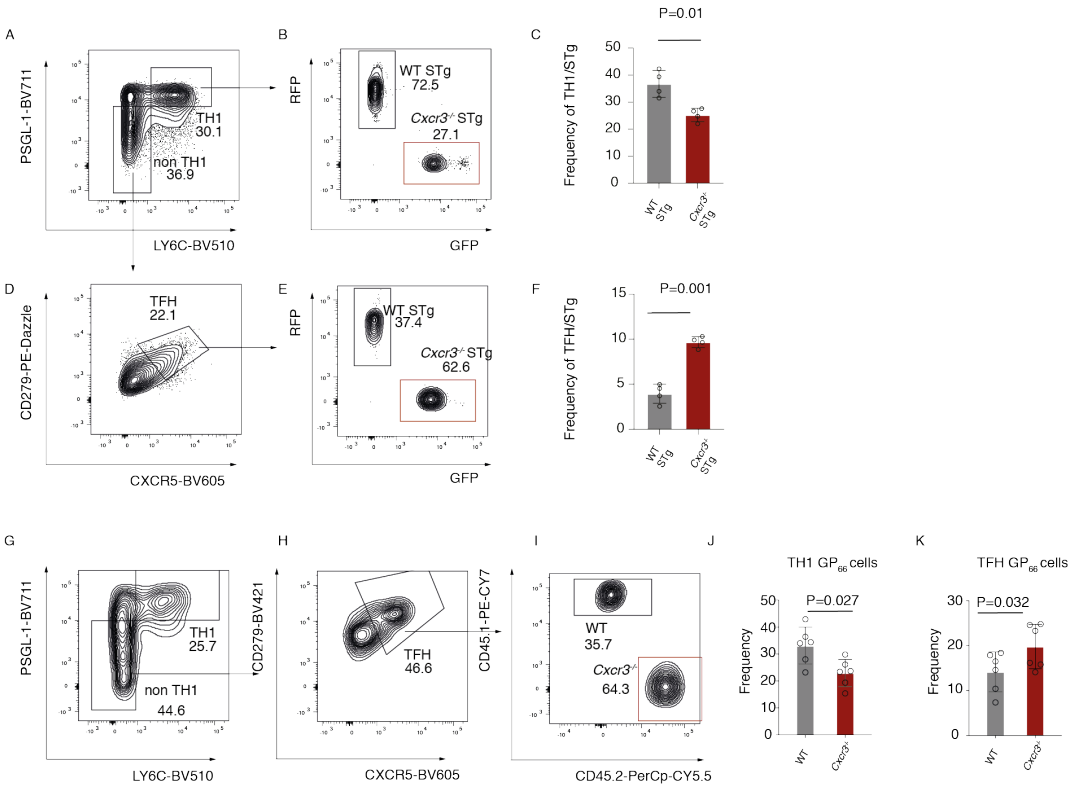

Figure 3.7 The microenvironment influences the ratio of WT versus CXCR3-deficient TFH cells

*(**A-F**) Naive RFP-labelled WT and GFP-labelled Cxcr3$^{-/-}$ STg cells were co-transferred into C57BL/6 mice prior to LCMV infection. Spleens were analysed at day 8 post infection. (**A-B**) Representative flow plots and frequency (**C**) of TH1 cells (CD162^{+} LY6C^{+}). (**D-E**) Representative flow plots and frequency (**F**) of TFH (CD162^{-} LY6C^{-} CD279^{+} CXCR5^{+}). Data are representative of one experiment and was done one time, n = 4 mice/group. Data show the mean ± SEM.*

*(**G-K**) Ly5.1 and Cxcr3$^{-/-}$ (Ly5.2^{+}) bone marrow chimeras were infected with LCMV. Splenic CD4^{+} CD44^{+} cells were analysed at day 8 post infection. (**G-I**) Representative flow cytometry plots and (**J**) frequency of TH1 (GP$_{66}^{+}$ CD44^{+} PSGL-1^{+} LY6C^{+}) and (**K**) TFH (GP$_{66}^{+}$ CD44^{+} PSGL-1^{-} LY6C^{-} PD-1^{+} CXCR5^{+}). Data are representative of one experiment and was done one time, n = 6 mice/group. Data show the mean ± SEM.*

3.2.8 CXCR3 ligands are spatially distributed in the draining lymph nodes

CXCR3 ligands (CXCL9 and CXCL10) have been shown to influence TH1 cell differentiation following immunisation [216], and I showed differential location of WT and *Cxcr3*$^{-/-}$ STg cells within LNs on day 4 post LCMV infection (**Figure 3.4**B-D). This raised the question whether CXCR3 ligands are expressed in the draining LNs during LCMV infection. To investigate this, I used a dual reporter of CXCR3 ligands, the REX3 transgenic mice [216] to generate bone marrow chimeras, where REX3^{+} cells reconstituted the hematopoietic cell compartment. Following reconstitution, naive GFP-labelled STg cells were transferred prior to LCMV infection. The inguinal LNs were harvested on day 4 post infection and were cleared, stained and imaged by LSFM (**Figure 3.8**). Specifically, day 4 was selected because at this day I observed difference in the location of WT and *Cxcr3*$^{-/-}$ STg cells within the LNs (**Figure 3.4**B-D). In an initial experiment, visualisation of micrographs indicated spatial distribution of CXCR3 ligands; CXCL9-RFP cells were positioned closer to the centre of the LNs and CXCL10-BFP cells were localised in the periphery including T:B border and interfollicular regions (**Figure 3.8**A). CXCL10-BFP cells were in close proximity to STg cells (**Figure 3.8**B). An in-press study from the lab demonstrated that CXCL10 is primarily produced by cDC2, which are important in TH1 cell differentiation [77, 79, 241]. Here, the initial experiment suggests that the cross talk between CXCL10-BFP cells and CD4^{+} T cells could mediate the position of TFH or TH1 cells to the T:B border.

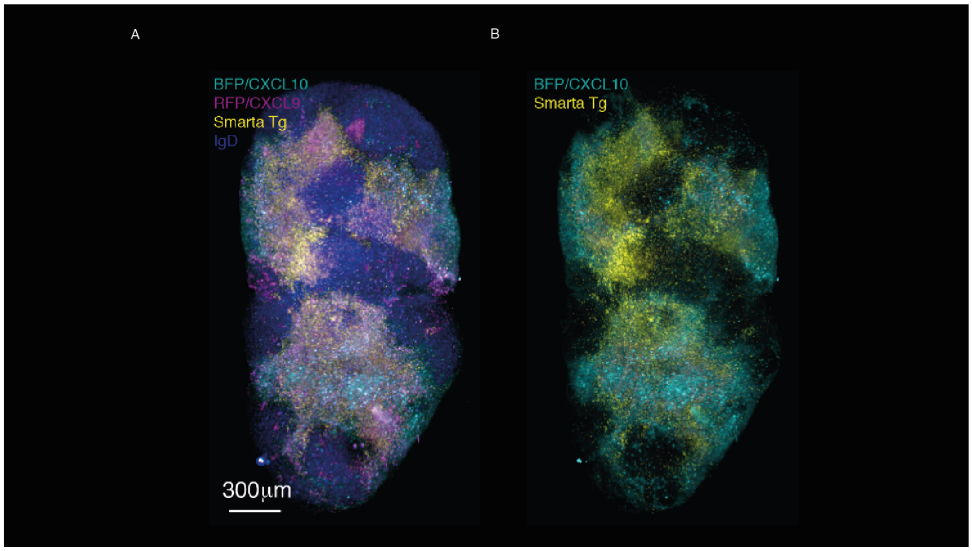

Figure 3.8 STg cells sit close to CXCL10 expressing cells

*(**A-B**) Naive GFP-labelled STg cells were adoptively transferred into REX3 bone marrow chimeric host prior to LCMV infection. Inguinal LNs were harvest at day 4 post infection, cleared by Ce3D method and imaged by LSFM. Representative micrographs showing (magenta, CXCL9 expressing cells; cyan, CXCL10 expressing cells; yellow, WT STg cells, blue; B cell follicles).*

Data are representative of two independent experiments, n = 3-4 mice/group.

3.2.9 CXCL10 inhibits early expansion of pre-TFH cells

Next, I asked whether loss of CXCR3 ligands regulate the differentiation of pre-TFH and pre-TH1 cells. To investigate this, GFP-labelled WT STg cells were adoptively transferred into naive WT, *Cxcl9*$^{-/-}$ or *Cxcl10* $^{-/-}$ mice and infected with LCMV (**Figure 3.9**). The results showed that absence of CXCL9 and CXCL10 led to an increase in the frequency of pre-TFH cells (**blue**) compared to WT mice (**grey**) with intact CXCR3 ligands (**Figure 3.9**A-B) and these findings resembled that observed in CXCR3-deficiency on day 4 post LCMV infection (**Figure 3.5**). In this setting both CXCL9 and CXCL10-deficiency promote TFH cell differentiation however, the imaging results showed that CXCL10-expressing cells were in close proximity to STg cells (**Figure 3.8**). Thus, I speculate that CXCR3/CXCL10 axis inhibits early expansion of pre-TFH cells, however if this differentiation is maintained or overcome later in infection is unknown.

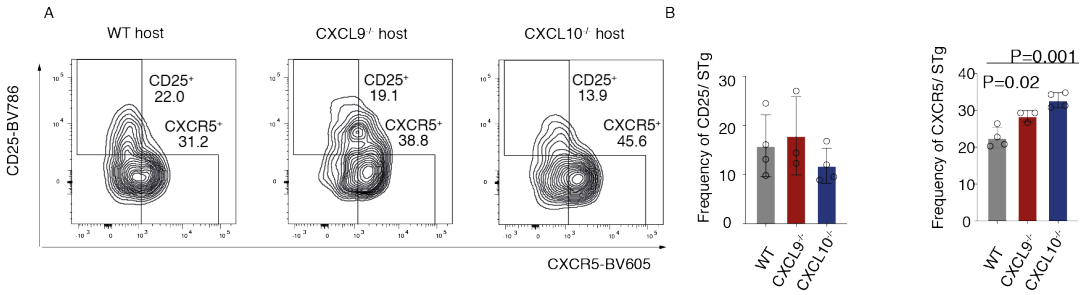

Figure 3.9 CXCL10 inhibits early expansion of pre-TFH cells

*(**A-B**) Naive WT STg cells were adoptively transferred into WT, Cxcl9*$^{-/-}$ *or Cxcl10* $^{-/-}$ *mice prior to LCMV infection. Spleens were harvested at day 4 post infection. (**A**) Representative flow plots and frequency (**B**) of pre-TH1 (CD25*$^{+}$ *CXCR5*$^{-}$*) and pre-TFH (CD25*$^{-}$ *CXCR5*$^{+}$*) STg cells in indicated mice.*

Data are representative of one independent experiment, n = 4 mice/group. Data show the mean ± SEM.

3.2.10 CXCR3 is not required for germinal centre formation

To understand if CXCR3 in TFH cells regulate GC B cell differentiation, naive WT and *Cxcr3*$^{-/-}$ mice (mice that lack CXCR3 in all cells) were infected with LCMV. Spleens were harvested from these mice on day 8 post infection. There was no overt change in GC B cell differentiation, B cell class switching or in the localisation of the GC B cells in the dark or the light zone (**Figure 3.10**A-F). In the setting, I analysed TFH cells and loss of CXCR3 had no overt change in GP_{66} tetramer^{+} *Cxcr3*$^{-/-}$ TFH cells (**red**) versus WT TFH cells (**grey**) (**Figure 3.7**G-H). Together, this data shows that in intact mice loss of CXCR3 has no effect on TFH cell differentiation and TFH-mediated GC B cell output. In addition, these findings emphasise that the role of CXCR3 in TFH cell differentiation is influenced by the microenvironment.

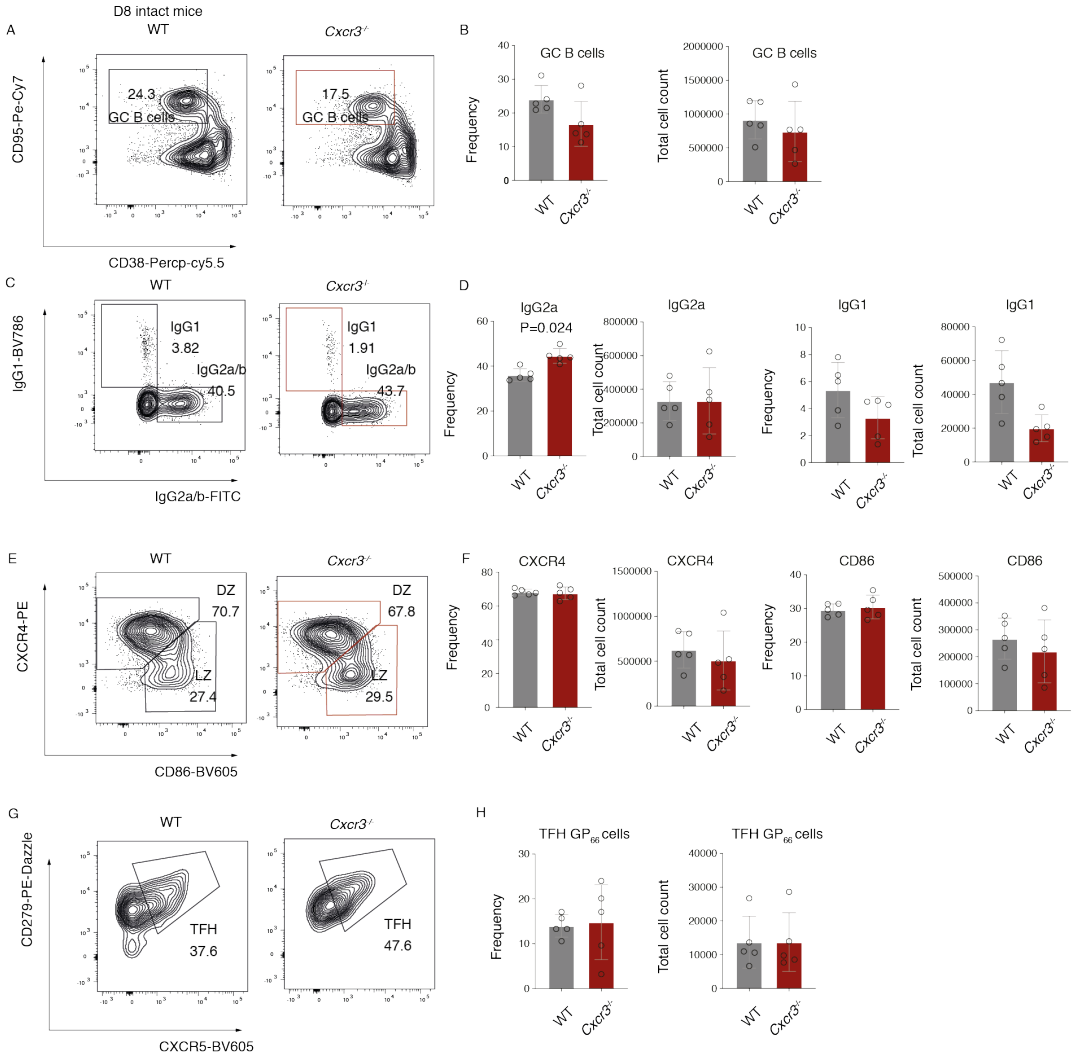

Figure 3.10 GC B cell response is independent of CXCR3

*(**A-H**) Intact WT and Cxcr3$^{-/-}$ mice were infected with LCMV. Spleens were analysed at day 8 post infection. (**A**) Representative flow cytometry plots and (**B**) frequency and total number of GC B cells (B220^{+} IgD^{+} CD95^{+} CD38^{-}).*

*(**C**) Representative flow cytometry plots and (**D**) frequency and total number of IgGa and IgG1 surface staining on GC B cells (B220^{+} IgD^{+} CD95^{+} CD38^{-}).*

*(**E**) Representative flow cytometry plots and (F) frequency and total number of DZ (CXCR4^{+} CD86^{-}) and LZ (CD86^{+} CXCR4^{-}) GC B cells.*

*(**G**) Representative flow cytometry plots and (**H**) frequency and total number of TFH cells (GP$_{66}^{+}$ CD44^{+} PSGL-1^{-} LY6C^{-} PD-1^{+} CXCR5^{+}).*

Data are representative of one independent experiment, n = 5 mice/group. Data show the mean ± SEM.

3.3 Discussion

Following infection, naive $CD4^+$ T cells proliferate and differentiate into TH1 and TFH cells. In turn these subsets play a central role in eliminating intracellular pathogens such as viruses [29, 119]. One primary transcription factor T-bet is required for TH1 and TFH cell differentiation in viral settings [23, 24], but the precise mechanism for T-bet in TFH is unknown. This study was aimed to dissect the contribution of T-bet in $CD4^+$ T cell proliferation and CXCR3-directed migration as critical regulators of TFH differentiation.

In this study, I identified that T-bet was induced in two distinct phases in $CD4^+$ STg cells following LCMV infection (**Figure 3.1**A). The first phase of T-bet induction occurred between day 0-1, while the second phase of T-bet induction was observed between day 2-4. During the second phase the level of T-bet expression was significantly increased as compared to the first phase of T-bet induction (**Figure 3.1**A). An earlier *in vitro* study demonstrated this two-phase T-bet induction in $CD4^+$ T cells but they showed no overt change in T-bet expression between the two phases [220], indicating that the outcome of *in vitro* study is not completely reflected in the *in vivo* system shown in here. The increase in T-bet expression during the second phase coincided with the upregulation of T cell activation marker CD25 (high affinity IL-2 receptor, IL-2rα) (**Figure 3.3**A). Previously, IL-2/CD25 signalling has been shown to promote the expression of T-bet and *Il12rb2* (which encodes IL-12 receptor (IL-12Rβ2)) in T cells [220], whereby IL-12/IL-12Rβ2 signalling upregulates T-bet expression in T cells [242]. Thus, the increase in T-bet expression in the second phase might be a combined effect of IL-2 and IL-12 signals in $CD4^+$ T cells.

Here, I showed that $CD4^+$ T cells start dividing following the first phase of T-bet induction (**Figure 3.1**A), suggesting a potential role of T-bet in cell proliferation. The higher percentage of $CD4^+$ T cells with enhanced T-bet and CD25 expression went through greater number of cell divisions in comparison to $CD4^+$ T cells with lower T-bet and CD25 expression (**Figure 3.1**B-C). T-bet-deficient $CD4^+$ T cells showed reduction in CD25 expression (**Figure 3.1**D). IL-2 signalling via CD25 has been shown to positively regulate T cell proliferation and differentiation [237]. Reduced frequency of CD25 expression, indicates that T-bet regulates pre-TH1 cell differentiation.

Unexpectedly, T-bet-deficient $CD4^+$ T cells showed greater cell division despite reduced CD25 expression in comparison to WT $CD4^+$ T cells (**Figure 3.1**D). Potentially, T-bet regulates pre-TH1 cell proliferation and not pre-TFH cells. Recent study investigated the role of T-bet in

$CD8^+$ T cell proliferation. In this study, mice were infected the mice prior to cell transfer and found that T-bet was not necessary for $CD8^+$ T cell proliferation [226]. In contrast, here, I infected mice after cell transfer and found a role of T-bet in $CD4^+$ T cell differentiation. Given the key difference in experimental set up between the two studies, it is highly possible that the early induction of T-bet plays a key role in T cell proliferation (supported by my study) but is dispensable at a later stage (shown in [226]).

I found that T-bet regulates CXCR3 expression in $CD4^+$ T cells in LCMV infection (**Figure 3.2**A). Unlike, T-bet induction that occurred before cells entered cell division (**Figure 3.1**A), CXCR3 was upregulated in $CD4^+$ T cells when $CD4^+$ T cells entered cell cycle post LCMV infection (**Figure 3.2**B). Comparing WT and *Cxcr3*$^{-/-}$ $CD4^+$ T cells, there was no overt difference in cell proliferation marked by CTV dilution on day 3 post infection (**Figure 3.2**C). Together, these findings suggest that CXCR3 does not act downstream of T-bet to regulate early $CD4^+$ T cell division but the role of CXCR3 in $CD4^+$ T cell proliferation at later time points is not yet clear.

CXCR3 directs $CD4^+$ T cell intranodal positioning, which facilitates their differentiation and function following immunisation [216]. Here, in an initial imaging experiment of whole intact LNs, I showed that *Cxcr3*$^{-/-}$ $CD4^+$ T cells were located in the centre of the LNs and not in the TFH niche which is the T:B border on day 4 post LCMV infection. In contrast, WT $CD4^+$ T cells were not just restricted to the centre of the LN but were distributed throughout the LN including the B cell follicles and T:B border (**Figure 3.4**A-D). The CXCR3 ligand CXCL10 expressing cells were located at T:B cell regions of the LNs following LCMV infection (**Figure 3.8**B), these results of the intact LNs need to confirmed and cell position quantified. A parallel study in the lab demonstrated that these CXCL10 producing cells located at T:B border were cDC2 in LCMV infection [241]. It has been shown that cDC2 cells present at T:B border are required for TFH differentiation following immunisation [77, 79]. The migration of TFH cells to T:B border is necessary for TFH cell differentiation and TFH-mediated B cell response [6, 76]. In this study, I demonstrated that T-bet promotes CXCR3 expression in pre-TFH cells following LCMV infection (**Figure 3.3**D). Thus, I hypothesised that CXCR3 acts downstream of T-bet to regulate migration of TFH cells to T:B border in response to CXCL10 chemokine gradient established by cDC2 at T:B border. However, while there was reduced frequency, there was no change in the total number of *Cxcr3*$^{-/-}$ TFH cells as compared to the WT in a less competitive environment following LCMV infection (**Figure 3.6**E). Furthermore, in a competitive environment there was a higher frequency of *Cxcr3*$^{-/-}$ TFH cells as compared to the WT TFH

cells (**Figure 3.7**). This suggests that the differentiation of TFH cells is a multifactorial process that could be tuned by antigens, cytokine and nutrients in the microenvironment. Although these experiments were done only once and need to be confirmed, overall, they indicate that CXCR3 is required for TFH cell differentiation following LCMV infection.

