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University
of Glasgow

The Impact of Inflammatory Context on the Metabolic Profile of T Follicular Helper Cells

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1.1 **Abstract**

T follicular helper (Tfh) cells are a subset of CD4⁺ T cells that are key in driving the Germinal Centre response, assisting B cells to produce high-affinity antibodies during the adaptive response. Tfh cell differentiation/function is metabolically regulated; however, characterisation of Tfh cell metabolism during a type 2 helminth infection (e.g. *H. polygyrus*), and its relationship to Tfh cells derived from type 1 inflammation, remains poorly understood. To date, Tfh cell metabolism in comparison to other T effector cells (Th1/Th2) remains understudied. This thesis aims to define the key metabolic pathways utilised by murine Tfh cells compared to other CD4⁺ T cell subsets, in Th1 (IAV Infection) and Th2-mediated inflammation (*H. polygyrus*). Both infections induce strong Tfh and T effector cell responses. Using metabolic profiling techniques (METFLOW, PHOSFLOW, lipid assays, bulk RNA sequencing), I demonstrated that Tfh cells are metabolically active, capable of lipid accumulation and show lipid-dependent gene expression profiles, albeit less so than Th2 cells in *H. polygyrus* infection. Thus, Tfh cells favoured increased glycolytic gene expression over Th2 cells; however, Th2 cells have increased expression of genes associated with lipid and cholesterol pathways. To assess variation in Tfh metabolism across inflammatory contexts, I used bulk RNAseq to compare metabolic gene expression in Tfh cells from both Th1- and Th2-driven infections. My analysis revealed that Tfh2 cells (type 2) consistently display increased gene expression for lipid metabolism compared to Tfh1 (type 1), regardless of anatomical location or infection. Further to this, I found that in human *in vitro*-derived Tfh cells, acute inhibition of SCD-1, a key enzyme in lipid biosynthesis, facilitated a reduction in CXCR5 (and possibly CCR7) expression on the surface of Tfh cells. This indicates that manipulation of lipid metabolism directly influences Tfh cell biology and could possibly prevent their migratory capacity. Overall, my data shows that Tfh cell metabolism does not exhibit a fixed phenotype and that it can alter depending on the immune environment. Furthermore, this increases understanding of key metabolic nodes of Tfh cells, like lipid biosynthesis and the role of SCD-1, suggesting that metabolic manipulation may be a viable strategy for the selective modulation of Tfh cells. This is crucial as, to date, no therapies specifically target Tfh cells; however, with further research, we may be able to target Tfh immunometabolism effectively to enhance vaccine efficacy or, in cases of aberrant Tfh cell responses, modulate these responses in favour of the patient's needs.

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1.5 **Abbreviations**

- TCR – T Cell Receptor
- DP – Double Positive
- CTECs – Cortical Thymic epithelial cells
- MECs – Medullary epithelial cells
- CD25 – Interleukin-2-receptor α chain
- p: MHC – Peptide: Major Histocompatibility Complex
- MHC – Major Histocompatibility Complex
- DC – Dendritic Cell
- HEVs – High endothelial venules
- APC - Antigen-presenting cell
- Th – CD4+ T helper Cell
- IL- Interleukin
- ILC-2 – Type 2 Innate lymphoid cells
- EAE - experimental autoimmune encephalomyelitis
- Treg (s) – T regulatory cells
- TSLP- Thymic Stromal Lymphopoietin
- GC -GCs
- NK – Natural Killer Cells
- Tfh- T follicular helper Cell
- Tfr – T follicular regulatory cells
- *H. polygyrus* - *Heligmosomoides polygyrus*
- FRC- Fibroreticular cells
- IFN- γ – Interferon γ
- RORYt- Retinoic Acid receptor-related orphan receptor Υ t
- GM-CSF- granulocyte/macrophage colony-stimulating factor
- mAB- Monoclonal Antibody
- TNF- α - Tumour Necrosis Factor α
- Bcl-6- B cell lymphoma 6
- FDC- follicular Dendritic Cell
- IFZ- Interfollicular Zone
- INFZ- Intrafollicular Zone
- Ab- Antibody

- DZ- Dark Zone
- LZ – Light Zone
- Pentose Phosphate Pathways (PPP)
- BCR- B cell Receptor
- HES- *H. polygyrus* excretory/secretory Antigen
- PI3K - phosphatidylinositol 3-kinase
- mTOR/C -mammalian target of rapamycin/complex
- OX40L- OX40 Ligand
- TF- Transcription Factor/s
- NA – neuraminidase
- HA- Haemagglutinin
- DMF- Dimethyl Fumarate
- TCA- Tri-carboxylic Acid
- FAS- Fatty acid synthesis
- FAO -Fatty Acid Oxidation
- S1P -Sphingosine-1-phosphate
- LCMV – Lymphocytic choriomeningitis Virus
- PtdE- phosphatidylethanolamine
- PtdC- Phosphatidylcholine
- SCD-1 -Stearoyl CoA Desaturase 1
- mLN – mesenteric LN
- mdLN – mediastinal LN
- IAV – Influenza A Virus
- PKM1/2- Pyruvate Kinase M1/2
- GUT1- Glucose Transporter 1
- HK2- Hexokinase 2
- G6PD – Glucose-6-Phosphate Dehydrogenase
- FAS – Fatty Acid Synthase
- ATPCL- ATP citrate Lyase
- ACC – Acetyl-CoA Carboxylase
- IDH2- Isocitrate Dehydrogenase 2
- ASA- Aspartate Aminotransferase
- CPT1A – Carnitine Palmitoyltransferase 1A
- ACADM- medium-chain acyl-CoA dehydrogenase

- VHL – Von Hippel- Lindau protein
- HIF – Hypoxia-inducible Factor
- SMH – somatic hypermutation
- ER- Endoplasmic Reticulum
- MDE – Multiple Pairwise Differential Expression
- DE- Differential Expression
- SL2 - Searchlight 2
- LN- Lymph node

1.6 Publications

Meehan, G.R., Gunes, U., Camargo da Rosa, L., Scales, H.E., Finney, G., Deehan, R., Sintoris, S., Athanasiadou, A., Jones, J., Perona-Wright, G. and Brewer, J.M., 2025. Spermidine suppresses DC activation via eIF5A hypusination and metabolic adaptation. *Discovery Immunology*, p.kyaf009

1.7 Graphical Abstract

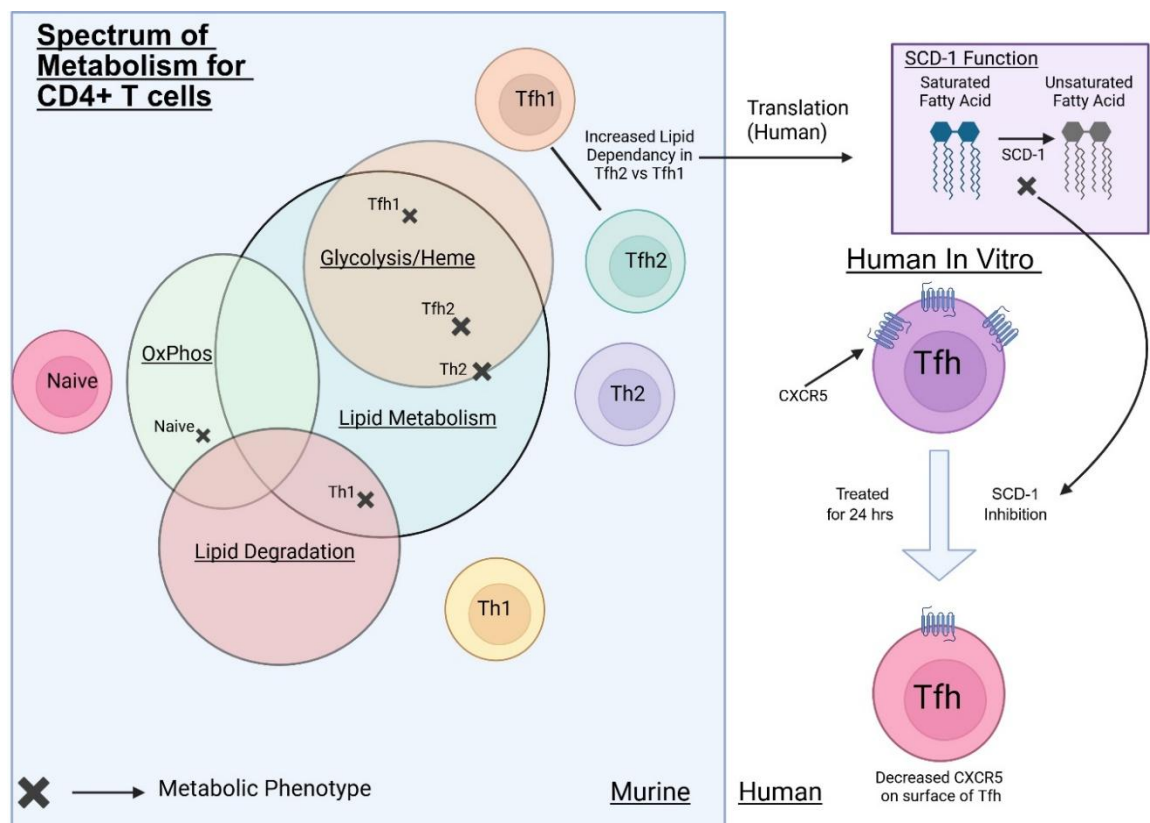


Figure 1.1. Graphical Abstract for Thesis.

This figure highlights the key findings in this thesis and shows the link between the murine and human aspects of work presented within. Created in BioRender.

1.8 Author's Declaration

I declare that, except where reference is made to the contribution of others, this thesis is the result of my work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Signature: Jack Jones Name: Jack Jones

Date: 12:08:2025

1.9 Introduction

1.9.1 Overview

T follicular helper (Tfh) cells were discovered less than 20 years ago by Dr Shane Crotty and Dr Martin Lipp (Crotty, 2014); however, the understanding of the requirement for T cell help to B cells was identified as one of the first responsibilities of T cells, well before the discovery of Tfh cells in the early 2000s. The first documented work was published by Claman et al, 1966, who showed that T cells were required for B cell activation and antibody (Ab) production (Bishop, 2016, Claman et al., 1966).

Primary work on human tonsillar GCs paved the way for Tfh cell biology. However, it was the understanding of B cell lymphoma 6 (Bcl-6) and its role in Tfh cell biology that led to the recognition of this specific population (Crotty, 2019). Like Tfh cell biology, immunometabolism had a similar fate in its early years of discovery. The first paper published by Erika Pearce marked a significant milestone, indicating that metabolism was more than just a cellular function and could have multiple, differing effects on a cell if manipulated (Pearce, 2010). This PhD bridges two areas of biology that were neglected, and through it, I will present novel findings of Tfh cell biology.

I hypothesise that Tfh cells in different immune contexts will employ divergent metabolic pathways and that their immunometabolic signature would be distinct from other effector T cells. This understanding is vital and has been overlooked to date, as understanding the metabolic difference between Tfh cells and other effector T cells may pave the way for new therapies to boost/attenuate Tfh cells' responses towards pathogens or to self during autoimmunity and allergy (Deenick and Ma, 2011). To understand the complex immunometabolism of Tfh cells, firstly this introduction will explore key areas of T cell biology, such as development and function, before delving deeper into the transcriptional and metabolic requirements of Tfh and effector T cells to give an overarching appreciation of the role of metabolism in T cells and how, as scientist we can manipulate these pathways to have beneficial outcomes.

1.9.2 CD4+ T Cell Development

T cells originate in the bone marrow, where haemopoietic stem cells give rise to early lymphoid progenitor cells. These early thymic progenitor cells migrate to the thymus via the bloodstream for the essential maturation process that allows the development of different immune cells. Once in the thymus, the early lymphoid progenitors differentiate into early thymic progenitors, then into T cell-committed thymic progenitors through interaction with stromal cells. The development of thymocytes relies on these interactions with Cortical thymic stromal cells (CTECs) and medullary epithelial cells (MECs), which are antigen-presenting cells (APCs) that co-localise to distinct areas of the thymus, known as the medulla or cortex. Interaction with CTECs drives signalling that suppresses alternative fates and activates key T cell lineage transcription factors (TFs).

T cells can develop one of two types of T cell receptor (TCR), making them either $\alpha\beta$ T cells, which are the most common T cells or $\gamma\delta$ T cells, which make up approximately 5% of the T cell pool (Spits, 2002). Most $\gamma\delta$ T cells are not MHC-restricted and recognise antigens differently compared to $\alpha\beta$ T cells (Born et al., 2013)

The thymus facilitates the functional development of T cells through thymic selection and facilitates TCR rearrangement to generate either CD4+ or CD8+ single-positive thymocytes (Anderson and Takahama, 2012). In the early stages of thymic maturation, T cells start to express varied T cell receptors (TCRs), which are then tested on a series of MHC: peptide combinations to select cells that are not self-reactive T cells, to prevent autoreactivity. This process is known as the stages of Thymopoiesis (Thapa and Farber, 2019).

1.9.3 Stages of Thymopoiesis

In the early stages of Thymopoiesis, thymocytes lack CD4+ and CD8+ and are double-negative cells (Figure 1.2). This population can be further subdivided based on CD44 (activation marker) and CD25 expression. Cells that express CD25 but not CD44 undergo β -selection, which results in the successful rearrangement of the TCR- β locus and the development of a TCR complex with CD3 and the pre- α chain. Through this nascent TCR, these cells can differentiate and drive upregulation of CD4+ and CD8+ to develop into double-positive (DP) cells (Falk and Eichmann, 2002).

If a thymocyte does not undergo β -selection, it will die via apoptosis. The development of the pre- α TCR chain precedes DP formation and enables the TCR to assume the

conventional $\alpha\beta$ phenotype. Once a functional $\alpha\beta$ TCR has been developed (Figure 1.2), the cells must undergo two processes to ensure the functionality of the TCR (Thapa and Farber, 2019, Hernandez et al., 2010).

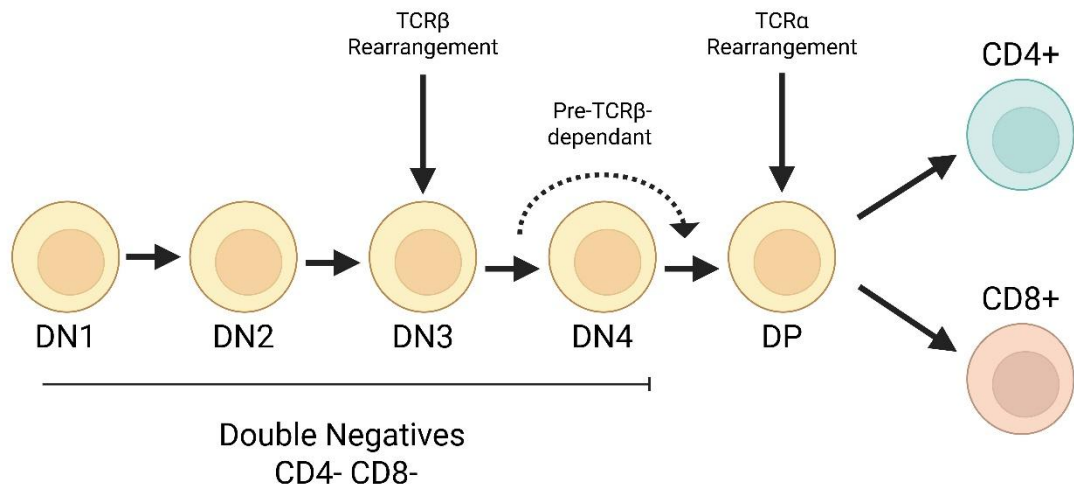


Figure 1.2. $\alpha\beta$ T cell development in the Cortex of the Thymus.

This is a stepwise process that T cells undergo during development in the thymus to create $\alpha\beta$ T cells, expressing the surface markers CD4+ or CD8+. This diagram was created using BioRender and adapted from (Spits, 2002).

Positive Selection occurs in the cortex of the thymus, whereby interactions with CTECs ensure that the TCR recognises peptides loaded onto the major histocompatibility complex (p:MHC). If the cell binds with the incorrect affinity (too weak) or does not recognise the presented antigen, it will undergo apoptosis via neglect due to no survival signal being received. This stage essentially checks if the TCR is functional and binds with the correct intermediate strength with the self-peptide. This intermediate binding strength indicates a good balance between self-recognition and the potential ability for pathogen antigen recognition. At this stage, thanks to positive selection, the T cells can either become CD4+ or CD8+ based on interactions with MHC I (CD8) or MHC II (CD4), then migrate to the medulla to undergo negative selection (Takada and Takahama, 2015).

In negative selection, newly single-positive T cells are subjected to the presentation of self-antigens by MECs, Dendritic Cells (DCs), or macrophages. This is a key TCR checkpoint, as if the T cells bind too strongly, they will undergo apoptosis or become anergic. This scrutinises TCR interactions and prevents the generation of self-reactive T cells, which could drive autoimmunity. The T cells that successfully navigate all these stages of thymic selection will then leave the thymus and migrate to the lymph node (LN) (Kondo et al., 2017).

Essentially, T cells that bind too weakly/do not bind are eliminated in negative selection, whilst those which bind too strongly are removed during positive selection. Interestingly, during thymic selection, regulatory T cells (Tregs) are formed when T cells bind with relatively high but not deletion-inducing affinity. These cells receive extra co-stimulation (CD28) and cytokine signals (IL-2), which promote the induction of FOXP3, which drives them to become Tregs to maintain self-tolerance in the periphery (Hsieh et al., 2012)

1.9.4 Migration/Integration of Naïve T Cells in the Lymph Node

Thymic emigrants migrate into the LNs through the bloodstream by entering specialised blood vessels known as high endothelial venules (HEVs); this process, known as transmigration, and requires the involvement of a series of adhesion molecules, such as L-selectin on the surface of the T cells, and also chemokines like CCL21 (Figure 1.3). Once transmigration has occurred, the T cells enter the paracortex, inside the LN, and migrate along the fibroreticular cell network. CD11c⁺ DCs are essential for maintaining the HEVs and supporting their function (Moussion and Girard, 2011, Luckheeram et al., 2012).

All naïve T cells continuously circulate throughout secondary lymphoid organs during their lives and use the same process as described above to enter the LN through HEV and transmigration. Upon entering an LN, they spend approximately 24 hours interacting with APC cells within the T cell zone, attempting to discover and engage with their cognate antigen to drive activation. If the T cell does not encounter the relevant foreign p: MHC II complex (Piconese et al., 2020), it will exit the LN via interaction with Sphingosine-1-phosphate (S1P), allowing it to enter the efferent lymphatic vessels and recirculate to another secondary lymphoid organ to repeat the process (Baeyens et al., 2015).

Naive T Cell Entry into Lymph Nodes

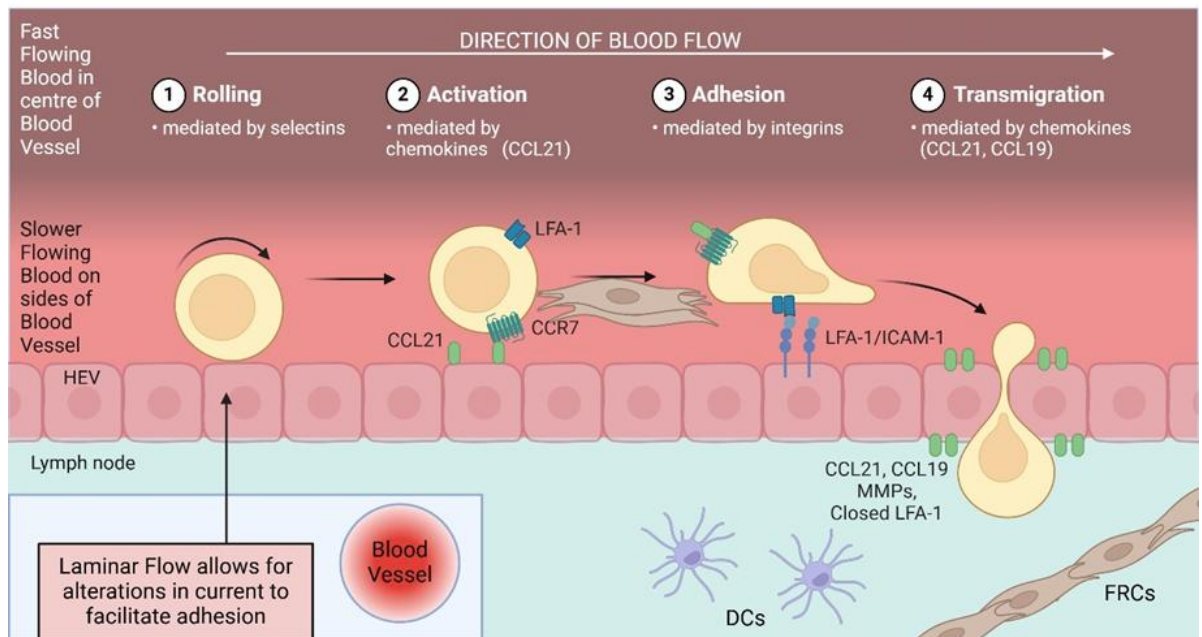


Figure 1.3. Naïve T Cell Migration into the Lymph Node.

The process involved in the migration of naïve T cells into the LN during recirculation. Created in BioRender with information from (Duan L, 2016, Moussion and Girard, 2011).

1.9.5 T Cell Activation

In homeostasis, when the T cells are naïve, the function of recirculation is to look for their cognate antigen presented on APCs to become activated. APCs carry the antigen from the site of infection to the draining LN. The APC can then present the antigen on either MHC class I or II, depending on the origin of the antigen. If the antigen was endogenous, it is processed and presented on class I, whilst an exogenous antigen is presented on MHC class II. The DC can express both MHC I and II at the same time, and depending on which cell it comes into contact with, this will dictate which MHC is utilised. This process allows APC to activate both CD4⁺ and CD8⁺ branches of the T cell adaptive immune response (Shah et al., 2021).

If a CD4⁺ T cell encounters its cognate antigen presented by an APC, the TCR will bind to p: MHC-II, the initial signal required for activation; however, complete activation requires several co-stimulatory signals, differentiation, and effector responses. Successive co-stimulatory signals between CD28 on the T cell and CD80/86 on the surface of the APC initiate proliferation and cytokine production. The cytokine environment also provides a signal that allows for the complete activation of the effector or memory response (Shah et

al., 2021). Cytokines produced by the activated T cells (e.g., IFN- γ) can also facilitate a positive feedback loop, which drives increased activation of the APC, thus increasing T cell activation also (Burke and Young, 2019). If the T cell only receives activation without co-stimulation, this drives T cell anergy, known as peripheral tolerance (Lechler et al., 2001).

When a T cell activates, it upregulates expression of CD44. This marker is maintained on the surface of the T cell throughout different stages of development and differentiation (Schumann et al., 2015). The expression of CD69 is also upregulated in response to activation; however, it is not maintained. During this process, the subsequent effector function of the CD4⁺ T cell into different T helper (Th) cells predominantly depends on the cytokine profile of the microenvironment (Cibrian and Sanchez-Madrid, 2017).

1.9.6 CD4⁺ T cells Subset Differentiation

The first documented research on different CD4⁺ T cell subsets was published in the late 1980s. This research described Th1 and Th2 cells and was fundamental to our understanding of CD4⁺ T cell biology, whereby Mossmann and colleagues defined T helper 1 (Th1) cells as Interferon-gamma (IFN- γ) producers, whereas Th2 cells produced Interleukin 4 (IL-4) (Mosmann et al., 1986). Since this discovery, several more Th cell subsets have been identified and well-characterised, including Th17, Tfh, and T regulatory cells (Tregs) (Zhu et al., 2010)

During T cell differentiation, the APC dictates the microenvironment inside the LN that determines the ability of a CD4⁺ T cell to develop into the particular differentiated form of T helper cell required. When in contact with cytokines in the environment, the receptor triggers a signalling cascade that drives a differentiation programme for the required lineage, whilst suppressing the differentiation programmes linked to other lineages. This drives the activation of different TFs to aid polarisation, which in turn drives effector function and the production of cytokines that can assist other CD4⁺ T cells in the differentiation process. The cytokine cocktail required to drive polarisation is unique to each Th cell type. These cytokines are the key mechanism required for differentiation, and this is highlighted in Figure 1.4 (Luckheeram et al., 2012).