An important feature of GC TFH cells is that they express high levels of PD-1 as compared to pre-TFH cells or TFH cells present in the B cells follicles [94]. PD-1 has been shown to downregulate CXCR3 that facilitates TFH cells to remain confined in the GCs [82]. Unlike B cells which are clonally restricted to one GCs, TFH cells have been shown to transit between GCs which promotes antigen variation of B cells in an immune response [94]. Potentially a CXCR3/PD-1 axis play a role in regulating the dynamic movement of TFH cells between the GCs which may facilitate diversification of antibody response.

Within GCs, TFH cells provide help signals to B cells that drives the differentiation of GC B cells into long-lived plasma and memory B cells [6]. Thus, I sought to investigate whether intrinsic CXCR3 in TFH cells facilitated B cell differentiation or B cell class switching. In this study, I found that in CXCR3-deficient intact mice, there was no change in GC B cell differentiation, B cell class switching or GC B cell localisation in the GC DZ or the LZ on day 8 post LCMV infection (**Figure 3.10**). To test this in depth, WT or *Cxcr3* $^{-/-}$ STg cells could be adoptively transferred into OT-I mice and infected with LCMV in future experiments. In this setting, STg cells will be the only one regulating B cell responses, with a total absence of host $CD4^+$ T cell competition. This will help to understand the intrinsic role of CXCR3 in TFH-mediated B cell affinity maturation and GC B cell differentiation into long-lived plasma and memory B cells. While this study still leaves questions over the precise role for CXCR3 in TFH, more work is required to establish the timing and context where CXCR3 may impact TFH differentiation, either positively or negatively.

3.4 Methods

3.4.1 Mice

Mice were maintained on a C57BL/6 background in specific-pathogen-free conditions at the Walter and Eliza Hall Institute animal facility. ZsGreen T-bet reporter [200], *Tbx21*$^{-/-}$ [243], Smarta TCR transgenic (STg) transgenic [235], *Cxcr3*$^{-/-}$ [244], *Cxcl9*$^{-/-}$ [245], *Cxcl10*$^{-/-}$ [246] and REX3 mice [216] have been previously described. Both male and female mice at 6-10 weeks of age were used in this study. All animal experiments were performed in accordance with the Walter and Eliza Hall Institute animal ethics committee (AEC approval # 2018.026).

3.4.2 Bone marrow chimeras

Mixed bone marrow chimeras were generated by lethally irradiating Ly5.1xC57BL/6 mice with two doses of (2x 550R), 3 hours apart and reconstitution with a mixture of CXCR3$^{-/-}$ and Ly5.1 bone marrow cells in a 1:1 ratio. Mice were allowed 8 weeks to reconstitute their haematopoietic system before infecting them with virus.

3.4.3 Viral infections and adoptive transfer

For adoptive transfer experimental setup, STg cells were isolated using naive CD4^{+} T cell isolation kit (Miltenyi Biotec, catalogue # 130-104-453) according to manufacturer's guidelines. Cells were single transferred or co-transferred into non-irradiated C57BL/6 mice using intravenous injection. Animals harvested on day 0-2 received 1×10^6 STg cells, day 3 received 4×10^5 STg cells, day 4 received 2×10^5 STg cells and day 8 received 2.5×10^4 STg cells. One day post adoptive transfer, mice were infected with LCMV Armstrong. Animals harvested on day 0-2 were infected with 1×10^6 PFU, day 3 infected with 5×10^5 PFU, day 4 infected with 2×10^5 PFU and day 8 infected with 3×10^3 PFU. Virus was prepared in PBS and given intravenously.

3.4.4 Antibodies and dyes for flow cytometry

For T cell analysis: anti-CXCR5 (clone L138D7; Biolegend), anti-CD25 (clone 3C7; BD Horizon), anti-PSGL-1 (clone 2PH; BD OptiBuild), anti-LY6C (clone HK1.4; Biolegend), anti-CD279 (clone RMP1-30; Biolegend), anti-CXCR3 (clone CXCR3-173; Biolegend), anti-CD44 (clone 1M7; BD PharMingen), anti-CD4 (clone GK1.5; BD Horizon) and Fixable Viability Stain eF506 (eBioscience). *For B cell analysis*: anti-CD86 (clone GL1; BD Horizon), anti-IgG1 (clone X56; BD OptiBuild,), anti-Ig2a/2b (clone R2-40; BD PharMingen) anti-B220 (clone RA3-6B2; BD Horizon), anti-CD38 (clone 90; eBioscience), anti-CD95 (clone Jo2; BD

PharMingen), anti-CXCR4 (clone 2B11; eBioscience), anti-IgD (clone 1126c; produced in house), anti-CD138 (clone 281-2; BD Horizon) and Fixable Viability Stain 700 (BD Horizon).

3.4.5 T cell labelling with division tracking dye

Isolated naive STg cells were CTV-labelled by using CellTrace Violet Cell Proliferation Kit (Molecular Probes, Thermo Fisher Scientific, catalogue # C34557) according to the manufacturer's instructions, with minor modifications. First, 5mM of CTV (stock concentration) was prepared by dissolving CTV powder in 20ml of DMSO. This stock solution was further diluted 1 in 10 in 0.1% BSA in PBS to prepare CTV solution (0.5mM) to minimise toxicity and cell death following CTV labelling. Then, 10ml of CTV solution (0.5mM) per 1ml was added to the cells resuspended in 0.1% BSA in PBS (< 2×10^7 cell/ml) and vortexed immediately. The cell suspension was incubated at 37°C for 20 min in a water bath with a lid to prevent light exposure. The staining process was stopped by resuspending cells in 20× volume of 5% cold FCS-RPMI and kept on the ice for 5 min before centrifugation. Cells were washed twice and resuspended in PBS prior to adoptive cell transfer.

3.4.6 Intact lymph node clearing and imaging

Inguinal LNs were fixed in PFA (4%) for 12 to 24 hours at 4°C. LNs were transferred into blocking solution (PBS containing 1% Normal Rat Serum (JacksonImmuno Research), 1% BSA (Sigma) and 0.3% Triton X-100 (Sigma)) for another 24 hours at 4°C. Followed by staining for 3 days at 4°C with primary antibodies 1 in a 100µl of blocking buffer. The antibodies used were: Alexa-Fluor-555-conjugated B220 (produced in house; clone RA3-6B2), Alexa-Fluor-647-conjugated CD31 (Biolegend; clone MEC13.3) or Alexa-Fluor-647-conjugated IgD (produced in house; clone 1126c). Whole LNs were then washed overnight in PBS (0.2% Triton X-100 and 0.5% 1-Thioglycerol (Sigma)) and immersed in Ce3D clearing solution [247] containing 1.455g/ml Histodenz (Sigma), 0.5% 1-Thioglycerol and 0.1% Triton-X100 in 40% N-Methyacetamide (Sigma) for overnight. The clearing solution was replaced with fresh solution and kept for 2 to 3 more days on a shaker at room temperature. Next, cleared LNs were embedded in 2% low melting agarose having 1:10,000 Fluoresbrite YG Calibration beads (1µm, Polysciences) in glass capillaries (2.15mm diameter; Zeiss). Lastly, LNs were kept submerged in clearing solution before imaging to allow refractive index to become consistent between the agarose and clearing solution. Images were then acquired on Zeiss Z.1 light-sheet Microscope, with a 5×/0.16 air objective and were processed using Imaris (Bitplane).

3.4.7 Statistical analysis

Flow cytometry was analysed using Flowjo (FlowJo LLC) and statistical significance was determined using the unpaired (two-tailed) and paired (for chimeric and co-transfer experiments) Student's t test. All experimental data is presented as mean ± standard error of the mean (SEM) with statistical analysis performed using Prism 9 (GraphPad Software).

Chapter 4 Transcriptional flexibility in T follicular helper cells in different infections

4.1 Introduction

GCs are discrete structures formed in the secondary lymphoid tissues following infection or immunisation where B cells undergo proliferation, somatic hypermutation, affinity-based selection and differentiation into long-lived antibody-secreting plasma and memory B cells [6, 9]. This differentiation allow B cells to produce high affinity antibodies that are tailored to provide protection against diverse pathogens such as viruses, bacteria, fungi and helminth [6]. In all these diverse pathogen settings, TFH cells co-exist with B cells to provide helper signals that drive maturation and differentiation of B cells [23, 24, 25, 26, 27, 28]. Of note, this process needs to be tightly regulated in all pathogen settings to prevent autoantibody production.

TFH cells help B cells through two known mechanisms including costimulatory molecules and cytokine signals. Each of these 'helper' signals have a unique role in driving B cell class switching, antibody production and GC formation [6, 9, 10, 11]. To provide help by costimulatory molecules, TFH-B cell form short, non-stable contacts [21]. Contact surface area between TFH-B cells is proportional to the help received by B cells. The extent of surface contact is augmented by binding ICOSL expressed by B cells with ICOS expressed on TFH cells. Ligation of B cell-ICOSL with TFH-ICOS promotes the upregulation of CD40L molecules on TFH cells [21]. A critical component of this T cell help is CD40L, a TNF family member that engages with B cell-CD40. CD40 signalling acts in a positive feedback mechanism to promote ICOSL expression by GC B cells [21]. Together, CD40 and ICOS co-stimulation increase the T:B surface contact, which leads to amplification of the help signals delivered through T cells and facilitates the selection of GC B cells, the formation of GCs and affinity maturation of GC B cells [22, 248, 249].

TFH cells secrete cytokines that facilitate GC B cell differentiation and maturation. The essential cytokine produced by TFH cells is IL-21 [23, 24, 25, 26, 27, 28]. On the basis of IL-21 expression, TFH can be divided into two multifunctional populations, namely IL-21$^+$ TFH and IL-21$^-$ TFH cells [209]. The IL-21$^+$ TFH cells express T-bet, GATA3, IFN-γ, IL-10 and IL-4, while IL-21$^-$ TFH cells express only GATA3 and IL-4, and lack T-bet or IL-10, suggesting transcriptional and functional heterogeneity within TFH cells [209]. TFH also produce IL-4 and IFN-γ depending on the infectious settings [29] and these TFH-derived cytokines play a critical

role in tailoring GC output [44]. Early studies have demonstrated that IFN-γ, IL-4 and IL-21 regulate B cell class switching and affinity maturation [209, 250]. Switching to IgG2 class antibodies is required to clear viral infections and, this is promoted by TFH-derived IL-21 [251]. Further, TFH-secreted IFN-γ is also correlated with IgG2 production following Zika virus infection [252]. In contrast, IgE and IgG1-switched B cells are critical in limiting helminth infections and here TFH-secreted IL-4 regulates IgE and IgG1 class switching [47, 143]. In helminth infections, TFH cells produce IL-21, however this is dispensable for B cell class switching [253]. In bacterial infection such as *C. rodentium,* TFH-derived IL-21 and IL-4 is associated with protective IgG1 antibodies [27]. In addition, TFH-derived IL-21 has been shown to promote an IgA response to commensal microbiota [254, 255]. Together, these studies highlight that TFH cells produce different cytokine signals dependant on the type of pathogen challenge.

The overarching hypothesis of this study is that pathogen-induced cytokine *milieu* drives pathogen-induced gene signatures in TFH cells that regulate TFH cytokine signals which shapes B cell antibody production and isotype switching. In support of this hypothesis there is evidence that pathogen-induced milieu upregulates the expression of lineage defining transcription factor which directs differentiation to one of the TH subsets TH1, TH2, TH17 cells [119, 120, 121]. Here the co-existing TFH cells share features of other TH subsets in their respective skewed settings which tune TFH cytokine signals [124, 125]. For example, in viral infections, TFH cells have been shown to express the prototypical TH1 transcription factor T-bet which regulates IFN-γ expression in TFH cells [23, 24]. However, TFH cells in TH2-skewed *Listeria monocytogenes* infection do not show T-bet expression [218]. Collectively, this suggests that T-bet is precisely expressed in some TFH cells, and that T-bet regulates IFN-γ expression in viral-induced *milieu.* In contrast, TH2-associated transcription factors BATF, IRF4, c-MAF and NFAT promote IL-4 in TFH cells following TH2-skewed helminth infections [150, 155, 156, 158, 178, 180]. In bacterial infections such as *C. rodentium,* less is known about the possibility that TH17 and TFH cells share transcriptional programs. Thus, the aim of this study is to determine pathogen-induced transcriptional signatures by TFH cells in viral, bacterial and helminth infections.

Following infection, a classical GC is divided into two zones the LZ and the DZ and each zone consist of distinct population of cells, whereby the LZ consist of GC B cells, TFR and TFH cells [9, 10, 25, 218]. In contrast, the DZ contains GC B cells that proliferate, and undergo somatic hypermutation [9, 10, 18, 19]. Somatic hypermutation is a process in which random mutations are added in the B cell receptor resulting in B cells with high affinity, lower affinity, or no change

in affinity for an antigen [10]. In the LZ, GC B cells are in contact with TFH cells which facilitates in the selection of high affinity GC B cells [9, 10]. There is a special subset of TREGs known as TFR cells which are present in close proximity to both GC B cells and TFH cells and these cells counter-regulate both TFH and B cells [10, 256, 257]. It has been shown that TFR-derived inhibitory factors such as TGF-β, IL-10, granzyme B and co-stimulatory molecule CTLA-4 suppress multiple GC processes including affinity maturation, production of IL-4 and IL-21 by TFH cells and T:B cell interactions [62, 123, 258, 259]. Importantly, in the LZ, GC subsets TFH, GC B cells and TFR cells are exposed to similar cytokine milieu, therefore, it is likely that GC cells confined in the LZ niche share similar gene signatures. Indeed, both GC B and TFH cells express T-bet in viral infections [23]. This suggest that pathogen-induced transcriptional signatures in TFH cells regulates TFH cytokine signals in GCs which probably leads to similar pathogen-specific gene signatures in GCs subsets tailoring GC output in context dependent manner. Therefore, the second and related hypothesis of this study is that within GC LZ, pathogen-induced TFH-derived cytokine signals shape GC B cell gene signatures according to the type of pathogen. This study aims to determine if TFH, GC B cell gene signatures are aligned between viral, bacterial and helminth infections. Understanding this will give insight into how the immune system tailors B cell response to eliminate broad range of pathogens and form a foundation to understanding this in antibody-mediated diseases such as Lupus and asthma.

To investigate the pathogen-associated transcriptional signatures in TFH cells and GC B cells, I will be conducting RNA sequencing (RNAseq) on collected TFH and GC B cells following virus, bacteria and helminth infections. The infection models listed in this study have previously been shown to generate robust GC response and these infections include viruses influenza A, strain HKx31 (H3N2) [23, 209, 260], LCMV Armstrong [24, 218], bacteria *C. rodentium* [261], fungus (Candida albicans (C. albicans)) and helminth (*H. polygyrus* [44], *Trichuris muris* (*T. muris*) [262]). To correlate pathogen-induced milieu with gene signatures in GC subsets, I found that influenza A and LCMV were TH1-skewed, *H. polygyrus* was TH2-skewed, *T. muris* was TH1-2-skewed and *C. rodentium* was TH1-17-skewed infection. Further, I confirmed, using T-bet that there are distinct pathogen-dependent gene signatures shared between non-TFH, TFH and LZ GC B cells. I have collected the following GC subsets IL-21–GFP^{+} TFH, IL-21–GFP^{-} TFH, non-TFH and B cells in the GC LZ and DZ at the early peak of GC formation in each pathogen setting. While my laboratory work was cut short due to the COVID-19

pandemic, these samples will be used to perform RNAseq analysis to further investigate my hypothesis.

4.2 Results

4.2.1 Identification of early peak of GC response in diverse infections

To investigate the pathogen-associated genes signatures of GC subsets by RNAseq, I first established the early peak of GC response to collect cells for each pathogen. The early peak of the GC response was to collect maximum number of GC cells to obtain high RNA yield. Further, TFH transcriptional programs have been shown to differ between early and late GC response in helminth infection [25]. An early collection time point limits the influence of transcriptional changes that may occur during the GC response in each infection. To determine the early peak of GC B and T cell response in each pathogen setting, I infected C57BL/6 mice with LCMV Armstrong, influenza A HKx31 (H3N2), *H. polygyrus*, *T. muris* and *C. rodentium* infections. Mice were analysed on the indicated days throughout infection (**Figure 4.1**).

In LCMV infection, I observed a higher percentage of TFH and GC B cells at day 10 than all other timepoints analysed. I found that the maximum percentage of TFH and GC B cells at day 12 post *H. polygyrus* infections and day 21 in *T. muris* infection as compared to other timepoints (**Figure 4.1**A-B). I observed that the early peak of TFH cell response in influenza A was on day 10, while at day 14 for *C. rodentium* infection. In contrast, I found the early peak of GC B cell response was on day 12 for influenza A and *C. rodentium* infections. Therefore, I selected the same day of an early peak for both TFH and GC B cells in influenza A and *C. rodentium* because the project aims to understand how pathogen-induced cytokine milieu tunes transcriptional signatures in both TFH and GC B cells at a given time. The time courses for LCMV and influenza A are short because the early peak of GC response was previously established in **Chapter 2.** The days established here as the early peak of TFH and B cell response were used to further characterise these infections and collect GC cells for RNAseq.

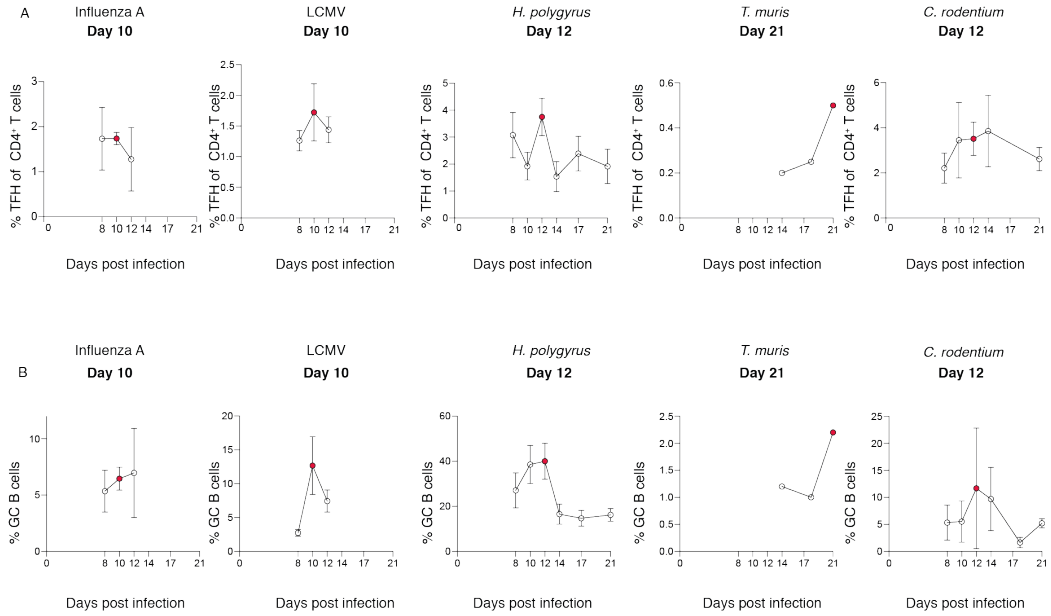

Figure 4.1 Early peak of GC response in diverse infections

*(**A, B**) C57BL/6 mice were infected with influenza A, LCMV, H. polygyrus, T. muris and C. rodentium pathogens. Lymph nodes (LNs) were harvested on the indicated days post infection. The percentage of (**A**) TFH cells and (**B**) GC B cells were determined over the course of infection and the selected day as an early peak for both TFH and GC B cells is highlighted in red.*

Data are representative of one independent experiment, n =3-4 mice/time/group. Data show the mean ± SEM.

4.2.2 Characterisation of pathogen-induced cytokine *milieu*

To investigate pathogen-induced cytokine milieu, I determined the cytokine production by $CD44^+$ $CD4^+$ T cells because in each pathogen setting naive $CD4^+$ T cells differentiate into either TH1, TH2 or TH17 cells. TH subsets are characterised by their cytokine production. For example, TH1 cells primarily produce IFN-γ, whereas TH2 cells produce IL-4, IL-13, IL-15 and TH17 cells mainly secrete IL-17 [119, 142, 161]. Here, C57BL/6 mice were infected with either influenza A, LCMV, *H. polygyrus*, *T. muris*, C. albicans or *C. rodentium* pathogens. At the early peak of the GC response (determined in **Figure 4.1**), I harvested the mice and restimulated all lymphoid cells with phorbol myristate acetate and ionomycin to induce maximal IFN-γ, IL-4 and IL-17 production in T cells (**Figure 4.2**). I observed the higher percentage of IFN-γ^+ $CD4^+$ $CD44^+$ T cells in LCMV infection (**Figure 4.2**D), thus consistent with previous study, LCMV was considered as pathogen that drives TH1-skewed responses [263]. Activated $CD4^+$ $CD44^+$ T cells in influenza A expressed high IFN-γ than all other infections

but not LCMV (**Figure 4.2**D), whereas $CD4^+$ $CD44^+$ T cells in influenza A demonstrated a lower percentage of IL-4 and IL-17 production (**Figure 4.2**D). Based on these results, I classified LCMV and influenza A infections as pathogens that drive TH1-skewed response. In *H. polygyrus* infection, about 1% of $CD44^+$ $CD4^+$ T cells expressed IL-4; although this represented a low level of IL-4 production, this frequency was still higher than all other infections examined in this study. It is possible that other TH2 cytokines such as IL-13 and IL-5 are more highly expressed than IL-4 in this *H. polygyrus* strain, however I was unable to stain for these due to lack of reagents. Based on this result and in agreement with previous studies [264, 265], I classified *H. polygyrus* as a pathogen that drives TH2-skewed response (**Figure 4.2**D). In contrast to the other infection investigated, approximately 0.2% and 2% of all $CD4^+$ $CD44^+$ T cells produced IL-17 and IFN-γ, respectively in *C. rodentium* infection (**Figure 4.2**D). From these findings, I categorised *C. rodentium* as a pathogen that induce TH1-17-skewed response. Activated $CD4^+$ $CD44^+$ T cells in C. albicans showed similar percentage of IL-17 production like in *C. rodentium* infection, thus this pathogen was also classified as TH17-skewed infection model. Previously, *T. muris* infection has been shown to drive both TH2 and TH1 immune responses [266]. Here in *T. muris* infection, I found 0.2% and 1.0% of the total $CD4^+$ $CD44^+$ T cells produced IL-4 and IFN-γ, respectively, whereas the expression of these cytokines was lower than in TH2-skewed *H. polygyrus* and TH1-skewed influenza A infection (**Figure 4.2**D). Potentially the low percentage of IL-4 and IFN-γ indicates an opposing action of T-bet and GATA3 in $CD4^+$ $CD44^+$ T cells in *T. muris* infection [207, 267]. Based on this result, *T. muris* infection was considered a pathogen that drives both TH1 and TH2-skewed response. Herein, on the basis of the cytokine production by $CD44^+$ $CD4^+$ T cells, I have characterised *C. rodentium* as a pathogen that drives a combination of TH1-17-skewed response and *T. muris* pathogen induces TH1-2-skewed response. Both influenza A and LCMV induce TH1-skewed response and *H. polygyrus* pathogen leads to TH2-skewed response. In addition to cytokine profiling in the secondary lymphoid tissues, in the future I plan to detect cytokines by BD CBA Mouse cytokine kit in the serum collected from the mice infected with diverse pathogens. This is because in the blood there are cTFH cells that are characterised as TH1 like TFH-1, TH2 like TFH-2 and TH17 like TFH-17 and are called TH like due to their similarities with conventional $CD4^+$ TH subsets [231, 232]. Among these subsets, cTFH-2 and cTFH-17 cells produce IL-21 whereas circulating cTFH-1 produce limited amount of IL-21 [231]. Thus, comparing cytokine profile within the tissue and in the serum for each infection might indicate

whether circulating TFH cells produce distinct or same cytokines as TFH cells in the lymphoid tissues.