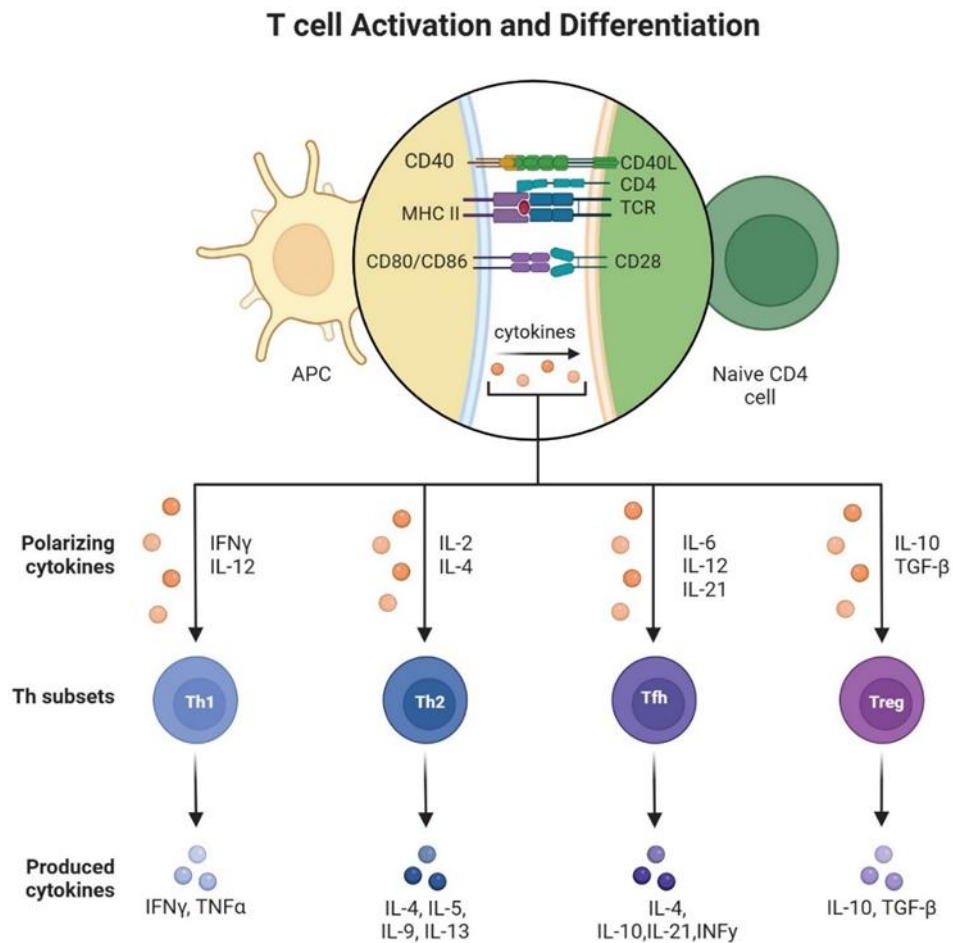


Figure 1.4. T cell Activation and Differentiation.

Diagram highlighting the key processes involved in the differentiation and activation of CD4⁺ T helper cells during differentiation into effector/regulatory cells. The cytokine profiles of each cell type are also represented. Created in BioRender, using information from (Hall, 2015, Guisier et al., 2020, Crotty, 2019).

1.9.7 Th1 Cells

Th1 cells are critical in responding to pathogens like bacteria, viruses, and protozoa. However, in certain conditions, they can also drive autoimmunity. To drive Th1 cell generation during activation, cytokines from the microenvironment produced by DCs and nearby innate immune cells bind to the receptors on the surface of the CD4⁺ T cell, triggering intracellular signalling, promoting differentiation. The predominant cytokine profiles responsible for Th1 cell generation are IL-12, IL-27 and IFN- γ and are secreted by a variety of different cells, such as macrophages/DCs, etc. (Butcher and Zhu, 2021).

Signalling from IFN- γ occurs via STAT1 and drives the expression of the master TF of Th1 cells, Tbet. The induction of Tbet can then drive further IFN- γ production, which works in

an autocrine manner to stabilise the Th1 phenotype (Lighvani et al., 2001). Th1 cells also require IL-12 to induce Tbet expression, and it is thought that IL-27 produced by nearby cells in the presence of infection can drive IL-12R expression on the surface of Th1 cells (Holscher, 2004). IL-12 signalling through IL-12R drives STAT4 expression, which further drives IFN- γ expression (Ullrich et al., 2020).

Research into DC subsets suggests that cDC1 expands are the main drivers of Th1 cells, as they are a major source of IL-12, and in models of Th1 inflammation without cDC1, Th1-mediated inflammation is severely attenuated (Martinez-Lopez et al., 2015). The generation of Th1 and Th2 cells shows distinct induction profiles, as T cells lacking STAT4 favour Th2 induction even in the presence of IL-12, confirming that IL-12R and STAT4 play crucial roles in the signalling pathways that result in Th1 differentiation. IL-12 must sustain STAT4 signalling for Th1 cells to develop (Nishikomori et al., 2002).

1.9.8 Th2 cells

Th2 cells play a critical role in coordinating responses to pathogens such as helminths, and are also responsible for hypersensitivity and allergic reactions. The cytokines most commonly produced by Th2 cells are IL-4, IL-5, IL-9, and IL-13, all of which play distinct roles in coordinating the type 2 immune response.

IL-4 is essential for Th2 differentiation. IL-4 is responsible for STAT6 phosphorylation, which in turn drives the induction of GATA3, the master regulator of Th2 cells (Luckheeram et al., 2012). GATA3 prevents Tbet expression through downregulation of STAT4. GATA3 also drives upregulation of STAT6 gene expression to solidify the transcriptional program essential for Th2 cell differentiation (Usui et al., 2003). Interestingly, GATA3 is also expressed at a low level in naïve T cells, as it is required for their development in the thymus (Wei et al., 2011).

Reports suggest that TCR signal strength may be responsible for Th1/Th2 induction, with weaker TCR stimulation sufficient to upregulate and drive GATA3 expression in the absence of IL-4/STAT6 (Yamane et al., 2005). This is unlike Th1 cells, which require exclusive cytokine microenvironments. Th2 differentiation can be IL-4 dependent or independent; however, the induction of GATA3 is critical to the phenotype (Zhu et al., 2004). GATA3 drives the remodelling of chromatin of the IL-4, IL-5 and IL-13 locus, facilitating the production of these canonical cytokines for function (Yamashita et al., 2004). STAT5 induction is also required, activated through IL-2, independent of IL-4, and

not involved in GATA3 induction. However, the coordination of GATA3 and STAT5 is required to induce IL-4 production (Luckheeram et al., 2012).

The source of IL-4 that drives Th2 differentiation remains unclear. Reports suggest that many cells, such as mast cells, eosinophils, basophils, and type 2 innate lymphoid cells (ILC2), can be producers of early IL-4. DCs are not believed to be the main source; however, once the first Th2 cells are made, they become the major source of IL-4, creating another critical positive feedback loop (MacDonald and Maizels, 2008). The cytokines produced by Th2 cells are also responsible for tissue repair, which is critical in the damage response initiated during helminth infection. These cytokines also contribute to B-cell proliferation and IgE class switching (Walker and McKenzie, 2018).

1.9.9 Other Th cell types – Th3/Th9/Th17/Th22

Many other Th cell subtypes exist, such as Th9, Th17 and Th22 cells. These cells are implicated in many responses to many pathogens and can contribute to the development of autoimmunity. In 1994, Schmidt et al. revealed that CD4⁺ T cells could be converted to IL-9-producing cells when activated in the presence of IL-2, IL-4 and TGF- β (Schmitt et al., 1994). These cells have been latterly coined Th9 cells (Dardalhon et al., 2008). Th9 is a relatively new subset of the Th cell. Still, interest in their role in many pathologies has rapidly expanded knowledge and interest in their differentiation and function (Li et al., 2017a). The signature cytokine involved in their phenotype is the pleiotropic IL-9, which has an expansive range of functions (Li et al., 2017a). Their main functions are to drive immune tolerance, protect against parasitic infections, and drive anti-tumour responses in line with autoimmunity and allergy, as IL-9 can recruit and activate mast cells, which are crucial for the induction of tolerance and are also responsible for the recruitment of effector cells during anti-helminth/tumour immunity (Kaplan, 2013).

Unlike other Th cells, which require a specific master TF, Th9 cells require numerous TFs for differentiation (O'Shea and Paul, 2010). It has been shown that Th9 cells are extremely unstable in *in vitro* conditions, as once induced, they lose the ability to express IL-9 within days (Tan et al., 2010). PU.1 serves as one of the key TFs associated with Th9 cells, as it is involved in Th9 differentiation by blocking Th2 cytokine expression and driving IL-9 expression through chromatin remodelling (Li et al., 2017a).

Unlike Th9, Th17 cells are a more well-defined subset of Th cells, responsible for driving the immune response against extracellular bacteria and fungi; however, they are also

implicated in various autoimmune diseases. The debate, however, around whether Th17 cells are an independent Th subset was discussed up until the late 1990s, when key experiments using IFN- γ /IFN- γ receptor-deficient mice developed magnified experimental autoimmune encephalomyelitis (EAE) in the absence of Th1 cells, highlighting the existence of another population of effector cells that were not Th2 cells (Ferber et al., 1996)

It was later discovered that the adoptive transfer of IL-17-producing Th cells drove this pathology, and Th17 cells were shown to be a distinct population of CD4⁺ Th cells (Steinman, 2007, Langrish et al., 2005). The identification of their master TF, retinoic acid receptor-related orphan receptor- γ t (ROR γ t), was then discovered along with the key roles of IL-6 and TGF- β in the differentiation of non-pathogenic Th17 cells. Research highlighted that TGF- β signalling in the presence of IL-6 drives ROR γ t expression (Yang et al., 2007). Like other CD4⁺ T cell subsets, Th17 cells can also be further divided into pathogenic and non-pathogenic subgroups, as determined by their production of different cytokines (e.g. pathogenic; produce pro-inflammatory IL-17, whilst non-pathogenic Th17 produce IL-10, etc) (Wu and Wan, 2020).

Th17 cells adopt a pathogenic phenotype by secreting high levels of TNF- α , IFN- γ , and GM-CSF. These cytokines aid in driving sustained inflammation and excessive recruitment of neutrophils and macrophages, culminating in tissue destruction. On the contrary, non-pathogenic cells boast mucosal defence through production of IL-17A/F, which recruits neutrophils and facilitates IL-6 and TGF- β release to drive pathogen clearance whilst limiting tissue damage (Peters et al., 2011). This functional divergence is also reflected metabolically: non-pathogenic Th17 cells rely on oxidative phosphorylation (OXPHOS), whereas pathogenic Th17 cells are highly glycolytic (Bystrom et al., 2022). This phenomenon is interesting, as even though these cells are from the same lineage, exposure to IL-21 and IL-1 β can promote Th17 cells to become pathogenic (Peters et al., 2011)

Th17 differentiation can be split into 3 distinct stages: TGF- β and IL-6 signalling to induce the phenotype, then self-perpetuation by IL-21 and finally the consolidation of the phenotype by IL-23 (Luckheeram et al., 2012). Th17 cells require IL-23 to facilitate the production of IFN- γ and Granulocyte/monocyte-colony-stimulating factor (GM-CSF) through STAT3/STAT4 activation, which drives their pathogenic phenotype (Langrish et al., 2005). Due to the heterogeneity of Th17 cells, they have a diverse range of functions, from conferring protection at mucosal barrier tissue sites (Weaver et al., 2013) to maintain tissue homeostasis through the production of IL-10 (Zhang et al., 2020).

The key cytokines produced by Th17 cells include IL-17A/F, IL-21 and IL-22. To date, many successful monoclonal therapies targeting IL-17 pathways have been used clinically in autoimmune conditions like psoriasis, in which these therapies reduce inflammation and improve skin lesions (Mosca et al., 2021). However, in Inflammatory Bowel Disease (IBD), anti-IL-17 monoclonal antibody (mAb) therapy was shown to exacerbate inflammation as it blocks the regulatory and homeostatic mechanism of Th17 cells at barrier tissues in the gut (Hueber et al., 2012, Lin et al., 2015). Th22 cells also exist and secrete IL-22 (Tamasauskiene and Sitkauskiene, 2018) And also Tumour Necrosis Factor Alpha (TNF α), granzyme B, IL-13, and IL-26. These cells are implicated in skin disease, autoimmune disease and maintenance of the mucosal barrier (Jiang et al., 2021).

1.9.10 T Regulatory Cells

Effector T cells play a crucial role in our protection. However, resolving these responses after the clearance of pathogens is essential and must be tightly regulated to prevent the development of disruptive immunopathological conditions and fibrosis. Previously, it was thought that tolerance was achieved through clonal deletion and anergy; however, pioneers such as Fiona Powrie, Shimon Sakaguchi, Herman Waldmann, Nicole Le Douarin, Juan Lafille, and others confirmed the presence of CD4⁺ T regulatory (Treg) cells with the suppressive capacity to mediate and resolve inflammation. Prior to the discovery of the signature TF, FOXP3, the presence of Tregs was highly debated among immunologists as to whether there was a distinct population of T cells that could resolve inflammation (Hori, 2021).

The concept of regulatory cells has been around since the 1970s; many were sceptical about their existence and regarded them as “suppressor T Cells”. Thanks to the pioneering work by Sakaguchi et al. (1995), which showed a distinct population of CD4⁺ CD25⁺ T cells had regulatory capacity, the paradigm shifted, and it was decided that “T suppressor cells” should be renamed Tregs to prevent any undue negativity regarding this population (Workman et al., 2009). Further research has highlighted numerous key findings regarding Treg differentiation and function, as well as their role as the primary orchestrators in the resolution phase of the immune response.

Tregs can control innate and adaptive responses towards self-antigens, pathogens, microbiota and multiple allergens. They maintain immune tolerance and homeostasis through various mechanisms. Tregs produce IL-10, TGF- β , and IL-35, which dampens

inflammation. Tregs also directly compete for IL-2 with effector T cells, which is crucial for preventing excess T cell proliferation. They are also able to modulate cAMP, CD39, and CD73 signalling, which can drive metabolic disruption in effector cells (Grover et al., 2021). In addition to this, Tregs can also drive cytotoxicity to induce cell death; this can be mediated through granzyme and perforin, as well as altering an APC's ability to activate T cells by mediating CTLA-4 binding to prevent CD80/86 co-stimulation (Gouirand et al., 2022). All these mechanisms act collectively to dampen down the immune response, and errors can result in tissue damage and autoimmunity (Boothby et al., 2020, Goswami et al., 2022).

Like Th17 cells, Tregs can also be subdivided into different groups; however, this is predominantly based on their origins. Some Tregs are induced as detailed above during the active immune response in the periphery, whilst others arise in the thymus during thymic selection. These are denoted as natural Tregs or thymic Tregs (Cheru et al., 2023). The main differences between these two subsets are their inception and also their reliance on the cytokine environment; induced Tregs require cytokine-dependent signals to a greater degree than natural Tregs (Povoleri et al., 2013).

Other Treg subsets do exist or are highly debated. Th3 cells have been suggested as a subset of regulatory T cells, which produce high volumes of TGF- β . Their main role is mediating oral tolerance to autoantigens and preventing a response to non-pathogenic bacteria (Corthay, 2009)

1.9.11 T Follicular Helper Cells

Tfh cells are a unique subset of CD4⁺ T cells located in the secondary lymphoid tissues and are required to drive the formation of germinal centres (GCs). GCs allow for the selection of high-affinity antigen-specific B cells and facilitate the clonal expansion and diversification of the humoral response. This diversification is driven by Tfh cells, which can skew Ab production to the correct isotype required for that response. Tfh cells provide B cells with the correct foundations to mount the adaptive response (plasma cells) and allow for long-term immunity (memory cells) for the host towards pathogens to be achieved (Haniuda et al., 2020, Crotty, 2014).

Tfh cells express a restricted receptor (cytokine/chemokine) and TF repertoire; this aids B cell development by ensuring that they are spatially positioned in the right microenvironment to provide precise help to antigen-specific B cells. Tfh are highly

selective in choosing which B cells to help. CXCR5 is critical for Tfh cell migration, while ICOS, PD-1, CD40L, and the TCR are critical for activation and costimulatory signals that facilitate B cell activation and diversification by driving intracellular signalling to activate key TFs essential for B cell maturation. They also modulate T-B cell interactions. CD40L is essential as it engages with CD40 on B cells and can drive activation, proliferation and class-switching.

Key cytokines, such as IL-21 and IL-4, are also fundamental to this process and are released by Tfh cells. These cytokines can drive B cell proliferation/survival/class-switching and somatic hypermutation (SHM), which is the process whereby the B cell can alter the affinity of its BCR through point mutations in the variable regions of its immunoglobulin genes. Together, this repertoire of receptors and TFs allows for the development and functional output of the germinal centre response (Crotty, 2019, Linterman and Vinuesa, 2010)

The main driver in Tfh cell development and function is the TF, Bcl-6. This drives the expression of key modulators of the GC response, whilst also being critical in the repression of other T cell lineages; however, it should be noted that it represses TFs and does not block their expression entirely, as Tfh cells have been shown to express low levels of different TFs such as GATA3 and Tbet (Choi and Crotty, 2021, Fang et al., 2018, Olatunde et al., 2021). Interestingly, Bcl-6 can also transcriptionally repress Tbet, STAT-6 and ROR γ t expression, and studies have shown that blocking the generation of Th1, Th2 and Th17 development can directly induce Tfh cell markers (CXCR5, PD-1, ICOS, CD40L) expression in CD4⁺ T cells and can facilitate Tfh Cell development (Kassiotis and O'Garra, 2009, Yu and Vinuesa, 2010).

1.9.12 Activation and Differentiation of Tfh Cells

During the activation of naïve T cells in the secondary lymphoid organs, an APC presents antigen to its cognate T cell. Through direct stimulation of the TCR and Costimulation via receptors and cytokines, the T cell becomes activated and undergoes rapid proliferation as previously described in section 1.9.5. This process normally lasts a few days to form effector T cell populations. Additionally, this process can result in the formation of pre-Tfh cells that express elevated levels of PD-1 and ICOS. Pre-Tfh cells also express prominent levels of the TFs T cell factor 1 (TCF-1), Bcl-6 and cMAF, and lower levels of B lymphocyte-induced maturation protein-1 (BLIMP-1), CCR7 and EBI2 (Webb and

Linterman, 2017). This process of pre-Tfh cell formation is critical for the early stages of Tfh cell development, and these activated pre-Tfh cells can aid B cells outside the germinal centre to become activated and produce low-affinity antibodies, known as the extrafollicular response (Qin et al., 2018).

Multiple classes of DC can prime Tfh differentiation. However, they also require a suitable microenvironment and B-cell assistance. In the initial phase of Tfh differentiation, interactions are essential to drive their differentiation. It has been shown that signalling through ICOS and OX40L is critical in early Tfh cell priming, along with cytokines secreted from the DC, like IL-6, IL-12 and type 1 Interferons, which can promote Bcl-6 induction and IL-21 expression (Ma and Nakayamada, 2021). Furthermore, IL-27 released by DCs can also drive Tfh cell differentiation through STAT1/STAT3-mediated pathways (Batten et al., 2010), whilst CD40/40L signalling can reinforce sustained ICOS expression. However, though, the direct combination of time microenvironment and DC states (metabolic and transcriptional) remains poorly defined, especially in vivo and across different immunological stimuli (Ma and Nakayamada, 2021).

During DC priming, Tfh cells upregulate the TF, Bcl-6, through interactions between ICOS and its ligand. A direct consequence of the induction of Bcl-6 is the increase in the expression of CXCR5 and a decrease in CCR7 expression. This enables the migration of the Tfh cells out of the T cell zone and to the border of the B cell follicle, as CXCR5 is a chemokine receptor that binds with CXCL13, which is expressed in the B cell follicle. Once at the T/B border, Tfh cells interact with antigen-specific B cells and initiate the GC response (Crotty, 2019, Vinuesa et al., 2016).

The interaction between the Tfh cell and the B cell promotes each other's differentiation, whereby the B cells provide the correct stimuli to the Tfh cells for them to differentiate to produce IL-21 and upregulate CD40L, which in turn allows for the generation of the germinal centre and further B cell maturation through somatic hypermutation and class-switching (Konforte et al., 2009). The initiation and stages of the germinal centre response are detailed in section 1.9.16

The costimulatory molecules on Tfh are essential for their role, for example, PD-1 promotes the formation of plasma cells through interaction with its cognate ligands PD-L1 and PD-L2, both expressed at the same time on GC B cells, whilst also being critical to Tfh positioning and function (Shi et al., 2018). SLAM-associated protein (SAP) allows Tfh cells to interact with B cells through the SLAM family of receptors, 2B4, Ly9, CD84 and CD150 (SLAMF1). These receptors are critical in controlling Tfh and B cell interaction:

for example, CD84 enhances the Tfh-B cell cross-talk, whilst SLAMF1 helps to drive IL-4 production in Tfh cells through SAP signalling (Sahoo et al., 2016). Defective SAP signalling can result in the inability to form GCs due to defects in Tfh cell differentiation, whilst its overexpression has been associated with the presence of autoantibodies in autoimmunity (Gartshteyn et al., 2024, Deenick et al., 2010).

Tfh cells are shown to be evolutionarily conserved between both mice and humans, both transcriptionally and functionally (Choi and Crotty, 2021). However, the cytokine profiles needed for Tfh induction appear distinct. IL-6 is a potent inducer of murine Tfh differentiation and IL-21 expression; however, this is not replicated in humans (Ma et al., 2009). Unlike during murine *in vitro* Tfh cell differentiation, which requires IL-6, *in vitro* human Tfh differentiation can be accomplished via co-culture of CD4⁺ naïve T cells with TGF- β or Activin A and IL-12, whilst blocking IL-2 (Locci et al., 2016). IL-2 is a direct inhibitor of Tfh differentiation as it drives the expression of Bcl-6 antagonists, including the TFs BLIMP-1 and STAT-5 (Papillion and Ballesteros-Tato, 2021).

1.9.13 Heterogeneity and Function of Tfh Cells

Initially, it was thought that Tfh cells were lineage independent of other Th cells, as Bcl-6 was shown to repress TF expression associated with other key Th cell lineages such as GATA3, Tbet and ROR γ t. However, this theory has been discounted by recent research, which shows Tfh cells can pick up the flavour of the microenvironment through the low-level expression of Tbet (Th1) and GATA3 (Th2), driven by the type (Type 1 /Type 2, etc.) of immune response (Zaini et al., 2023). This heterogeneity can be further exploited after the cessation of the GC response, whereby Tfh can repolarise into other Th cell subsets (Th1/Th2, etc.) to carry out effector function. This adopted flavour allows for Tfh cells to share cytokine production and chemokine receptor expression akin to Th counterparts (Song and Craft, 2024).

Indeed, in the type 2 immune response towards the helminth *H. polygyrus*, Tfh cells are the main producers of IL-4, not Th2 cells, in the secondary lymphoid organs (King and Mohrs, 2009). The production of IL-4 is seen throughout Type 2 immune responses, such as asthma and other parasitic infections. However, the intrinsic mechanisms for inducing IL-4 production are different between Tfh and Th2 cells. Notch pathways and the CNS2 enhancer mediate IL-4 production in Tfh cells, whilst in Th2 cells, it is regulated by GATA3 (Kubo, 2021).

A similar profile of Tfh cells producing conventional Th cell cytokines is also observed in type 1 immune responses, whereby Tfh can exhibit Tbet expression and produce IFN- γ through STAT4 phosphorylation driven by IL-12 and type 1 Interferons (Weinstein et al., 2018). Interestingly, loss of Tfh cells during viral infection is compensated for by Th1 cells through CD40L and production of IL-21, which can drive B cells to help produce class-switched antibodies (Chen et al., 2022). This highlights the plasticity of the immune response between Tfh and Th cells, as both can substitute for each other.

These findings have led to the naming of Th1-like (Tfh1) and Th2-like Tfh (Tfh2) subsets, and more recently, further classes of Th-like Tfh have been reported. Th17 and Treg-dominated immune responses have given rise to Th17-like Tfh or Type 3-like Tfh (similar to Tregs), and also a population of FOXP3⁺ Tfh cells. FOXP3⁺ Tfh cells are independent of T follicular regulatory cells (Tfr cells), as Tfr cells regulate Tfh cell responses, whilst FOXP3⁺ Tfh cells regulate the resolution of the GC response (Song and Craft, 2024) highlighting the plasticity of Tfh generation.

The generation of Tfh and Th17 is similar in that IL-6 signalling drives production of IL-21 by a STAT3-mediated mechanism (Zhou et al., 2007). As with Tfh cells, IL-2 is also repressive to the Th17 phenotype (DiToro et al., 2018). Likewise, with Th1 cells assisting B cells when there is a lack of Tfh cells, Th17 cells can also assist in inducing B cell proliferation, class-switching and contribute to forming the germinal centre. This is most likely due to IL-21 production and CD40L expression; however, this was not addressed in the research (Mitsdoerffer et al., 2010). Tfh cells have been reported to be able to produce IL-17 and are associated with autoreactive autoantibody production in autoimmune BXD2 mice, as removal of IL-17-producing Tfh cells can prevent autoreactive GC formation and reduce disease burden (Hsu et al., 2008).

As the GC reaction proceeds, and high-affinity plasma cells are selected for and leave the LN, FOXP3-expressing Tfh cells emerge. These Tregs arise from Tfh cells and are not the same as T follicular regulatory (Tfr) cells, which are thymically derived and subsequently gain access to the GC (Linterman et al., 2011). FOXP3⁺ Tfh cells drive the resolution of the GC response; however, their mechanism of action remains elusive (Le Coz et al., 2023). Tfr cells (thymically derived) differentiate from Tregs and provide a similar mechanism of action to FOXP3⁺ Tfh cells; however, the two cell types are transcriptionally different. FOXP3 Tfh-like cells have low expression of IL-2R, whilst Tfr cells have increased IL-2R expression. Unlike Tfr cells, which are a defined subset that co-express FOXP3, CXCR5 and Bcl-6, and regulate the GC response, FOXP3⁺ Tfh-like cells

are more ambiguous and are a transient subset of Tfh cells only observed in certain diseases and experimental models. Tfr cells are mainly involved in preventing autoreactive antibodies whilst suppressing/terminating GC formation (Clement et al., 2019, Sage and Sharpe, 2015, Jacobsen et al., 2021).

Lymph Node Architecture and the Germinal Centre Response

1.9.14 Organisation and Function of the Lymph Node

The immune system is composed of primary, secondary, and tertiary lymphoid organs, interconnected with the proximal blood and lymphatic systems. The lymphatic system comprises a system of vessels responsible for transferring lymph around the body to the lymphoid organs (Moore and Bertram, 2018). LNs are found throughout the body and are connected to the lymphatic system through the afferent and efferent lymphatic vessels. The antigen (soluble or being carried by an APC) enters the afferent lymphatic vessels and flows into the lymphatic sinus, known as the subcapsular and medullary sinus. In addition to the lymphatic vessels, HEVs permeate and intertwine throughout the LN and are the main blood source in these organs, as well as the entry point for lymphocytes. The LN is split into distinctive areas, which house key players in the immune response. The compartmentalisation of areas of the LN is largely due to stromal cell populations (Grant et al., 2020).

The three main LN regions are the cortex, the paracortex, and the medulla. The cortex is located on the outermost side of the LN and contains B cells and follicular Dendritic Cells (FDCs). This area is segmented into areas known as interfollicular zones (IFZ). The inner area of the LN, known as the paracortex, is the T cell zone. The paracortex comprises T cells and fibroreticular cells (FRCs), the latter of which are essential for T cell migration as they make up the branching network through which they traverse. The medulla is closer to the efferent lymphatic and contains the medullary sinuses (Ozulumba et al., 2023, Pikor et al., 2021). The medullary sinus drains lymphatic fluid and allows lymphocytes and antibodies to enter the bloodstream through the thoracic duct into the left subclavian vein (Ozawa et al., 2022)

During homeostasis, the LNs act as central hubs for T cell trafficking from the blood. They enter through the paracortex through HEVs and scan for cognate antigens (Arasa et al.,

2021). Between the T and B cell borders lies an area, known as the intrafollicular zone (ITFZ), abundant with lymphoid cells such as natural killer (NK), $\gamma\delta$ T cells, NK T cells and innate-like CD8⁺ T cells (Gregoire et al., 2007, Rampoldi et al., 2020, Kurioka et al., 2018). In the INFZ zone, migratory DCs activate CD4⁺ T cells. Tregs cluster close to these areas to prevent aberrant activation or the generation of autoimmune cells (Wang and Zheng, 2013).

1.9.15 Mesenteric (Intestine) and Mediastinal (Lung) Lymph Nodes

LNs are encapsulated secondary lymphoid organs, and despite this common structure, differences are observed in LNs at different sites within the body (Grant et al., 2020). The mesenteric LNs (mLN) comprise a network of multiple LNs that drain different areas of the intestine. These sites are primarily responsible for initiating and perpetuating the adaptive immune response in the intestine (King et al., 2017, Dubey et al., 2017).

Due to their constant exposure to antigens, the intestine's LNs are the largest draining LNs in the body and understanding their site-specific function is crucial in studying immune responses in the gut. Using Chicago blue dye, it was demonstrated that different anatomical locations within the gut drain into distinct LNs (Carter and Collins, 1974).

The duodenum lymph drains to the LNs found within the pancreatic tissue, whilst the jejunum drains to the middle mLN. The distal ileum, caecum, and colon drain the end LN of the mLN (Figure 1.5). Interestingly, the transverse colon drains to the pancreatic LNs, whilst the descending colon and rectum drain into the caudal LNs (Carter and Collins, 1974, Mowat and Agace, 2014), highlighting that understanding the location and the correct location of the reactive LNs is critical in understanding certain pathogenic models of disease.

Conversely, the mediastinal LNs (mdLN/s) are the primary sites for draining the lungs (Figure 1.5). They are found in the mediastinum, between the lungs and the chest cavity. Like the mLN, the mdLN significantly increase in size (during infection, etc.), due to the influx and proliferation of immune cells, predominantly T and B cells (Stolley et al., 2024). It has also been shown that interactions between DCs and fibroblastic reticular cells through the CLEC2-podoplanin (gp38) axis are the main driving forces to increase the size of the LN through relaxation of the actomyosin contractility and network tension caused by CLEC2 Disruption, allowing FRCs to relax (Acton et al., 2014). Further to this, it was shown that podoplanin-associated proteins CD44 and CD9 as essential for FRC

morphological remodelling, mediating lamellipodial and filopodial protrusions required for effective network spreading (de Winde et al., 2021). Together, these studies show that DC–FRC crosstalk via CLEC-2–podoplanin signalling controls both the mechanical relaxation and structural reorganisation required for LN expansion during immune responses, emphasising the active contribution of stromal cells to immune system plasticity

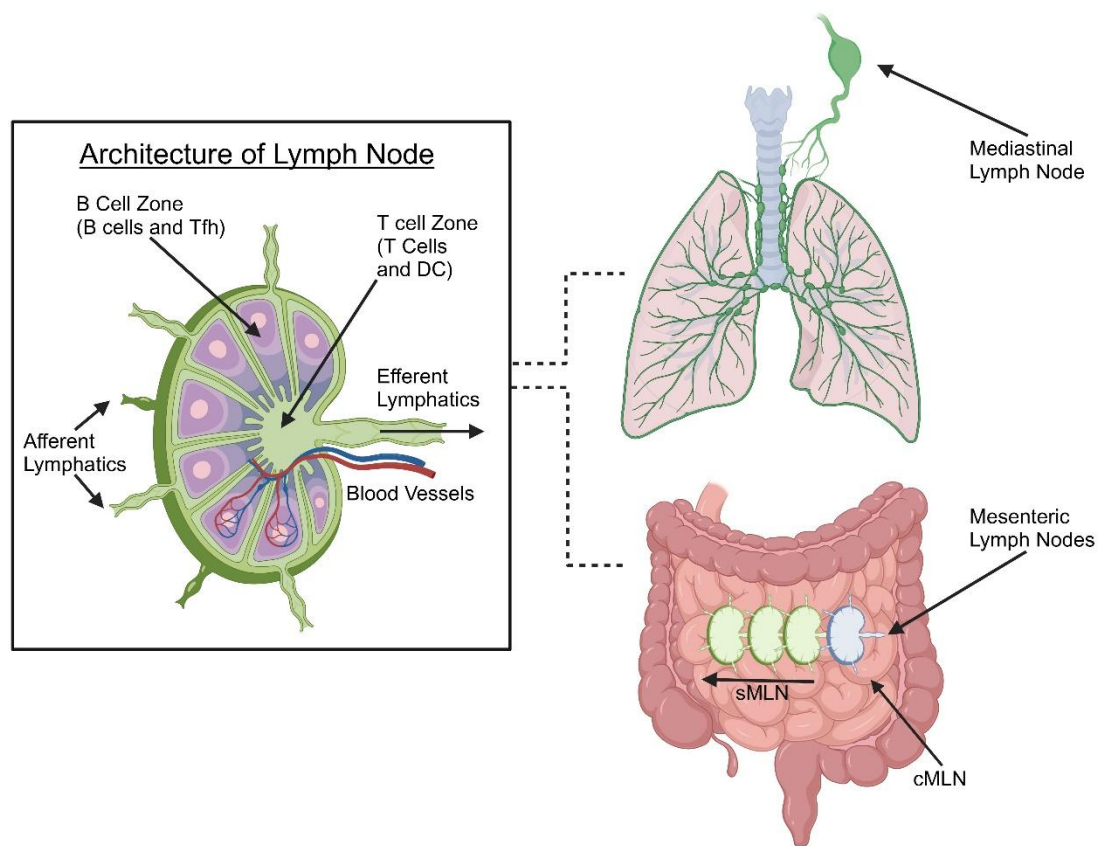


Figure 1.5. Mediastinal and Mesenteric Lymph Node Architecture.

The mLN consists of the small intestine-draining and the colon-draining LNs, which sit in the abdominal cavity of the mouse. The mediastinal LNs are found behind the lungs in the thoracic cavity of the mouse and drain the lungs. The LN architecture (Left) is presented, highlighting key areas and lymphatic vessels essential in the induction and delivery of an effective adaptive response. sMLN- small intestine draining mLN, cMLN – colonic draining mLN (large intestine draining mLN). Diagram created in BioRender, adapted from (Houston et al., 2016) and (Reed and Ager, 2022).

1.9.16 Activation and the Germinal Centre Response

B cells are like T cells in that they are constantly circulating in the bloodstream and enter through the HEV into the LN cortex. Once inside the LN, they migrate to the B cell follicles under CXCL13 chemokine gradients (Pereira et al., 2010). For a B cell to

participate in the GC response, it must undergo initial activation by recognising its cognate antigen presented by either FDCs, macrophages, or conventional DCs extrafollicularly. This drives upregulation of CCR7, a chemokine receptor, allowing the B cell to migrate to the T/B cell border. Here, the B cells interact with Tfh cells, which drives co-stimulation of both cells along with survival signals. The B cell has two choices at this stage: 1) proliferate and drive activation of the GC, or 2) differentiate into short-lived extrafollicular plasma or memory B cells. The extrafollicular plasma cells provide an early, lower-affinity antibody response towards the stimulus (i.e. infection, etc.), whilst the GC reaction occurs. The extrafollicular plasma cells mediate control awaiting the development of a functional GC response, whereby high-affinity plasma cells are released and drive a strong, highly specific humoral response (Stebegg et al., 2018).

GCs form at the T/B cell border and reside inside the B cell follicle of secondary lymphoid organs once formed. The response relies heavily on T cell help, provided by Tfh cells. Tfh cells aid in choosing the correct long-lived, antigen-specific, high-affinity class-switched B cell. Once the B cell has first proliferated/differentiated in the GC, the plasma cells subsequently migrate to the tissue affected (Foster et al., 2022).

The microstructure of the GC allows for a specialised area that aids B cells during their maturation (Figure 1.6). The GC is split into the Dark Zone (DZ) and the Light Zone (LZ). Each zone is distinct in its function. The DZ consists of highly mitotic proliferating mutating B cells (centroblasts), whilst the LZ consists of non-dividing B cells (centrocytes), FDCs, and Tfh cells and is pivotal in the process of BCR selection (De Silva and Klein, 2015). Centroblasts undergo somatic hypermutation (SHM) in the DZ to increase the affinity of the BCR. SHM is the process whereby point mutations are introduced into the variable regions of immunoglobulin genes in B cells. This allows for variation in the hope that a favourable mutation will drive increased affinity towards the antigen presented. Post maturation, they downregulate CXCR4 and migrate to the LZ to undergo a form of positive selection, whereby they interact with FDCs and Tfh cells (Stebegg et al., 2018). Tfh cells are involved in both the initial activation/differentiation of B cells by driving the GC reaction, and also help in the selection of BCR with the highest affinity in the later stages of B cell maturation (Olatunde et al., 2021).

B cells normally undergo several cycles of LZ affinity selection and DZ SHM. FDCs can also interact with Tfh cells to provide signals influencing B cell differentiation. FDC and Tfh are essential for the positive selection of centrocytes, and Tfr cells control the output of the whole GC response. FDCs are key in allowing the B cells to test their affinity for their

BCR and drive affinity maturation (van der Poel et al., 2019). FDCs maintain the response through antigen presentation to GC B cells and upregulation of key adhesion molecules such as ICAM and VCAM (van der Poel et al., 2019).

Whilst FDCs are the architects of the GC response, Tfh cells act as the chaperones of the GC response. They secrete cytokines such as IL-4 and IL-21, which aid in class-switching and are key in controlling B-cell proliferative ability. Most GC B cells cannot trigger BCR signalling due to increased phosphatase activity that dampens BCR signalling (Khalil et al., 2012). Therefore, Tfh cells help supply proliferative and survival signals critical to maintaining the response (Crotty, 2014). This is mainly due to Tfh-derived signals through CD40/CD40L, ICOS/ICOSL and TCR; MHC II interactions, which drive an increase in calcium flux in the B cell to drive maturation (Shulman et al., 2014).

Tfh cells are also critical in choosing the highest-affinity B cells and controlling which cells proliferate, are selected for apoptosis due to low affinity, or undergo another round of SHM. This differs from the FDC selection, as FDCs measure affinity through the binding strength of the BCR to the antigen presented. In contrast, Tfh cells provide help based on the levels of peptide presented by B cells on their MHC-II, which is a proxy for higher BCR affinity, as B cells with a higher affinity BCR present more peptide-MHC-II, so they get more Tfh cell help (Vinuesa et al., 2010).

Tfh cells can be divided into two subsets based on their CD90 expression. The expression of CD90 has been shown to dictate their anatomical location within the GC reaction. CD90 low/negative Tfh cells require antigen-specific B cells expressing MHC II to develop and reside in the GC, whilst CD90 high Tfh cells do not and continuously proliferate and are found outside the GC reaction (Yeh et al., 2022). It is believed that CD90 becomes downregulated through chronic TCR stimulation, as it is observed on chronically stimulated or exhausted T cells. The requirement of B-cell help may suggest that MHC-II/TCR signalling directs the downregulation whilst driving transcriptional changes through TCR signalling in the Tfh to promote survival (Mackerness et al., 2010)

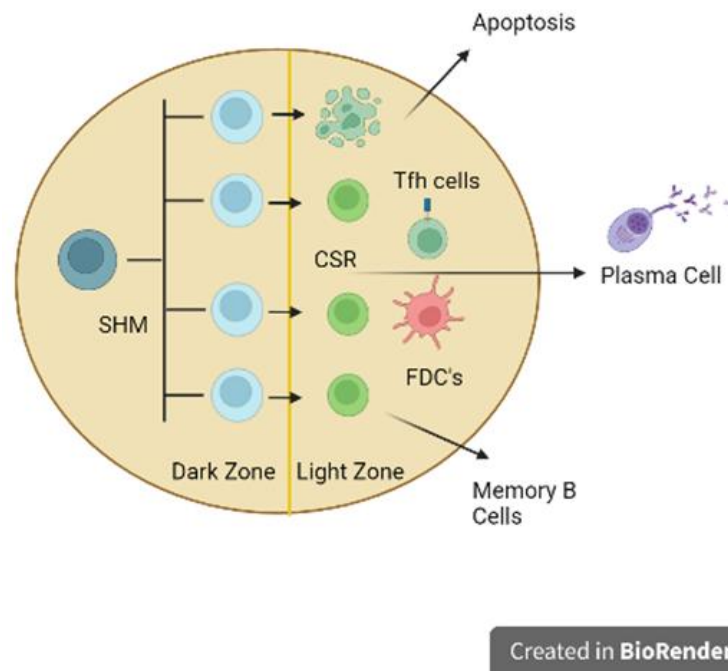


Figure 1.6. Development of Functional Germinal Centre.

Activated B cells undergo clonal expansion and SHM in the GC dark zone and become centroblasts as described in the main text. Once SHM occurs, the centroblasts differentiate into centrocytes and downregulate CXCR4 and migrate into the light zone under a chemokine gradient, where they interact with Follicular Dendritic Cells (FDCs) and T follicular helper cells (Tfh) to evaluate the affinity of their newly rearranged BCR. High-affinity BCRs are chosen, whilst B cells with low-affinity BCRs undergo apoptosis. High-affinity receptors allow cells to undergo class-switch recombination (CSR) or sequential rounds of SHM in the DZ. Once the BCR has been optimised, the centrocytes differentiate into memory or plasma B cells. Created in BioRender and adapted from (Klein and Dalla-Favera, 2008)

In summary, together, all these aspects of the LN response facilitate the initiation of the T, B and antibody responses that are critical to defence against many infections. The next sections will explore different infections (helminth, viral, etc.) and the role of CD4⁺ T cells, particularly Tfh, Th2 and Th1 cells. This will then proceed to explore how their immunometabolism is tailored to each cell's metabolic demands to facilitate their effector functions during pathogen-induced adaptive immune responses.

The Type 2 Immune Response

1.9.17 Helminth Infection

Globally, parasitic helminth infections are a major health obstacle, affecting around 1 to 2 billion people (Specht and Keiser, 2023). Prevalence is often associated with severe poverty and frequently results from inadequate sanitation practices. Helminthiasis can also decrease how individuals' immune system responds to infectious agents such as malaria, HIV/AIDs, whilst also decreasing vaccine efficacy, leading to a cycle of morbidity, which can put significant pressure on the socioeconomic capacity of low-income regions of the planet (World Health and Infections, 2012).

Mass efforts to treat individuals to try to clear helminth infection have had varying success. While programs using anthelmintic medication like Albendazole and Mebendazole are generally successful at decreasing the burden of disease, many challenges remain due to geographic factors, compliance with treatment, and public health infrastructure. The WHO aims to address these issues by 2030, whereby they have set global targets, which effectively look to control soil-transmitted helminth (STH) infection, eliminate disease in certain groups, increase sanitation and decrease the amount of tablets required for preventative chemotherapy for STH (Montresor et al., 2020).

Helminth infection primarily affects children due to behavioural habits, exposure to contaminated environments, nutritional vulnerability and immature immune responses. This can result in stunted growth and can affect educational attainment (Hotez et al., 2008). Another consequence of helminth infection is malnourishment, likely caused by impaired nutrient absorption in the intestine (gut helminth infection), resulting in deficiencies in key vitamins and minerals. This, in turn, creates a cycle which ultimately increases an individual's susceptibility to acquiring helminth infection due to a weakened immune system (Stephenson et al., 2000).

Research into helminth infections has increased significantly over the past decade, with a focus on neglected tropical diseases. However, many initiatives do not address the need to improve our understanding of basic helminth biology. Enhancing our knowledge of these diseases will enable the development of novel therapeutic approaches, which may, in turn, lead to more effective treatments and control of helminthiasis. However, this will require significant investment (King, 2019).

The most common STH infections in humans are roundworm (*Ascaris Lumbricoides*), whipworm (*Trichuris trichiura*) and 2 Hookworm infections (*Necator americanus* and *Ancylostoma duodenale*). Most helminth infections do not replicate in humans, except for *Strongyloides stercoralis* (Hotez et al., 2008). Mortality is rare. Most infections are asymptomatic due to low worm burden, and research suggests that the long co-evolution with helminths has driven polarised immune response towards regulatory responses, to live with the parasite rather than drive expulsion, and this results in long-term infection of the host (Wammes et al., 2014).

This co-evolution with parasites has been considered a driving force in the development of the type 2 immune response. However, recent research has offered alternative views, as there are several innate mechanisms that favour a type 2-like mechanism irrespective of infectious stimuli (Torow et al., 2017), coupled with the fact that, as neonates, we favour skewing our immune response towards the type 2 response. This is evident in the first days after birth, when IL-33-mediated type 2 eosinophilic recruitment in the lungs is essential for establishing lung homeostasis, which drives anti-inflammatory responses, favouring alveolar macrophages to be recruited and seeded into the lung (Saluzzo et al., 2017). This may suggest that the type 2 immune response developed to prevent damage and protect the barrier functional integrity towards multicellular organisms, as type 2 elicited cytokine release can drive tissue homeostasis and wound repair during an active response, highlighting the system's ability to mitigate disturbance (Harris and Loke, 2017).

Even though helminth infection differs between host species, their commonality is that they predominantly drive type 2 immunity in their host for control and clearance. In the intestine, a mechanism known as “weep and sweep” is a response initiated by Th2-mediated immunity, whereby the body increases mucus production to entrap the helminth-“weep”-whilst enhancing muscular contractions, “sweep” them out of the body (Harris and Loke, 2017)

Helminth infection can also decrease vaccine efficacy through decreased quantity and quality of antibodies; one proposed mechanism is the direct result of increased production of IL-10-producing Tregs, which can still affect vaccine efficacy even after the helminth infection has cleared (Hartmann et al., 2019). In areas where helminth infections are not endemic, we see increased rates of autoimmunity and allergy (McSorley and Maizels, 2012). This suggests a protective mechanism elicited by Tregs or through skewing of the immune response from type 1 to type 2 by helminth infection that protects the individual from autoimmunity/allergy. This has led to interest in using helminths as an

immunotherapy or helminthic secretions as novel therapeutic drugs (Smallwood et al., 2017). One particular area of interest is the use of *Heligmosomoides polygyrus* (*H. polygyrus*)-derived secretions, known as TGF- β mimics (TGM), which have been shown to preferentially bind to TGF- β receptor on the surface of CD4⁺ T cells to drive Treg induction (White et al., 2021), or through binding with CD44, CD49d and TGF- β receptors to target myeloid cells in a cell specific manner that mammalian TGF- β is unable to achieve, highlighting interesting therapeutic potential as these secretions may be able to target multiple different receptors on immune system cells (Singh et al., 2023).

1.9.18 The Hygiene Hypothesis/Old Friends

The ability of parasitic infections to modulate the immune response has led to the development of key theories, such as the hygiene hypothesis, which was presented by D.P. Strachan in 1989. He proposed that children with a lower incidence of childhood infection could be the key driver of the rise in allergy in the Western world (Strachan, 2000, Strachan, 1989).

The hygiene hypothesis provides a rationale for the significant increase in the prevalence of allergies and autoimmunity since the 1980s and alludes to immune defects driven by a lack of exposure to infection, resulting in poorer immune tolerance. This lack of antigen exposure can be shown geographically, whereby in Western countries, where infection is controlled and prevalence is low, there are higher rates of allergy than those in areas where infection is more common. The prevalence of food allergy in preschool children is roughly 10% in Western countries compared to 2% in areas such as mainland China (Prescott et al., 2013). This phenomenon is not limited to allergy, as inflammatory bowel disease (IBD) is two-fold higher in Western Europe than in Eastern Europe (Burisch et al., 2014)

Updates to the hygiene hypothesis have been developed to augment the specific microbes associated with this theory, such as the “Old Friends” theory presented by G.Rock in 2003, which suggests the co-evolution of humans and parasites, such as helminths, may provide a more rational argument due to the immunomodulatory effects of helminths on the host immune system, suggestive that a lack of helminth infection in more developed countries is driving the increase in allergy and autoimmune disorders (Frew, 2019). Thanks to research, we now understand more about the role of excretory products produced by helminths, and current research is looking at whether there is a therapeutic rationale for using these molecules in clinics. To date, many of the early clinical trials using helminth-derived

products have failed to show efficacy or reach a conclusive answer over whether the placebo effect drove results. However, in these trials, the treatments were shown to be safe and tolerated well by patients ((Shields and Cooper, 2022, Ryan et al., 2020)

1.9.19 *H. polygyrus* - Murine Model of Roundworm Infection

Using murine models to study helminth infection has allowed us to gain valuable insight into the complex nature of helminths and their hosts. Models such as *H. polygyrus* have enabled us to study both acute and chronic immune responses to these infections and have pioneered the use of helminths and their products as potential immunotherapies. *H. polygyrus* is a trichostrongyloid nematode helminth parasite that primarily naturally infects rodents. The infection can persist in several mouse models for several months, and in certain cases, it can be chronic (Maizels et al., 2012). *H. polygyrus* shares common chronicity with members of the *Ascaris*, *Trichuris*, and hookworm species, in that it elicits similar immunosuppressive capabilities through induction of Tregs, drives type 2 immune responses, and shares their ability to establish long-term infection (Smits et al., 2010).

The *H. polygyrus* life cycle is completely enteric and direct. Larvae enter the host orally, where they travel to and invade the duodenal/small intestine mucosa and mature in the serosal layer of the intestine wall for between 8-10 days, known as the “Tissue-dwelling stage”. Maturation involves the larvae developing into the adult worm and involves several moults and a period of growth. After maturation, the adult worm migrates from the serosa across the lamina propria into the lumen, where it wraps around the villi to facilitate its long-term survival in the gut. In the lumen, the worm mates to produce eggs, which will be transported via stool to reinfect (Figure 1.7).