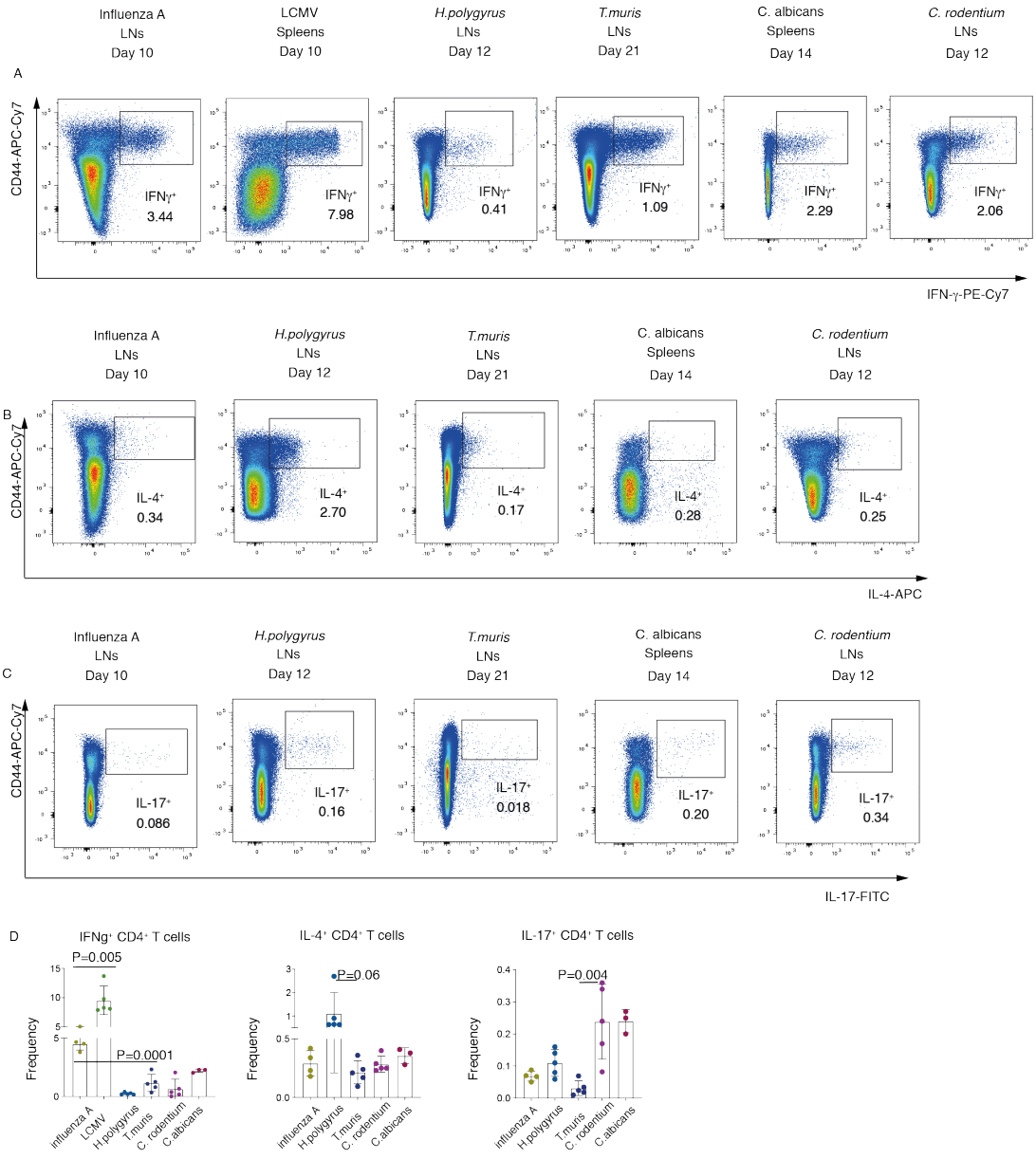

Figure 4.2 Characterisation of pathogen-induced cytokine milieu

*(**A-D**) C57BL/6 mice were infected with either influenza A, H. polygyrus, T. muris, C. albicans or C. rodentium pathogens. Lymph nodes and spleen were harvested on days established as the early peak of GC B and T cell response.*

*(**A-C**) Representative flow cytometry plots of intracellular expression of IFN-γ^+, IL-4^+ and IL-17^+ cytokines in $CD44^+$, $CD4^+$ T cells.*

*(**D**) Percentage of IFN-γ^+, IL-4^+ and IL-17^+ cytokines in $CD44^+$, $CD4^+$ T cells was quantified.*

Data are representative of one independent experiment, n =3-5 mice/group. Data show the mean ± SEM.

4.2.3 Lack of GC response in Candida albicans infection

C. albicans fungus was added in the pool of infection as TH17-skewed infection (**Figure 4.2**). However, I found that the percentage of TFH and GC B cells were similar between infected and uninfected spleen sample and the percentage of TFH and GC B cells seem inconsistent between infected mice (**Figure 4.3**A-B). This indicated that the GC response was not efficiently induced in C. albicans and therefore this infection was removed from the study. A study demonstrated that splenic $CD4^+$ T cells produce high levels of IL-2 in C. albicans infection [268]. IL-2 is a potent negative regulator of TFH differentiation [73]. It is likely that in here, there are fewer TFH cells due to high IL-2, thus, GC B cell response is impaired.

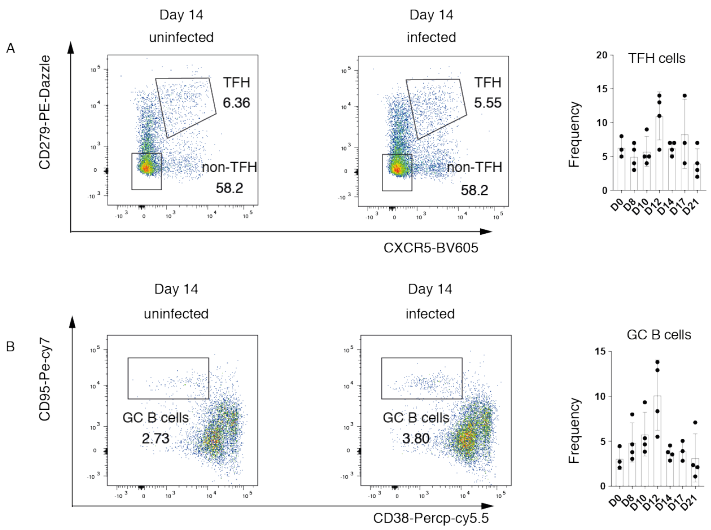

Figure 4.3 Lack of GC response in C. albicans

*(**A-B**) C57BL/6 mice were infected with C. albicans and spleens were analysed on the indicated days.*

*(**A**) Representative flow plots of the percentage of TFH cells in uninfected and infected mice and the percentage of TFH cells were quantified at the indicated days post infection.*

*(**B**) Representative flow plots of the percentage of GC B cells in uninfected and infected mice and the percentage of GC B cells was quantified at the indicated days post infection.*

Data are representative of one independent experiment, n = 3-5 mice/group. Data show the mean ± SEM.

4.2.4 T-bet induction in GC subsets is influenced by pathogen microenvironment

High T-bet was induced in TFH cells following LCMV infection as compared with influenza A infection (as shown in **Chapter 2**), this suggests that T-bet is differentially expressed in different infections. Another study compared T-bet expression in TH1, TH2, TH17 polarising conditions and showed that T-bet was more highly induced in TH1 cells than TH2 and TH17 cells [200]. Together, these studies indicate that T-bet is a pathogen-induced transcription factor in TFH cells and non-TFH cells. Here, I analysed T-bet expression in TFH and non-TFH effector cells in diverse infections to support the hypothesis that pathogen-induced gene signatures are shared between TFH and non-TFH cells in pathogen-dependent manner. To investigate this, ZsGreen_T-bet reporter mice [200], which faithfully report the expression of *Tbx21* transcript were infected with either influenza A, LCMV, *H. polygyrus*, *T. muris* or *C. rodentium.* LNs from the infected mice were analysed at peak of GC response (established in **Figure 4.4**). Consistent with my previous findings (**Chapter 2)**, I found higher percentage of T-bet$^+$ non-TFH in LCMV infection compared with non-TFH in other infections, however, the MFI intensity of T-bet$^+$ non-TFH in LCMV was similar to non-TFH in all infections (**Figure 4.4**C-D). Previous studies have shown that transcription factors including GATA3 and Bcl6 are co-expressed with T-bet in TH1 cells [269, 270]. Here, I found low levels of T-bet expression by TH2 cells in *H. polygyrus* infection, potentially, T-bet is co-expressed with GATA3 in both TH1 and TH2 cells (**Figure 4.4**C-D). Comparison of LCMV*, T. muris* and influenza A, revealed a similar percentage of T-bet$^+$ TFH cells. However, the gMFI was higher in *T. muris* than the other two infections (**Figure 4.4**C-D). The low levels of T-bet expression in TFH cells in both LCMV and influenza A infections may indicate a common mechanism of transcription repression that is absent in TFH cell in *T. muris* infection.

Within the GCs, T-bet^{+} TFH provide IFN-γ signals to GC B cells, which has been shown to induce T-bet in B cells [23, 24, 271]. Next, I sought to dissect whether T-bet expression in TFH compartment can regulate T-bet in B cells following different infections. I identified high percentage of T-bet expression in GC B cells, which aligned with T-bet expression in TFH cells following LCMV infection (**Figure 4.4**E**)**. In *T. muris* infection, despite higher percentage of T-bet in TFH and non-TFH, B cells demonstrated a lower percentage of T-bet. It is likely that IL-4 may inhibit T-bet expression in B cells. This is because *T. muris* was characterised as TH1-2-skewed pathogen (**Figure 4.2**). It is likely that TFH cells produce both IFN-γ and IL-4 whereby IL-4 has been shown to antagonize T-bet expression in B cells [271]. In contrast to other infections, I observed low levels of T-bet expression in T and B cells in both *C. rodentium* and *H. polygyrus* infections (**Figure 4.4**C-E**)**. Consistent with earlier studies [23, 272, 273], I found that the transcription factor T-bet was selectively expressed in both TFH and GC B cells during TH1-skewed LCMV and influenza A infection. Using T-bet expression as an exemplar, this data suggests that there are pathogen-induced transcriptional programs shared between TFH and non TFH cells. In turn pathogen-induced gene signatures in TFH cells influence transcriptional programs in GC B cells.

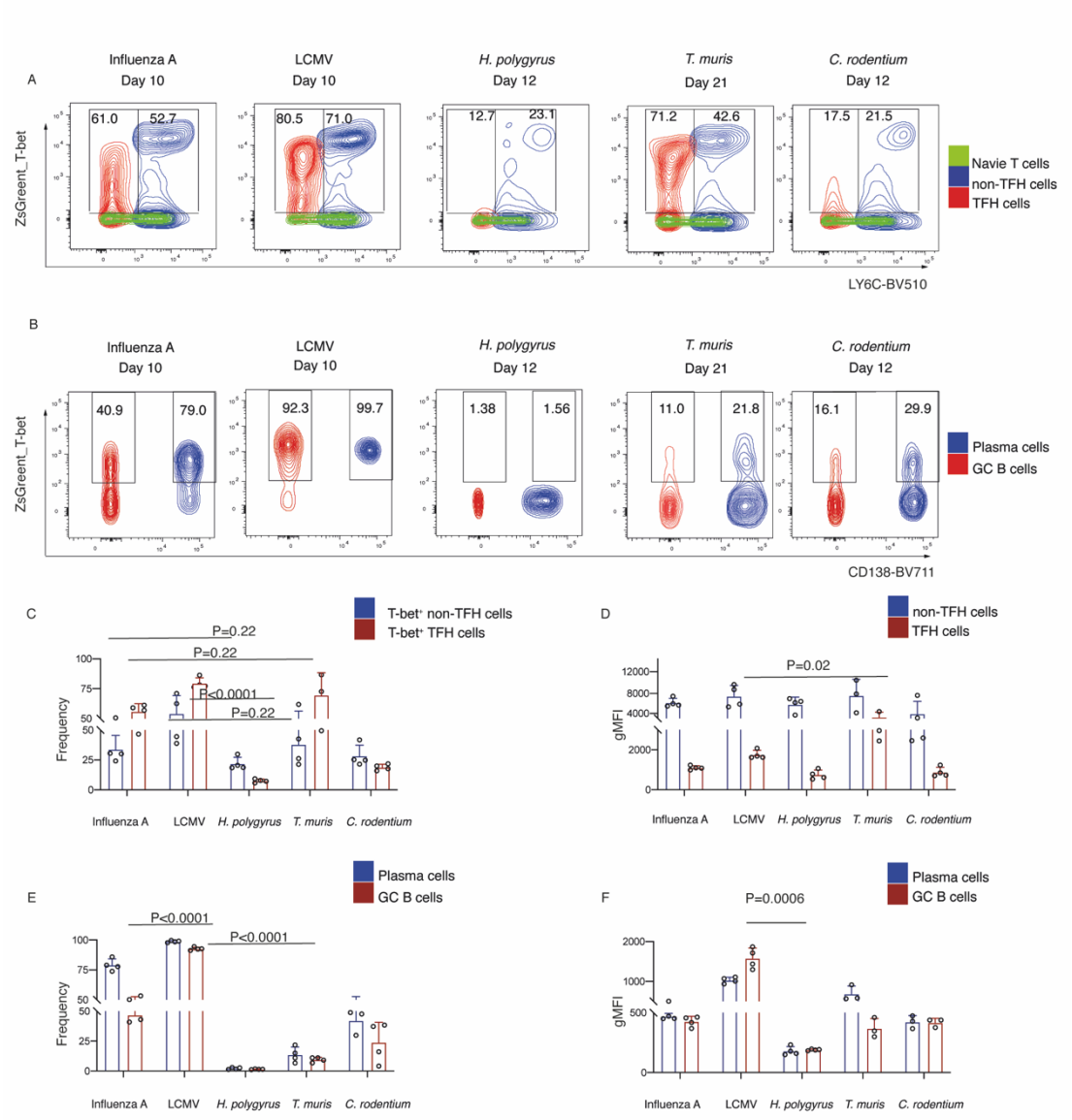

Figure 4.4 T-bet expression in GC subsets in pathogen-dependent manner

*(**A-F**) ZsGreen_T-bet reporter mice were infected with either influenza A, LCMV, H. polygyrus, T. muris or C. rodentium. LNs and spleens were harvested on days determined as early peak of GC B and T cell response for all infections.*

*Representative flow cytometry plots (**A**), frequency (**C**) and MFI of T-bet (**D**) in non-TFH cells (Blue, $CD44^+$, $Ly6C^+$), TFH (Red, $CD44^+$, $Ly6C^-$) and naive T cells (Green, $CD44^-$, $CD4^+$). Representative flow cytometry plots (**B**) frequency (**E**) and MFI of T-bet (**F**) in plasma cells (Blue, $CD44^+$, $B220^-$, $CD138^+$) and GC B cells (Red, $CD138^-$, $B220^+$, IgD^-, $CD95^+$).*

Data are representative of one independent experiment, n =3-5 mice/group. Data show the mean ± SEM.

4.2.5 Gating strategy of GC subsets in different infections

To determine the pathogen-induced gene signatures in TFH cells through RNAseq, I infected IL-21^{GFP} Foxp3^{RFP} reporter mice with either influenza A, LCMV, *H. polygyrus*, *T. muris* or *C. rodentium* pathogens. Lymphoid tissues were harvested from infected mice at the early peak of GC formation from each pathogen setting, and the TH subsets were collected according to the gating strategy shown in (**Figure 4.5**A).

TH subsets were gated into a total of four populations including IL-21–GFP^{+} TFH, IL-21–GFP^{-} TFH, TFR and non-TFH cells from infected mice for different reasons. TFH cells can either be IL-21 producing (IL-21–GFP^{+} TFH) or non-producing (IL-21–GFP^{-} TFH), and these two TFH cells show functional diversification [209]. Thus, to compare the gene signatures of these two TFH populations between different infections, I sorted TFH cells as IL-21 positive (IL-21–GFP^{+} TFH) and IL-21 negative (IL-21–GFP^{-} TFH) (**Figure 4.5**A). TFR cells share expression of CXCR5 and CD279 with TFH subset, while FOXP3 is exclusively expressed by TFR cells. Thus, IL-21^{GFP} Foxp3^{RFP} reporter was used to identify TFR (CXCR5^{+}, PD-1^{+}, Foxp3-RFP^{+}) and to gate TFR cells out from TFH cells subsets to focus on cells that are driving protective immune response (**Figure 4.5**A). Importantly, I sorted non-TFH cells (CXCR5^{+}, PD-1^{+}, IL-21-GFP^{-}, Foxp3-RFP^{-}) (**Figure 4.5**A) to determine pathogen-induced TFH gene signatures. This is because gene signature of non-TFH are regulated by pathogen-induced *milieu* and according to our hypothesis non-TFH pathogen-induced gene signatures are shared with TFH cells. Thus, comparing gene signatures of TFH and non-TFH cells, I can determine pathogen-induced gene signatures in each pathogen settings.

To investigate the gene signatures of LZ GB cells that are regulated by TFH helper signals, WT mice were infected with different pathogens. At the early peak of the GC response, cells were collected from LNs pooled from 6-8 C57BL/6 mice infected for each pathogen. Here, both GC B cells that are localised in the LZ (CD86^{+}, CXCR4^{-}) and DZ (CD86^{-}, CXCR4^{+}) were collected according to the gating strategy shown in (**Figure 4.5**B). GC B cells in the LZ, are in close contact with TFH cells and potentially have gene signatures influenced by TFH cell cytokines. Comparing gene signatures of LZ and DZ GC B cells will inform us about the genes signatures in GC B cells (LZ) that are directly regulated by TFH cells.

All collected TH cells and GC B cells from different infection are listed in **Table 4.1 and 4.2**. Future RNAseq analysis on these collected cells will give insight into pathogen-induced gene signatures of TFH cells. In addition, it will inform about those GC B cell genes shaped by TFH

cells according to the type of pathogen. This is an important step for understanding how humoral immune response is tailored to fight different infections.

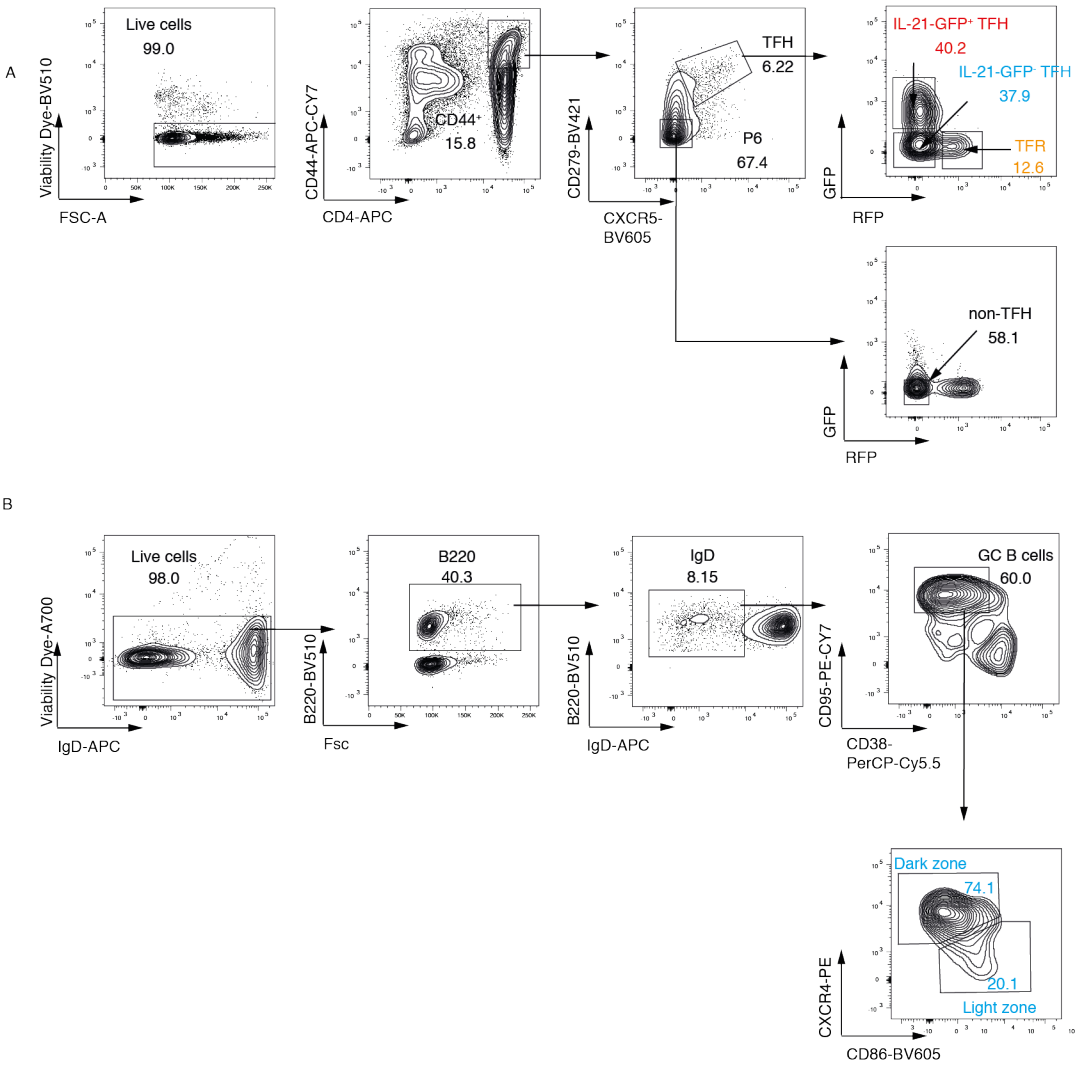

Figure 4.5 Gating strategy of GC subsets in different infections

*(**A**) IL-21^{GFP}Foxp3^{RFP} reporter mice were infected with influenza A. LNs (cervical and mediastinal) were analysed at day 10 post infection. CD4$^+$ T cell subsets were distinguished as IL-21-GFP$^+$ TFH cells (Red, CD44$^+$, CD279$^+$, CXCR5$^+$, IL-21-GFP$^+$), IL-21-GFP$^-$ TFH (Blue, CD44$^+$, CD279$^+$, CXCR5$^+$, IL-21-GFP$^-$), TFR cells (Orange, CD44$^+$, CD279$^+$, CXCR5$^+$, Foxp3$^+$RFP) and non-TFH (Purple, CD44$^+$, CD279$^-$, CXCR5$^-$, Foxp3-RFP$^-$, IL-21-GFP$^-$).*

*(**B**) C57BL/6 mice were infected as in A and GC B cells subsets were marked as GC LZ B cells (Blue, CXCR4$^-$, CD86$^+$) and DZ B cells (Blue, CXCR4$^+$, CD86$^-$).*

Data are representative of one independent experiment, n = 4-5 mice/group.