Mice are coprophagic – the act of eating faecal matter, so transfer occurs naturally in the wild easily. The name *polygyrus*, as in many gyrations, describes the adult worm's behaviour, which tightly wraps around the intestinal villi to remain in constant contact with the epithelium. Direct sustenance does not occur through this contact, as they do not facilitate blood feeding like hookworms (Reynolds et al., 2012). *H. polygyrus* feeds directly on the intestinal tissue and uses the villi as a platform for mating and laying eggs (Maizels et al., 2012).

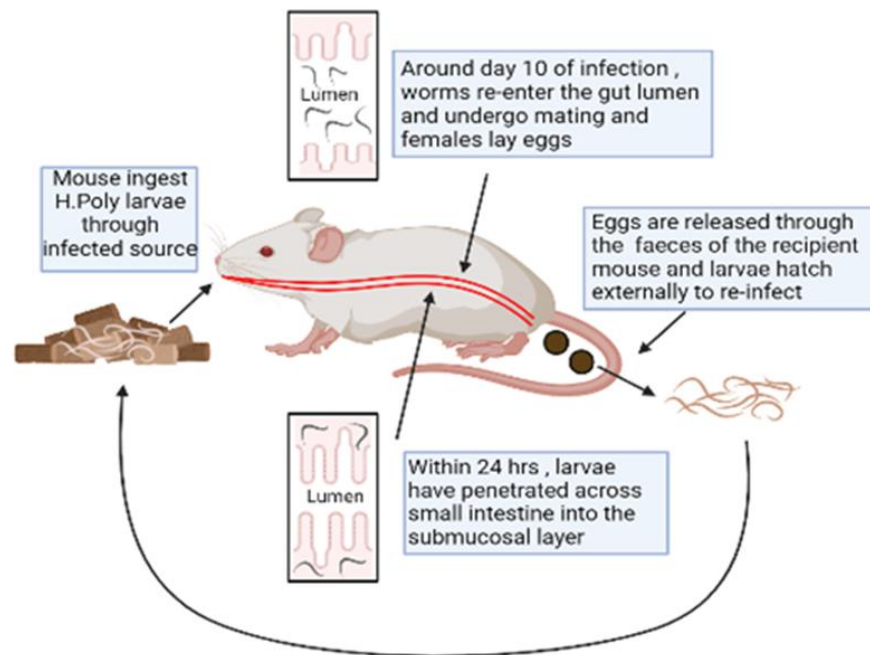


Figure 1.7. *H. polygyrus* Life Cycle.

Schematic diagram of the life cycle of *H. polygyrus* (*H. Poly*) employed in a murine host (Adapted from (Reynolds et al., 2012). Created using BioRender.

Anti-Helminth Immune Response

1.9.20 Innate Immune Response

The mechanism by which *H. polygyrus* larvae invade the submucosal layer of the small intestine remains unknown. In this tissue-dwelling stage, the epithelial cells around the larvae release Alarmins in response to damage associated with the trans-mucosal migration of helminth and drive inflammatory cell influx into the intestine (Ariyaratne et al., 2022). The Alarmins consist of IL-25, IL-33 and thymic stromal lymphopoietin (TSLP), which are stored in epithelial cells and released upon damage accrued to their cell membranes (Gauvreau et al., 2023). These cytokines are important in activating innate lymphoid cells to mount a strong inflammatory response (Stanbery et al., 2022).

For example, IL-25 is fundamental in the clearance of *H. polygyrus* infection, as it stimulates ILC2s to release type 2 cytokines like IL-5 and IL-13, which are important for clearing infection (Smith et al., 2018). IL-33 is fundamental in the initiation of the type 2 immune response, as it drives the activation of ST2, its cognate receptor on various immune cells, including mast cells, Th2 cells, DCs, and ILC2s. This drives activation and

production of canonical type 2 cytokines such as IL-4, IL-5, and IL-13 (Yi et al., 2022). The immunomodulatory effects of *H. polygyrus* can target IL-33 production to prevent the induction of a strong type 2 response (McSorley et al., 2014).

The release of TSLP is also crucial in activating various innate immune cells, including ILC2s, with the assistance of IL-25 and IL-33. This epithelial-derived cytokine may signal through distinct receptor pathways; however, their signalling synergises on downstream transcriptional programs (e.g., GATA3, STAT5, NF- κ B), driving stronger activation and functional capacities in these cells. Convergence between the innate and adaptive immune systems is critical for a response towards a pathogen. It has been shown that TSLP can also indirectly act to drive Th2-mediated inflammation by promoting Th2 responses in DCs through OX40 Ligand (OX40L) (Stanbery et al., 2022, Ito et al., 2005).

In response to *H. polygyrus* infection, macrophages, mast cells and ILC2s become activated to mediate and try to expel the helminth infection. Mast cells can become activated in response to IgE binding to Fc ϵ receptor on their surface (driving crosslinking), or in response to ATP or IL-33 being released from damaged cells. In turn, mast cells release mediators through degranulation, like histamine (Jordan et al., 2021). Histamine causes tight junctions between cells to loosen, particularly in blood vessels, resulting in local oedema, whilst IL-13 drives contractions of smooth muscle cells, forming the basis of the “weep” and “sweep” mechanism. This mechanism uses increased luminal fluid from local oedema and smooth muscle contractions to move the helminth infection through the intestine so that worms can be expelled (Vacca and Le Gros, 2022)

IL-4 and IL-13 drive activation of fibroblasts to aid wound repair by promoting collagen deposition and fibrosis. Similarly, macrophages are involved in tissue repair and mediating inflammation (Lekki-Jozwiak and Baska, 2023). IL-33 from mast cells drives ILC2s to release IL-13, which results in goblet cell hyperplasia and mucin production, used for expulsion of the worm (Shimokawa et al., 2017). Granulocytes (basophils and eosinophils) are also recruited to the site of infection by IL-5 and assist in the immune response and secrete pro-inflammatory cytokines and mediators that can damage the helminth (Lekki-Jozwiak and Baska, 2023, Smith et al., 2012)

1.9.21 Bridging the Innate and Adaptive Immune Response

To initiate the adaptive immune response, APCs, most commonly DCs, migrate from the infected tissue, process and present antigens from the pathogen to orchestrate the activation of T and B cells in the draining LN. In the case of *H. polygyrus* infection, DCs capture antigen from the lumen of the submucosa and migrate to the small-intestine draining mLNs.

The literature extensively defines our understanding of DC's role in activating cognate T cells in *H. polygyrus*, particularly in generating Th2 and Tfh cell responses towards the parasite. CD11b⁺ cDC2s promote Th2 cell differentiation, thanks to their expression of the TF, KLF4 (Krüppel-like factor 4), which promotes the expression of IL-10 and OX40L in DCs (Tussiwand et al., 2015). cDC2s also promote Tfh cell generation, which is highly dependent on contextual signals and the local microenvironment. This is highlighted in recent research by Tatsumi et al. (2025), who demonstrated that CD301b⁺ DCs produce IL-2 through CD40 interactions, which drives increased CD25 expression in CD4⁺ T cells, thereby facilitating the skewing towards Th2 cells. Th2 cells require IL-2 for differentiation, whilst conversely, Tfh cells differentiation is repressed by IL-2, through Bcl-6/BLIMP-1 mediated inhibition. Hence, highlighting that possibly in IL-2-rich environments, this CD25/IL-2/CD40 axis drives Th2 cells, whilst also in an IL-2-dependent mechanism could block Tfh cell differentiation (Tatsumi et al., 2025).

1.9.22 Role of Th2 cells in *H. polygyrus* Infection

Th2 cells are critical in the host's adaptive response towards *H. polygyrus* infection. Th2 cells migrate to the small intestine from the mLN and are the main source of key cytokines such as IL-5 and IL-13. These cytokines work to activate key cellular components of the type 2 response, such as macrophages and ILC-2s (McManus, 2022, Maizels et al., 2012).

Interestingly, blocking Th2-mediated responses in *H. polygyrus* does not drive a compensatory revision to the pro-inflammatory Th1 response, as expected and previously defined in other infection models. This inhibition of the type 2 adaptive response drives an immunoinhibitory phenotype, which suppresses immune responses through increased Treg differentiation, a process that has been shown to diminish Th1 responses. This highlights a crucial mechanism employed by *H. polygyrus* to drive chronic immunosuppression, to persist and thrive within the host (Liu et al., 2004). This increases the damage inflicted, as

the type 2 response also protects the host from tissue destruction caused by *H. polygyrus* infection, mediated through wound healing macrophage activation driven by Th2 cytokine release (Vacca and Le Gros, 2022).

1.9.23 Role of B cells in *H. polygyrus* Infection

Concurrent with the Th2 response, B cell responses are critical in mounting a strong immune response toward *H. polygyrus*. It has been shown that B-cell antibody responses can control the development and expansion of Th2 cells (Wojciechowski et al., 2009). B cells are critical in the immune response towards helminth infection, as aside from acting as APC to Th2 cells, they also secrete pro-inflammatory cytokines such as IL-2, IL-4 and TNF- α , which are used by many innate and adaptive immune cells to drive protective immunity (Wojciechowski et al., 2009). Particularly, IL-2, which Th2 cells require for their proliferation and differentiation (Alugupalli and Abraham, 2009).

B cells also secrete Abs such as IgG1, which is the dominant isotype generated during type 2 immunity towards *H. polygyrus* compared to IgE, IgM or IgA (McCoy et al., 2008). It has been suggested that the Abs can bind to helminths; however, more commonly, they adopt an indirect mechanism to confer protection through activation and recruitment of immune cells through binding with Fc γ receptors on the surface of granulocytes to drive cross-linking and degranulation or through cytokine production, which activates and recruits immune cells towards the helminth (Harris and Gause, 2011). Abs can limit egg production and adult worm development in *H. polygyrus* infection through neutralisation of enzymes required by the helminth for larval invasion, feeding and migration (Esser-von Bieren et al., 2013, Harris and Loke, 2017).

1.9.24 Role of Tfh cells in *H. polygyrus* Infection

To generate a robust B cell response, Tfh cells are required to drive the GC reaction to create high-affinity antibody-secreting plasma cells (Zaini et al., 2023). The ability to drive an effective humoral response is dictated by Tfh cells and their ability to produce IL-4, which drives Ab production towards IgG1 and IgE isotypes, crucial for type 2 immune responses towards helminth infection. This cytokine is also crucial for forming the GC and facilitating SHM. The IL-4 produced by Tfh cells is required to maintain the type 2 immune microenvironment in the reactive mLN during *H. polygyrus* infection (Meli et al.,

2017). Evidence also suggests that, in the context of *H. polygyrus* infection, Th2 cells can become Tfh-like cells to produce IL-4 inside the B cell follicle (Glatman Zaretsky et al., 2009).

Like IL-4, IL-21, produced by Tfh cells, is also crucial for driving an effective adaptive response during *H. polygyrus* infection. Research has shown that IL-21-deficient mice infected with *H. polygyrus* exhibit significantly hindered intestinal granuloma formation and impaired T cell expansion and survival. This drives the development of a diminished type 2 immune response. Moreover, IL-21^{-/-} mice have a diminished capacity to respond to secondary infection with *H. polygyrus*, emphasising the importance of cytokines in the generation of memory responses. Furthermore, these mice also displayed decreased basophil and eosinophil populations, which are critical in controlling this pathogen, highlighting the convergence between the adaptive and innate immune systems and how they synergistically require each other to facilitate effector functions. It has been shown that IL-21 is critical for plasma cell differentiation but is expendable for GC formation and isotype switching (King et al., 2010). This indicates that IL-21 is multifaceted in regulating both innate and adaptive responses to *H. polygyrus* infection.

Whilst the role of the cytokines produced by Tfh cells has been explored in detail in the literature, the direct effect of Tfh cells has only recently been described. Using a CRE system that specifically depleted Bcl-6 in only CD4⁺ T cells to remove the Bcl-6⁺ CD4⁺ T cell population, it was shown that the mice completely lost the ability to drive GC responses and the subsequent ability to produce high-affinity IgG1 antibody responses (Meli et al., 2023). IgG1 responses are required to prevent superinfection through a process known as concomitant immunity, whereby infection with a persistent pathogen protects the host from subsequent infection with the same pathogen. This was not achieved in the CD4^{cre}Bcl6^{fl/fl} mouse model, highlighting the requirement for Tfh cells to drive preventive measures that protect the host from subsequent infection (Meli et al., 2023)

Bcl-6 can be expressed at low levels in other CD4⁺ T cell subsets. Interestingly, it was shown that the removal of Bcl-6-expressing cells enhanced *H. polygyrus* infection clearance due to increased production of IL-10 by Th2 cells. Meli et al., 2023, showed that increased IL-10 resulted in enhanced goblet cell differentiation, which drives increased helminth expulsion through the “Weep and Sweep” mechanism (Meli et al., 2023).

The role of IL-10 in protecting against *H. polygyrus* infection has been explored, as it was shown that IL-10 can drive an increased regulated removal of the pathogen (tailored adaptive response) with stronger epithelial barrier functions (Filbey et al., 2014). The two

papers from Meli et al. (2023) and Filbey et al. (2014) show that increased IL-10 production can drive pathogen clearance through the “weep and sweep” mechanism and enhance barrier integrity. However, it should be noted that enhanced clearance is most likely an indirect consequence of loss of Tfh cells, as it was previously shown that loss of Bcl-6 in Th1 also resulted in increased IL-10 production, which can alter innate immune responses and lead to diminished responses towards visceral leishmaniasis, highlighting the complex nature of low level expression of Bcl-6 in both Th1 and Th2 cells (Meli et al., 2023).

1.9.25 Immunomodulation of *H. polygyrus*

H. polygyrus can elicit strong immunomodulatory effects on various branches of the innate and adaptive immune response. The infection triggers a strong Treg response and immunosuppressive macrophage and DC populations, which decrease the Th2 response towards the helminth parasite. The main molecular basis of the immunomodulatory effects is exerted through secreted products known as HES (*H. polygyrus* excretory/secretory antigen), which involve TGF- β -like ligands known as TGMs (TGF- β mimics), proteases and proteins similar to venom allergen/Ancylostoma secreted protein-like (VAL) family. The secreted proteins, similar to the VAL family, are highly immunodominant. This means that they stand out to the immune system and drive a strong immune response towards them, even if multiple epitopes are present (Maizels et al., 2012).

Helminth secretions, like HES’s ability to modulate immune responses, are not a solely unique characteristic of *H. polygyrus* infection. Many different helminth infections can affect such modulation, for example, in the secretory product of *Acanthocheilonema viteae*, ES-62, which can prevent allergy and autoimmunity in many disease models, but in a Treg-independent manner (Pineda et al., 2014, Doonan et al., 2019).

Overall, there are many mechanisms employed by the immune system to target and try to expel helminth infections like *H. polygyrus*. These include both innate and adaptive immune responses, which all work together to try to control the infection; however, *H. polygyrus* is also able to employ strategies to regulate the immune response through secretions such as HES, which can suppress branches of the immune response through driving increased differentiation of cells involved in the resolution phase of immunity, such as Tregs. Overall, *H. polygyrus* is an excellent murine model of helminth infection and serves as a formidable method for understanding Th cell responses during type 2 immunity, due to the robust immune response that this infection drives.

The Type 1 Immune Response

1.9.26 Influenza A Virus Infection

Influenza A Virus (IAV) is the most common influenza respiratory infection in humans and animals. This contagious viral infection can lead to devastating consequences for individuals and can cause chronic decreased pulmonary function (Keeler et al., 2018). IAV typically occurs in seasonal winter epidemics; however, in tropical regions, it can occur at any time of year. IAV presents as a febrile acute respiratory illness but can have systemic effects; symptoms normally include fever, chills, headache, weakness/lethargy, cough, dry throat and increased nasal mucus production (Javanian et al., 2021). The World Health Organisation predicts that IAV infects around a billion people annually, resulting in around 500k deaths and 3-5 million people developing severe illness (Clayville, 2011, Krammer et al., 2018). The virus most commonly spreads from person to person through aerosol droplets, direct contact with an infected person or objects containing viral particles (Javanian et al., 2021).

Influenza viruses are part of the *Orthomyxoviridae* family of RNA viruses. They are grouped into 4 types: A to D. They are single-stranded negative RNA viruses that give rise to the surface proteins NA (neuraminidase) and HA (hemagglutinin). HA is used to adhere to the target cell surface, allowing entry, while NA enzymatically aids viral replication and release. The genome is composed of 8 RNA segments, each encoding 1 or more viral proteins, such as HA and NA; however, IAV also produces a range of internal and accessory proteins.

These include the polymerase complex proteins PB1, PB2, and PA, which are critical for viral RNA transcription and replication; the nucleoprotein (NP), which facilitates the encapsulation of the viral RNA; the matrix proteins M1 and M2, involved in virion structure and uncoating; and the non-structural proteins NS1 and NS2, which can control the host's antiviral responses and nuclear export of viral RNA. The RNA segments can also produce accessory proteins such as PB1-F2, PA-X, and others, depending on the strain, which contribute to virulence (Robb et al., 2011). For example, using the laboratory-adapted viruses of IAV infection, PR8 (A/Puerto Rico/8/1934) and X31 (A/Hong Kong/1/1968 × A/PR8/34 (X31; H3N2)) share similar internal gene segments but differ in their surface HA and NA, which define their subtype (H1N1 for PR8, H3N2 for X31) (Slepushkin et al., 2001).

The IAV PR8 (H1N1) strain is a modified mouse-pathogenic virus routinely used in studies due to its robust replication in mice. In contrast, IAV X31 (H3N2) is a reassortment virus that has 6 identical genes to PR8 (8 in total), but the HA and NA surface glycoproteins are derived from the human H3N2 Hong Kong 1968 strain of IAV. This results in the ability to replicate similar to PR8, however confers reduced virulence, allowing for severe (PR8) and milder (X31) to be contrasted and understood immunologically in vitro and in vivo (Rutigliano et al., 2014).

Due to the nature of IAV having very high mutation rates, creating vaccines to target it is challenging, as the expression of mutated NA and HA proteins constantly evolves. Most vaccine strategies target HA and NA antigens on the viral surface, which can be blocked by Abs; however, as the virus mutates, these Abs cannot bind with the same affinity/or don't bind at all, with NA/HA, and the vaccine becomes less/effective. This means that predictive modelling of IAV dominant strains is used, and seasonal vaccines are created against those strains (Lou et al., 2024). The IAV seasonal vaccines are often quadrivalent (4 strains included) in the hope of creating broader protection (Chan et al., 2021).

Due to this, reports on vaccine efficacy for the European Winter 2023/2024 IAV infection season show that 53% of individuals in primary care and 44% in hospital settings were protected against mild and severe IAV infection, similar to what was observed in the 2022/2023 winter IAV season vaccination results (Maurel et al., 2024). However, this efficacy falls short of that of other vaccine successes, and hence, understanding the GC reaction, especially in Tfh cells, may enhance the Ab response to vaccination.

Antiviral therapies are also available for IAV infection; however, prevention is better than cure, as incubation of the virus can still result in significant lung damage. There are 4 currently approved antivirals for IAV infection, 3 of which block NA to prevent the enzyme's ability to aid viral reproduction or be released into the periphery. The 4th antiviral for the treatment of IAV infection, Baloxavir Marboxil, inhibits the viral endonuclease enzyme in IAV, which prevents the virus from being able to hijack the host cells' mRNA processing machinery, blocking viral replication (Kosik and Yewdell, 2019).

IAV can “overpower” the host cell's mRNA machinery through a process known as cap snatching. IAV has its own RNA polymerase (PB1, PB2, and PA) to synthesise viral mRNAs; however, translations cannot occur as transcripts require a 5'-methylated cap structure to be recognised by the host's ribosomes. To ensure translation, PB2 binds the 5' cap of the host pre-mRNAs and PA cleaves 10–13 nucleotides downstream, providing a capped RNA fragment as a primer for PB1 to make viral mRNA-essentially stealing the 5'

cap off host pre-mRNAs. This cap-snatching strategy allows viral mRNAs to mimic host mRNAs and access the cell's translational machinery. Simultaneously, viral proteins like NS1 degrade host mRNAs and inhibit host gene expression, redirecting resources toward viral replication and suppressing antiviral responses (De Vlugt et al., 2018)

IAV confers two mechanisms to confer increased virulence – antigenic drift and antigen shift – and the ability of these differs significantly in their epidemiological impact. Antigenic drift refers to the ability to accumulation of point mutations in the viral genome, particularly in genes involved in NA and HA proteins, allowing for minor antigenic changes to evade detection by the immune system, essentially the basis of seasonal IAV epidemics. However, in antigenic shift, which is responsible for the ability of IAV to drive pandemic strains of the virus, the mechanism is different. Antigenic shift occurs when two different IAV strains (can be animal and human variants) infect the same host cell, and their genome segments can reassort, creating a novel IAV with new HA and/or NA genes, that the human population has no pre-existing immunity towards, making it highly virulent (Wikramaratna et al., 2013).

When a new variation of IAV emerges, such as the H1N1 IAV pandemic of 1918-1919 or when it jumps across species, as seen in the avian IAV (H7N9) epidemic in China (2013), mortality can increase significantly. The 2013 Chinese Avian IAV epidemic had a mortality rate of 35%, while the 1918 H1N1 pandemic resulted in over 40 million deaths. Epidemiology research has highlighted the key groups associated with increased mortality towards IAV infection, these include: the elderly, immunocompromised, children, and pregnant women (Nguyen et al., 2024).

1.9.27 Type 1 Immune Response To IAV Infection

IAV primarily infects the airway epithelial cells. Once infected, these cells will produce viral particles as IAV overpowers the cells' mRNA machinery to aid in its propagation. This hijacking and release of viral particles does not go unnoticed by the immune system, and innate mechanisms of action are deployed to counteract this foreign invasion of the epithelium, which are all employed to drive an effector response and bridge the gap between the innate and adaptive response (Chen et al., 2018).

Aside from innate and adaptive responses, physical barriers and physiologic processes prevent IAV infection. For example, covering your mouth and nose can prevent IAV infection, whilst mucus, produced by goblet cells, traps IAV through sialic acids and

prevents its ability to infect epithelial cells. However, IAV has developed a strategy to evade this mucus-trapping mechanism, whereby NA can cleave sialic acids to allow for their release (Cohen et al., 2013). The anti-viral therapy, Oseltamivir, inhibits NA, which prevents viral entry into cells and increases the amount of virus trapped in mucin to reduce the burden of infection (Hama, 2016).

If the virus evades the mucin layer and infects epithelial cells, intracellular sensing through Toll-like receptors (TLRs) and RIG-1 (Retinoic Acid-Inducible Gene-1) initiates the response to the virus and engages pattern recognition receptor intracellular signalling. This, in turn, drives activation of type I and type III Interferon response, consequently releasing IFN- $\alpha/\beta/\lambda$ to limit viral spread in the upper airways through activation of epithelial cells, which switch on interferon-stimulated genes to increase resistance to infection (Lazear et al., 2019). The release of INF results in leukocyte migration into the infected tissue and the release of cytokines, further aiding in controlling the infection (Reed and Ager, 2022)

Neutrophils are the first line of defence against viral infection and are rapidly recruited to the site of infection; here, they drive clearance through phagocytosis of dead/dying cells, and the production of granzyme and perforin to break down infected cells, although not to the same degree as NK and CD8⁺ T cells (Lamichhane and Samarasinghe, 2019).

Neutrophils also use Neutrophil extracellular traps to trap viral particles (George et al., 2021).

Neutrophil migration is crucial for successfully recruiting T cells to the area, as they deposit CXCL12 chemotactic guidance trails, which serve as signposts for virus-specific CD8 T cells during migration from the mdLN (Lim et al., 2015). Macrophages are critical in the response to IAV. There are two key populations: resident and monocyte-derived, which work together to drive phagocytosis and increase the cytokine milieu TNF- α , IL-1 β , IL-6, IFN- α and IFN- β) in the local microenvironment (Nguyen et al., 2024).

DCs, like macrophages, are susceptible to IAV infection directly; however, when uninfected, they play a crucial role in bridging the innate and adaptive immune responses towards IAV. They secrete pro-inflammatory cytokines whilst also carrying antigens from the tissue microenvironment to the lung-draining LN, where they can activate the adaptive response towards the pathogen (Waithman and Mintern, 2012).

1.9.28 Role of T cells in IAV Infection

T cells play a vital role in managing and eradicating IAV infections from the host. Both CD4⁺ and CD8⁺ T cells directly contribute to reducing the virus burden and the severity of the disease through multiple mechanisms, including cytotoxicity and the release of pro-inflammatory cytokines and mediators, which aid B cells in producing antibodies. CD8⁺ T cells are excellent at recognising and killing virally infected cells and also contribute to decreased viral shedding, whilst CD4⁺ T cells elicit other effector functions such as cytokine release, cytotoxicity, memory and B cell help (Sun and Braciale, 2013).

When the T cells arrive at the lung from the draining LNs, they infiltrate the tissue and initiate effector functions, which are observed almost instantly. The presence of CD4⁺ and CD8⁺ cells in the respiratory tract leads to a significant reduction in overall viral titres. The clearance of IAV is not solely dependent on T cells; however, complete removal through transgenic or acute depletion renders mice unable to clear the infection, particularly in CD4⁺ T cells (Topham et al., 1996, Hufford et al., 2015). This is likely because CD4⁺ T cells can drive local microenvironmental effects and direct strong Ab responses.

The main CD4⁺ T cell response during IAV infection is dictated by Th1 cells, which produce IFN- γ , TNF- α , and IL-2. These cytokines activate macrophages and aid in driving stronger CD8⁺ T cell responses. CD4⁺ T cells can also target infected epithelial cells through MHC-II/TCR interactions and lyse infected epithelial cells (Chen et al., 2018, Takeuchi and Saito, 2017). A strong antibody response is critical in the adaptive response towards IAV infection. Robust GC responses involving Tfh, B cells and FDCs are required to create high-affinity Abs towards IAV, which drive neutralisation (binds to and prevents the virus from infecting cells) and opsonisation, which enhances phagocytosis of infected cells to prevent spread (Devarajan et al., 2022). The cycle in which the adaptive immune responses work to control and eliminate IAV infection is clearly described below in Figure 1.8.

Adaptive Immune Response in Influenza A Infection

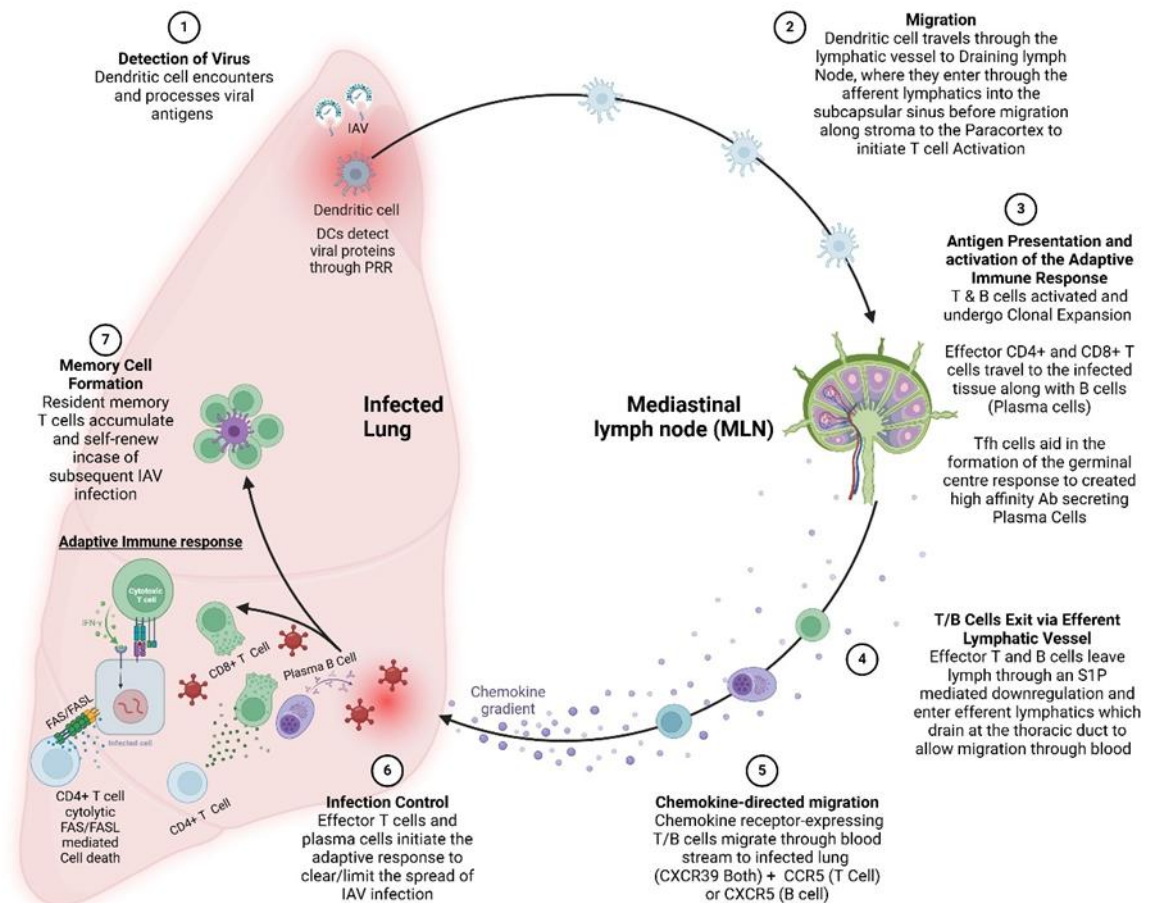


Figure 1.8. Initiation and Function of Adaptive Immune response to IAV Infection.

This diagram highlights the multiple stages in the process of antigen recognition to memory cell formation during the adaptive immune response towards IAV infection in the Lung. Created in BioRender and adapted from (Humphries et al., 2021)

1.9.29 Role of Tfh cells in IAV Infection

Unlike conventional CD4⁺ T cell responses initiated early in infection with IAV infection, Tfh cells appear later. Previous research suggests that Tfh cell populations in the draining LNs peak around days 8-12 of IAV infection (Boyden et al., 2012). They are crucial to driving the formation of GC reactions to produce high-affinity Ab-secreting plasma cells. Tfh cells are vital in the production of antibodies, as shown by the significant attenuation

of IgG responses to the vaccinia virus, by approximately 98%, when Tfh cells were absent (Xiao et al., 2014).

A strong Tfh cell response to IAV requires CD4 effector cells to continually recognise antigens in the LN microenvironment while receiving stimulatory signals from the continuing infection. Tfh cells release IL-21 and IFN- γ in the context of IAV infection. It has been shown that such IFN- γ production by Tfh cells is instrumental for the development of memory B cells (Arroyo-Diaz et al., 2023). IFN- γ , like IL-4 in *H.polygyrus*, can also instruct class switching in B cells to produce IgG2a and IgG2b during IAV infection (Mohr et al., 2010). IL-21 is also critical in the response towards intracellular viral pathogens, as it was highlighted that during Lymphocytic choriomeningitis (LCMV) infection, it was critical in maintaining the CD8+ T cell response and subsequent control of the infection (Zander et al., 2022).

Moreover, it has been shown previously that mice lacking IL-21 had attenuated IAV-specific IgG1 and IgG2 compared to wild-type mice (Eto et al., 2011). However, the role of Tfh cells is not clear, as Miyauchi et al.(2016) showed that in the absence of Tfh cells, IgG2 could be created in response to IAV vaccination, and that IL-21 and IFN- γ from Th1 cells were the main drivers of this protective response (Miyauchi et al., 2016). This could also highlight redundancy, whereby deletion of Tfh cells is minimised by other effector cells stepping in to fill the void left by the lack of Tfh cells.

Recent research has shown that protein arginine methyltransferase 5 (PRMT5), a post-translational protein modifier, can diminish Tfh and Tfr populations in mice during IAV infection. This is because PRMT5 alters gene expression programs such as Bcl-6, which are vital for Tfh cell differentiation. This then leads to reduced B cell activation and high-affinity antibody production towards the virus; however, the effect of this on the survival of the mice or disease severity was not assessed (Read et al., 2024). The TF, TCF-1, is also critical for Tfh cell differentiation in IAV infection, as it acts upstream of Bcl-6/Blimp-1 axis, to prevent repression of Bcl-6 and allow for Tfh cell differentiation (Xu et al., 2015).

Perhaps surprisingly, it has also been demonstrated that Tfh cells require a functional Treg response to elicit a robust IAV-specific humoral response. This appears to be due to the Tregs preventing a large accumulation of IL-2 inside the LN, favouring Tfh cell differentiation (Leon et al., 2014). Furthermore, the generation of Tfh cells during IAV infection is regulated by antigen availability within the specific CD4 T cell pool; increased competition among T cells leads to attenuated Tfh cell response (Olson et al., 2016)

Interestingly, it has been shown that metabolic hormones such as Leptin can promote the function of Tfh cells in response to IAV in immunisation and infection responses. This is directly driven through the STAT3/mammalian target of rapamycin (mTOR) pathways, leading to increased Tfh cell differentiation and IL-21 production. This is not specific to Tfh cells alone, as Th1 cells can be activated by leptin through STAT3/phosphatidylinositol 3-kinase (PI3K) /mTOR pathways to increase IFN- γ production. The ability of leptin to control Tfh and Th1 function as well as the association of low leptin levels with poor vaccine responses highlights the importance of metabolism in the immune response and, therefore, is an area of specific interest to this project (Deng et al., 2021) (Maurya et al., 2018).

1.9.30 The Importance of Targeting Immunometabolism Therapeutically

For many years, the metabolism of cells during an immune response was overlooked and regarded only as a core cellular function until the seminal work by Erika Pearce and others, which unravelled the importance of metabolism on immune cell function and hence gave rise to the field coined Immunometabolism (Pearce et al., 2009). However, well before these findings, Borregaard and Herlin documented the role of energy metabolism in neutrophils during phagocytosis (Borregaard and Herlin, 1982).

Several therapeutics, including Dimethyl fumarate (DMF), Methotrexate, Rapamycin, and Metformin, are used to suppress inflammation. It is now clear that one of their mechanisms of action is to modulate the metabolism of immune system cells, resulting in anti-inflammatory effects. This is because when immune cells get activated, they induce a metabolic switch that favours particular effector responses. For example, pro-inflammatory cells mainly favour aerobic glycolysis, similar to that observed in the Warburg effect in cancer, whilst anti-inflammatory cells rely more on oxidative phosphorylation (OXPHOS) during their responses. The Warburg effect is when cancer cells preferentially use glycolysis, even in the presence of oxygen, rather than OXPHOS (Vander Heiden et al., 2009). The modulation, therefore, of immune cell function through metabolic manipulation may be therapeutically advantageous and could be more specific than generalised systemic immunosuppression (Palsson-McDermott and O'Neill, 2020).

Metabolic manipulation of immune cells can drive reprogramming to elicit a different functional phenotype, such as in the treatment of Multiple Sclerosis, where DMF can

convert pro-inflammatory macrophages and T cells into anti-inflammatory phenotypes (Gross et al., 2016, Bomprezzi, 2015). Likewise, Rapamycin can convert pro-inflammatory Th17 cells to Treg cells (Kopf et al., 2007). Although metabolic inhibitors have been shown to achieve broad immune suppression, it has not been fully defined whether specific effector functions can be manipulated metabolically in Tfh cells.

The Role of Metabolism in CD4+ T cells

1.9.31 Overview of Key Pathways in T Cell Immunometabolism

Cellular metabolism is critical for the functioning of all cells, as it enables the generation of energy through catabolic reactions that break down complex organic compounds into key metabolites through a series of enzyme-mediated reactions, which are required for all cellular processes (Palsson-McDermott and O'Neill, 2020). Reflecting this, multiple pathways are upregulated when a T cell becomes activated through TCR stimulation, such as glycolysis, lipid, and amino acid metabolism. The integration and precise metabolites of these pathways activated can dictate the flavour of the T cell generated, while also being critical for its effector function (Geltink et al., 2018)

Glycolysis is the process in which glucose is converted to pyruvate in the cytoplasm of the cell. Pyruvate can then progress into anaerobic respiration to form lactate, which is released by the cell. Pyruvate can also be converted to acetyl-CoA and be shuffled into the tricarboxylic acid (TCA)/Citric Acid cycle inside the mitochondria. Once inside the TCA cycle, the acetyl-CoA is broken down and then reformed. During this process, NADH and FADH₂ are reduced from their oxidised forms, and acts as electron donors for the Electron Transport Chain (ETC) used to drive OXPHOS. These electron donors are then used to generate large quantities of ATP (~32 per glucose molecule)(Liu et al., 2023a). However, acetyl-CoA can also be exported back into the cytoplasm as acetyl-CoA or as citrate to create lipids through fatty acid synthesis (FAS) (Figure 1.9).

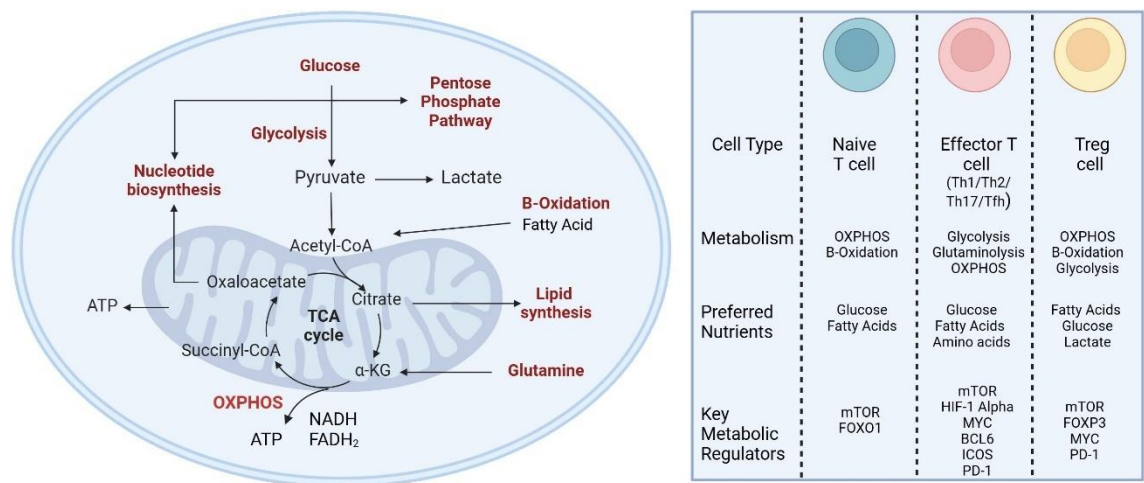


Figure 1.9. T Cell Metabolism

This diagram provides a simplistic overview of the key pathways involved in T cell metabolism and summarises the similarities and differences between distinct T cell states and lineages. Created in BioRender and adapted from (Yang et al., 2022)

Both glycolysis, FAS and OXPHOS have benefits to T cells (ATP generation, nucleotide and amino acid assembly, ribosome activity, etc); however, in activated T cells, it is widely considered that glycolysis and FAS are favoured over OXPHOS, which requires high oxygen consumption and takes longer to generate ATP than glycolysis. This shift also facilitates FAS, as T cells favour biosynthetic pathways over efficient energy production (Lochner et al., 2015).

OXPHOS can generate large amounts of ATP and is particularly important for long-term survival, which is required for naïve and memory T cell populations. In contrast, glycolysis and FAS benefit activated, proliferating T cells. FAS has an important role in activation, as it allows the biosynthesis of new cellular membranes, supports cytokine production and generates less ROS than OXPHOS. Furthermore, intermediates from FAS can drive epigenetic remodelling and post-translational modifications that support the induction of TFs essential for effector cell function (Ma et al., 2024, Zhou et al., 2023).

FAS can be fueled by sequestering lipids from external and internal sources or by using acetyl-CoA to build lipids. However, like everything in metabolism, the control of lipids is also critical, as lipid peroxidation and/or accumulation can also be detrimental to a cell. Lipid peroxidation occurs when free radicals like ROS attack lipids and can drive ferroptosis, a form of cell death. In contrast, lipid accumulation occurs when cells store lipid as neutral lipid droplets (Cai et al., 2021).

Lipid peroxidation and accumulation can be controlled through β -oxidation pathways, which break down lipids into acetyl-CoA and α -ketoglutarate, which can be used by the TCA cycle. A single molecule of palmitate can yield 106 ATP in comparison to roughly 32 ATP created through 1 molecule of glucose, during the β -oxidation of palmitate and the OXPHOS of glucose (Cai et al., 2021, Lochner et al., 2015).

Glutamine serves as a key metabolic substrate that fuels the TCA cycle and supports OXPHOS, contributing to cellular energy requirements. Beyond its role in bioenergetics, glutamine-derived intermediates support various anabolic pathways, facilitating the biosynthesis of essential macromolecules, including nucleotides, non-essential amino acids, cholesterol, and other effector lipid species. These processes sustain growth and division by synthesising cell membranes and key lipid mediators. Moreover, metabolite derivatives of glutamine can influence cell signalling and function by facilitating post-translational modifications (PTMs) of proteins, like glycosylation, methylation, and acetylation, which are essential for regulating protein activity, localisation, and interaction networks (Yang et al., 2022).

During steady-state, naïve T cells have low metabolic requirements and primarily use OXPHOS to generate energy. This process is fueled through glucose uptake and β -Oxidation. The main drivers of naïve T cell metabolism are tonic TCR-stimulation and IL-7 produced by APC and stromal cells. These two requirements converge to regulate metabolism, particularly through the upregulation of glucose and mitochondrial metabolism, and prevent apoptosis (Yang et al., 2022).

Glucose metabolism in effector cells is significantly higher in effector than in naïve T cells. The key glucose transporter GLUT1 facilitates glucose uptake, and a deficiency can result in decreased effector cell generation (Th1, Th2, Th17, and Tfh) (Wik and Skalhegg, 2022). Additionally, TCR stimulation upregulates other key transporters involved in the transport of lipids and amino acids (Almeida et al., 2016). The major signals required for the effector metabolic shift from OXPHOS to glycolysis are provided by the PI3K/AKT/mTOR pathway.

During T cell activation, the PI3K/Akt/mTOR signalling pathway is rapidly activated downstream of key receptor and costimulatory signals. The main upstream signals involve TCR activation and costimulatory signalling via CD28, both recruiting and activating PI3K. The activated PI3K catalyses the formation of downstream effectors like phosphatidylinositol (3,4,5)-trisphosphate (PIP₃), which drives recruitment of Akt and its activator PDK1. Furthermore, Akt phosphorylation drives downstream activation of mTOR

complex 1 (mTORC1), a central metabolic regulator that promotes nutrient uptake, glycolysis, lipid synthesis, and protein translation in favour of T cell growth and proliferation. Additional cytokine and co-stimulatory signals can also boost this response for examples through the common γ -chain family of cytokines (e.g., IL-2, IL-7), which further sustains Akt and mTOR activation via JAK–STAT–PI3K cross-talk. Collectively, these upstream cues activate the PI3K–Akt–mTOR axis to direct the increased metabolic and biosynthetic requirements of activated T cells. Fine-tuning is achieved through the differential expression of key receptors/TFs upregulated in each distinct population of CD4⁺ T cells. Increasing evidence suggests that each CD4⁺ T cell subset has a different metabolic requirement, and this will be explored in the subsequent sections (Yang et al., 2022).

1.9.32 Tfh cell Immunometabolism

To date, characterisation of Tfh immunometabolism remains incomplete. Nevertheless, previous work has emphasised the importance of the mTOR complex in Tfh cells, as demonstrated by the impaired Tfh function, GC formation and IgA production following RAPTOR and RICTOR deletion, towards foreign antigen immunisation and LCMV infection in the Peyer's patches (Yang et al., 2016a). RAPTOR and RICTOR are both proteins that, along with mTOR, form complexes known as mTORC1(RAPTOR) and mTORC2 (RICTOR), and deletion results in loss of either one of these complexes.

mTORC1 is formed of mTOR, RAPTOR, mLST8 (G β L), PRAS40, and DEPTOR, which act together to control kinase activity, substrate specificity, and complex stability. mTOR complex 2 (mTORC2), is composed of mTOR, RICTOR, mLST8, SIN1, PROTOR1/2, and DEPTOR, is instrumental in driving Akt phosphorylation at Ser473 and cytoskeletal organization (Kennedy and Lamming, 2016)

In CD4⁺ T cells, RAPTOR is crucial for T cell activation, proliferation and metabolism, whilst RICTOR is involved in T cell survival, differentiation and localisation (Zeng et al., 2016). It has been suggested that mTOR activation in Tfh cells enhances glycolysis and glucose uptake (Zeng et al., 2016, Choi et al., 2018). Dong et al. (2019) provided further evidence supporting the use of glycolysis by Tfh cells during IAV PR8 infection. They highlighted that HIF1 α -mediated glycolysis and succinate-mediated OXPHOS are necessary for GC responses and Tfh cell differentiation (Dong et al., 2019). HIF-1 α has been shown to stabilise glycolytic gene expression (e.g. GLUT1, HK2, etc.) (Zhang et al.,

2025a). Interestingly, though, it has also been suggested that degradation of HIF-1 α by Von Hippel–Lindau protein (VHL) is required for proper Tfh cell development, highlighting the complex interplay between VHL–HIF-1 α –mTOR axis in fine-tuning Tfh cell generation (Huang et al., 2022)

Tfh cells have been shown to exhibit decreased levels of glycolysis and mitochondrial respiration compared to Th1 cells (Ray et al., 2015). This difference may be attributed to a balance between PD-1, which can inhibit glycolysis, and ICOS, which can induce GLUT1 activation and glucose uptake, as Tfh cells express high levels of both receptors on their surface (Patsoukis et al., 2015). Interestingly, glucose deprivation can induce Bcl-6 expression in T cells, leading to the upregulation of AMPK, which in turn drives the upregulation of Tfh markers (PD-1, CXCR5, ICOS) in CD4 T cells (Xie et al., 2017).

Inhibition of glycolysis in Tfh has been shown to decrease the expression of IL-21 in Tfh cells, whilst GLUT1 expression is required for differentiation (Mayberry et al., 2022). Recent research demonstrated that enforced tetramerisation of the glycolytic enzyme pyruvate kinase M2 (PKM2) resulted in a marked decrease in glycolytic activity and differentiation of Tfh cells (Lin et al., 2024). This effect underscores the essential role of aerobic glycolysis in supporting Tfh cell development and function. Mechanistically, PKM2 tetramerisation allows PKM2 to translocate to the nucleus and interact with Bcl-6, a master regulator of Tfh cells. Moreover, this disrupts downstream signalling through the AKT/GSK-3 β pathway, which is vital for Tfh cell survival and differentiation. These findings emphasise the dual role of PKM2 as both a metabolic enzyme and a signalling regulator in the orchestration of Tfh cell responses (Lin et al., 2024).

Interestingly, research showed that deletion of the OXPHOS regulator cytochrome c oxidase in Tfh cells did not affect their overall numbers. However, it impaired GC formation, highlighting a loss in the functional capacity of Tfh cells to support this essential aspect of humoral immunity (Tarasenko et al., 2017). Further to this, it has been shown that Tfh cells have low lactate levels when compared to other effector CD4⁺ T cell subsets, suggesting a reduced reliance on anaerobic glycolysis in lupus-prone mice (Gong et al., 2023)

The role of glutaminolysis in Tfh cells is debated. Many studies of glutamine metabolism focus on using 6-diazo-5-oxo-L-norleucine (DON) inhibitors, which block glutamine utilisation enzymes such as glutaminase and inactivate them. DON inhibition of glutamine metabolism has been shown to reduce Tfh cell differentiation and expression of Bcl-6, CD40L and ICOS (Choi et al., 2024), with Tfh cells increasing SLC1A5 (amino acid

transporter for glutamine) during activation (Ren et al., 2017). However, converse results have also been observed, such as inhibition of Glutaminase, GLS1, fails to affect Tfh differentiation or Bcl-6 induction (Zou et al., 2022). This suggests that the use of glutamine metabolism may be context-specific or compensatory to support function, rather than an absolute core requirement for lineage differentiation.

A recent publication highlighted the key differences between activated T cells and polarised Tfh cells *in vitro* from C57BL/6 mice. The main finding was that Tfh cells favoured an accumulation of the end products of anabolic pathways responsible for synthesising phosphatidylethanolamine (PtdE) and phosphatidylcholine (PtdC)—namely, cytidine diphosphate-ethanolamine and sn-glycero-3-phosphocholine. This accumulation relates to an increase in metabolic flux through these pathways. In parallel, levels of key intermediates such as glycine and serine were reduced, further supporting the idea of active utilisation within these biosynthetic routes. Fundamentally, this study also underscores the pivotal role of lipid metabolism in reinforcing T cell fate decisions. PtdE can also function as a post-transcriptional regulator, while PtdC inhibits Th17 differentiation and promotes Treg lineage commitment (Gong et al., 2023; Fu et al., 2021).