Table 4.1 List of TH subsets collected in different infections

#	Date	Infection	TH cell skewed infection	Harvest day (post infection)	Secondary lymphoid tissues	IL-21$^+$ TFH cell count	IL-21$^-$ TFH cell count	Non-TFH cells cell count
1	06192018	influenza A	TH1	Day 10	Cervical, Mediastinal LNs	$7.6x10^4$	$8.7x10^4$	$80x10^4$
2	07082018	influenza A	TH1	Day 10	Cervical, Mediastinal LNs	$6.5x10^4$	$15x10^4$	$85x10^4$
3	11022018	LCMV	TH1	Day 10	Axillary, Brachial, Inguinal LNs	$2.54x10^4$	$5.5x10^4$	$72x10^4$
4	01182019	LCMV	TH1	Day 10	Axillary, Brachial, Inguinal LNs	$7.4x10^4$	$8.0x10^4$	$140x10^4$
5	04012020	*H. polygyrus*	TH2	Day 12	Mesenteric LNs	$17x10^4$	$57x10^4$	$250x10^4$
6	04012020	*H. polygyrus*	TH2	Day 12	Mesenteric LNs	$21x10^4$	$68x10^4$	$300x10^4$
7	11292020	*T. muris*	TH1-2	Day 21	Mesenteric LNs	$6.4x10^4$	$9.8x10^4$	$300x10^4$
8	11292020	*T. muris*	TH1-2	Day 21	Mesenteric LNs	$15x10^4$	$14x10^4$	$370x10^4$
9	02052019	*C. rodentium*	TH1-17	Day 12	Mesenteric LNs	$16x10^4$	$31x10^4$	$244x10^4$
10	04262019	*C. rodentium*	TH1-17	Day 12	Mesenteric LNs	$5.2x10^4$	$13x10^4$	$140x10^4$
11	05272019	*C. rodentium*	TH1-17	Day 12	Mesenteric LNs	$9.5x10^4$	$9.9x10^4$	$140x10^4$

Table 4.2 List of GC B cells collected in diverse infections

#	Date	Infection	TH cell skewed infection	Harvest day (post infection)	Secondary lymphoid tissues	GC B cells (LZ) cell count	GC B cells (DZ) cell count
1	10192018	influenza A	TH1	Day 10	Cervical, Mediastinal LNs	$45x10^4$	$92x10^4$
2	11192018	influenza A	TH1	Day 10	Cervical, Mediastinal LNs	$32x10^4$	$78x10^4$
3	11262018	LCMV	TH1	Day 10	Axillary, Brachial, Inguinal LNs	$23x10^4$	$28x10^4$
4	11302018	LCMV	TH1	Day 10	Axillary, Brachial, Inguinal LNs	$26x10^4$	$40x10^4$
5	02122020	*H. polygyrus*	TH2	Day 12	Mesenteric LNs	$86x10^4$	$200x10^4$
6	02122020	*H. polygyrus*	TH2	Day 12	Mesenteric LNs	$180x10^4$	$400x10^4$
7	01102020	*T. muris*	TH1-2	Day 21	Mesenteric LNs	$10x10^4$	$20x10^4$
8	01102020	*T. muris*	TH1-2	Day 21	Mesenteric LNs	$6.9x10^4$	$5.7x10^4$
9	06032019	*C. rodentium*	TH1-17	Day 12	Mesenteric LNs	$19x10^4$	$37x10^4$
10	07022019	*C. rodentium*	TH1-17	Day 12	Mesenteric LNs	$29x10^4$	$75x10^4$

4.3 Discussion

My overarching hypothesis is that TFH cells express pathogen-specific transcriptional programs which in turn regulate B cell responses including antibody production and isotype switching in a pathogen-dependent manner. Investigation of this hypothesis will give insight into how the immune system generates protective humoral immune response against different infections. While I am still waiting to perform the detailed RNAseq analysis on collected GC subsets and non-TFH cells from distinct infection models, here, I discuss some of the potential TFH transcription factors that are either core (expressed in most of the pathogen settings) or pathogen-induced depending on the type of pathogen settings that work in concert to fine-tune TFH cell function (**Figure 4.7**).

In most of the pathogen settings, transcription factors associated with TFH cells regulate production of IL-21, IL-4, IFN-γ and the expression of contact dependent molecules CD40L, ICOS, which are essential for B cell differentiation and antibody production (**Figure 4.6**) [6]. One primary TFH transcription factor is Bcl6 which has an indispensable role in TFH cell differentiation and function [6, 25, 274]. The ultimate mechanism implemented by Bcl6 is to suppress other transcription factors that promote alternate fate at the expense of TFH fate. One key example of Bcl6 mediated suppression is that it negatively regulates KLF2 by directly binding to the promoter region of *Klf2* gene (which encodes KLF2) [56]. Recently, KLF2 was shown to suppress TFH-associated factors such as IL-4, IL-21 and ICOS [56]. Furthermore, Bcl6 has been shown to suppress Runt-related transcription factor 2 and 3 transcription factors that inhibit ICOS expression. These findings strongly suggest that Bcl6 regulates multiple transcription factors that negatively regulate TFH function. Other transcription factors such as TCF-1 and STAT3 have been shown to play an essential role in TFH function by promoting IL-4, IL-21 and ICOS expression following viral and bacterial infections [74, 275, 276]. Another transcription factor, c-MAF was shown to contribute significantly to TFH function by fine-tuning the production of TFH-derived cytokines IL-21, IL-4, IL-17 [59, 157]. A few other studies demonstrated IRF4 transcription factor at the centre of regulating TFH-dependent B cell help signals because of its role in promoting Bcl6, ICOS, IL-21 and IL-4 expression [277, 278]. Importantly, TFH cells express CXCR5 which is critical for TFH migration to the B cell follicles [6] and CXCR5 is regulated by a key transcription factor Achaete-scute homologue 2 (ASCL2) [279]. Based on these findings, I speculate that these core set of transcription factors Bcl6, TCF-1, IRF4, c-MAF, STAT3 and ASCL2 and chemokine receptor CXCR5 regulate

differentiation, migration and function in most of the TFH cells in each distinct infectious setting (**Figure 4.6**).

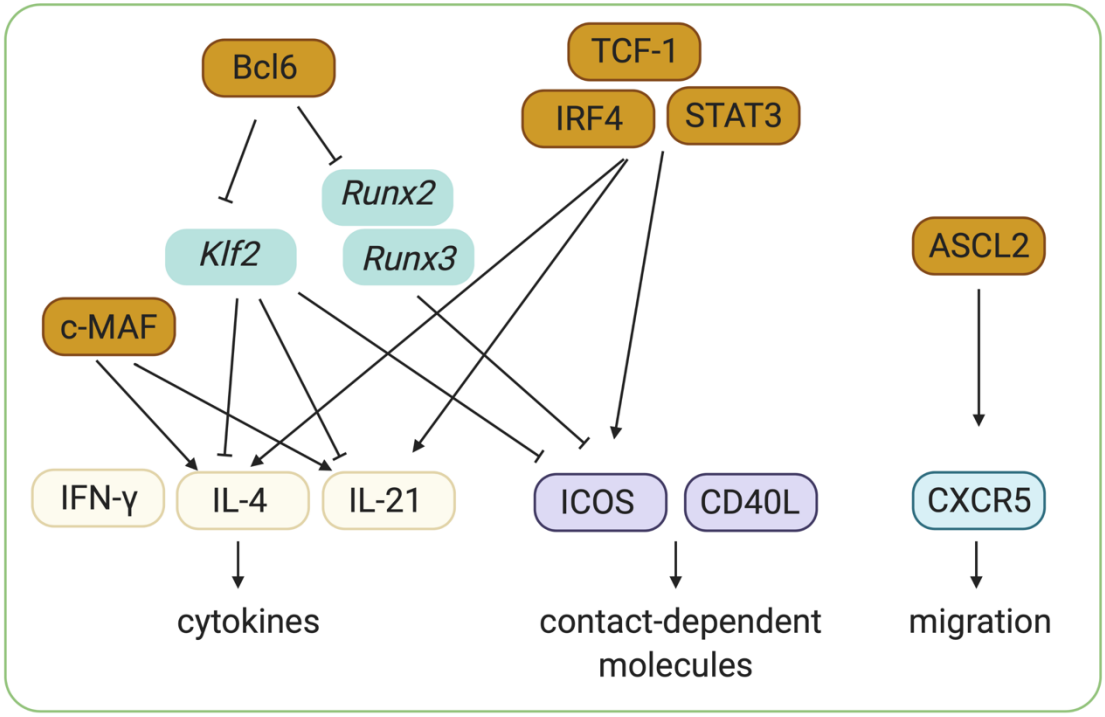

Figure 4.6 Proposed core TFH-associated molecules

In most of the pathogen settings TFH cells express several transcription factors including Bcl6, TCF-1, STAT3, IRF4, c-MAF and ASCL2. The coordinated action of these transcription factors promotes expression of TFH-derived cytokines (IFN-γ, IL-4, IL-2), contact dependent molecules (ICOS, CD40L) and TFH migratory factor CXCR5.

In viral infections, TH1 cells differentiate in parallel with TFH cells and TFH cells express prototypical TH1 transcription factor T-bet and TH1 associated cytokine IFN-γ [23, 24, 198, 280]. To support that T-bet is pathogen-induced transcription factor in TFH cells, I analysed T-bet expression in non-TFH and TFH cells during infection with pathogen that drive either TH1, TH2, TH1-2 or TH1-17-skewed response (**Figure 4.4**). Indeed, I found higher percentage of cells expressing T-bet in both TFH and non-TFH effector cells following TH1-skewed LCMV infections (**Figure 4.4** A). In contrast, there was a lower percentage of T-bet^{+} non-TFH and TFH cells following *H. polygyrus* and *C. rodentium* infection which skew responses in a TH2 direction (**Figure 4.4** A). Together these findings suggest that T-bet expression in non-TFH cells is reflected in TFH cell in pathogen-induced cytokine *milieu*. Thus, I have collected both non-TFH cells and TFH cells for RNAseq and comparing non-TFH gene signatures with TFH gene signatures will indicate pathogen-associated gene signatures in TFH cells.

T-bet positively regulates expression of TH1 associated cytokine IFN-γ in TFH cells in viral infections [23, 24]. Early studies have demonstrated that T cell-derived IFN-γ induces T-bet in B cells which regulates IgG2a class switching in B cells, while T-bet expression in TFH cells suppressed IgG1 production [23, 51, 281]. Another important role of T-bet in B cell is the generation of long-lived antibody secreting plasma and memory B cells following viral infection [50]. Furthermore, T-bet in GC B cells has been shown to promote the localisation of B cells in the dark zone of the GCs which facilitates B cell proliferation and affinity maturation in malaria infection model [273]. TFH cells share GC LZ niche with GC B cells, thus, I investigated whether T-bet in TFH cells could regulate T-bet expression in GC B cells. Consistent with T-bet expression in TFH cells, T-bet was highly expressed in GC B cells in LCMV infection as compared to influenza A infection (**Figure 4.4** B-E). Potentially higher percentage of T-bet$^+$ TFH cells induced higher levels of IFN-γ in LCMV than influenza A. This in turn led to higher T-bet expression in GC B cells in LCMV versus influenza A infection. Similar to TFH cells, GC B cells lacked T-bet expression in TH2-skewed *H. polygyrus* infection. This supports the notion that pathogen-induced transcription factors in TFH cells tailor TFH cytokine signals which shape transcription factor expression in B cells. Therefore, I will perform RNAseq on GC B cells in both LZ and DZ because GC LZ cells are in close proximity to TFH cells and exclusion of GC B cells (DZ) gene signatures from GC B cells (LZ) will indicate gene signatures directly regulated by TFH cells. To investigate this, I plan to use confocal microscopy to analyse the localisation of T-bet-expressing cells in the GCs in different infections. This temporospatial mapping will give insight into whether T-bet expression in TFH cells reflects the positioning of GC B cells in the LZ within a shared GC LZ niche. I speculate that T-bet will be highly expressed in GC LZs in the secondary lymphoid tissues from LCMV infected mice in comparison to *H. polygyrus* infected mice.

In helminth infection such as *H. polygyrus*, CD4$^+$ TH cells differentiate into TH2 cells which are characterised by IL-4, IL-13 and IL-15 production [47]. Consistent with a previous study [47], I found higher IL-4 production by activated CD4$^+$ TH cells as compared to other infections, suggesting that these IL-4 producing cells are TH2 cells (**Figure 4.2** B). TH2 cell differentiation is regulated by lineage defining transcription factor GATA3 which is activated downstream of IL-4 signalling [148, 149, 282]. In addition, GATA3 regulates TH2 cytokine production by directly binding to the promoter regions of the *Il4* and *Il13* gene loci (which encode IL-4, IL-13) and enhancer regulatory elements of *Il4* gene locus (which encodes IL-4), promoting expression of IL-4 and IL-13 [150]. Other transcription factors including NFAT, AP-

1, JunB, c-MAF and IRF4 are also associated with IL-4 production in TH2 cells [283, 284]. Similar to TH2 cells, TFH cells have shown to secrete IL-4 which regulates B cell class switching to IgE instead of TH2-derived IL-4 in helminth infections [143]. In contrast to TH2 cells where GATA3 is a central regulator of IL-4 expression, other transcription factors c-MAF and IRF4/BATF complex directly bind to IL-4 locus, promoting IL-4 transcription in TFH cells [156, 285]. In TFH cells, the expression of BATF is initiated by both IL-4/STAT6 and IL-6/STAT3 signalling [150], whereby STAT6 is activated in TFH in IL-4 enriched cytokine *milieu*. Potentially, STAT6 is another pathogen-associated transcription factor shared between TFH and TH2 cells in helminth infections [150]. Transcription factor growth factor independence 1 (GFI1) acts downstream of STAT6 and promotes TH2 differentiation and IL-4 production [286]. Currently, it is unknown whether GFI1 is expressed in TFH cells or if it can promote TFH-derived IL-4 production in TH2-skewed infections which will be investigated by RNAseq in TFH cells in *H. polygyrus infection.* Based on these studies, I speculate that GATA3, STAT6, IL-4 in TH2 and STAT6, IL-4 in TFH cells are pathogen-induced transcription factors and cytokine that will be upregulated specifically in TFH and non-TFH cells following RNAseq analysis on TH cells *following H. polygyrus* infection (**Figure 4.7**).

A seminal study by King and Morus (2009) demonstrated that IL-4 producing cells localised in the B cells follicles are TFH cells following *H. polygyrus* infection [47]. TFH derived-IL-4 has been shown to control the generation of IgG1 and IgE switched B cells, affinity maturation of IgG1-expressing B cells and GC formation during helminth infections [47, 143]. IL-4 regulates these multiple B cell processes through STAT6, whereby IL-4 activated STAT6 directly promotes the transcription of immunoglobulin heavy chain germline gamma1 and epsilon which are required for isotype switching to IgG1 and IgE respectively [287]. In addition, STAT6 indirectly promotes the transcription of immunoglobulin heavy chain germline epsilon through NFIL3 in cultured B cells and following immunisation [288]. However, the role of NFIL3 in B cell class switching during *H. polygyrus* infection is unexplored and will be investigated by RNAseq analysis in GC B cells collected from *H. polygyrus* infected mice. STAT6 regulates GC formation by downregulating EBI2 in B cells which leads to GC formation in helminth infection while it is not required for GC formation in LCMV infection [46]. EBI2 is G-protein chemotactic receptor, and its ligand is highly expressed in the outer regions of the B cell follicle, thus downregulation of this receptor allows B cells to migrate to the centre of the B cell cells follicles initiating GC formation [116, 117]. Based on these studies, I predict that TFH-

derived IL-4 will induce STAT6 expression in GC B cells and STAT6 will be upregulated in RNAseq analysis in GC B cells isolated from *H. polygyrus* infected mice (**Figure 4.7**).

In *T. muris* helminth infection, TH cells demonstrated high T-bet, moderate IFN-γ and low IL-4 expression. Thus $CD4^+$ T cells, in this model were classified as both TH1-2 cells (**Figure 4.2-4.4**). In this setting, non-TFH cell have characteristics of both TH1 and TH2, potentially the pathogen-induced milieu will upregulate transcription factors and cytokines in TFH and B cell similar to that in *H. polygyrus* and LCMV infection.

Activated $CD4^+$ T cells showed low levels of IFN-γ and T-bet expression whereas higher IL-17 production in *C. rodentium* in comparison to influenza A, thus, this model was characterised as a pathogen that drives TH1-17-skewed response (**Figure 4.2-4.4**). Currently these studies were unable to detect IL-17 production either by human tonsillar TFH or murine TFH cells following immunisation or infection [57, 58, 209]. Thus, in this setting, I speculate that IFN-γ instead of IL-17 will be upregulated in TFH cells during RNAseq analysis. Earlier studies demonstrated that T-bet^+ TFH provide IFN-γ signals to GC B cells which result in upregulation of T-bet in B cells [23, 24, 271]. In this study, I found higher percentage of T-bet^+ B cells in *C. rodentium* than *H. polygyrus* (where TFH cells are potentially IL-4 and IL-21 producing), this result supports the speculation that in this model TFH cells are probably expressing IFN-γ. Together, in this setting, I speculate that several pathogen-induced factors will be identified in each cell compartment during RNAseq analysis including IL-17, RORγT, T-bet, IFN-γ, STAT4, STAT3 for TH17 cells whereas T-bet, IFN-γ, STAT4 for TFH cells and T-bet, STAT4 in B cells (**Figure 4.7**). Here, I have mentioned some transcription factors and cytokines expressed by non-TFH, TFH and GC B cells in each type of infection that I will be identify in RNAseq analysis but also identify new, as yet uncharacterised transcription factors. The identification of pathogen-associated gene signatures that lead to distinct TFH cytokines in diverse infections can be used to develop transcriptional targets to control TFH-derived cytokines that might lead to autoimmune diseases and allergies.

In this study, I have proposed that in addition to the core transcription factors, TFH cells express pathogen-induced transcription factors and cytokines which are different for each type of infection. During TFH cell differentiation phase there are multiple other transcription factors that negatively regulate genes which are critical for TFH migration, TFH related costimulatory molecules, inhibitory receptors and cytokines [56]. Instead, Bcl6 inhibits these transcription factors by binding to the DNA sequences in the regulatory regions of their respective genes

and recruits corepressor complexes which leads to the suppression of their transcription [289, 290, 291]. Potentially, both TFH inhibitory transcription factors and Bcl6 binding sites will be different for each pathogen challenge. Thus, future RNAseq results will be complemented with Cleavage Under Targets and Tagmentation (CUT&Tag) analysis whereby CUT&Tag is a molecular technique that helps to identify transcription factors DNA binding sites throughout the genome [292]. CUT&Tag is similar to chromatin immunoprecipitation assay but has several advantages as it requires a much lower cell number, gives lower background and is more cost-effective than chromatin immunoprecipitation assay [292]. Using the CUT&Tag technique, Bcl6 antibody will be used to identify unique Bcl6 cofactor binding sites in TFH cells following viral, bacterial and helminth infections. I speculate that there will be common Bcl6 DNA binding sites in all pathogen settings. However, Bcl6 will not bind and suppress the proposed pathogen-induced gene signatures in different infections.

T-bet is differentially expressed in TFH cells following LCMV and influenza A infections as demonstrated in **Figure 4.4.** In **Chapter 2**, I showed that T-bet is required for TFH cell differentiation in LCMV infection while it is not necessary in influenza A infection. These findings indicate that T-bet has a role in TFH differentiation in context dependent manner and potentially T-bet works in collaboration with Bcl6 in regulating TFH fate in LCMV infection. The interplay between T-bet-Bcl6 has been shown in TH1 cells where T-bet physically recruits Bcl6 to the promoter region of the gene of interest resulting in the suppression of the gene [210, 270]. Thus, T-bet DNA binding sites in TFH cells will be determined by CUT&Tag and then compared with Bcl6 DNA binding sites to investigate whether T-bet-Bcl6 forms a complex to bind to Bcl6 DNA binding sites or T-bet works independently to control TFH cell differentiation and function. This information is important to understand how combination of lineage defining transcription factors work together while maintaining TFH phenotype yet adding additional functional properties. Recently, a seminal study found a rare population of IL-13 secreting TFH cells that co-expressed Bcl6 and GATA3 in wildtype mice following Alternaria allergen immunisation but not in helminth infection [293]. Here IL-13 was implicated in the generation of high affinity IgE producing B cells that led to allergen induced anaphylaxis [293]. IL-13^{+} TFH were also found in cTFH cell compartment of peanut allergic or aeroallergen patients than the healthy controls [293]. Another study demonstrated TFH cells produce high level of IL-17 and expressed prototypical TH17 transcription factor RORγT in autoimmunity prone BDX2 mice model [294]. In this study and another study, IL-17 was implicated in the development of autoantibody producing B cells in autoimmune BXD2 mice [60, 294].

Mechanistically, IL-17 was shown to promote the expression of *Rgs16* and *Rgs13* in the B cells which impaired migration of B cell between the LZ and DZ, and prolonged retention of B cells in the GCs that promoted autoantibody production [60]. Furthermore, TFH-derived IL-4 and IL-21 was shown to mediate acute anaphylaxis in response to peanut allergen [295]. There is emerging evidence that IL-4 and IL-21 produced by TFH cells lead to IgE antibody class switching which results in pathogenesis of asthma [296]. In addition, increased frequency of cTFH cells and higher serum IL-21 levels were associated with rheumatoid arthritis patients [297]. These studies highlight that TFH cells are transcriptionally and functionally flexible. Understanding the role played by the microenvironment in tuning TFH heterogeneity might provide valuable inside in the development of transcriptional targets to prevent the generation of pathogenic autoantibody producing B cells in autoimmunity and allergy.

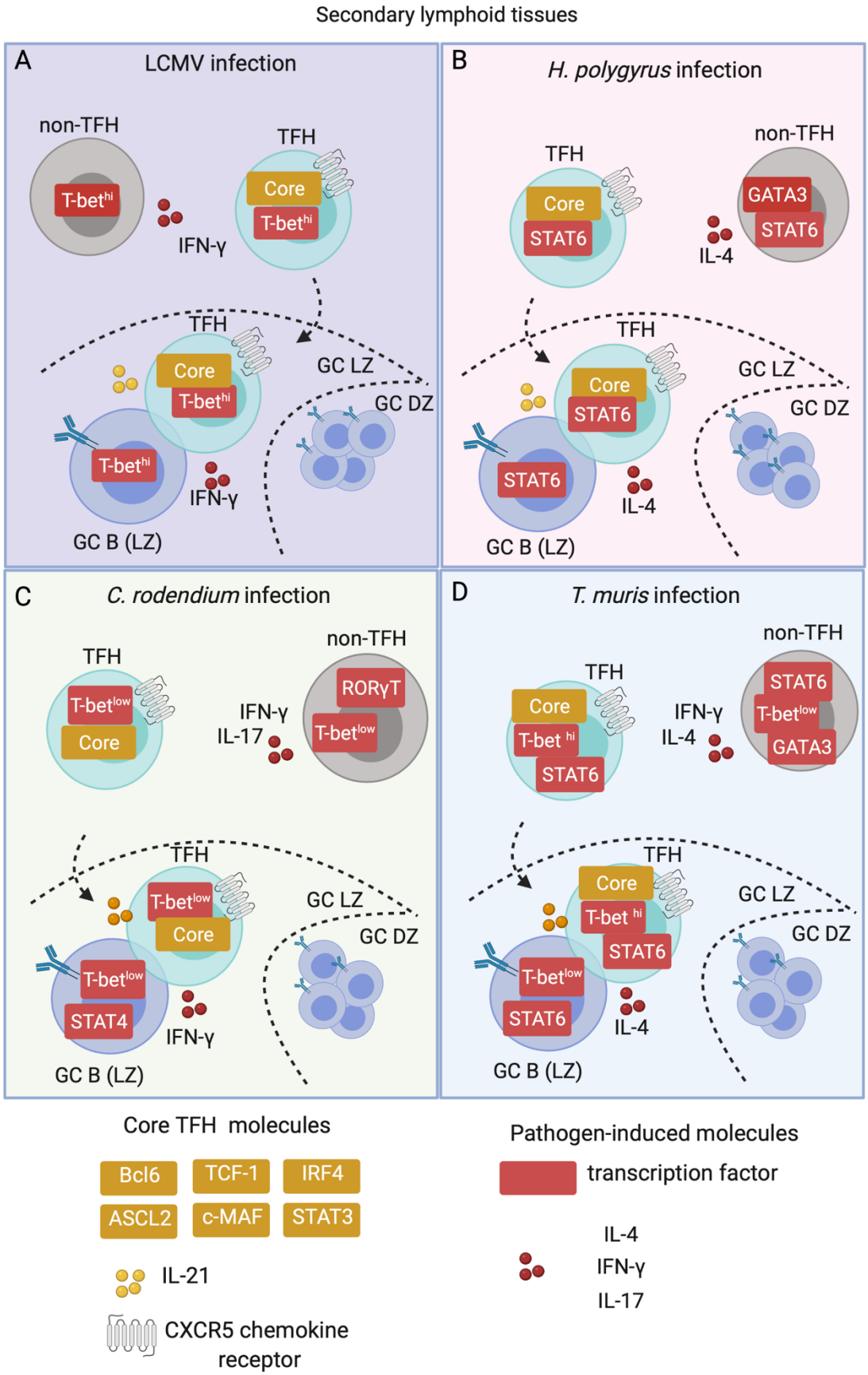
Secondary lymphoid tissues
A
LCMV infection
non-TFH
T-bethi
IFN-γ
TFH
Core
T-bethi
GC LZ
GC DZ
GC B (LZ)
B
H. polygyrus infection
TFH
Core
STAT6
non-TFH
GATA3
STAT6
IL-4
GC B (LZ)
C
C. rodendium infection
TFH
T-betlow
Core
IFN-γ
IL-17
non-TFH
RORγT
T-betlow
STAT4
GC B (LZ)
D
T. muris infection
TFH
Core
T-bet hi
STAT6
IFN-γ
IL-4
non-TFH
STAT6
T-betlow
GATA3
GC B (LZ)
Core TFH molecules
Bcl6
TCF-1
IRF4
ASCL2
c-MAF
STAT3
IL-21
CXCR5 chemokine receptor
Pathogen-induced molecules
transcription factor
IL-4
IFN-γ
IL-17

Figure 4.7 Proposed shared pathogen-induced factors in non-TFH, TFH and GC B (LZ) cells signals in different infections

*(**A-D**) Following infection, activated $CD4^+$ T cells differentiate into non-TFH and TFH cells in the secondary lymphoid tissues. In most of the pathogen settings, TFH cells express core TFH molecules including transcription factors, cytokines, chemokine receptors (shown in **Figure 4.6**). I speculate that pathogen-specific factors induce similar transcriptional factors in non-TFH and TFH cells. TFH cells migrate to the GCs which are divided into the LZ and DZ. In the LZ of the GC, TFH cells provide pathogen-specific help signals to GC B (LZ) cells which shapes GC B (LZ) transcription factor profile. (**A**) In LCMV infection, TFH cells share pathogen-induced high T-bet expression with non-TFH and GC B (LZ) cells. (**B**) Non-TFH, TFH and GC B (LZ) cells express H. polygyrus-associated STAT6 transcription factor (**C**) In C. rodentium bacterial infection, TFH cells share low T-bet expression with counterpart non-TFH cells and GC B (LZ) cells. (**D**) T. muris-associated factors regulate STAT6 and low T-bet expression in TFH, non-TFH and GC B (LZ) cells.*

4.4 Methods

4.4.1 Mice

Mice strains described below were maintained on C57BL/6 background in specific-pathogen-free conditions at the Walter Eliza Hall Institute. IL-21^{GFP} mice [209], Foxp3^{RFP} mice [298], ZsGreen_T-bet reporter [200] mice have previously been described. IL-21^{GFP} mice were crossed with Foxp3^{RFP} mice to generate IL-21^{GFP}Foxp3^{RFP}. Female mice were used at 6-8 weeks of age.

4.4.2 Infections

Influenza A infection: mice were humanely anesthetized with Penthrane and inoculated intranasally with 1x10^4 plaque-forming units (PFU) of influenza A strain HKx31 (H3N2). *LCMV infection*: mice were infected with 3x10^3 PFU of LCMV Armstrong by intravenous injection into the tail vein. *H. polygyrus infection*: mice were inoculated with 200 infective third-stage larvae by oral gavage. *T. muris infection:* mice were infected with 200 *T.muris* eggs via oral gavage and helminth burdens were analysed on various days post-infection as previously described [299]. *C. rodentium infection*: mice were inoculated with 2x10^9 colony-forming unit of *C. rodentium* by oral gavage. Mice were assessed between 5 to 8 days after infection and bacterial dissemination was assessed by cultures of livers and spleens from infected mice on agar plates containing nalidixic acid. *C. albicans infection:* mice were injected intravenously with 1x10^5 CFU of *C. albicans.*

LCMV, influenza A and *C. rodentium* experiments were performed in accordance with the Walter and Eliza Hall Institute animal ethics committee, while *T. muris* and *H. polygyrus* infections were conducted in accordance with Monash university animal ethics committee.

4.4.3 Cell staining for flow cytometric analysis

For identification of TFH cells: single cells were incubated with anti-CXCR5 for 20 min at 37° C, then washed and stained with additional surface markers at 4° C for 20 min. *For B cells analysis:* single cells were incubated with surface markers for 20 min at 4°C. *For intracellular analysis:* samples were stimulated for 4 hours at 37°C in RPMI containing 10% FCS, 100ng/ml PMA (phorbol 12-myristate 13 acetate), 500ng/ml ionomycin, GolgiStop (BD Biosciences) and GolgiPlug (BD Biosciences). Staining for cytokines was performed by Cytofix/Cytoperm kit. The flow cytometry analysis was done on BD LSRFortessa X-20 cell analyser (BD Biosciences) and data analysis was performed with FlowJo v.10 (FlowJo LLC).

4.4.4 Antibodies and dyes for flow cytometry

For T cell analysis: anti-CXCR5 (Clone L138D7; Biolegend), anti-CD279 (Clone RMP1-30; Biolegend), anti-CD44 (Clone 1M7; BD PharMingen), anti-CD4 (Clone GK1.5; BD Horizon) and Fixable Viability Stain eF506 (eBioscience). *For B cell analysis*: anti-CD86 (Clone GL1; BD Horizon), anti-B220 (Clone RA3-6B2; BD Horizon), anti-CD38 (Clone 90; eBioscience), anti-CD95 (Clone Jo2; BD PharMingen), anti-CXCR4 (Clone 2B11; eBioscience), anti-IgD (Clone 1126c; produced in house), anti-CD138 (Clone 281-2; BD Horizon) and Fixable Viability Stain 700 (BD Horizon). *For Cytokine analysis*: anti-IFNγ (Clone XMG1.2; BD Horizon), anti-IL-4 (Clone 11B11; BD Biosciences), anti-IL-17 (Clone TC11-18H10; Biolegend).

4.4.5 Storage of cell pellets for RNAseq

Stained cells were sorted using Aria fusion (BD) and resuspended in RLT buffer containing β-mercaptoethanol according to the manufacturer's directions (Qiagen RNeasy Plus Micro Kit-cat# 74034) and stored at -80° C for further processing.

4.4.6 Statistical analysis

Statistical significance was determined using the unpaired (two-tailed) Student's t test. All experimental data is presented as mean ± standard error of the mean (SEM) with statistical analysis performed using Prism 9 (GraphPad Software)

Chapter 5 General Discussion

The overarching hypothesis of the studies contained in this thesis is that pathogen-induced factors promote unique TFH transcriptional gene signatures which in turn shape B cell antibody production. To date, knowledge on the transcriptional programs required for TFH cell differentiation and function is largely based on the interpretations from individual infections without direct comparison between infection models. This study aimed to delineate the controversial role of pathogen-induced T-bet and CXCR3 molecules in TFH cell differentiation and migration in viral infection models and then investigate this more broadly in diverse infection types. This information will help to shape a comprehensive model of TFH gene program that caters for different infections.

5.1 Context-dependent role of T-bet in TFH differentiation

Despite being phenotypically and functionally distinct, the TFH and TH1 share a common precursor that is characterised by the co-expression of two lineage specifying transcription factors, Bcl6 and T-bet. Bcl6 and T-bet are mutual competitors and antagonise the expression of each other. Ultimately, one of these transcription factors wins the differentiation race, to determine the formation of TFH and TH1 cells respectively (as reviewed in Sheikh and Groom [1], Appendix 1). In line with this model of TFH/TH1 differentiation, T-bet-deficient mice showed expansion of TFH cells with a reciprocal decrease in counterpart TH1 cells following Toxoplasma gondii infection in mice [198]. Contrary to TFH/TH1 differentiation model, T-bet-deficient STg cells demonstrated that T-bet is required for both TFH and TH1 cell differentiation following LCMV infection [24, 218]. Thus, in this study I have demonstrated a context-specific role for T-bet in TFH differentiation (**Chapter 2**). Specifically, although distinct viral infections induce T-bet, the threshold of this expression is distinct between infections. In influenza A infection, low T-bet was induced, and this promoted preferential TFH lineage formation *in vivo* (**Chapter 2; Figures 2 & 4**). In contrast, when T-bet was highly expressed within TFH cells, T-bet-deficiency limited the formation of both TH1 and TFH cells in LCMV infection (**Chapter 2; Figures 3 & 4**). These findings highlight that the ratios of Bcl6 to T-bet within TH1/TFH shared precursor cell is a decisive factor in shaping $CD4^+$ T cell fate trajectory.

The ratio of Bcl6 to T-bet is established by, and interconnected with, cytokine signals supporting transcriptional networks in newly activated $CD4^+$ T cells that undergo differentiation (as reviewed in Sheikh and Groom [1], Appendix 1). One primary cytokine IL-2

has been shown to regulate the ratio of Bcl6 and T-bet by downregulating Bcl6 expression while promoting T-bet expression in TH1 cells [270]. I proposed that IL-2 signalling through STAT5 in $CD4^+$ T cells could be different between LCMV and influenza A infection that led to different outcomes of T-bet-deficiency in $CD4^+$ T cells. To test this, IL-2 signalling was enhanced in $CD4^+$ T cells following LCMV infection in comparison with influenza A infection (**Figure 5**). Recently, DiToro et al [73] established a new paradigm whereby early TFH precursors produce IL-2, while IL-2 is consumed by early $CD25^+$ TH1 precursors (CD25 is a high affinity IL-2 receptor). The equilibrium between IL-2 producers/consumers keep IL-2 levels in check and imbalance leads to either the promotion or inhibition of TFH differentiation. Thus, in the absence of T-bet during LCMV, the loss of IL-2 consumers (TH1) results in excessive IL-2 and STAT5 activity, and thus cells are blocked from both TH1 and TFH fates [23]. In contrast, during influenza A infection, there is likely a competing cytokine, potentially IL-6, that may upregulate Bcl6 expression and promote TFH, even when the IL-2 consumers are lacking in the absence of T-bet [23]. Consistent with this, recent research led by the Ballesteros group [300] found that TFH utilized cell intrinsic IL-6 signalling, which blocked STAT5 from binding to the *Il2rb* locus (encodes CD122, a chain of IL-2 receptor), in turn TFH lacked expression of IL-2 receptor and remained insensitive to IL-2 [300]. Together, this explains how high IL-2 may tip the Bcl6-T-bet axis and act as a cytokine switch between TFH and TH1 fate commitment (as reviewed in Sheikh and Groom [1], Appendix 1).

Several other factors may also explain the different transcriptional requirements for $CD4^+$ T cells during between LCMV and influenza A infections. In addition to IL-2, other cytokines have shown to play a role in tipping Bcl6-T-bet axis such as IL-12, IFN-γ, TNF-α, type I interferons which promote T-bet, while TGF-β and IL-6 induce Bcl6 expression (as reviewed in Sheikh and Groom [1], Appendix 1). The DC sources of these cytokines act to facilitate TFH and TH1 fate commitment have recently been reviewed [301]. Recently, high level of the proinflammatory cytokine TNF-α was correlated with an increase in $T\text{-}bet^+$ TH1 cells with reciprocal decrease in $Bcl6^+$ TFH cells in the spleen of diseased COVID-19 patients [302]. Further, during malaria infection, characterised by high TNF-α and IFN-γ, TFH cells expressed T-bet, thus, suggesting that the ratios of Bcl6-T-bet in TFH cells is tuned by this cytokine *milieu* [303]. In a study, the timing of IL-6 production by DCs was shown to direct the TFH/TH1 dichotomy, whereby an early wave of type I IFN induced DCs to produce IL-6. In turn, this promoted precursor cells to commit to the TFH fate at the expense of TH1 fate [304]. In contrast, late production of type I IFN resulted in DCs becoming insensitive to type I IFN;

hence, DCs failed to produce IL-6. In this setting, precursor cells adopted a TH1 fate trajectory instead of a TFH trajectory [304] (as reviewed in Sheikh and Groom [1], Appendix 1). These findings indicate that several cytokines may play an important role in setting the ratio of Bcl6-T-bet and regulate TH1 and TFH cell differentiation in LCMV or influenza A infection.

The infiltration of cytokine-secreting cells into the secondary lymphoid tissues could be another factor different between LCMV and influenza A infection. A comparative study demonstrated impaired B cell responses in the draining LNs of LCMV infected mice as compared with vesicular stomatitis virus [305]. Here, it was reasoned that in LCMV infection inflammatory monocytes ($CD11b^+$ $Ly6C^{hi}$) were recruited to the draining LNs that led to reduced B cell responses [305]. This suggests that inflammatory monocytes are selectively recruited in LCMV infection versus vesicular stomatitis virus. It has been shown that inflammatory monocytes promote TH1 but not TFH cell differentiation following plasmodium and salmonella infection [213, 306]. Inflammatory monocytes are recruited to the lymphoid tissues in CCR2-dependent manner where they secrete IL-12 and stimulate TH1 ontogeny following immunisation [307]. Influenza A infects the lungs whereas LCMV infection is systemic. It is possible that the infiltration of inflammatory monocytes to the lymphoid tissue may differ between LCMV and influenza A infection. This can be confirmed by comparing the recruitment of inflammatory monocytes in the secondary lymphoid tissues of LCMV and Influenza A infected WT mice. If recruitment of inflammatory monocytes is distinct between infections, blocking via the deletion of CCR2 will promote TFH instead of TH1 cells by altering the ratio of Bcl6-T-bet in differentiating $CD4^+$ T cells. IL-12 has been shown to promote T-bet whereby high T-bet enhanced TH1 cells at the expense of TFH cells following salmonella infection [308]. Thus, I speculate that infiltrating inflammatory monocytes tip Bcl6-T-bet balance by promoting T-bet levels in IL-12 dependent manner. My results to date highlight that while LCMV and influenza A are characterised under the umbrella of viral pathogens that induce TH1-skewed response, IL-2 levels are different in each pathogen challenge. This means that inflammatory responses in different infections play a role in determining whether TH1-mediated cellular response or TFH dependent antibody response dominates the other.

My work is consistent with the concept that inflammatory factors alter the ratio of T-bet-Bcl6 which in turn shapes TH1 and TFH dichotomy [23, 304]. Currently, multiple cytokines are being considered as an immunotherapy against pathogens. For example, IL-6 blocking is being tested to treat severely affected Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV)-2

patients [309]. Furthermore, a study correlated type I IFNs treatment with the clearance of virus in patients infected with SARS-CoV-1[310]. While current knowledge of the interplay between cytokine and transcriptional gene networks would suggest both of these immunotherapies heighten TH1 differentiation, this may come at the cost of TFH differentiation and therefore be detrimental to the development of neutralizing humoral immunity [6] (as reviewed in Sheikh and Groom [1], Appendix 1). Thus, in-depth understanding of what inflammatory factors and how these factors are regulated in pathogen-dependent manner will indicate suitable immunotherapies without compromising on either TH1 or TFH cell differentiation.

5.2 T-bet-dependent TFH differentiation is influenced by CXCR3

T-bet was shown to promote both TH1 and TFH cell differentiation following LCMV infection (as shown in **Chapter 2**), however, the downstream mediator of T-bet expression required for TFH differentiation in this infection setting remains unknown. In TH1 cells, T-bet upregulates CXCR3 which orchestrates intranodal placement of TH1 cell to T:B border and access to this niche is important for TH1 cell differentiation and function [216, 239]. I found that the majority of pre-TFH cells (known as early TFH precursors) expressed CXCR3. T-bet expression controlled CXCR3 upregulation in pre-TFH cells following LCMV infection (**Chapter 3; Figure 3.3**). Current findings indicate a conflicting role of CXCR3 in TFH cells whereby CXCR3-CXCL10-dependent $CD4^+$ T cell interactions in the interfollicular regions were shown to promote GC formation and protective antibody responses following vaccination [217]. Conversely, another study demonstrated that PD-1 downregulated CXCR3 to maintain the localisation of TFH cells in the GCs [82]. Given that T-bet is expressed in TFH cells, I hypothesised that CXCR3 acts downstream of T-bet to regulate TFH cell positioning at T:B border and differentiation. On day 4 post LCMV infection, deficiency in T-bet or CXCR3 led to expansion of pre-TFH as compared with WT cells at the expense of pre-TH1 cells (**Chapter 3; Figure 3.5**). This implies that CXCR3 is not required for early differentiation of TFH cells. Importantly CXCR3-deficiency in STg cells phenocopies that of T-bet. Later in infection, CXCR3-deficient $CD4^+$ T cell had a reduced frequency of TFH cells, while there was no change in the total number of *Cxcr3*$^{-/-}$ TFH as compared to the WT cells following LCMV infection (**Chapter 3; Figure 3.6**). Visual observation of the GCs in the whole intact LNs by LSFM indicated presence of fewer *Cxcr3*$^{-/-}$ TFH cells as compared to WT cells (**Chapter 3; Figure 3.6**). Fewer *Cxcr3*$^{-/-}$ TFH in GCs by LSFM contradicts no change in the number of *Cxcr3*$^{-/-}$ TFH result and thus these experiments need to be repeated. Together, these findings to date indicate that CXCR3 is not required for pre-TFH but is necessary for TFH cell

differentiation (on a per cell number basis) following LCMV infection.