Lipid metabolism appears to be key to the function of Tfh cells. A recent study employing CRISPR-Cas9 *in vivo* screening assays highlighted that crucial enzymes associated with the cytidine diphosphate-ethanolamine pathway play a key role in controlling Tfh and humoral immunity. This pathway is a direct result of increased fatty acid synthesis. The genetic deletion of Pcty2 – a key enzyme in synthesising PtdE, a form of phospholipid, caused attenuated Tfh differentiation. PtdE is a strong post-translational regulator of Tfh differentiation and prevents Tfh CXCR5 internalisation/degradation (Fu et al., 2021). PtdE is intricately linked to central lipid synthesis, and incorrect PtdE biogenesis can result in increased SREBP-1 activation and increased TAG biosynthesis, highlighting a possible positive feedback loop present in Tfh cells to maintain CXCR5 expression (Porstmann et al., 2005). It has also been highlighted that CD90 neg/low GC Tfh cells are particularly enriched in activated lipid and steroid metabolic pathways compared to CD90 high Tfh that reside outside the GC reaction (Yeh et al., 2022).

Tfh cells appear to favour biosynthetic pathways such as fatty acid synthesis, particularly Stearoyl CoA Desaturase 1 (SCD-1). SCD-1 is a critical enzyme in lipid metabolism, whereby it catalyses the desaturation of saturated fatty acids into monounsaturated fatty acids. SCD-1 converts 1) stearoyl-CoA to oleoyl-CoA and 2) palmitoyl-CoA to

palmitoleoyl-CoA. This conversion is essential for the synthesis of various lipids such as cholesterol esters, phospholipids and triglycerides (Kalyesubula et al., 2025)

Research has shown that SCD-1 inhibition can drive endoplasmic reticulum stress and Tfh apoptosis *in vitro* and *in vivo* during IAV X31 immunisation (Son et al., 2020). This highlights that Tfh cells rely on FAS, particularly the conversion of saturated fatty acids to monounsaturated fatty acids, to maintain ER health. This ability to control lipid balance mitigates endoplasmic reticulum (ER) stress, which in turn prevents apoptosis.

Interestingly, though, FASN inhibition, important in synthesising long-chain fatty acids, did not affect Tfh differentiation. It was also shown that the balance of Tfh to Tfr cells was increased following SCD inhibition as the relative population of Tfh cells decreased, whilst Tfr did not. The study did not address whether this was, in fact, a conversion from Tfh to Tfr cells. Interestingly, this study also showed a decrease in Tfh cell magnitude following statin treatment, an HMG-CoA Reductase inhibitor that is important for cholesterol synthesis; however, the authors did not investigate this further (Son et al., 2020).

The role of cholesterol in Tfh cells has been explored. Findings from Li et al. (2016) showed that cholesterol plays a significant role in the ability of Tfh cells to function and differentiate. The oxysterol, 7- α , 24-hydroxy cholesterol, facilitates the migration of Tfh cells to the B cell zone by regulating the receptor EBI2 (Li et al., 2016). It has also been highlighted that cholesterol biosynthesis was decreased in Raptor-deficient T cells, highlighting how mTOR can dictate certain metabolic requirements of Tfh cells (Zeng et al., 2016).

The role of lipid metabolism in Tfh cells is fundamental to their ability to control oxidative stress that may stem from the choice of metabolic pathway Tfh cells employ, such as glycolysis and the TCA cycle, which produce large amounts of reactive oxygen species (ROS). The correct breakdown of lipids prevents ROS-induced lipid peroxidation, which otherwise could lead to cell death. Moreover, extended Tfh-GC B cell interactions result in ferroptosis, which is an iron-dependent form of apoptosis resulting from lipid peroxidation, caused by excessive production of ROS linked with TCR, CD28 and PD-1 stimulation (Yao et al., 2021). The role of iron in Tfh is also defined, as it has been shown that iron overload promotes Tfh cell expansion, cytokine secretion, and autoantibody production in lupus-prone mice (Gao et al., 2022), whilst labile iron accumulation in CD4⁺ T cells has been shown to promote Tfh cell differentiation through Bcl-6 demethylation (Scindia et al., 2022).

Furthermore, other studies have highlighted the upregulation of the pentose phosphate pathways in Tfh cells (Huang et al., 2021b), which are critical for generating NADPH, a key reducing agent involved in antioxidant defence. Moreover, increased levels of itaconate and 2-hydroxyglutarate have been reported in Tfh in lupus-prone mice (Gong et al., 2023); these metabolites have been defined as having key roles in modulating immune responses and redox homeostasis. The upregulation of these pathways and metabolites suggests a coordinated cellular response aimed at preventing oxidative stress, thereby enhancing antioxidant capacity and maintaining redox homeostasis under inflammatory or stress-related conditions (Mayberry et al., 2022)

In summary, the consensus is that Tfh cells rely on mTOR signalling, which facilitates their active glycolytic, iron and lipid metabolic phenotypes. Tfh cells have also been shown to favour iron and other forms of metabolism depending on the context from which they are derived; however, there remain many unanswered questions about Tfh cell immunometabolism. To date, we are still unaware if Tfh cells' metabolism is similar or different in type 1 or type 2 immune responses and whether this differs between anatomical locations or pathogenic challenges. Further to this, understanding how murine Tfh immunometabolism relates to human Tfh cells is also understudied, and whether the translation of findings between species is relevant and could be used to drive therapeutic discovery to target Tfh cells through their metabolism.

1.9.33 Th1 Immunometabolism

It has been shown that Tfh cells do not possess the same metabolic requirements as effector CD4⁺ cells. Th1 cells are more glycolytic than Tfh cells (Ray et al., 2015). Th1 cells require glycolysis to function; this is most likely due to the migration requirements of Th1 cells, which need to migrate to the tissue rather than Tfh cells, which migrate to the border of the T/B follicles. This increase in glycolysis may also be a result of Tbet, which is pro-glycolytic, whilst Bcl-6 in Tfh cells is repressive of this metabolic pathway (Oestreich et al., 2014).

When Th1 cells are cultured in galactose-containing media, without glucose, this forces reliance on oxidative phosphorylation by limiting glycolysis. The resulting Th1 cells exhibit significantly reduced expression of the lineage-defining TF, T-bet (Wik and Skalphegg, 2022) and the effector cytokine IFN- γ (Chang et al., 2013). This impairment can be partially rescued through supplementation with mannose, highlighting that

glycolysis-derived metabolites are important for optimal Th1 differentiation and function (Zygmunt et al., 2018).

Th1 cells also require glutaminolysis, and this metabolic pathway has been shown to control the balance between Th1 and Treg cells, as glutamine restriction decreases α -ketoglutarate, promoting Treg differentiation and hindering that of Th1 cells (Yang et al., 2022). It is well known that mTOR signalling promotes Th1 differentiation, as mTORC1 phosphorylates T-box, which allows for the expression of Th1 master TF, Tbet (Chornoguz et al., 2017) (Figure 1.9). Inhibition of mTOR can also decrease the ability of Th1 cells to produce IFN- γ (Geltink et al., 2018).

In summary, effector Th1 cells favour glycolysis and glutaminolysis to drive rapid proliferation and IFN- γ production. Active mTOR signalling is also required to induce the main TF Tbet to be expressed in Th1 cells, showing the close link between metabolism and the desired Th1 phenotype.

1.9.34 Th2 Immunometabolism

Th2 cells have higher glycolytic demands than other Th cell subsets due to their heavy reliance on mTORC2 signalling (Waickman and Powell, 2012). This increased glycolytic demand created by mTORC2 in Th2 is largely due to the regulatory role of mTORC2 in driving increased c-MYC expression, a key TF which controls expression of glycolysis-related genes (Masui et al., 2014). However, the expression of the glucose transporter, Glut1, in Th2 cells shows comparable levels with that of Tfh cells (Ray et al., 2015, Heieis, 2019). The small GTPase RHOA controls and drives the glycolysis required for Th2 differentiation and the propagation of allergic airway inflammation, highlighting the key role of glycolysis in Th2-mediated immunity (Yang et al., 2016b).

It has been widely considered that high glycolysis and low fatty acid oxidation are the preferred choice of this Th2 subset during activation (Patel and Powell, 2017); however, the role of lipid metabolism is now being explored due to the role of PPAR- γ , which is critical in FAS later in Th2 cell differentiation. It has been suggested that PPAR- γ is important in the expression of ST2, which is key to Th2 cell function during infection, particularly in the tissue (Stark et al., 2019, Heieis, 2019).

FAS pathways are critical for Th2 cell differentiation and require both endogenous and exogenous lipid/acetyl-CoA generation (Kabat et al., 2023). Interestingly, citrate from the

TCA cycle supports FAS predominantly by driving activation of mevalonate pathways for cholesterol and sterol synthesis. Cholesterol in Th2 cells has been shown to be important in their effector functions, as Th2 cells express Cyp11a1, which is responsible for the conversion of cholesterol to pregnenolone, which is secreted by Th2 cells and is shown to drive restoration of immune homeostasis through inhibiting T helper cell differentiation and class-switching in B cells (Mahata et al., 2014). This highlights a possible regulatory mechanism of Th2 cells to prevent tissue damage through dampening excessive immune responses in settings such as allergy and helminth infection, where pregnenolone has been shown to be secreted (Mahata et al., 2014)

Furthermore, the uptake of exogenous amino acids and nutrients has been shown to affect Th2 cell differentiation and function. It has been demonstrated that glutamine metabolism is essential for IL-4 production by Th2 cells, but not for the expression of GATA3 (Klysz et al., 2015). Interestingly, although unlike in Tfh cells, iron inhibits the activation and differentiation of Th2 cells, possibly highlighting a novel difference in these cell types (Ni et al., 2022)

Interestingly, it has been reported that there is a context-dependent metabolic switch that arises as Th2 cells migrate from reactive LNs to peripheral tissues during infection. Our lab has proposed a mechanism involving the PD-1/ST2 axis, in which PD-1 signalling drives fatty acid oxidation (FAO), which subsequently enhances expression of the IL-33 receptor (ST2) in Th2 cells. In addition, the findings from this study suggest that Th2 cells favour OXPHOS over glycolysis. However, rather than challenging the established link between glycolysis and cytokine production in Th2 cells as previously defined, these observations may instead reflect location-specific metabolic reprogramming between different microenvironments, highlighting a metabolic plasticity within the Th2 lineage (Heicis, 2019).

Overall, Th2 cells are highly glycolytic cells inside the LN that favour upregulation of pathways such as FAS to drive cholesterol biosynthesis to support proliferation and cytokine production. This cholesterol pathway upregulation is linked to the production of pregnenolone to exert immune regulation to prevent excessive responses from being initiated. Interestingly, though, it is reported that Th2 cells undergo a metabolic switch from leaving the lymph node to entering the affected tissue, whereby they favour FAO and OXPHOS pathways to support their function, highlighting the plasticity in the immunometabolism of Th2 cells.

1.9.35 Other T cell Subset Immunometabolism

Glycolysis and glucose transport via GLUT1 are higher in Th1, Th2 and Th17 cells than in Tregs (Patel and Powell, 2017). Tregs do not favour the excessive use of glycolysis, or uptake of glucose by Glut1, as inhibition of Glut1 uptake through AMPK activation in CD4⁺ T cells results in increased FAO and the expression of the master TF for Tregs, Foxp3. Tregs favour FAO pathways to fuel their function (Michalek et al., 2011). This difference in favoured metabolism is likely due to the nature of Treg biology, whereby they prefer to proliferate less intensely and have decreased cytokine production (Zeng and Chi, 2015) more akin to the metabolism of a naïve T cell. However, this is not to say that glycolysis is not a critical requirement for Tregs, as they engage this form of metabolism during activation, migration, and proliferation; however, they shift away from glycolysis as they develop to utilise other bioenergetic pathways like OXPHOS and FAO (Tomaszewicz et al., 2023).

Several studies have shown the importance of FAO in Treg cells (Kurniawan et al., 2020). However, genetic deletion of CPT1A, an enzyme essential in FAO, showed no effect on the development or function of murine Tregs *in vivo* (Raud et al., 2018). It should be noted that not all FAO require CPT1A; short and medium-chain FAO use CPT1A-independent pathways (Field et al., 2020). Unlike FAO, OXPHOS is critical in Treg function, as deletion of the Rieske iron-sulfur protein, a component of the electron transport chain complex III, specifically in Tregs, drives loss of function and causes autoimmunity in mice (Weinberg et al., 2019).

Interestingly, in naïve T cells, mTOR signalling is a negative regulator of *de novo* FOXP3 expression (Delgoffe et al., 2009). It has been shown that a lack of mTOR signalling can cause naïve T cells to differentiate into Treg cells (Kurniawan et al., 2020); however, recent reports have highlighted the role of mTORC1 as being crucial for Treg function, as *in vivo* deletion of RAPTOR leads to the development of a fatal inflammatory disorder in mice, due to loss of suppressive capacity by Treg (Chapman et al., 2018). Overall, this highlights the very different nature of Treg immunometabolism compared to effector CD4⁺ Th cells, which require active mTOR signalling for their differentiation and function. In contrast, in Tregs requirements for mTOR signalling appear more complex (Zeng and Chi, 2015).

Conversely, Th17 cells rely heavily on glucose and aerobic metabolism but also require activation of Fatty acid and cholesterol biosynthesis pathways. Cholesterol biosynthesis is particularly important as the Th17 lineage dependent TF, ROR γ t, can use sterol ligands to

perform effector functions. They also utilise key amino acids such as glutamine as a source of energy (Wang et al., 2022). Similarly to Th17 cells, Th9 lymphocytes also depend heavily on fatty acid metabolism (Nakajima et al., 2024) and also glucose metabolism, through the PPAR- γ -mTOR1-IL-9 pathway (Peesari and McAleer, 2024).

Overall, this introduction highlights to us that understanding the immunometabolism of T cells can be complex due to the nature of T cells' plasticity and adaptability to their surroundings. It could be suggested that the microenvironment that surrounds a T cell dictates its immunometabolic phenotype. This is where this thesis hopes to address the unknowns about Tfh cell metabolism and ask the question: Is Tfh cell metabolism indistinguishable between infections/anatomical location, or does the microenvironment in which a Tfh cell is derived drive a distinct metabolic signature?

1.10 Hypothesis and Aims

My PhD was funded by the industrial partnership PhD programme between the University of Glasgow MVLS and Sitryx Therapeutics Ltd., who, as a company, are interested in the ability to manipulate inflammation through targeting the immunometabolism to create therapeutics. This ability to change effector function interested us, as currently, there are no specific inhibitors against Tfh cells in the clinic; even though Tfh cells have been implicated in numerous allergic and autoimmune diseases, as the key mediators in the pathologies (Cicalese et al., 2020). Ultimately, I wanted to see if I could identify key pathways involved in the immunometabolism of murine Tfh cells that could be translated into human Tfh cells, in the hope of identifying areas of interest for therapeutic intervention (Yan et al., 2017).

To date, Tfh metabolism is still not fully understood, and we do not have any indication of whether Tfh metabolism is regulated differently in the context of contrasting types of inflammation, such as in type 1 or type 2 immunity. To address this knowledge gap (Tfh in Th1 and Th2 immunity), we used two well-characterised murine models of *H. polygyrus* and IAV infection to understand the similarities and differences in Tfh cell immunometabolism between these two distinct immune responses. This thesis aims to provide the first information on whether Tfh cells will be metabolically similar or different in different contexts.

Understanding these distinctions in metabolism may enable novel areas of future research aimed at therapeutically manipulating the metabolism underlying these responses to

facilitate more favourable outcomes in infection and immune-mediated disease. I expect the immunometabolism of Tfh cells to be distinct, as it was recently shown that Tfh cells from type 1 and type 2 immune responses have distinct transcriptional repertoires (Zaini et al., 2023), that may facilitate alternative metabolic signatures.

The main objectives and hypotheses are as follows;

Specifically, it was hypothesised that **Tfh cells in different immune contexts would use different metabolic pathways and that these immunometabolic signatures would be distinct from other effector T cells.**

To test this hypothesis, 3 complementary objectives were addressed:

1. To characterise the immunometabolism of Tfh cells, compared to naïve and effector T cells, using a murine infection with the helminth *H. polygyrus* as a driver of Tfh expansion.
2. To assess the influence of immune context on Tfh metabolism, by comparing the metabolic signatures of Tfh cells in *H. polygyrus* infection (Th2 context) to those occurring during infection with IAV (Th1 context).
3. To test the therapeutic potential of manipulating metabolic pathways in Tfh cells, using a human *in vitro* Tfh cell culture assay.

Together, these objectives enabled me to understand Tfh metabolism whilst addressing the real-world application of that knowledge. The findings from these aims will allow me to highlight whether manipulation of Tfh cell metabolism can facilitate functional alteration in Tfh cell biology that could be used therapeutically to benefit patients' needs.

Materials and Methods

2.1 Murine Materials and Methods

2.1.1 Animal Models

All animals used in this investigation were cared for in compliance with local ethical and UK Home Office License Regulations, and non-adverse handling methods were used to minimise animal stress. The mice were housed and maintained under standard housing conditions at the University of Glasgow. Infection protocols are compliant with Home Office and University of Glasgow guidelines and were carried out under project licenses held by either Rick Maizels (*H. polygyrus*, PPL number PP4096415) or Gerard Graham (IAV, PPL number PP6655603)

Female C57BL/6J mice (8 weeks old) were purchased from Envigo (Huntingdon, UK) and kept at the Wolfson Research Facility (WRF) in individually ventilated cages with unlimited access to food, water, and environmental enrichment. The animals were randomly assigned to each experimental group and had their ears notched for identification purposes. Upon arrival in the animal unit, all mice were acclimatised for 1 week before use.

Mice were euthanised using cervical dislocation or, where the mediastinal LN was required, they were given a lethal intraperitoneal injection of Dolethal (200 mg/ml) (Covertus). Death was confirmed by femoral artery incision.

Prof. Judi Allen (University of Manchester) kindly provided B6-4get mice on a C57BL/6J background. These mice were bred in-house, and female age-matched mice, approximately 8-10 weeks old, were used in experiments. The WRF staff cared for the colony, and Georgia Perona-Wright and Jack Jones oversaw colony management.

B6-4get mice were first developed by (Mohrs et al., 2001). These animals are knock-in mice designed using a viral IRES (internal ribosome entry site) containing enhanced green fluorescent protein inserted between the translational stop codon and the 3' UTR of the IL-4 gene. This allows for the expression of eGFP when IL-4 is transcribed (Figure 2.1). The addition of EGFP to IL-4 does not affect the activity or expression of the cytokine.

Essentially, the B6-4get mice are genetically modified so that when the IL-4 gene is transcribed, the IRES allows for the production of both IL-4 and eGFP from the same

mRNA, but as separate proteins. The eGFP acts as a marker to show when IL-4 is being made. Homozygous B6-4GET mice are viable, normal in size and weight compared to C57BL/6J counterparts, fertile and do not display physical or behavioural abnormalities.

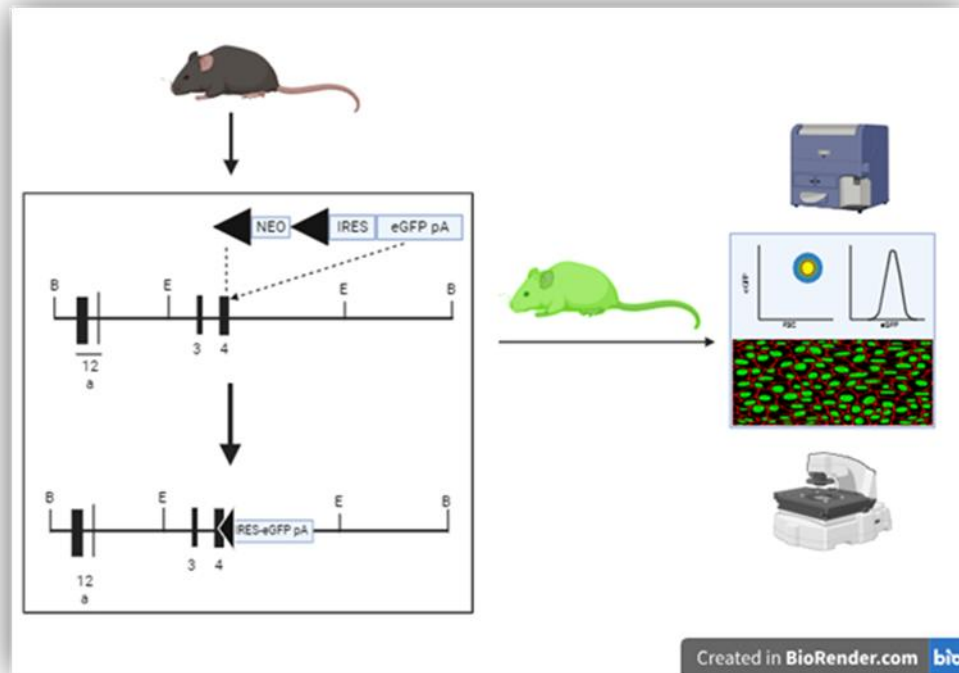


Figure 2.1. Overview of the Development of B6-4GET Mice.

The B6-4get mice were developed using a vector containing a loxP-flanked Neomycin resistance cassette, with an IRES (internal ribosome entry site), EGFP gene, and a Bovine growth hormone polyadenylation signal. Electroporation allowed insertion of the target vector into 129S4/SvJae-derived PrmCre embryonic stem (ES) cells between the endogenous translation stop codon and IL-4 exon 4 at the 3'UTR, and resulting mice were bred onto the C57BL/6 background. Image created by the author, adapted from (Mohrs et al., 2001), in BioRender.

2.1.2 *H. polygyrus* Infection

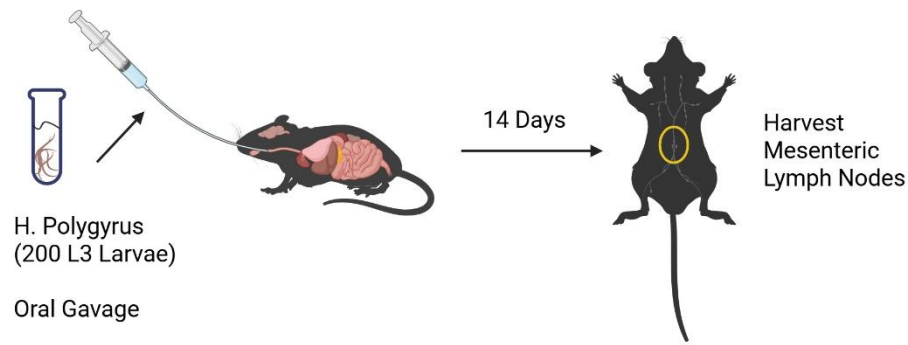
H. polygyrus (also known as *H. polygyrus bakeri* or *H. bakeri*) was maintained as described (Johnston et al., 2015). The life cycle for *H. polygyrus* was maintained by Claire Ciance and Anne-Marie Donnachie (Maizels Lab, University of Glasgow), and L3 larvae were provided for each experiment. Larvae were counted and resuspended to a concentration of 1 larva/ μ l under sterile conditions. Mice were then orally gavaged with 200 L3 Larvae in 200 μ l of sterile water. Oral gavages were performed by trained animal unit staff to prevent animal discomfort using a round-ball stainless steel-tipped 24-G

gavage needle (Figure 2.2). Mice were weighed at day 0 (prior to infection), day 7 and day 14 time points to monitor health status.

2.1.3 IAV Infections

IAV(A/Puerto Rico/8/1934 (H1N1), known as PR8) was propagated through infection of Madine-Darby Canine Kidney (MDCK) cells, utilising a stock reservoir generously donated to us by Dr. Edward Hutchinson (MRC-University of Glasgow Centre for Virus Research). MDCK (T75) cells at 80% confluence were infected with IAV at an MOI of 0.001 PFU per cell. The cells were incubated at 37 °C and 5% CO₂ for 48-72 hours, until the cell attachment reached approximately 10%. The cell supernatant was then pelleted at 1500 rpm for 5 minutes. IAV aliquots were then prepared (250 µl volume) and stored at -80 °C until required. IAV infection of mice was achieved by delivering 100-110 PFU of IAV intranasally under brief general anaesthesia induced by isoflurane, administered by Dr Marieke Pignen (Figure 2.2). Mice were monitored daily from day 3 of infection, including measurement of weight, and a supplemented diet was provided if a 10% weight loss threshold was reached. The supplemented diet consisted of a soft diet (standard food pellets soaked in distilled H₂O) or supermarket infant food (Item code: 7858501, Sainsbury's).