In this study, I found that the CXCR3 ligand CXCL10 expressing cells were located at T:B cell regions of the LNs and STg cells were in close proximity to these CXCL10 expressing cells (**Chapter 3; Figure 3.8**). These results are yet to be confirmed and cell positions quantified, however this initial observation suggests that CXCR3 exerts its role downstream of T-bet induction to regulate TFH intranodal migration to T:B border in response to CXCL10. Recent studies in our laboratory indicate that CXCL10 producing cells located at T:B border are cDC2 following LCMV infection [241]. cDC2 cells located at T:B border are required for TFH differentiation following immunisation [77, 79]. Collectively, the current findings propose a new mechanism that T-bet$^+$ TFH cells rely on CXCR3/CXCL10-dependent migration to T:B border. In this niche pre-TFH cell interaction with cDC2 provide additional differentiation signals to TFH cells. However, this path may only be required when T-bet is induced in pre-TFH, and it is this complexity that has resulted in the unresolve role for CXCR3 in TFH differentiation.

Due to CXCR5 expression, a population of peripheral CD4$^+$ T cells are considered circulating memory TFH cells [311]. In the past few years these cells have gathered a lot of interest due to their potential use as a biomarkers of protective antibody response to vaccination or infection and autoantibody production in autoimmune diseases [311]. On the basis of CXCR3 and CCR6 surface expression, circulating TFH cells are characterised into three distinct populations, TH1-like cTFH-1 (CXCR3$^+$ CCR6$^-$), TH2 like cTFH-2 (CXCR3$^-$ CCR6$^-$) and TH17 like cTFH-17 (CXCR3$^-$ CCR6$^+$) and are called TH like due to their similarities with conventional CD4$^+$ TH subsets [231, 232]. Among these cTFH cells, cTFH-1 cells (CXCR3$^+$ CCR6$^-$) were correlated with influenza specific antibody producing cells following seasonal influenza vaccination [232]. Recent study showed that inactivated influenza vaccine initially induced short-lived antibody secreting cells and later elevated levels of CD21^{lo} and CD21^{hi} influenza specific memory B cells. Here, cTFH-1 cells were associated with early short-lived and later memory B cell response following immunisation [233]. Increased frequency of cTFH-2 and cTFH-17 cells than cTFH-1 were found in rheumatoid arthritis patients [312], while augmented cTFH-2 cells were correlated with disease activity in lupus patients [313]. Given that cTFH cells predict protective antibody response, it is unclear whether cTFH cells originate from GC TFH cells. There are a few similarities between human cTFH and tonsillar GC TFH cells, which include shared T cell receptor sequence and expression of c-Maf, PD-1 and CXCR5 [311, 314, 315]. GC TFH cells are Bcl6$^+$, CCR7$^-$, while cTFH cells lack Bcl6 expression but express CCR7 which allows them to

migrate to the secondary lymphoid tissues [316]. In LCMV experiment, I showed high CXCR3 expression in TFH cells, which led to a question whether TFH cells exit the GCs and secondary lymphoid tissues in CXCR3-dependent manner and form pool of TH1-like cTFH-1 in the blood. This can be investigated by analysing cTFH in the blood and TFH in lymphoid tissues in mice where CXCR3 is specifically deleted after GC have formed in LCMV infection. CXCR3 deletion could be induced by tamoxifen in hCXCR3$^{flox/flox}$ ERT2Cre mice line which has been established in the laboratory by crossing hCXCR3 $^{flox/flox}$ mice [317] with ERT2Cre [318]. Understanding the link between cTFH and GC TFH cells is important for the development of vaccines and assessment of the role of TFH in autoimmunity. As it is not feasible to directly monitor GC TFH activity in patients, identifying the ontogeny of cTFH cells in blood will allow more insight into vaccine responses and autoimmune diseases.

5.3 Transcriptional flexibility in TFH cells in different infections

The coordination between cell-mediated and humoral arms of the adaptive immunity is a prerequisite for the clearance of different pathogens including viruses, bacteria and helminth. Each pathogen-associated factor directs the differentiation of activated CD4$^+$ T cells to either TH1, TH2 or TH17 [122, 123]. These TH subsets regulate cell-mediated arm of the adaptive immunity which involves activation of other immune cells including macrophages, natural killer cells and eosinophils [122, 123]. In most pathogen challenges, TFH cells provide help signals to B cells to shape the humoral arm of the adaptive immunity best suited for each type of infection. As exemplified by TFH-secreted IFN-γ regulates B cell switching to IgG2a which is important to clear viral infections, while TFH produced IL-4 promotes B cell class switching to IgE and IgG1 that helps to eradicate helminth infection [23, 24, 47, 218]. The underlying mechanism that regulates TFH cells to produce cytokines according to the type of infection is unclear. In viral infections, TFH and TH1 cells co-express T-bet which promotes IFN-γ expression in TFH cells (as shown in **Chapter 2; Figure 6)** and by others [24, 218]. This finding may support the hypothesis that pathogen-influenced cytokine *milieu* upregulates similar gene signatures in TFH cells and counterpart CD4$^+$ T cell subsets. This facilitates humoral and cell mediated immune response to work in synergy against multiple infections. I found that TH1 associated transcription factor T-bet is differentially induced between influenza A and LCMV infection whereby high T-bet expression is required to regulate TFH cell differentiation in LCMV infection (as shown in **Chapter 2; Figure 4**). This indicates that pathogen-induced cytokine *milieu* is a key switch that regulates the level of gene expression in TFH cells. Prior to investigating this hypothesis, I sought to determine if T-bet expression was related to

pathogen-induced cytokine *milieu* in different infection models. As I had previously observed differentially T-bet expression in LCMV and influenza A, suggesting that T-bet expression is regulated by the pathogen microenvironment (**Chapter 4; Figure 4.4**). Multiple infection models were selected according to their cytokine skewing including virus influenza A, LCMV, bacteria *C. rodentium* and helminth *H. polygyrus* and *T. muris*. I found that the higher percentage of T-bet$^+$ non-TFH cells in LCMV infection compared with non-TFH in other infections (**Chapter 4; Figure 4.4**). The percentage of T-bet$^+$ TFH cells in LCMV, *T. muris* and influenza A was similar. In contrast, TFH and TH1 cells showed lower levels of T-bet expression in *C. rodentium* and *H. polygyrus* infection (**Chapter 4; Figure 4.4**). It can thus be suggested that pathogen-specific cytokine milieu regulates distinct gene programs in TFH cells.

TFH and GC B cells are co-localised in the LZ of the GCs, here they are exposed to similar cytokine *milieu* [6]. Indeed, both GC B and TFH cells express T-bet in viral infections (**Chapter 2; Figure 1**). This indicates that pathogen-induced transcriptional factors in TFH cells are reflected in GC B cell gene signatures which are confined in the LZ. The other related hypothesis of this study was that TFH-derived cytokines shape GC B cell gene signatures according to the type of pathogen. To support this hypothesis, I first analysed T-bet in B cells following different infections. I found high T-bet in GC B cells in LCMV as compared to influenza A infection (**Chapter 4; Figure 4.4**). High level of T-bet in GC B cells was aligned with T-bet expression in TFH cells in LCMV infection. T-bet has been shown to regulate IFN-γ expression in TFH cells (**Chapter 2**). I speculate that TFH-derived IFN-γ promotes elevated T-bet in GC B cells in LCMV infection as there is evidence that IFN-γ-mediates T-bet expression in B cells [271]. In *C. rodentium* and *H. polygyrus* infection, both TFH and GC B cells demonstrated low T-bet expression (**Chapter 4; Figure 4.4**). Therefore, in multiple infections, I observed that the levels of T-bet are matched in non-TFH, TFH and GC B cells but are distinct in different infections. To formally analyse pathogen-induced gene signatures in TFH cells, I will conduct RNAseq on collected TFH, non-TFH and GC B cells in the LZ and DZ in viral, bacterial and helminth infections. Pathogen-induced factors promote CD4$^+$ T cells to differentiate into either TH1, TH2 or TH17 [122, 123]. Therefore, comparing gene signatures of TFH and non-TFH cells during RNAseq, I will be able to determine pathogen-induced gene signatures in TFH cells. Two subsets of GC B cells were collected, the LZ GC B cells that are in close proximity to TFH cells and GC B cells in the DZ. The variation of gene expression during RNAseq analysis in LZ GC B cells as compared with GC B cells in the DZ will indicate the genes that are directly influenced in LZ GC B cells by TFH cells. The data generated from

this study will clearly indicate the pathogen-specific gene programs in TFH cells and how these pathogen-associated genes influence antibody producing B cells. A population of Treg cells, TFRs have been shown to share similar tissue niche as LZ GC B cells and TFH, while negatively regulating both GC B cells and TFH responses [319]. The transcriptional profile of TFR cells has been shown to be influenced by the microenvironment [137]. It is plausible that the transcriptional similarities between LZ GC B cells and TFH would be extended to TFR cells according to the infection settings. In this study, identification of gene signatures of TFH, LZ GC B cells and including TFR cells may indicate potential transcriptional targets that can be used to either enhance or control humoral immune responses against different infections.

5.4 Concluding remarks

In summary, the key finding of this thesis is that high T-bet in TFH cells regulate TFH cell differentiation in LCMV, whereas low T-bet in TFH cells is not required for their development in influenza A infection. This led to the investigation of CXCR3 as a downstream target of T-bet to regulate TFH cell differentiation and localisation in the GCs in LCMV. Together, T-bet and CXCR3 data indicates that there is a heterogeneity in TFH cell differentiation and migration paradigm in pathogen-dependent manner. This led me to a broader hypothesis that pathogen-induced microenvironment promotes expression of additional genes in TFH cells. In turn, TFH cells provide help signals to B cell tailored according to the type of pathogen. Importantly, transcriptional and functional flexibility of TFH cells has been correlated with the development of autoimmune diseases and allergies. Recent study identified TFH co-expressed Bcl6 and GATA3 and produced IL-13 in mice challenged with Alternaria allergen immunisation. Here, TFH-derived IL-13 regulated the generation of high affinity IgE producing B cells that led to allergen induced anaphylaxis [293]. Instead, TFH cells secreted IL-4 in helminth infection was implicated in the development of low affinity IgE [293]. One important thing to consider here is that both Alternaria allergen immunisation and helminth drive TH2-skewed response [293]. This study again supports the notion that the microenvironment regulates unique gene programs in TFH cells which in turn shape B cell class switching or antibody production. This may lead to either protective or dysregulated humoral immune response in infection, autoimmunity and allergy. Therefore, dissecting the interplay between microenvironment and transcriptional programs represents a key step in identifying novel targets for the development of vaccines or drugs to treat allergy and autoimmune diseases that exhibit a specific skewing of TFH populations

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Appendix 1

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REVIEW ARTICLE OPEN

Transcription tipping points for T follicular helper cell and T-helper 1 cell fate commitment

Amania A. Sheikh[1,2] and Joanna R. Groom[1,2]

During viral infection, immune cells coordinate the induction of inflammatory responses that clear infection and humoral responses that promote protection. CD4$^+$ T-cell differentiation sits at the center of this axis. Differentiation toward T-helper 1 (Th1) cells mediates inflammation and pathogen clearance, while T follicular helper (Tfh) cells facilitate germinal center (GC) reactions for the generation of high-affinity antibodies and immune memory. While Th1 and Tfh differentiation occurs in parallel, these CD4$^+$ T-cell identities are mutually exclusive, and progression toward these ends is determined via the upregulation of T-bet and Bcl6, respectively. These lineage-defining transcription factors act in concert with multiple networks of transcriptional regulators that tip the T-bet and Bcl6 axis in CD4$^+$ T-cell progenitors to either a Th1 or Tfh fate. It is now clear that these transcriptional networks are guided by cytokine cues that are not only varied between distinct viral infections but also dynamically altered throughout the duration of infection. Thus, multiple intrinsic and extrinsic factors combine to specify the fate, plasticity, and function of Th1 and Tfh cells during infection. Here, we review the current information on the mode of action of the lineage-defining transcription factors Bcl6 and T-bet and how they act individually and in complex to govern CD4$^+$ T-cell ontogeny. Furthermore, we outline the multifaceted transcriptional regulatory networks that act upstream and downstream of Bcl6 and T-bet to tip the differentiation equilibrium toward either a Tfh or Th1 fate and how these are impacted by dynamic inflammatory cues.

Keywords: Cytokines; Infection; T follicular helper cells; T-helper 1 cells; Transcription factors

Cellular & Molecular Immunology _####################_; https://doi.org/10.1038/s41423-020-00554-y

INTRODUCTION

CD4$^+$ T cells form a bridge between the cell-mediated and humoral arms of the adaptive immune response to pathogens. Following infection, naive CD4$^+$ T cells can differentiate into distinct T-helper (Th) subsets, including Th1, T follicular helper (Tfh), Th2, Th17, and regulatory T cells as well as memory cell precursors.[1] Naive and newly activated CD4$^+$ T cells sense changes in the microenvironment and integrate those signals through the upregulation of lineage-defining transcriptional factors. The balance of these transcription factors then directs CD4$^+$ T cells down a particular development path. The flexibility of CD4$^+$ T cells to diverge into distinct subsets, which is guided by pathogen-specific inflammatory cues, enables tailored immune responses against diverse immune challenges.

Tfh and Th1 cells are key players in orchestrating CD4$^+$ T-cell-dependent cell-mediated and humoral adaptive immune responses to intracellular pathogens, such as viruses. Tfh cells migrate into germinal centers (GCs), which are specialized microanatomical structures that form in secondary lymphoid organs following vaccination and infection and are sites of robust humoral immune responses. Within GCs, Tfh and B cells interact to allow affinity maturation of B cells and their differentiation into memory and high-affinity antibody-secreting B cells. Dysregulated Tfh cell differentiation profoundly impacts immune responses and can lead to immunodeficiency and systemic autoimmune disease.[2]

In contrast, cell-mediated immune responses during viral infection are elicited by Th1 cells. Unlike Tfh cells, Th1 cells leave secondary lymphoid organs to infiltrate peripheral tissues and facilitate cell-mediated responses to localized inflammation or infection. Here, they produce cytokines, which in turn activate macrophages and CD8$^+$ T cells to promote clearance of intracellular pathogens and tumors.[3] Given this role in orchestrating the humoral and cellular arms of the adaptive response, a fundamental goal is to understand the factors that initiate Tfh and Th1 ontogeny.

In Th1-skewed infections, CD4$^+$ T cells differentiate into Tfh cells, Th1 cells, and memory cell populations in parallel. The choice between these fates follows antigen presentation by DCs and is based on T-cell receptor signal strength, the microenvironment and costimulatory receptor signaling induced by cellular interactions. Prior to this developmental bifurcation and the formation of mature effector subsets, Tfh and Th1 cells share a common precursor stage (Fig. 1);[4,5] therefore, the differentiation path of Tfh and Th1 cells overlaps, and CD4$^+$ T cells select either fate at the expense of the other. Currently, the precise timing of this CD4$^+$ T-cell bifurcation is contested. While some studies indicate that CD4$^+$ fate decisions are imprinted early, prior to the first cell division following activation,[6,7] others indicate that this branching occurs later, between days 2 and 4 post infection.[5] These discrepancies may represent differences in infection models,

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Received: 25 June 2020 Accepted: 2 September 2020
Published online: 30 September 2020

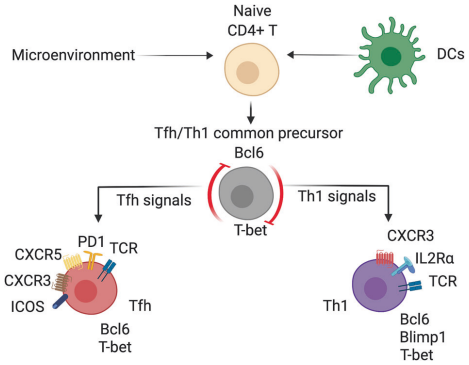

Fig. 1 Tfh and Th1 fate trajectories. Naive CD4$^+$ T cells following antigen presentation by dendritic cells and additional signals from the microenvironment develop into common Tfh/Th1 precursor cells that coexpress Bcl6 and T-bet, which are expressed competitively. In the presence of Tfh signals, common Tfh/Th1 precursor cells differentiate into Tfh cells that co-express key transcription factors (Bcl6 and T-bet), chemokine receptors (CXCR5 and CXCR3), and molecules (PD-1 and ICOS). In contrast, common Tfh/Th1 precursor cells that differentiate into Th1 cells in response to Th1 signals express the canonical Th1 transcription factors Blimp-1, T-bet, and Bcl6; chemokine receptors (CXCR3); and IL-2 receptor (IL-2Rα)

experimental approaches to define precursors, or plasticity in differentiating CD4$^+$ T cells. In this review, we discuss how transcriptional factors work independently and together to direct Tfh/Th1 bifurcation. We propose that a Bcl6–T-bet axis exists in parallel with the Bcl6–Blimp-1 paradigm for Tfh/Th1 ontogeny. Importantly, these transcriptional networks are context-dependent and tuned by dynamic changes in the environment to tip the balance toward either Tfh or Th1 cell formation. Furthermore, we discuss recent advances and anomalies in the field that shed new light on how the unique cytokine milieu in different infections is a key decisive factor in determining Tfh versus Th1 fate. Understanding the multifactorial process of the Tfh/Th1 dichotomy will pave the way to rationally develop immunotherapeutics to direct pathogen clearance and vaccines that promote the formation of neutralizing antibodies following viral infections.

THE BCL6–T-BET AXIS: THE LINCHPIN OF TFH/TH1 EQUILIBRIUM

Despite being phenotypically and functionally distinct, Tfh and Th1 cells share precursors characterized by the coexpression of two lineage-specifying transcriptional factors, Bcl6 and T-bet (Fig. 1).[4,5,8–11] Unlike mature effector populations, CD4$^+$ T-cell memory precursors express Bcl6 and T-bet at low to intermediate levels, and Bcl6 deficiency leads to loss of CD4$^+$ central memory cells along with Tfh cells.[12–15] Within the precursors of effector cells, Bcl6 and T-bet are competitively co-expressed and antagonize the expression of each other. Ultimately, one of these transcription factors wins the differentiation race to determine the formation of Tfh or Th1 cells. It is therefore important to understand the characteristics that allow these transcriptional factors to either be co-expressed or show dominate expression to mediate CD4$^+$ T-cell fate commitment in the context of infection.

Bcl6

Bcl6 is a proto-oncogene zinc-finger transcriptional repressor that has a profound role in the function and differentiation of multiple immune lineages. Bcl6 expression is essential for robust humoral immunity. GC B cells express high levels of Bcl6, which prevents their differentiation into plasma and memory cells.[16,17] Consistent with this, Bcl6-deficient mice display impaired GC formation, lack of antigen-specific antibodies against T-cell-dependent antigens, and impaired affinity maturation.[18] In addition, Bcl6 regulates the generation and maintenance of memory CD8$^+$ T cells.[19] A decade ago, studies illustrated the role of Bcl6 in Tfh fate commitment.[2] Initial studies highlighted that the interleukin 6 (IL-6) and interleukin 21 (IL-21) cytokines promote Tfh differentiation in vitro and that this differentiation is associated with the upregulation of Bcl6 in both murine and human Tfh cells.[11,20–22] CD4$^+$ T cells deficient in Bcl6 failed to develop into Tfh cells and were insufficient to support GC reactions in vivo, thus definitively showing that Bcl6 is required for Tfh differentiation.[11,21,23] Since the discovery of Bcl6 as a lineage-defining transcription factor, studies on the Tfh transcriptional differentiation axis have been focused on the antagonistic relationship between Bcl6 and Blimp-1, (a transcription factor encoded by *Prdm1*).[11,21,23,24] Bcl6 and Blimp-1 antagonize and inversely regulate each other's expression in both GC B cells and Tfh cells. In T cells, Blimp-1 is downregulated in Tfh cells but it is maintained at high levels in non-Tfh CD4$^+$ T cells.[11] The overexpression of Blimp-1 inhibits CD4$^+$ T cells to acquire the Tfh phenotype by inhibiting the expression of canonical markers, including CXCR5, ICOS, and PD-1.[11,21,23,25] While it was previously proposed that Tfh cell formation was a default differentiation state for CD4$^+$ T cells, this has recently been shown to not be the case, as cells deficient in both Bcl6 and Blimp-1 fail to form Tfh cells in vivo following both immunization and viral infection.[25] As discussed below, this study confirmed previous work highlighting that Bcl6 acts as a hub for the transcriptional repression of pathways that inhibit Tfh differentiation.[25] Importantly, this and other studies have recently demonstrated that Bcl6 repression of these transcriptional networks occurs independently of Blimp-1, further highlighting the indispensable role for Bcl6 in Tfh fate commitment.[25,26]

Several unique structural features of the Bcl6 protein allow it to interact with diverse transcription factors and chromatin modifiers to form transcriptional complexes. These interactions permit Bcl6 to control gene expression in CD4$^+$ T-cell precursors and in mature Tfh cells. The Bcl6 protein consists of an N-terminal POZ (or BTB, broad complex, tramtrack, bric-a-brac) domain, a middle domain also known as RDII, and a C-terminal zinc-finger domain. The BTB domain mediates interactions with Bcl6 corepressors, including N-COR, SMRT, and BCOR.[27–29] These cofactors compete to bind to the Bcl6 N-terminus and can recruit histone deacetylase (HDAC) protein complexes to form a transcription-repressing complex at the target gene. HDACs are enzymes that modify chromatin structure, and this in turn prevents the ability of transcription factors to bind to regulatory regions and activate the transcription of target genes.[30–32] Mutations in the BTB domain inhibit the differentiation of Tfh cells.[33] Furthermore, BCOR-deficient CD4$^+$ T cells fail to differentiate into Tfh cells.[34] Taken together, these results suggest that mutations in the BTB domain prevent BCOR binding. While N-COR and SMRT are also expressed in CD4$^+$ T cells, further research is needed to determine their role in Tfh differentiation; however, recently, N-COR was shown to be recruited to the Bcl6 promoter to negatively regulate Bcl6 expression.[25] Bcl6 utilizes its largest domain, the RDII domain, to associate with the corepressor MTA3. This interaction leads to the repression of Blimp-1.[35] The C-terminus of the Bcl6 protein harbors six Kruppel-like C2H2-type zinc-fingers that can bind to a nine-base-pair DNA sequence (TTCCT(A/C)GAA) that shares sequence homology with STAT (signal transducer and activator of transcription) and activator protein 1 (AP-1) DNA binding sites.

In 2015, a landmark study led by Hatzi et al. mapped the cis-acting targets of BCL6 in human GC Tfh cells. This research outlined that BCL6 is directly or indirectly recruited to loci of

multiple key genes actively involved in Tfh fate.[22] First, BCL6 is indirectly recruited to non-BCL6 DNA binding sites by other transcription factors in Tfh cells.[22] In accordance, BCL6 is enriched at AP-1 DNA binding motifs in Tfh cells. This is mediated by the physical association of BCL6 and AP-1 in $CD4^+$ T cells, which enables BCL6 to be recruited to cis-regulatory regions of many genes in an AP-1-dependent manner.[22] AP-1 is a collective term used for transcription factors that consist of Jun, Fos, or ATF (activating transcription factor) subunits that form dimers. These transcriptional activators play a vital role in T effector cell differentiation, proliferation, and function.[36–38] It is suggested that recruitment of BCL6 to the AP-1 DNA binding motif converts AP-1-dependent gene activation to repression. Notably, BCL6 and AP-1 colocalize at the *Prdm1* locus, which contains an AP-1 DNA binding motif.[22] It could be that BCL6 exploits AP-1 to establish the Tfh transcription program through suppression of Blimp-1. A secondary mechanism of BCL6 occurs via direct binding to the enhancer and promoter regions of genes important in T-cell migration. The relocation of Tfh precursor cells to the B-cell follicle is a prerequisite for an effective GC response.[39] BCL6 regulates multiple T-cell migration factors to establish Tfh cell homing to B-cell follicles and to prevent Tfh cell egress from secondary lymphoid tissues. Specifically, BCL6 binds to the promoter and enhancer of *Ccr7* (encoding CCR7) and *Selplg* (encoding PSGL-1 proteins), which are known to regulate the migration of T cells to the T zone of secondary lymphoid tissues.[22] *Selplg* was shown to be directly repressed by Bcl6 following LCMV infection.[25] Furthermore, BCL6 binds to the gene encoding EBI2, which may lead to repression of its expression.[22] In both B and Tfh cells, EBI2 has been shown to play a role in the localization of cells to the extrafollicular regions of secondary lymphoid tissues.[40,41] Moreover, BCL6 promotes the expression of the key Tfh cell markers IL-21R and CXCR5 in $CD4^+$ T-cell culture, and mutations in the Bcl6 zinc-finger DNA binding domain restrict BCL6-mediated upregulation of Bcl6, IL-21R, and CXCR5 in $CD4^+$ T cells.[21] Recently, Bcl6 repression of Gata3, Runx2, and Klf2 was confirmed to increase the expression of CXCR5 to promote the migration of $CD4^+$ cells into B-cell follicles in vivo.[25] Overall, these actions of BCL6 on T-cell migration facilitate the movement of cells toward the follicle, into environmental niches that further promote Tfh differentiation.

One of the most critical roles of Bcl6 in imprinting Tfh fate is to block the differentiation of alternate Th cell types. For example, in human Tfh cells, BCL6 binds to the promoter regions of genes important for alternate Th fates, including *GATA3*, *RORA*, and *IFNGR1*, and the enhancer regions of the *TBX21* gene (which encodes T-bet).[22] In addition, Gata3, Tbx21, and Id2 constitute a transcriptional signature of Bcl6-repressed genes in antigen-specific mouse T cells.[25] Mature $CD4^+$ T cells also have BCL6 binding sites that are depleted of the enhancer histone marks H3K4me1 and H3K27ac in comparison to naive $CD4^+$ T cells, suggesting that these regulatory regions are in an inactive state. It is likely that BCL6, along with its corepressors N-COR, SMRT, and BCOR, recruit HDACs to these sites to dynamically modify histone marks.[42] Furthermore, Bcl6-deficient cells cultured in Th1 conditions demonstrated increased expression of T-bet and RORγt,[21] suggesting that this mechanism may be at play even in non-Tfh cells. In summary, Bcl6 controls Tfh fate commitment via direct repression of alternative fates by regulating the coercion of cofactors and epigenetic factors and inhibiting alternate Th cell positioning and cytokine signaling. Together, these studies show that Bcl6 is highly involved in establishing Tfh fate.

T-bet

The transcription factor T-bet is expressed in numerous immune lineages and plays an essential role in regulating antiviral immunity. In $CD8^+$ T cells, T-bet preferentially promotes effector T-cell differentiation over memory precursor differentiation.[43] B cells deficient in T-bet failed to produce IgG2a following acute and chronic viral infections.[44–46] Furthermore, T-bet expression in GC B cells plays a role in the localization of these cells to the GC dark zone during malaria.[47,48] In addition, T-bet is required for the differentiation of several ILC populations, including NK cells,[49–51] in which it instructs interferon (IFN)-γ production.[52–54] Despite these pleotropic roles, T-bet is best known for its essential function in Th1 cell differentiation and driving the production of the canonical Th1 cytokine IFN-γ.[55] T-bet binding sites exist in the *Ifnγ* locus along with permissive H3K4me3 and H3K36me3 histone modifications in Th1 cells.[4] T-bet also prevents Th cell precursors from adopting non-Th1 effector fates.[56,57] Indeed, retroviral gene transduction of T-bet into Th2 cells converted cells to IFN-γ-producing Th1 cells,[55] demonstrating that T-bet can direct Th1 fate in fully polarized non-Th1 helper cells. In addition, T-bet directly binds to the loci of the gene encoding tumor necrosis factor (TNF). TNF and IFN-γ are key cytokines regulating Th1 cell effector function. T-bet also regulates the expression of the chemokines CCL3 and CCL4 and the chemokine receptor CXCR3, which are indispensable for Th1 development and migration of Th1 cells to the site of inflammation.[3,58] In addition to directly binding to these loci and activating their transcription, T-bet has been shown to bind hundreds of immune regulatory genes across the mouse and human genomes.[4,59–61] Like all other T-box proteins, T-bet contains two functional domains.[62] The T-box domain, which binds a 24-bp palindromic DNA sequence, consists of the T-bet recognition sequence TCACACCT. The unique quaternary structure of T-bet enables the binding of two distinct DNA sites, potentially allowing T-bet to mediate DNA loop formation and long-range DNA interactions.[62] Meanwhile, the transactivation domain facilitates the binding of T-bet-interacting proteins and transcriptional cofactors, such Mediator and P-TEFb, which are recruited to form the super elongation complex to activate Th1 gene expression.[61] As described below, this domain also allows T-bet to coopt the function of other transcriptional regulators.

Understanding how T-bet imprints the Th1 gene program at the expense of an alternative Th cell program has been a topic of interest for more than a decade.[63] T-bet encourages the Th1 fate by preventing alternate Th gene programs and negatively regulating the transcription of lineage-defining transcription factors and prototypical alternate Th genes. In addition to the previously mentioned co-expression of T-bet and Bcl6, T-bet can also be co-expressed with other lineage-specifying transcriptional factors in both precursor and committed cell subsets, such as its coexpression with RORγt in Th17 cells and with GATA3 in Th2 cells.[63] In these settings, T-bet uses a similar mechanism to sequester these alternate Th cell transcriptional factors during in vitro T-cell differentiation.[56,57] TCR signaling via tyrosine protein kinase (ITK) phosphorylates the motif at the C-terminal domain of T-bet. This promotes the formation of the T-bet-GATA3 complex in $CD4^+$ T cells cultured in Th1-polarized conditions. As a result, GATA3 is sequestered in Th1 cells, which prevents GATA3 from activating the Th2 gene program.[56] Furthermore, T-bet directly binds to the sites in the *Gata3* locus that are in close proximity to H3K27me3 repressive chromatin modifications, thus inhibiting Gata3 expression in Th1 cells.[59] Unlike other lineage-defining transcription factors, GATA3 is expressed in naive $CD4^+$ T cells,[64] and its expression is substantially reduced in Th1 cells.[59] In addition, T-bet-RUNX3 and T-bet-NFAT1 complexes limit RUNX3- and NFAT1-mediated Th2 signature cytokine expression (interleukin-2 (IL-2), IL-4, IL-5, and IL-13).[65,66] Similarly, T-bet physically interacts with Runx1 in Th17-polarizing conditions. This blocks Runx1 binding to the *Rorc* promoter, thereby inhibiting its transcription. This in turn cripples RORγt-mediated Th17 differentiation.[57] Thus, the constitutive expression of T-bet in Th cell precursors leads to reduced RORγt, blocking Th17 and promoting Th1 differentiation. T-bet in $CD4^+$ T cells can directly bind to its own *Tbx21* locus, and this binding site is associated with

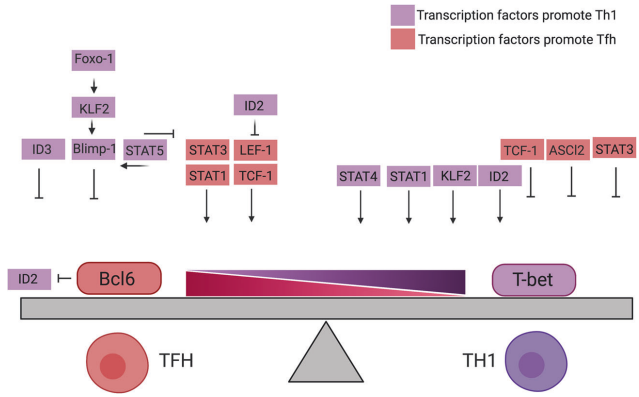

Fig. 2 Transcriptional networks leading to Tfh and Th1 differentiation. The lineage-defining transcription factors Bcl6 and T-bet maintain equilibrium in Tfh/Th1 precursors. The interplay between a secondary set of transcription factors either promotes Bcl6 (*pink*) and T-bet (*purple*) or inhibits Bcl6 (*purple*) and T-bet (*pink*) expression, which tips the Bcl6–T-bet axis and directs Tfh and Th1 fate commitment

permissive H3K4me1 histone modifications.[60] However, T-bet induction is unchanged in T-bet-deficient cells when stimulated with IL-12 and IFN-γ and following *Toxoplasma gondii* infection.[59] Conversely, the T-bet expression in T-bet and Stat4 double-deficient mice is substantially lower than that in Stat4-deficient mice during *Toxoplasma gondii* infection, suggesting that T-bet may regulate its own expression in certain circumstances.[59]

Bcl6–T-bet complexes

Considering the multiple mechanisms that both Bcl6 and T-bet use to transcriptionally imprint $CD4^+$ T cell differentiation, it may appear counterintuitive for these two factors to not only be competitively expressed but also work together to direct Tfh and Th1 differentiation and function. However, T-bet collaborates with Bcl6 to prolong non-Th1 helper cell program repression even in fully in vitro-differentiated Th1 cells.[8] While it is possible that T-bet–Bcl6 complexes may bind to Bcl6 DNA binding sites, leading to the Tfh fate, it appears that T-bet dominates Bcl6 in these interactions and utilizes the transcriptionally repressive actions of Bcl6 to promote Th1 identity. This dominance occurs due to the C-terminus of T-bet, which masks the Bcl6 DNA binding site while leaving the T-bet, T-box DNA binding domain exposed.[8] Among others, the T-bet-Bcl6 complex can be recruited to the *Ifnγ* locus and *Socs1* and *Socs3* promoters in Th1 cells.[8] As the name (suppressor of cytokine signaling) suggests, Socs1 is involved in blocking the IFN-γ and STAT1 signaling pathways. These signaling pathways are critical for acquisition of the Th1 gene program.[67,68] However, following the establishment of the Th1 fate, T-bet-Bcl6 complexes act to decrease these signals. In this way, the recruitment of Bcl6 to the *Ifnγ* locus prevents an excessive amount of IFN-γ in Th1 culture.[8] Potentially, this action may limit the immune pathology and autoimmunity caused by excessive Th1 signals; however, further studies are required to determine if this molecular mechanism is relevant in vivo and disrupted during immune pathology. In addition, Blimp-1 is highly expressed in Th1 cells, and as mentioned above, Blimp-1 antagonizes *Bcl6* expression. Therefore, Blimp-1 further prevents the expression of Bcl6 and Tfh genes in Th1 cells.[63] Together, these findings unravel potential mechanisms implemented by T-bet to block non-Th1, and particularly Tfh, differentiation.

As discussed, Bcl6 is constitutively expressed in early Th1 precursors at low levels. Importantly, the reverse is also true, in that T-bet can be coexpressed with Bcl6 in precursor Tfh cells both in culture and during infection.[4] Originally, it was suspected that T-bet coexpression was transient during Tfh differentiation; however, we and others have demonstrated high expression of T-bet in Tfh cells following infection.[5,46,69] The molecular mechanisms of T-bet-Bcl6 coexpression are yet to be defined within Tfh cells. It is likely that they form functional complexes, similar to those described in Th1 cells, in these cells. Of note, Tfh cell expression of T-bet is required to regulate the expression of key Th1 factors, IFN-γ, and CXCR3, although a recent fate-mapping study demonstrated that Tfh cells could continue to express IFN-γ even when T-bet expression was transient.[46,69,70] T-bet-deficient Tfh cells and their precursors promote B-cell isotype switching toward IgG1 during influenza infection, potentially through the loss of IFN-γ and other alterations of Tfh cytokine production.[46]

Intriguingly, we have demonstrated a context-specific role for T-bet in Tfh differentiation. Specifically, while distinct viral infections induce T-bet, the degree of this expression is distinct between infections. In settings in which low T-bet is induced, its loss results in the promotion of the Tfh lineage in vivo. In contrast, when T-bet is highly induced within Tfh cells, T-bet deficiency limits the formation of both Th1 and Tfh cells.[46] This work highlights that the ratio of Bcl6 to T-bet within precursor cells is critical for precursor cell fate decisions. How these ratios are established by and interconnected with environmental signals and supporting transcriptional networks to instruct not only the fate commitment of $CD4^+$ T cells but also their overall function, and impact on humoral immune responses remains to be determined.

TRANSCRIPTIONAL NETWORKS OF THE TFH/TH1 DICHOTOMY: FINE-TUNING THE LINEAGE-DEFINING FACTORS

High mobility group (HMG) transcription factors

The interplay between multiple transcription factors is highly regulated and forms the backbone of T-cell differentiation. These multifaceted regulatory networks act both upstream and downstream of Bcl6 and T-bet to tip the differentiation equilibrium to bias either a Tfh or Th1 fate (Fig. 2). Key regulators of the Bcl6–T-bet axis are the HMG family of transcription factors, T-cell factor (TCF-1), and lymphoid enhancer factor (LEF-1). TCF-1 is encoded by the *Tcf7* gene, and LEF-1 is encoded by *Lef1*. Several studies delineate the profound role of TCF-1 and LEF-1 in T-cell responses. Both TCF-1 and LEF-1 support memory $CD8^+$ T-cell formation by the expression of another T-box protein, Eomes.[71,72] In addition, TCF-1 favors the expression of GATA3, a canonical Th2 transcription factor.[73] It has also been demonstrated that TCF-1 dampens

the inflammatory effects of Th17 cells by reducing IL-17A expression.[74] This implies that HMG transcription factors favor a non-effector T-cell phenotype. In keeping with these observations, TCF-1 and LEF-1 expression promotes Tfh development. TCF-1 and LEF-1 are highly expressed in naive $CD4^+$ T cells, and they remain high in Tfh precursors and mature Tfh cells during LCMV, vaccinia virus, and blood stage *Plasmodium* infection in mice.[5,75,76] In contrast, TCF-1 and LEF-1 are rapidly downregulated in effector $CD8^+$ and Th1 T cells.[75–77] While culture of $CD4^+$ T cells in either Tfh-polarizing (αIFN-γ, αIL-12, and rmIL-6) or Th1-polarizing (αIL-4, αTGF-β, and rmIL-12) conditions did not result in changes in Lef1 or Tcf7 transcription,[75] in $CD8^+$ T cells, the downregulation of TCF-1 in effector cells compared with memory precursors was driven by IL-12.[78] This indicates that either there are differences in IL-12 signaling between $CD4^+$ and $CD8^+$ T cells or that IL-12 works with other factors to independently instruct between Th1/Tfh and effector/memory differentiation. Thus, additional studies are needed to resolve these differences.

TCF-1 instructs Tfh differentiation through multiple mechanisms. First, TCF-1 directly promotes *Bcl6* and inhibits *Prdm1* transcription in Tfh cells during LCMV infection.[76,79] Second, TCF-1 is enriched at the IL-6 receptor gene locus (*Il6ra* and *Il6st*).[75] This likely enables enhanced responsiveness of Tfh cells to IL-6 signaling, which is important in Tfh differentiation.[80] In accordance, TCF-1-deficient mice demonstrated reduced expression of other factors that are involved in Tfh cell commitment and growth, including Bcl6 and ICOS. Conversely, loss of TCF-1 promoted the expression of Th1 cell-associated factors in Tfh cells, including T-bet, Blimp-1, and CD25 protein.[75,76] Finally, dually expressed p33 (an isoform of TCF-1) and Bcl6 proteins physically interact and act to recover Bcl6-mediated autorepression.[76] However, whether Bcl6 forms a heterodimer with p33 or Bcl6-p33 recruits another transcriptional regulator to reverse Bcl6 autoregulation in the context of viral infection remains unclear. Although some of the mechanisms by which TCF-1 promotes Tfh differentiation act side-by-side with Bcl6, overexpression of Bcl6 overcomes deficiency of TCF-1, indicating that TCF-1 acts upstream of Bcl6 in promoting *Bcl6* expression.[76] Interestingly, ablation of TCF-1 during the late phase of Tfh differentiation is redundant for Tfh ontogeny but is necessary for Tfh-dependent GC B-cell development, which highlights the role of TCF-1 in Tfh cell function in GCs.[76] LEF-1 recognizes a similar DNA consensus motif to TCF-1. This enables LEF-1 to exploit the same mechanisms as TCF-1 to initiate Tfh differentiation. It has been shown that ectopic expression of LEF-1 enhances the transcription of Tfh regulatory genes (*Il6ra*, *Il6st*, *Bcl6*, and *Cxcr5)* in Th1 cells. In addition, LEF-1-deficient, antigen-specific $CD4^+$ T cells fail to initiate Tfh differentiation early during LCMV infection.[75] In keeping with the similar mechanisms enacted by these HMG factors, LEF-1 and TCF-1 show functional redundancy. Indeed, the defects in Tfh differentiation in *Lef1* and *Tcf7* double-deficient mice is more profound than those in single-deficient mice, indicating that LEF-1 and TCF-1 coordinate to regulate Tfh differentiation.[75] Collectively, these results imply that TCF-1 and LEF-1 act together as key players in tipping the Tfh/Th1 equilibrium and an essential prerequisite for Tfh fate commitment.

Id transcriptional regulators

Id2 and Id3 are inhibitors of DNA binding proteins and are differentially expressed in Th1 and Tfh cells.[81–83] The Id family of proteins consists of four proteins, Id1, Id2, Id3, and Id4. They are functional inhibitors that act to reduce the DNA binding activity of E-protein transcription factors (E2A, E2-2, and HEB).[84] Among the Id proteins, Id2 and Id3 play a profound role in the ontogeny of several immune cells, including innate lymphoid cells, regulatory T cells, natural killer cells, invariant NKT cells, and effector and memory $CD8^+$ T cells.[85] In addition, IL-12 signaling negatively regulates Id3 expression in antigen-specific $CD8^+$ T cells. However, IL-2 signaling via STAT4 and STAT5 positively mediates Id2 expression. These STATs were found to be enriched at the Id2 promoter in $CD8^+$ T cells.[81] In comparison, little is directly known about the environmental cues that influence Id2 and Id3 expression in $CD4^+$ T cells. Although it is likely that Id proteins in $CD4^+$ T cells require similar cytokine regulatory pathways as those in $CD8^+$ T cells, further studies are needed to investigate this hypothesis. Naive $CD4^+$ T cells express high amounts of Id3. During LCMV infection, Tfh cells retained high levels of Id3. In contrast, Th1 cells preferentially expressed Id2.[83,86] Impaired Id2 expression in viral-specific $CD4^+$ T cells restricts T-bet expression and the expression of other Th1-associated genes (granzyme B and IFN-γ), which results in a reduced Th1 population.[86] Moreover, in the absence of Id2, E proteins circumvent Id2-imposed inhibition and induce several key Tfh genes, including *Cxcr5*, *Il6ra*, *Tcf7*, and *Lef1*, resulting in Th1 cells adopting a strong Tfh signature gene profile.[86] Nevertheless, it is unclear how Id2 regulates T-bet expression. In this model, it is plausible that high TCF-1 and LEF-1 expression feeds back to downregulate T-bet expression, as mentioned earlier. TCF-1 and LEF-1 play a key role in curtailing T-bet expression. Indeed, Bcl6 expression is unchanged in Id2-deficient Th1 cells, suggesting that Id2 regulation of the Tfh/Th1 differentiation axis may occur exclusively via Tcf7/Lef1 mechanisms and not through the direct regulation of either Bcl6 or T-bet.[86] Furthermore, Id3 negatively regulates Ascl2-mediated CXCR5 expression. Ascl2 acts by upregulating Cxcr5 and downregulating canonical Th1 gene (*Il12rb1, Tbx21, Ifnγ*, and *Gzmb)* transcription by directly binding to their loci.[87] Therefore, Id3 deficiency in $CD4^+$ T cells promotes Tfh development, which has been observed both in viral infection[86] and following immunization.[87]