H. polygyrus Infection



Influenza A (IAV PR8 (H1N1)) Infection

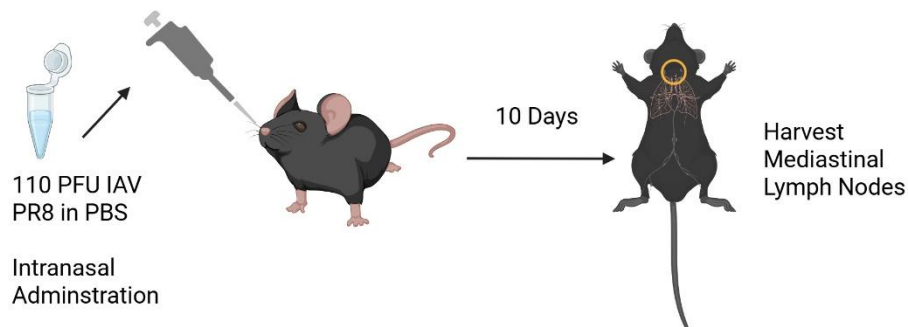


Figure 2.2. Overview of Infection Models for both *H. polygyrus* and IAV.

C57BL/6 or B6.4get mice were infected with *H. polygyrus* or IAV (PR8), and the reactive draining LNs were removed during the peak T cell response. The author created the diagram in BioRender.

2.1.4 Flow Cytometry

Lymphocytes were collected from the mesenteric or mediastinal LNs of experimental mice, and LNs were processed through a 70- μ m filter to generate a single-cell suspension. Cells were then resuspended in a known volume and counted to distinguish live/dead cells, using a haemocytometer. After the concentration of cells was determined, the equivalent 2×10^6 cells live were aliquoted into FAC tubes to allow for further staining. The cells were washed once in PBS to remove protein contamination before staining with Fixable Viability Dye eFluor 506 (EBioscience) and Fc block (Anti-mouse CD16/32 Antibody; Clone 93 Biolegend). This fixable dye allowed for the future determination of live and dead cells, while the Fc block prevented non-specific antibody binding. The eFluor 506 dye was diluted in PBS, and cells were stained for 20 minutes. Cells were then washed and surface marker staining was performed in FACS Buffer (PBS with 2% FBS (pH) and 2 mM EDTA (Ultrapure 0.5M EDTA Cat. 15575020, Invitrogen) or 45 min at 4 °C in the dark, using the following surface antibodies (Figure 2.3):

Marker	Fluorochrome	Clone	Dilution	Manufacturer
CD3	FITC	17A2	1/200	Biolegend
CD3	PE	17A2	1/200	Biolegend
CD4	APC	RM4-5	1/200	Biolegend
CD4	BUV395	RM4-5	1/100	Biolegend
CD44	APC-CY7	IM7	1/200	Biolegend
CD44	PE-CY7	IM7	1/200	Biolegend
TCR- β	BUV395	H57-597	1/100	Biolegend
TCR- β	Percp5.5	H57-597	1/100	Biolegend
TCR- β	BV421	H57-597	1/100	Biolegend
CXCR5	BV605	L138D7	1/100	Biolegend
CXCR5	PE	L138D7	1/100	Biolegend
PD-1	BV711	29F.1A12	1/100	Biolegend
PD-1	BV785	29F.1A12	1/100	Biolegend
GATA3	APC	TWAI	1/50	eBioscience
GATA3	PE-CY7	TWAI	1/50	eBioscience
FOXP3	eFLUOR 450	FJK-16s	1/100	Invitrogen

Figure 2.3. Table of Antibodies used for Cell Surface and Intracellular Flow Cytometry

Cells were fixed and permeabilised for intracellular staining using the eBioscience FoxP3 TF staining Buffer Set (Cat. No. 00-5523-00) according to the manufacturer's protocol. Following fixation and permeabilisation, the cells were washed and then stained in 1x permeabilisation buffer. Staining was for 1 hour at 4 °C. Cells were then washed with FACS Buffer and resuspended in 300 μ l of FACS Buffer for subsequent acquisition of fluorescence data using the BD LSR Fortessa running FACS DIVA Software (BD Biosciences). All analyses were performed using FLOWJO software (Becton Dickinson, v10.10.0).

All antibodies were used at previously described optimal concentrations, as per our lab's operating protocols or following titration, as shown below (Figure 2.4). Antibodies were selected at a concentration of saturation, where the signal was optimal and did not increase with further increases in antibody concentration.

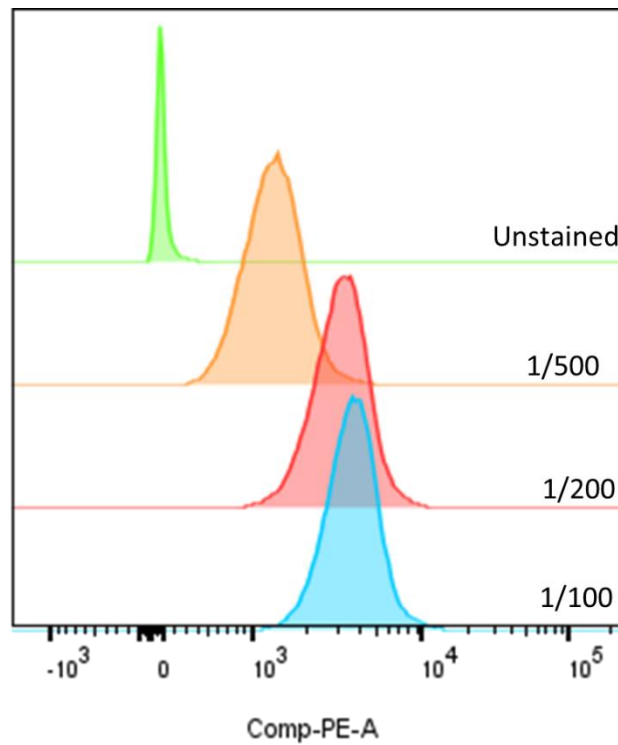


Figure 2.4. Titration of Anti-FAS antibody on PE (Abcam – EPR7465 -Ab 223964).

This diagram was created using Flowjo V10.10.0 and shows the titration of a METFLOW PE-conjugated anti-fatty-acid synthase antibody (Abcam ERP7465) on CD4⁺ T cells isolated from the mLN of an *H. polygyrus* Day 14-infected mouse.

2.1.5 METFLOW

METFLOW is a flow cytometry-based assay used to evaluate the metabolism of immune cells at single-cell resolution. The method utilises antibodies against various metabolic proteins and rate-limiting enzymes in distinct branches of the metabolic pathways to assess changes in their expression and hence their potential impact on cellular metabolism. Following fixation and permeabilisation as described above (Chapter 2.1.4), cells were subsequently stained using intracellular antibodies previously described by (Ahl et al., 2020). The following antibodies were used (Figure 2.5):

Marker	Protein	Fluorochrome	Clone	Dilution	Manufacturer
Glut1	Glucose Transporter type 1	AF594	ERP3915	1/100	Abcam
ACC	Acetyl-CoA Carboxylase Alpha	AF488	ERP4971	1/100	Abcam
HK2	Hexokinase 2	AF647	ERP20839	1/100	Abcam
CPT1A	Carnitine Palmitoyltransferase 1A	AF488	8F6AE9	1/100	Abcam
ACADM	Medium-chain specific (C4 to C12 straight chain) acyl-Coenzyme A Dehydrogenase	AF647	EPR3708	1/100	Abcam
PKM	Pyruvate Kinase M1/M2	APC	ERP10138	1/100	Abcam
G6PD	Glucose-6-phosphate Dehydrogenase	*PerCP 5.5	ERP20668	1/100	Abcam
FAS	Fatty Acid Synthesis	PE	EPR7465	1/100	Abcam
ASA+FA BP1	Aspartate Aminotransferase + Fatty Acid Binding Protein 1	FITC	ERP12145	1/100	Abcam
SCD-1	Stearoyl-CoA Desaturase 1	*APC	ERP21963	1/100	Abcam
ATP CL	ATP Citrate Lyase	PE	EP7047	1/100	Abcam
IDH2	Isocitrate Dehydrogenase 2	PE	ERP7577	1/100	Abcam
CD71	Transferrin Receptor 1	BV421	RI7217	1/100	Biolegend
CD98	Solute Carrier Family 3 Member 2 or CD98	PE-Cy7	4F2,Ly10	1/100	Biolegend
KI-67	Ki-67	FITC	SolA15	1/100	EBioscience

Figure 2.5. Table of Metflow Flow Antibodies used.

Asterisk highlight antibodies that were conjugated in-house using Abcam antibody conjugation kits for PerCp5.5 (ab102911) and APC (ab201807).

2.1.6 PHOSFLOW

Phos-flow is a flow cytometry-based assay to evaluate the activation status of signalling molecules regulated by phosphorylation. The assay was highly time-sensitive due to the dynamic nature of cell signalling. To minimise changes in phosphorylation from euthanasia to cell fixation, samples were maintained at 4 °C immediately following harvest and throughout the pre-fixation steps. Cells were first stained with a viability dye (live/dead discrimination) and incubated with an Fc receptor blocking reagent to prevent non-specific antibody binding. Fixation was then performed, as highlighted in Chapter 2.1.4 above, before both cell surface and intracellular staining steps. This step was crucial for maintaining phosphorylation signals before proceeding with antibody staining.

Experimental controls were included to validate the staining. mTOR signalling was inhibited using Rapamycin at a final concentration of 100 nM, or activated using the eBioscience™ Cell Stimulation Cocktail x500 (Cat. 00-4970) (containing PMA and Ionomycin (40.5 µM and 670 µM (x500)) however final concentration 2 µL cocktail per mL (1: 500 dilution) in complete RPMI 1640 medium supplemented with 10% foetal

bovine serum (FBS) (RPMI). A volume of 100 μ l of the 1:500 diluted stimulation cocktail in RPMI + 10% FBS was added to each sample and incubated for 30 min at 37 °C. The following antibodies were used (Figure 2.6).

Marker	Fluorochrome	Clone	Dilution	Manufacturer
S6	PE	A16009C	1/100	Biolegend
PHOS-S6	BV421	A17020B	1/100	Biolegend
AKT	APC	281046	1/100	Invitrogen
PHOS-AKT	FITC	DE9	1/100	Cell Signalling

Figure 2.6: Table of Phos Flow Antibodies used

2.1.7 Lipid Uptake and Neutral Lipid Staining

Bodipy FL C16 (D3821, Invitrogen) is a green (emission maximum, 512nm and wavelength of 503nm), fluorescent Fatty Acid, a synthetic analogue of c-16 palmitic acid used to monitor fatty acid uptake. Bodipy was reconstituted in DMSO at a stock concentration of 10mM. Cells were incubated at 37 °C for 30 minutes in 200 μ l RPMI without FBS with a 1:1000 dilution of Bodipy, before undergoing the normal staining protocol previously mentioned in chapter 2.1.4.

Lipidtox Green Neutral Lipid Stain (H34475, Invitrogen) is a neutral lipid stain. Neutral lipids are hydrophobic molecules that lack charged groups. These include triacylglycerols (TAGs), diacylglycerols (DAGs), cholesteryl esters (CEs), and cholesterol (Athenstaedt and Daum, 2006). Cells were fixed and permeabilised before staining. Cells were stained with the neutral lipid stain at a 1:200 concentration in cRPMI media at 37 °C for 30 minutes, then washed twice and analysed. The stain binds with high-affinity to neutral lipid droplets.

2.1.8 Fluorescence Assisted Cell Sorting (FACS)

Before sorting, cells were stained as outlined in Chapter 2.1.4. Tfh, Th1, Th2, and naïve T cells were isolated through sorting using the FACS Aria IIU cell sorter, using an 85 μ L nozzle size into 500 μ L of RPMI. The following antibodies were used to achieve this (Figure 2.7).

Marker	Fluorochrome	Clone	Dilution	Manufacturer
PD-1	BV785	29F.1A12	1/100	Biolegend
PD-1	BV711	29F.1A12	1/100	Biolegend
CXCR5	PE	L138D7	1/100	Biolegend
CD4	APC	RM4-5	1/100	Biolegend
CD4	FITC	RM4-5	1/100	Biolegend
CD3	BV421	17A2	1/100	Biolegend
TCR-β	BV421	H57-597	1/100	Biolegend
TCR-β	Percp5.5	H57-597	1/100	Biolegend
Fixable viability Live Dead Dye	BV510	N/A	1/1000	EBioscience
CD44	APC-CY7	IM7	1/100	Biolegend
CXCR3	PE-CY7	CXCR3-173	1/100	Biolegend
FC Block	N/A	N/A	1/100	Biolegend

Figure 2.7. Table of FACs Antibodies used for Fluorescence-Activated Cell Sorting for Bulk Transcriptomics.

All antibodies were titrated to achieve optimal staining levels for each cell and minimise impurities in the sorting process.

The antibody panels all included PD-1, CXCR5, CD4, CD3/TCR-β, Fixable viability Live/Dead dye, and CD44. However, CXCR3 was used for the identification of Th1 cells, whilst the IL-4-eGFP provided by the B6.4get mice was used to identify Th2 cells. Representative staining and sorting/validation for both models can be found in Chapter 4 (Figure 4.1) (*H. polygyrus*) and Chapter 5 (Figure 5.1) (IAV).

All lymphocytes were gated as live single cells before defining their receptor expression. The following CD4⁺ T cells studied in this thesis were defined as ;

- Tfh - CD4⁺ CXCR5^{High} PD-1^{High}
- Naïve T cells - CD4⁺ CXCR5^{Low} PD-1^{Low} CD44^{low} - CXCR3^{Low}/IL-4-eGFP^{low} for transcriptomics.

CXCR3^{low} was used in C57BL/6 (IAV infection), whilst IL-4-eGFP^{low} was used for B6/4get mice (*H. polygyrus* infection).

- Th2 - CD4⁺ non-Tfh (CXCR5^{low}, PD-1^{low}), CD44^{high}, GATA3⁺/IL-4 eGFP⁺
- Tregs - CD4⁺ non-Tfh, FOXP3⁺ cells

- Th1 cells - CD4⁺ CXCR5^{Low} PD-1^{Low} CD44^{High} CXCR3^{High}.
- Th2 - CD4⁺ non-Tfh (CXCR5^{low}, PD-1^{low}), CD44^{high}, GATA3⁺ or IL4-eGFP⁺ Cells

2.1.9 RNA Isolation

RNA was extracted from sorted T cells using the Qiagen Micro Plus RNA extraction kit or the Invitrogen Picopure RNA extraction kit. RNA was eluted in 8 µl of RNase/Nuclease Free H₂O. The concentration and purity of RNA were determined using the Nanodrop 1000 or Agilent 2100 Bioanalyzer. The eluted RNA was stored as frozen pellets at -70 °C until needed.

The Nanodrop spectrophotometer was used to assess the quantity and purity of RNA. Purity was calculated based on the 260/280 and 260/230 absorbance ratios. The 260/280 ratio highlights protein contamination, with optimal values ranging from 1.8 to 2.0. The 260/230 ratio indicates the presence of contaminants, such as salts and ethanol. Values above 2.0 are considered highly pure. Ratios under this threshold suggest sample contamination.

The Agilent 2100 Bioanalyser also allows for the qualitative and quantitative analysis of RNA. The RNA integrity score (RIN) indicates the integrity/degradation of the RNA. A RIN score of 10 indicates fully intact RNA, whilst anything below 5 is degraded. The concentration is also measured, along with the 28S/18S ratio, which highlights the relative abundance of large (28S) versus small (18S) ribosomal subunits.

2.1.10 Bulk RNA-Seq

Following the isolation of RNA from the FAC-sorted T cell populations. RNA was eluted and snap-frozen using solid CO₂ pellets to prevent loss of RNA integrity. Samples were then kept frozen at -80 °C for a maximum of 24 hrs before transportation under dry ice to NOVOGENE Geonomics UK. Samples were sequenced using the Illumina High Throughput Sequencing Platform using the NOVOGENE pipeline (Figure 2.8).

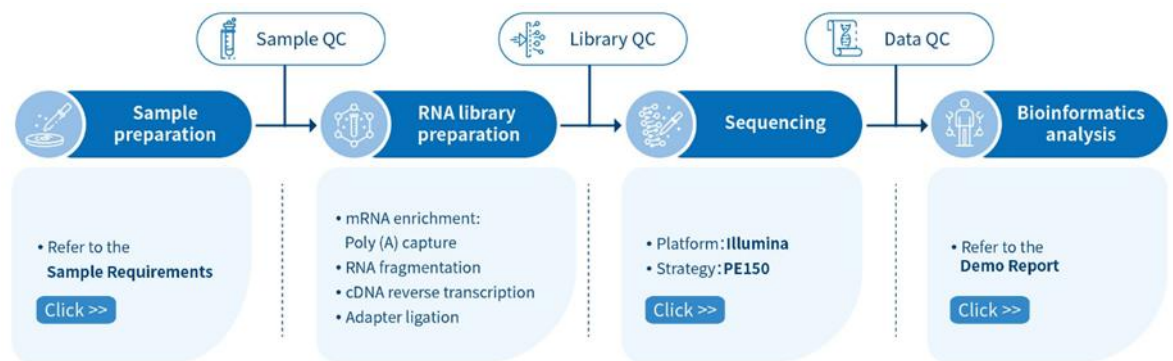


Figure 2.8: Novogene pipeline for processing of Bulk RNA samples

Data was processed through Searchlight2 (SL2) (Cole et al., 2021) by John Cole, Head of NGS & Bioinformatics at the University of Glasgow. This pipeline enables the rapid analysis and visualisation of bulk RNA-Seq data using differentially expressed gene lists created by DESeq2 for all comparisons. The data was further processed using RStudio and DAVID analysis, as explained in Chapters 4 & 5.

DAVID refers to the Database for Annotation, Visualisation and Integrated Discovery (Huang et al., 2009). This online tool enables the comprehensive analysis of large gene lists to understand the biological relevance of specific pathways or functional genetic enrichment for statistically upregulated or downregulated bulk RNA-Seq gene lists created using DESeq2. DAVID also provides pathway analysis to understand the relevance of specific gene signatures within the gene list to understand biological relevance. This tool allowed us to gain extra depth into the data that was not provided by the SL2 pipeline.

RStudio was used to analyse the data and create figures, where the data was curated into various graphs, such as box plots and volcano plots. The code is located in Appendix 1.

2.2 Human Materials and Methods

2.2.1 Human CD4⁺ T Cell Isolation and Storage

Human CD4⁺ T cells were isolated from commercially available blood cones, a byproduct from human convalescent plasma donations. This work was carried out in accordance with the 2004 Human Tissues Act and its associated codes of practice. All samples were accounted for, and confidentiality was observed at every stage of the process.

The ethical approval for the work carried out at Sitryx is as follows: Sitryx has approval from the Human Tissue Authority for the Storage of relevant material that has come from the Human body (Licence number is 12691 and the licence is granted under Section 16 (2) (e) (ii) of the Human Tissue Act 2004). Due to the nature of the work at Sitryx Therapeutics Ltd. They do not require NHS REC approval.

To isolate and cryopreserve human naïve CD4⁺ T cells, blood cones were obtained from the NHSBT and transferred to a 50 mL Falcon tube. The cone was flushed with EDTA-PBS until the majority of the blood had been washed out of the cone and into the 50 mL Falcon tube. The tube was then filled to 50 mL with PBS and carefully mixed.

Using SepMate™ tubes (2 tubes per donor), with 13 ml of Lymphoprep added, then 25ml of blood was passed through each of them vertically to allow gravity to pull the content through the tubes (two tubes were required, as the donor samples contained 50ml of blood). The blood must be placed above the Lymphoprep at this stage. The tubes were then centrifuged at 1200 x g for 20 minutes at room temperature. Following this, the top layer was poured off (mononuclear cell layer) into a new tube. The samples were then centrifuged again at 400 × g, room temperature, for 5 minutes.

The supernatant was discarded, the cell pellet (containing CD4⁺ T cells) was resuspended, and the two pellets from each sample were amalgamated. Cells were then counted and resuspended in a known volume. Aliquots were then taken for a purity check.

Isolation of CD4⁺ T cells was performed using negative selection with the EasySep™ Naive Human CD4 Isolation Kit (StemCell, catalogue #17555) in 14 mL tubes, as per the manufacturer's guidelines. The final isolated CD4⁺ T cells were centrifuged at 400g, room temperature, for 5 min, resuspended in 5 mL PBS, and counted. Again, aliquots were taken for a purity check. For freezing, cells were resuspended at 2×10^7 cells/mL in freezing media (90% foetal bovine serum [FBS]/10% dimethyl sulfoxide [DMSO]) and

frozen in 1 mL aliquots in labelled cryovials in a CoolCell at -80 °C. The paperwork was then completed on the front of the -80 °C for tracking of HTA samples temporarily stored in this location. Cryovials were stored in a -150 °C locked freezer, as per HTA guidelines.

2.2.2 Human CD4+ T Cell Activation and Differentiation

To obtain activated and differentiated cells, the frozen isolated CD4+ T cells were thawed by submersion in 37 °C water until the cell pellet had thawed. Once defrosted, cells were added to warmed cRPMI medium (10% FBS, 1% Penicillin (10,000 units/ml), 1% Streptomycin (10 mg/ml), and 1% L-glutamine (2 mM)). A 1 ml volume of medium was reserved to wash out the vials and harvest all cells. The cells were then centrifuged at 300 x g for 10 minutes. The excess RPMI was decanted, and the cell pellet was resuspended in 5 ml of warmed media. The cell count was performed using AO/PI and an Nexcelom automated cell counting system. Acridine orange (AO) and propidium iodide (PI) are nucleic acid-binding dyes that measure cell viability.

The cells were centrifuged again and resuspended in the desired volume of cRPMI medium. This was then added to 96-well plates. Tfh and Th0 cell cultures were plated at 75,000 cells per well, while Th2 cells were plated at 50,000 cells per well. The differences in plating concentrations were due to the optimisation of both assays: the Th2 assay was developed in-house by Sitryx at this concentration of cells, whilst the Tfh cell protocol was based on previously described research (Zhang et al., 2023b). Optimisation work was initially carried out by Ryan Kyle, Sitryx Therapeutic Ltd., whereby multiple combinations of Tfh-skewing factors were assessed before I chose two preferred stimulation/skewing cytokine cocktails to optimise further. Cells were stimulated using CD3/CD28 Dynabeads (Gibco) at a 1:1 bead-to-cell ratio. Differentiation into Tfh, Th2, or Th0 subsets was induced using the following cytokine cocktails:

Cytokine Supplier/Catalogue No.

- Human IL-21 - Peprotech 200-21-100uG
- Human IL-12 - Biotechne (R&D Systems) 219-IL-005/CF
- Human IL-6 - Peprotech 200-06-20uG
- Human TGF- β - Peprotech 100-21-10uG
- Purified NA/LE Rat Anti-Human IL-2 - BD Pharmingen™ 554562

- Human IL-4 – Peprotech 200-04 100ug
- Human IL-2 – Peprotech 200-02 500ug
- Anti-Human-IFN- γ – Biotechne (R&D Systems) MAB285

Cytokine Skewing cocktail

- **Tfh:** IL-21 (20 ng/mL), IL-12 (5 ng/mL), IL-6 (20 ng/mL), TGF- β (1 ng/mL), and anti-IL-2 (180 ng/mL)
- **Th2:** IL-4 (50 ng/mL), IL-2 (5 ng/mL), and anti-IFN- γ (5 μ g/mL)
- **Th0:** CD3/CD28 beads only, with cRPMI added to correspond to volumes across conditions.

All the cytokines used were human recombinant cytokines, whilst the antibodies were either mouse or rat anti-human antibodies. The cells were cultured at 37°C for three days to facilitate complete activation and differentiation. All procedures were conducted using sterile techniques within a tissue culture hood.

Following the three-day culture, the cells were subjected to centrifugation (400 xg for 5 minutes) and subsequently washed twice with 200 μ L of FACS buffer to eliminate any residual RPMI or stimulation mix. The cells were then stained with Live/Dead Near Infra-red (L/D NIR) reactive dye (Catalogue number L34976A - Invitrogen) in PBS containing FC block (Biolegend Trustain FCx: Catalogue No. 422302) for 30 minutes in the dark. After staining, the cells underwent centrifugation at 400g and were washed once more to remove any remaining stain. Thereafter, the cell surface antibody staining was performed at 4 °C for 60 minutes in the dark.