STAT family transcription factors

The transcriptional regulators of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) family are central to the Tfh and Th1 bifurcation. This pathway is initiated when cytokine ligands bind their respective cognate receptors; as a result, a conformational change occurs that induces receptor rearrangements, leading to JAK activation. JAKs phosphorylate STATs, which then translocate to the nucleus, where they either silence or activate several transcriptional programs.[88] Therefore, JAK/STAT signaling is an important mechanism by which cells integrate external environmental signals. Interestingly, STAT4 appears unbiased toward differentiation of either Tfh or Th1 cells during early T-cell differentiation; however, it is required for cells to move past the common precursor stage during differentiation, leading to the promotion of Th1 differentiation.[4,69] In addition, STAT4 induces IL-21 and IFN-γ expression in $CD4^+$ T cells both in vivo and in vitro.[4,69] Among the STAT family of transcription factors, STAT3 influences the Bcl6–T-bet axis toward Tfh development.[20,89] STAT3-deficient $CD4^+$ T cells fail to differentiate into early Tfh cells.[80] Furthermore, another study demonstrated a profound defect in Tfh subsets due to loss of STAT3 much later in LCMV infection.[90] STAT3 positively regulates Bcl6 expression first by directly binding to its promoter. Furthermore, the related zinc-finger transcription factors Ikaros and Aiolos positively correlate with Bcl6 expression in Tfh cells during Th1 polarization and in response to *Listeria monocytogenes* infection.[91] Mechanistically, these factors act together with STAT3 to form a transcriptional complex at the *Bcl6* promoter that initiates conformational changes in chromatin structure and results in gene activation.[91] In addition, IL-6 signaling also activates STAT1 in $CD4^+$ T cells. STAT1 is additionally activated downstream of type I IFNs (such as IFNα and IFNβ), which enables STAT1 binding in the *Bcl6* locus to contribute to Tfh development in in vitro studies.[92] The combined deficiency of STAT1 and STAT3 in $CD4^+$ T cells leads to a complete failure of early Tfh cell development following viral infection.[80] STAT5 and STAT3 have

common binding sites in the *Bcl6* locus, and the ratio of STAT3 and STAT5 is therefore critical, as in higher STAT5 conditions, similar to the scenario with high IL-2, STAT5 can mask the binding site in the *Bcl6* locus, preventing STAT3-dependent *Bcl6* transcription.[63,90] Furthermore, STAT5 also upregulates Blimp-1 to indirectly suppress Bcl6 expression during the T-cell priming phase in LCMV infection.[93] STAT3 can also counter this suppression via its own downregulation of T-bet and CD25, the high-affinity receptor for IL-2, indicating that this pathway not only promotes Tfh differentiation but also deters Th1 differentiation.[80] Taken together, these findings highlight the complex integration of the cytokine milieu that is mediated by the STAT family. However, some work remains to reveal how this is mediated in humans. Unlike in mice, patients with inborn errors of immunity show a conserved role for STAT3, and STAT3 deficiency leads to reduced Tfh cell numbers; in contrast, STAT1 deficiency does not impair human Tfh differentiation.[94]

KLF2

Another transcription factor, KLF2, takes a wide range of approaches to impair Tfh and promote Th1 differentiation. KLF2 is immediately downregulated after T-cell receptor stimulation in activated $CD4^+$ T cells.[95] KLF2 expression is maintained at low levels in Tfh cells in contrast to non-Tfh cells in viral infection and after immunization.[95,96] KLF2 directly binds to the promoter region of *Prdm1*, which in turn increases Blimp-1 expression, which, as described, leads to the repression of *Bcl6* expression to further restrict Tfh differentiation.[96] In addition, KLF2 is enriched at the regulatory region of the *Tbx21* locus, and overexpression of KLF2 results in an increased number of $T\text{-}bet^+$ and fewer $Bcl6^+$ antigen-specific $CD4^+$ T cells following immunization.[96] In addition to these direct transcriptional mechanisms, KLF2 also initiates the expression of sphingosine-1-phosphate receptor 1 (S1PR1) and L-selectin (CD62L) in T cells. Expression of these cell surface receptors facilitates T-cell egress and entry into lymphoid tissues.[97] Low levels of KLF2 in Tfh cells coincided with reduced S1PR1 expression, similar to that seen in memory $CD8^+$ T cells, which downregulated S1PR1 to establish a tissue-resident pool.[96,98] This suggests that downregulation of KLF2 in Tfh cells may be essential to block egress from lymphoid tissues and to encourage localization within the GCs. In addition, the transcription factor FOXO-1 further acts to promote *Klf2* transcription by binding to its promoter in human T cells, leading to Th1 differentiation.[99] Conversely, T- and B-cell crosstalk facilitated by ICOS-ICOS-L interactions allows the development of early Tfh cells to overcome KLF2-mediated T-cell migration through the inhibition of FOXO-1.[39,95] Furthermore, KLF2 has recently been confirmed to be part of the Bcl6-repressed transcriptional circuitry, along with TCF-7, which inhibits key Tfh genes encoding PD-1, ICOS, CD200, IL-6Rα, IL-21, and IL-4.[25]

Control of Th1 and Tfh identity and plasticity via interacting gene networks

Collectively, a secondary set of transcription factors help coordinate Bcl6 or T-bet expression in Tfh/Th1 cell precursors. These factors act both upstream and downstream of the central lineage-defining factors to tune their expression (Fig. 2). As a consequence, the ratio between Bcl6 and T-bet is disturbed, and precursor cells adapt a fate determined by the dominant lineage-specifying transcription factor. While some connections between different transcription factor families have been established, it is important to understand how these transcription factor networks act together to guide the cell toward one differentiation fate or the other. Furthermore, in accordance with the hypothesis that the balance of T-bet and Bcl6 determines cell fate, the loss of T-bet in T cells promotes Tfh ontogeny at the expense of Th1 differentiation both in vitro and in multiple Th1-biased infection models (including *Toxoplasma gondii*, *Plasmodium berghei* ANKA, and influenza).[4,46,100] However, it remains important to investigate how the cumulative effect of these secondary transcription factors impacts gene signatures and functional consequences within Th1 and Tfh cells. The relationship between these transcriptional regulators during in vivo T-cell differentiation is starting to emerge,[25] and it is likely that these transcription factors may play distinct roles within each effector subset. Similar to their behavior in a Th1-biased environment, in a Th2- or Th17-biased cytokine milieu, Tfh cells differentiate in parallel with Th2 and Th17 cells. Tfh cells can secrete multiple cytokines, such as IL-21, IL-4, IL-2, IL-9, IL-10, IL-13, and IFN-γ. It is now apparent that distinct functional Tfh subpopulations hold the potential to produce specific combinations of cytokines.[2,9,96,101–104] Indeed, Tfh subpopulations have been described both in humans and mice, and these may play distinct roles in mediating humoral responses in allergy and asthma and lead to specific protection following vaccination and infection.[94,102,105,106] In a key study, Eisenbarth and colleagues demonstrated that IL-13-producing Tfh (Tfh13) cells co-express Bcl6 and GATA3 in mice and humans with IgE antibodies against allergens. Tfh13 cells triggered the production of high-affinity IgE, which led to the induction of anaphylaxis.[102] In addition, over the course of Th2-skewed infection (with the helminth *Nippostrongylus brasiliensis*), the cytokine profile of Tfh cells changes from one dominated by the production of IL-21 to one favoring IL-4-producing Tfh cells. These subsets are transcriptionally, phenotypically, and functionally distinct and provide different helper signals to GC B cells, suggesting that the role of Tfh cells may change dynamically over the course of an infectious challenge.[107] However, we are only starting to appreciate this functional heterogeneity and plasticity within Tfh populations and potentially other T effector lineages. Furthermore, within mature Tfh and Th1 populations, it is unclear how epigenetic regulators establish and maintain the balance of lineage-defining transcription factors. As the *Bcl6* and *Tbx21* loci are kept in a permissive histone state within mature Tfh and Th1 cell pools, respectively, it is apparent that these gene networks remain responsive to changing environmental cues throughout infection.[10,108] This likely acts to maintain identity and function or to generate plasticity between $CD4^+$ effector populations and may mediate the functional heterogeneity within Tfh and Th1 populations throughout infection and disease.[102,105,107] Thus, the integration of and balance between transcription factor instruction with inflammatory mediator signaling is of undeniable interest.

CYTOKINE MEDIATORS OF THE TH1/TFH DICHOTOMY: TRANSLATING INFLAMMATION INTO CELL FATE

We recently reported the transcriptional heterogeneity that underlies the flexibility in Tfh differentiation in distinct viral infections.[46] Specifically, T-bet is required in a context-dependent manner for Tfh cell generation during LCMV and influenza infection. The essential role of T-bet is determined by variations in its expression level that are set by distinct inflammatory cues in these individual infection types.[46] The impact of the distinct cytokine milieu in various viral infections was confirmed in a study that investigated the inflammatory cytokines that distinctly impact Tfh and Th1 differentiation during vesicular stomatitis virus and LCMV infection.[109] Taken together, these works propose that each viral infection elicits unique inflammatory cues that work independently or together to regulate transcriptional networks that promote either T-bet or Bcl6 and control the Th1/Tfh bifurcation (Fig. 3).

IL-6

IL-6 was one of the first indispensable cytokine signals shown to drive Tfh cell formation.[80] This multifunctional cytokine is secreted by various cell types, including DCs, T and B cells, macrophages, fibroblasts, endothelial cells, glial cells and keratinocytes.[110]

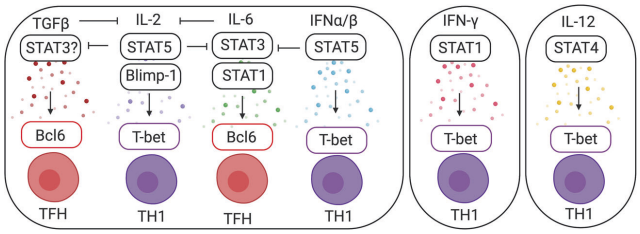

Fig. 3 Environmental cues that instruct CD4+ T cell differentiation. Individual cytokines activate downstream transcriptional factors, which in turn regulate Bcl6 or T-bet expression to imprint either the Tfh or Th1 fate

In LCMV infection, IL-6 deficiency impaired early Bcl6 expression and Tfh differentiation.[80] IL-6 signals through STAT3, which is sufficient to trigger initial Bcl6 expression and the Tfh fate trajectory. However, STAT3 deficiency does not directly phenocopy the outcome of IL-6 deficiency in Tfh development, implying that an alternate signaling cascade is needed to maintain Tfh fate commitment during LCMV infection. In addition to STAT3, IL-6 stimulation also activates STAT1 in $CD4^+$ T cells.[111] Antigen-specific $CD4^+$ T cells dually deficient for STAT1 and STAT3 completely fail to form Tfh cells, replicating the outcome of IL-6 deficiency and confirming that STAT1 and STAT3 act in collaboration downstream of IL-6 signaling to promote Tfh ontogeny.[80] Of note, during infection, cytokines such as IL-6 are not present in isolation. IL-6 has additional signaling mechanisms to guard against other factors that may promote Th1 differentiation. Indeed, via STAT3, IL-6 also negatively regulates the surface expression of CD25 during LCMV infection.[80] As mentioned earlier, high IL-2 sensitivity inversely correlates with Bcl6 expression and Tfh differentiation;[112] this dual signaling is therefore important to reinforce IL-6-directed Tfh differentiation. IL-6 is secreted by conventional DCs in response to CD40 stimulation and in the presence of type 1 IFN in viral infection.[113] In a recent study, the timing of IL-6 production by DCs was shown to direct the Tfh/Th1 dichotomy, whereby an early wave of type I IFN induced DCs to produce IL-6. In turn, this promoted precursor cells to commit to the Tfh fate at the expense of the Th1 fate.[109] In contrast, late production of type I IFN resulted in DCs becoming insensitive to type I IFN; hence, DCs failed to produce IL-6. In this setting, precursor cells adopted a Th1 fate trajectory instead of a Tfh trajectory.[109] Another study showed that activated follicular B cells also secrete IL-6 early in influenza infection, which is sufficient to drive Tfh differentiation.[114] In accordance with these findings, within GCs, follicular DCs produce IL-6 in the late stage of Tfh development.[115,116] Critically, this late induction of IL-6 is required and sufficient to clear chronic infection.[117] Interestingly, previous studies have reported that in the absence of IL-6, there is no difference in the frequency of Tfh cells between early LCMV infection and later points after the acute phase.[118,119] Taken together, these studies confirm that while IL-6 does promote Tfh differentiation, this is regulated in a context-dependent and spatiotemporal manner between and during infection.

IL-21

IL-21 is the cardinal Tfh cell-derived cytokine. While IL-21 alone is not required in regulating Tfh differentiation, in combination with IL-6, IL-21 promotes Tfh differentiation by activating the STAT3 signaling cascade in vitro.[20,119] IL-21 is a part of the IL-2 family of cytokines, including IL-2, IL-4, IL-7, IL-9, and IL-15, that share the common γ-chain (γ_c) IL-2R subunit.[120] Both IL-6 and IL-12 can induce IL-21 expression in murine $CD4^+$ T cells in vitro and in vivo.[4,121,122] However, mice deficient in IL-21R have no defect in Bcl6 expression or in Tfh differentiation during viral infections.[80,114,123]

TGF-β

Transforming growth factor-β (TGF-β) augments Bcl6 and deters T-bet expression. This tips the Bcl6–T-bet equilibrium in favor of Tfh differentiation at the expense of Th1 differentiation.[124] TGF-β receptor-deficient $CD4^+$ T cells had higher levels of IL-2Rα expression and STAT5 activity than wild-type cells in the early stage in LCMV-infected mice,[125] indicating that TGF-β restricts IL-2 responsiveness before $CD4^+$ T cells bifurcate into Tfh or Th1 cells. As a result, there were fewer Tfh and more Th1 cells in the absence of the TGF-β receptor after viral infection.[125] Human naive T cells cultured in the presence of TGF-β demonstrated increased expression of Tfh-related genes, including BCL6 and CXCR5, and decreased expression of Blimp-1.[124] TGF-β signals through STAT3 to drive the differentiation of human Tfh cells;[124] however, this has not been established in mice. In keeping with this essential role, TGF-β signaling in Tfh cells is critically required for GC formation and for the generation of influenza-specific antibodies.[125]

IL-2

IL-2 is a well-documented inhibitor of Tfh differentiation.[93,126] Currently, the expression of CD25 (the α chain of the high-affinity IL-2 receptor) is the earliest marker of cell fate between Tfh and Th1 cells. Early in infection, CD25 expression is downregulated in early Tfh precursors, making the cells insensitive to the inhibitory effect of IL-2 signaling. In contrast, the opposite is true for early Th1 precursors, which can be distinguished by their expression of CD25.[7] Thus, cells with higher CD25 expression are destined to become $Blimp1^+T\text{-}bet^{hi}IFN\text{-}\gamma^{hi}$ Th1 cells,[7] while cells with lower CD25 expression are early Tfh precursors, which are fated to become $Bcl6^{hi}CXCR5^{hi}$ Tfh cells.[7] Recently, DiToro et al. showed that the expression of IL-2 coincided with that of Bcl6 in $CD4^+$ T cells.[6] Thus, this established a new paradigm whereby early Tfh precursors are IL-2 producers and the Th1 precursors that express CD25 early are the IL-2 consumers.[6] The equilibrium between IL-2 producers/consumers maintains IL-2 levels, and any imbalance leads to either the promotion or inhibition of Tfh differentiation. We recently reported that T-bet-deficient $CD4^+$ T cells adopt the Tfh fate instead of the Th1 fate in influenza infection.[46] However, the role of T-bet in Tfh cells is not recapitulated during LCMV infection.[46,69] We proposed that there is a difference in IL-2 and subsequent STAT5 activity between these settings. Indeed, CD25 expression and STAT5 activity are augmented in LCMV infection in comparison with influenza infection.[46] In the absence of T-bet during LCMV infection, the loss of IL-2 consumers (Th1 cells) results in excessive IL-2 and STAT5 activity, and thus, cells are blocked from both the Th1 and Tfh fates.[46] Together, these results explain how high IL-2 may regulate Bcl6 expression to act as a cytokine switch between Tfh and Th1 fate commitment. In contrast, during influenza infection, there is likely a competing cytokine, such as IL-6, that may allow the Tfh fate to be promoted, even when IL-2 consumers are lacking in the absence of T-bet.[46] Consistent with this, recent research led by the Ballesteros group

found that GC Tfh cells utilized cell-intrinsic IL-6 signaling, which blocked STAT5 from binding to the *Il2rb* locus (encoding CD122, a chain of the IL-2 receptor); in turn, GC Tfh cells lacked expression of the IL-2 receptor and remained insensitive to IL-2.[127]

Type I IFNs

Type I IFNs (IFN-α and IFN-β) regulate the STAT5 signaling pathway in $CD4^+$ T cells to promote Th1 differentiation while inhibiting the Tfh fate trajectory.[90] Type I IFNs are a pleiotropic family of cytokines that regulate cell-type-specific signaling pathways. These cytokines signal through the type I IFN receptor, which consists of two subunits, IFNAR1 and IFNAR2.[128] IFNAR-deficient cells have increased Bcl6 expression and a Th1-like gene profile,[90] and $CD4^+$ T cells treated with type I IFNs show increased CD25 expression and STAT5 activity. STAT5 activity is a robust inhibitor of Bcl6 expression and promotes Blimp-1 expression in $CD4^+$ T cells.[63] By blocking type I IFN signaling, the Tfh phenotype is partially recovered in STAT3-deficient $CD4^+$ T cells during LCMV infection. This indicates that the STAT3 and type I IFN signaling pathways have an opposite role in Tfh development following viral infection. Concurrently, STAT3-deficient Tfh cells have elevated expression of a number of IFN-stimulated genes.[90] However, as discussed above, the immune population directly responding to type I IFN is critical, as signaling through IFNAR1 on DCs leads to enhanced IL-6 production, which subsequently promotes the Tfh fate.[109]

IL-12

It has been known for more than two decades that the IL-12-STAT4 signaling cascade promotes T-bet expression, which directs Th1 development.[129] The idea of IL-12 initiating both Tfh and Th1 differentiation was first observed in human $CD4^+$ T cells.[130,131] Later, murine $CD4^+$ T cells cultured in IL-12 demonstrated the expression of both canonical Th1 (IFN-γ and T-bet) and Tfh markers (IL-21 and Bcl6).[4] This suggests that both Tfh and Th1 cells have a common transitional state of differentiation. Although IL-12 predominantly acts through STAT4 signaling, STAT4-deficient $CD4^+$ T cells exhibit impaired Th1 but intact Tfh differentiation, potentially suggesting a temporal role for IL-12 in Tfh differentiation, where it is not needed late during infection.[4,69] In addition, IL-12-dependent STAT4 signaling is required for the expression of T-bet, IL-21, and IFN-γ in Tfh cells, indicating a role in fine-tuning Tfh subpopulations.[69] In addition, T-bet expression can also be induced by IFN-γ-STAT1 signaling in an IL-12-independent manner both in vitro and in vivo.[4,132,133] Interestingly, however, despite the close association between IFNγ, IL-12, and Th1 differentiation, dual deficiency of Ifnγr1 (the IFN-γ receptor) and STAT4 still permitted the expression of T-bet during *Toxoplasma gondii* (*T. gondii*) infection. Another IL-12-related cytokine, IL-27, binds to WSX-1, a class I cytokine receptor family member that shares similarities to the IL-12 receptor, and this signaling pathway has been shown to play a role in STAT1-dependent T-bet induction.[134] Taken together, these findings underscore that the unique cytokine milieu is critical in the regulation of lineage-defining transcriptional factors and, in turn, the tailoring of the Tfh/Th1 bifurcation in pathogen-specific ways.

CONCLUDING REMARKS

At present, it is not clear whether dominant inflammatory cytokines create an overwhelming milieu in which lymphoid organs are awash with a key, specific cytokine. More likely, however, niches exist within organs in which cytokines are restricted due to their precise expression by distinct cellular sources.[109,135–137] The DC sources of these cytokines that act to facilitate Tfh and Th1 fate commitment have been recently reviewed elsewhere.[127,136] It is, however, important to note that the cellular sources of cytokines, such as IL-12 and IL-6, are not restricted to a single DC subset. Therefore, continual changes in cytokine stimulation and fate directing through multiple T cell–DC interactions may regulate the gene regulatory programs that ultimately tip the balance between Tfh and Th1 differentiation.[138] Furthermore, it remains unclear how individual cell interactions or cytokines regulate heterogeneity within Tfh subpopulations, and how this is dynamically regulated in the leadup to or within GC reactions.[2,102,107]

As described herein, the balance of the type I IFNs IL-6 and IL-2 appears to be essential to govern the transcriptional networks that promote either Bcl6 or T-bet and shape the Tfh and Th1 dichotomy. Ultimately, these are the key cytokines that establish the equilibrium between the promotion of humoral or cellular adaptive immunity.[46,109] While several studies have linked the context-specific interplay between cytokine environment and $CD4^+$ T-cell differentiation, there is still a gap in knowledge regarding how these cytokines are differentially regulated in a pathogen-specific manner. Of note, these considerations appear to be of critical importance for understanding the immunopathogenesis in the current Coronavirus Disease 2019 pandemic. Profiling T cell responses of severe COVID-19 patients has demonstrated elevated T-bet expression which coincides with limited TFH differentiation.[139,140] A study conducted in 2003 that treated patients with type I IFNs during active severe acute respiratory syndrome coronavirus 1 (SARS-CoV-1) infection showed promising results for the promotion of viral clearance.[141] Furthermore, a trial blocking IL-6 to disrupt the cytokine storm in severely affected SARS-CoV-2 patients is underway.[142] While our current knowledge of the interplay between cytokine and transcriptional gene networks would suggest that both of these immunotherapies heighten Th1 differentiation, this may come at the cost of Tfh differentiation and therefore be detrimental to the development of neutralizing humoral immunity.[2] Thus, understanding how altering the cytokine milieu directly modulates the multiple gene transcriptional networks that underpin $CD4^+$ T-cell differentiation in a pathogen-specific manner is of critical importance when considering new therapeutic targets to both promote viral clearance and drive protective immunity.

ACKNOWLEDGEMENTS

We thank Stephen L. Nutt for critical reading of the manuscript. This work was supported by National Health and Medical Research Council (NHMRC) grants to J.R.G., an Ideas Grant (GNT1182649) and a Project Grant (GNT1137989). A.A.S. is supported by a University of Melbourne research scholarship. J.R.G. is supported by an Australian Research Council Future Fellowship (FT130100708). This work was made possible through Victorian State Government Operational Infrastructure Support and the Australian Government NHMRC Independent Research Institute Infrastructure Support Scheme (IRIISS). Figures were generated using Biorender.

AUTHOR CONTRIBUTIONS

A.A.S. wrote the manuscript and drafted the figures; J.R.G. provided edits and comments, and both authors agreed on the final manuscript and figures.

ADDITIONAL INFORMATION

Competing interests: The authors declare no competing interests.

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