Following staining, the cells were washed and fixed using the FOXP3 Fixation/Permeabilisation Kit (eBioscience) according to the manufacturer's protocol. Post-fixation, intracellular staining was conducted in permeabilisation buffer and incubated at 4 °C for an additional 60 minutes. The cells were then centrifuged at 500 $\times$ g for 5 minutes, washed, and resuspended in the appropriate volume of FACS buffer. Samples were analysed utilising the Invitrogen Attune NxT Flow Cytometer equipped with plate reader capabilities. The following antibodies were employed for staining (Figure 2.9):

Marker	Fluorochrome	Clone	Dilution	Manufacturer
L/D NIR Reactive Dye	633-635nm (APC-Cy7)	N/A	1/1000	Invitrogen
PD-1	PE	EHR2H7	1/50	Biolegend
GATA3	AF647	16E10A23	1/40	Biolegend
CCR7	AF488	G043H7	1/30	Biolegend
FOXP3	BV421	206D	1/30	Biolegend
CD40L	Percp-Eflour710	24-31	1/25	Invitrogen
Tbet	PerCP5.5	4B10	1/20	Biolegend
ICOS	PE-Cy7	ISA-3	1/30	Invitrogen
CXCR5 (Human)	BV421	RF8B2	1/30	BD Bioscience
IL-21 (Human)	PE	eBio3A3-N2	1/20	EBioscience
IgG1 X isotype control	PE	MOPC-21	1/50	Biolegend
FC Block	N/A	N/A	1/200	Biolegend
Bcl-6	AF647	K112-91	1/20	BD Bioscience

Figure 2.9. Table of FACs Antibodies used for Analysis of human *in vitro* derived Tfh, Th0 and Th2 cells.

All antibodies were used in excess to achieve optimal staining levels for each cell and minimise impurities in the sorting process.

2.2.3 IL-21 Restimulation Flow Cytometric Assay

To determine the production of IL-21 by cultured T cells, cells were restimulated using the Invitrogen Cell Stimulation cocktail X500 (Cat no. 00497093) in the presence of a protein transport blocker (Invitrogen Protein transport Inhibitor cocktail X500: Cat no. 00498093) for 4 hours at 37 °C. The stimulation cocktail contained PMA and Ionomycin (40.5 µM and 670 µM (x500)); however, the final concentration was 2 µL cocktail per mL (1:500 dilution) in complete cRPMI. This was also the same for the protein transport blocker, which was diluted 1:500 and added to the cells. Following such restimulation, cells were washed and stained for flow cytometry, as previously described in Chapter 2.2.2. The cells were stained with an intracellular antibody against IL-21 to measure cytokine levels within the cells

2.2.4 Western Blot

The purpose of the Western blot was to detect and quantify the protein levels of human SCD-1 from human *in vitro* derived Tfh, Th2 and Th0 cells. Cells were washed with cold PBS, and lysates were created using lysis buffer (Cell Signalling (Cat. #9803S)) in the presence of phenylmethylsulfonyl fluoride (PMSF) (Sigma Aldrich (Cat. 10837091001) – a serine protease inhibitor – and phosphatase inhibitor (ThermoFisher (Cat. 78440). To perform the western blot, initially, 15mg of protein from each sample was heated to 75 °C in the presence of a solution containing Bolt LDS (ThermoFisher, Cat. B0007)) + DTT to denature the proteins for 10 minutes.

To set up the gel electrophoresis, 1 L of 1x Blot MES (ThermoFisher (Cat. B0002) running buffer was prepared by diluting 50 mL of 20x Blot MES running buffer with 950 mL of MilliQ water. The chamber of the electrophoresis tank was pre-filled with 1X Blot Running Buffer to the level of the cathode. The tape was removed from a Bolt 4-12% Bis-Tris Plus gel, which was then assembled into the electrophoresis tank. The cassette was clamped before removing the gel comb, and wells were rinsed in 1x Blot MES running buffer using a syringe and needle. 20 µl (10mg) of protein samples were loaded using gel loading tips and 2µl of PAGERuler NIR protein ladder. 1X Blot MES running buffer was added to the fill line level. The gel was run for 22 min at 200V.

A PVDF Transfer Stack was used to transfer the protein from the gel to the PVDF membrane for Western blotting. The electrical contacts on the tray were aligned with the corresponding electrical contacts on the blotting surface of the iBlot™ 2 Gel Transfer Device. The pre-run gel(s) were wetted and placed on the transfer membrane of the Bottom Stack. A pre-soaked (in deionised water) iBlot™ Filter Paper was then placed on the gel, and air bubbles were removed using the Blotting Roller. An Absorbent Pad was placed on top of the top stack, ensuring the electrical contacts were aligned with the corresponding contacts on the blotting surface of the iBlot 2 Gel Transfer Device. The lid was then closed, and the blot was transferred for 7 minutes.

Following the transfer, a total protein stain was carried out. The membrane was removed and washed in MilliQ water for 5 minutes. The Stain Solution was prepared as follows: 125 µl of room-temperature AzureRed Dye was diluted in 50 mL Stain Buffer and mixed well. The blot was placed protein side down in the Stain Solution and rocked gently for 15–30 min. Next, the blot was placed in the fix Solution and incubated with gentle rocking

for 5 minutes before being rinsed three times with 100% ethanol for 2–3 minutes each time.

Finally, the blot was blocked for 45 minutes at room temperature with Azure Chemi Blot blocking buffer (Azure Biosystems (Cat SKU: AC2148)). The block was removed and the blot washed three times with Tris-buffered saline with Tween (TBST) for 5 minutes each. The primary antibody to detect SCD-1 (Cell Signaling (Cat. #2794T)) (Dilution: 1/500) was diluted in 5% BSA TBST and applied to the blot for 45 minutes at room temperature. The primary antibody solution was removed and stored for further use, and the membranes were washed three times for 5 minutes each with 25 mL of PBST wash buffer. To detect the SCD-1 protein band on the membrane, it was subsequently incubated with 20 ml of 1:1000 goat anti-rabbit 700 (Azure Biosystems (Cat. ACS128) in fluorescent blocking solution for 1 hour at room temperature.

The secondary antibody was removed, and the membrane was washed three times for five minutes each with 25 mL TBST wash buffer, followed by 10 minutes in Tris-buffered saline (TBS). The blot was visualised and imaged using a Sapphire FL Biomolecular Imager with the Cy5 and Cy7 channels to detect the protein of interest (SCD-1) and the total protein stain. Band sizes were compared to the PAGE ruler, along with the pre-stained protein ladder. Data were analysed using ImageJ and presented using GraphPad Prism. All SCD-1 data were standardised to the total protein stain.

2.2.5 Addition of Compounds

To understand the role of SCD-1 in Tfh cells, the following commercially available compounds were used to block SCD-1 enzymatic activity: CAY10566 and A939572. The compounds were added to cell cultures using a Tecan D300e Automated digital dispenser. The compounds were used at a range of final concentrations from 5 to 500 nM in our cell culture systems. They were added on day 0 or day 2 of the 3-day Tfh cell culture system to understand the effects of SCD-1 inhibition on T cells during early differentiation, and on established Tfh cells.

2.2.6 Data Analysis

Statistical analysis was performed using GraphPad Prism (Version 9, GraphPad Software, La Jolla, CA). The Shapiro-Wilk tests were used to determine the normality of the data.

Flow cytometry analysis was performed using FlowJo Software (Treestar).

Statistical significance was determined using one-way ANOVA or the T-test with Welch's correction, depending on whether normality was assumed. Welch's Correction was used as it could not be assumed that the data had the same standard deviation. P values of ≤ 0.05 were considered significant, whereby * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Data is shown as the mean and standard deviation.

For transcriptomic analysis, data analysis was performed through the Searchlight 2 (SL2) platform by John Cole. The significance of the data, as determined using Deseq2, was assessed by identifying genes that were significantly differentially expressed ($p_{\text{adj}} < 0.05$, absolute $\log_2$ fold change > 0.5). This analysis was performed using DESeq2, where the matrix of expression values and the differential expression table were uploaded into SL2. The SL2 pipeline incorporated statistical analysis as part of the program. Unless indicated in the figure, all transcriptomic statistical analyses were performed by SL2, as described by John Cole. DAVID analysis was also utilised (<https://davidbioinformatics.nih.gov/>), and the statistical analysis was completed by this pipeline independently, without user preference.

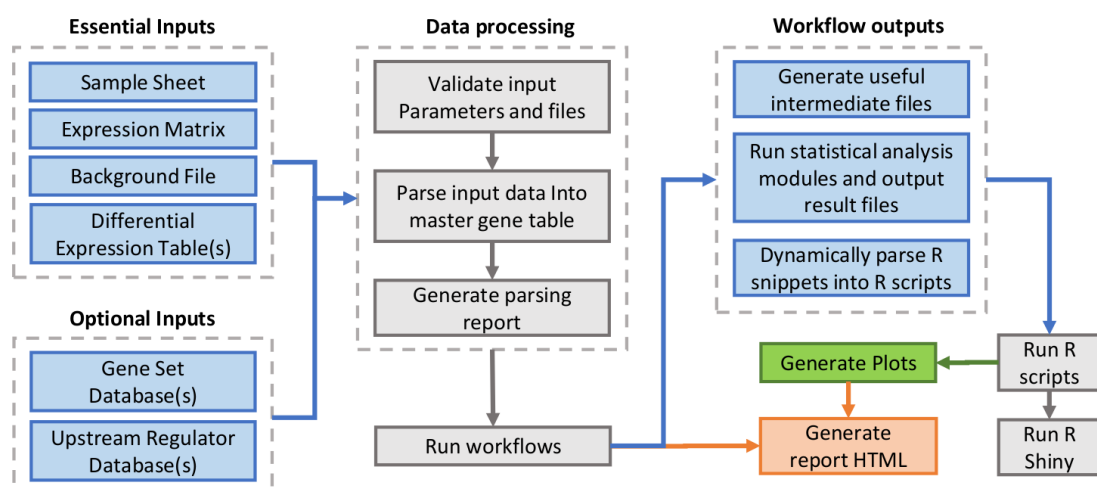


Figure 2.10. Schematic of Searchlight2 Pipeline.

This figure was created by (Cole et al., 2021)

Chapter 3 - Characterising the Immunometabolism of Tfh Cells During Active *Heligmosomoides polygyrus* Infection in the Mesenteric Lymph Nodes

3.1 Introduction

The role of metabolism in immunology had been overlooked for many years, but this changed significantly following work from Craig Thompson's lab, which showed that the CD28 receptor signalling regulated glucose metabolism in T cells, aiding cell growth and further functional metabolism (Frauwirth et al., 2002). Since then, our understanding of the importance of Immunometabolism has surged, and the role of metabolism, particularly in T cells, has become a significant focus (Makowski et al., 2020). Like immunometabolism, the role of Tfh cells was only defined at the turn of the century (Kim et al., 2001). Our understanding of how both fields align has since been explored in different immune contexts, (Fu et al., 2021, Dong et al., 2019, Son et al., 2020), but perhaps surprisingly, the immunometabolism of Tfh cells in Th2-mediated helminth disease has still not been well-characterised.

Previous work in the Perona-Wright lab showed that during *N. brasiliensis* infection, Tfh cells increase glucose and fatty acid uptake compared to non-Tfh cells in the mdLNs (Heieis, 2019). However, this research has limitations with respect to the current study, including the different anatomical location of *N. brasiliensis* infection compared to *H. polygyrus*, and certain assays have since become redundant following the completion of the research. It has been shown that the use of 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-d-glucose (2-NBDG) is not specific for monitoring glucose uptake and hence is unreliable for understanding this aspect of cellular transport of nutrients (Sinclair et al., 2020).

Thus, to date, limited work has been carried out to characterise the immunometabolism of Tfh cells in the intestinal draining LNs, in a type 2 immune response; rather, most work has focused primarily on defining their role (Meli et al., 2017). This thesis aimed to expand our knowledge of their distinctive role and to determine whether the immunometabolism of Tfh cells is similar or divergent to that displayed by those in the *N. brasiliensis* model and other diverse pathogenic challenge systems.

This unmet understanding could be a critical node in research on helminth infection and how to improve immune responses and increase vaccine efficacy against these parasitic maladies (Linterman and Hill, 2016). As described in Chapter 1, the global burden of helminth infection affects a significant proportion of the world; however, despite this, strategies to eradicate and prevent the disease are often ineffective. Using the model *H. polygyrus* allows us to create a robust murine model of Th2-mediated helminthic immunity in the intestine, facilitating exploration of whether immune evasion techniques employed by the pathogen can direct metabolic adaptations to favour pathogenicity (Maizels et al., 2012).

This chapter sought to understand the critical pathways associated with Tfh cell metabolism in the mLNs at day 14 of *H. polygyrus* infection during the peak T cell response (Perona-Wright et al., 2010). The initial focus was to determine whether the metabolic processes suggested by the previous work in this lab, including the role of glucose and fatty acids in Tfh cells, are common to gut helminths and to investigate whether rapid cell division is a characteristic of Tfh compared to effector cell populations and how this relates to metabolism (Heieis, 2019). This chapter will further explore the key areas of Tfh cell metabolism to understand the commonalities between infection models and to identify whether distinct metabolic profiles are required by Tfh and Th2 cells to facilitate their differentiation and function during Th2-skewed parasitic infections.

3.2 Aims

- To analyse and confirm that the location and magnitude of Tfh cell expansion in the mLNs during *H. polygyrus* infection is robust, concurrent with previously defined research (Meli et al., 2017, Heieis, 2019).
- To understand the level of Tfh cell proliferation compared to other T cell subsets in the mLN during *H. polygyrus* infection and how this relates to metabolic requirements
- To develop our understanding of Tfh Immunometabolism, particularly regarding activation, glycolysis and FAS metabolism, as previously highlighted by (Heieis, 2019) in a different model of helminth disease.
- To understand how, in addition to glycolysis and FAS, Tfh cells potentially utilise different metabolic pathways/transporters during a type 2-mediated immune response to helminth infection.

3.3 Results

3.3.1 Tfh cell expansion in the mLNs during *H. polygyrus* Infection

The model *H. polygyrus* was used as it provides a robust T cell expansion in the mLN and drives a strong IL-4-producing Tfh cell response (King and Mohrs, 2009). To understand the factors that regulate Tfh cells in the mLN during infection, I first mapped their expansion at the time of peak T cell responses during *H. polygyrus* Infection, as previously determined by our lab, which is indicative of active GC responses (Perona-Wright et al., 2010; Hewitson et al., 2011).

To define CD4⁺ T cells using flow cytometry, cells were first gated on single live lymphocytes, then sub-gated based on CD3 or TCR- β , with CD4 used to define this population of T cells (Figure 3.1A). To assess whether they were activated, I used the surface marker CD44⁺ (Figure 3.1 B), which is expressed at high levels during and after activation. I found that during infection, we observed an increased frequency of both naïve CD44^{low} and activated CD44^{high} CD4⁺ T cells in the mLN of *H. polygyrus* -infected mice compared to the uninfected controls, as well as an increase in the total number of T lymphocytes in the infected group (Figure 3.1D).

This is consistent with previous reports, whereby *H. polygyrus* drives robust migration of T cells into the mLN during infection (King et al., 2017). In addition, I observed an increase in the size of mLNs in the infected samples compared to the uninfected chain (Figure 3.1 C). After successfully staining and assessing the naïve and activated cells isolated from both uninfected and infected mLNs, I aimed to understand the dynamics of Tfh cells in active disease.

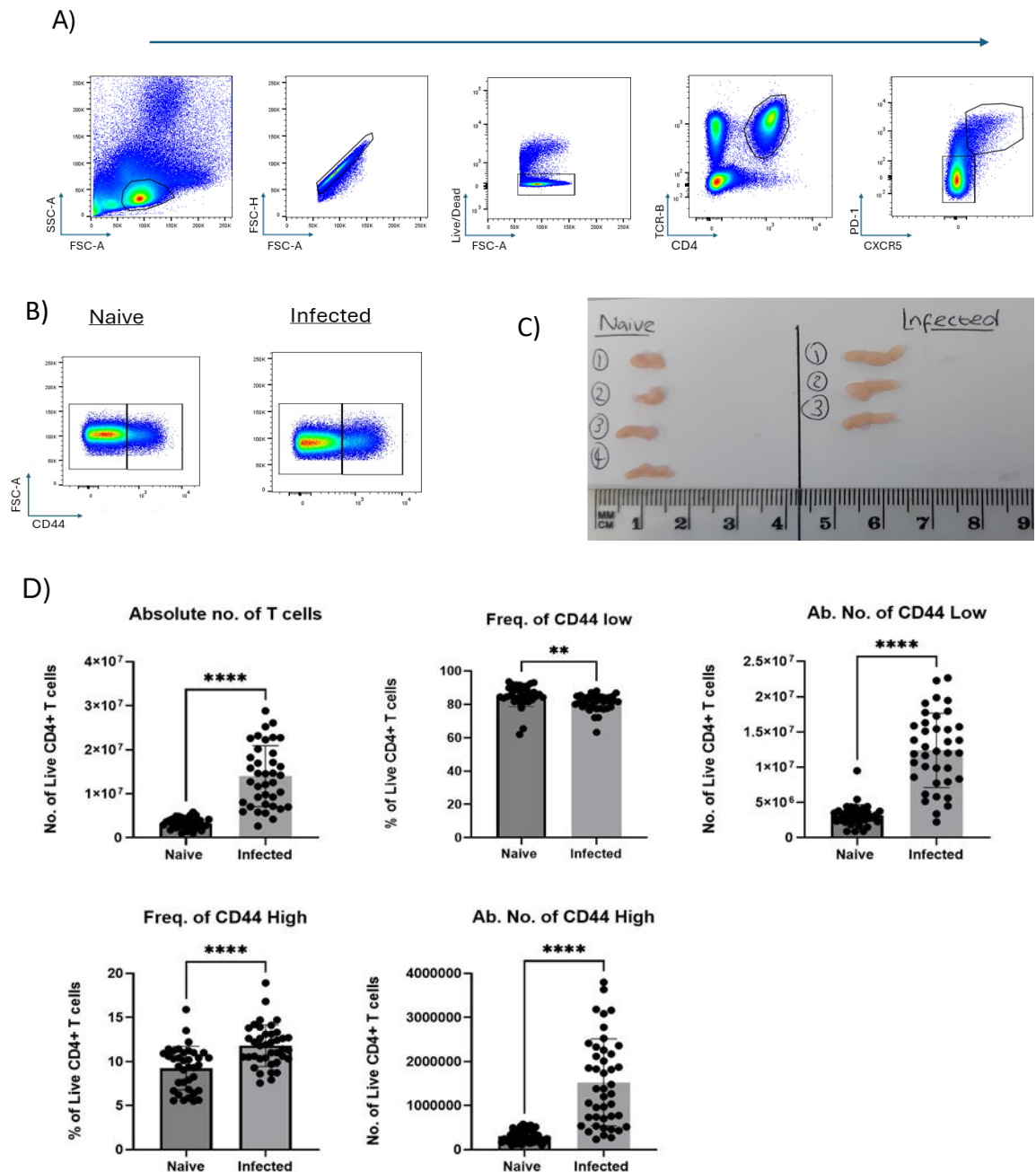


Figure 3.1: *H. polygyrus* infection Elicits Robust CD4⁺ T cell Expansion and Activation.

C57BL/6 mice were infected with 200 L3 *H. polygyrus* larvae, and 14 days post-infection, the mLN were removed. Naïve in this chapter figures refers to uninfected mice. A) Representative gating strategy of Tfh cell (Live CD4⁺ CXCR5^{High}, PD-1^{high} cells) from mLN samples of Infected mice. B) Representative Flow scatter plots of CD44 activation staining in non-Tfh CD4⁺ T cells from naïve and Infected mice. C) Representative images of the mLN chain in naïve and day 14 post-infection with *H. polygyrus*. D) Absolute number of T cells (naïve and infected), Frequency and absolute number of CD44^{Low} and ^{High} CD4⁺ T cells. Graphed data is from 1 experiment (n=5), shown with mean +/- SD. This data represents three repeated experiments with n = 5 mice per experiment. Statistical significance was determined using the Unpaired Student T-test, where

data were deemed normally distributed as distinguished through the Shapiro-Wilk test. Significance was denoted by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

Tfh cells were defined as live $CD4^+$ T cells, characterised by their high expression of the surface receptors CXCR5 and PD-1 (Figure 3.1A). My results highlight the increase in the frequency and total number of Tfh cells in the *H. polygyrus* infection compared to the uninfected counterparts, which was visually striking through flow cytometry and statistically significant when quantified (Figure 3.2). Having also established a robust protocol for naïve, activated and Tfh cell staining, I wanted to assess the specific location of Tfh cell expansion, as *H. polygyrus* has been shown to have distal effects in other tissues irrespective of infection in that location, such as in the spleen (Brailsford and Mapes, 1987), so I wanted to assess if Tfh expansion could be present in other anatomical locations.

H. polygyrus infects the small intestine of rodents. However, due to the proximity of the small intestine and large intestine's draining LNs in the mLN chain, I wanted to check for distal expansion in LNs not directly infected with *H. polygyrus*. It was evident that the Tfh cells' expansion was restricted to the LN draining the small intestine, whilst no expansion was present in the LNs draining the large intestine (Figure 3.3); this is consistent with the location of the infection (Maizels et al., 2012).

To understand how Tfh cells' metabolism relates to that of effector $CD4^+$ T cells in *H. polygyrus* infection, I aimed to optimise staining for both Th2 and Treg cells to complete our understanding of the activated T cell subsets within the LN. Th2 were defined as $CD4^+$ non-Tfh (CXCR5^{low}, PD-1^{low}), $CD44^{\text{high}}$, $GATA3^+$ cells, whereas Tregs were classified as $CD4^+$ non-Tfh, $FOXP3^+$ cells. Both $GATA3$ -positive and negative Tregs were included in our gating, as $GATA3^+$ Tregs are important in *H. polygyrus* infection, to control the immune response (Figure 3.4) (Wang et al., 2011b). Also, Th2 cells are defined as non-Tfh as Tfh cells can express $GATA3$ and produce high amounts of IL-4, so we gated them out to prevent misrepresentation or contamination of my results (King and Mohrs, 2009). Previously in our lab, we also saw that Th2 and Tregs peaked at day 14, increasing from day 7, coupled with previous research, which showed that IL-4 producing Tfh cells peaked at day 14 (Zaini et al., 2023) This meant that I captured the strongest magnitude of response in each T cell subset at this time point. However, it should be noted that the largest abundance of Th2 and Tregs is typically found in the tissue, as the effector cells leave the mLN to carry out their required functions (Webster, 2021).

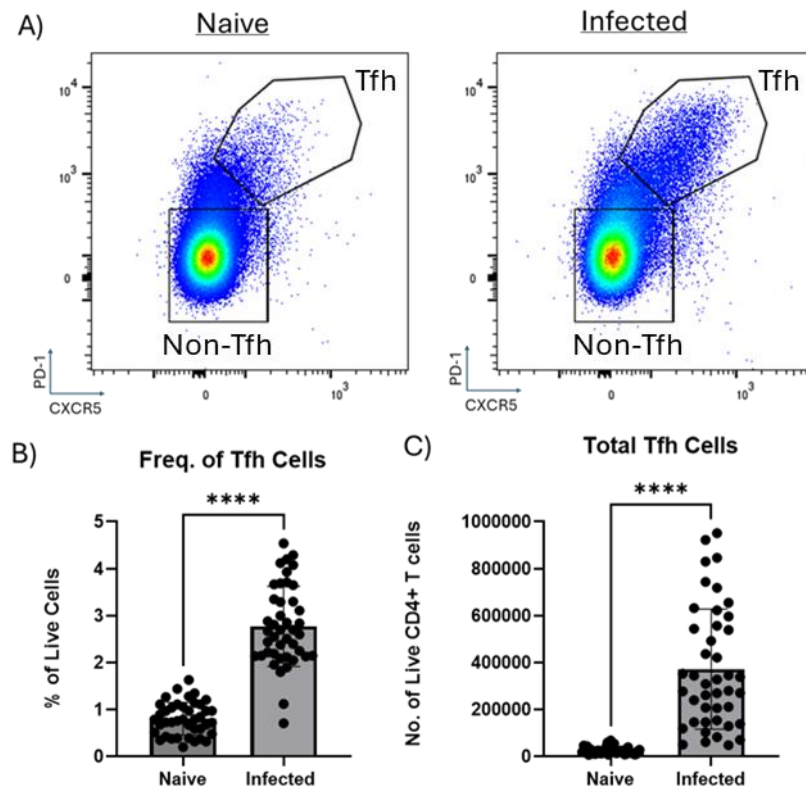


Figure 3.2: *H. polygyrus* elicits a strong Tfh cell expansion in the mLNs.

C57BL/6 mice were infected with 200 L3 *H. polygyrus* larvae, and 14 days post-infection, the mLNs were removed, and cells were processed and stained in preparation for flow cytometric analysis. A) Representative Flow scatter images of CXCR5 and PD-1 CD4⁺ Live T cells to highlight the increase in Tfh cells (CXCR5^{high} PD-1^{high}) between naïve (Uninfected) and infected mice. B) Graphed data of Tfh cells' absolute number and frequency between naïve and infected mice. Graphed data is from 1 experiment (n=5), shown with mean $\pm$ SD. This data represents three repeated experiments with n = 5 mice per experiment. Statistical significance was determined using the Unpaired Student T-test, where data were deemed normally distributed as distinguished through the Shapiro-Wilk test. Significance was denoted by * p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001.

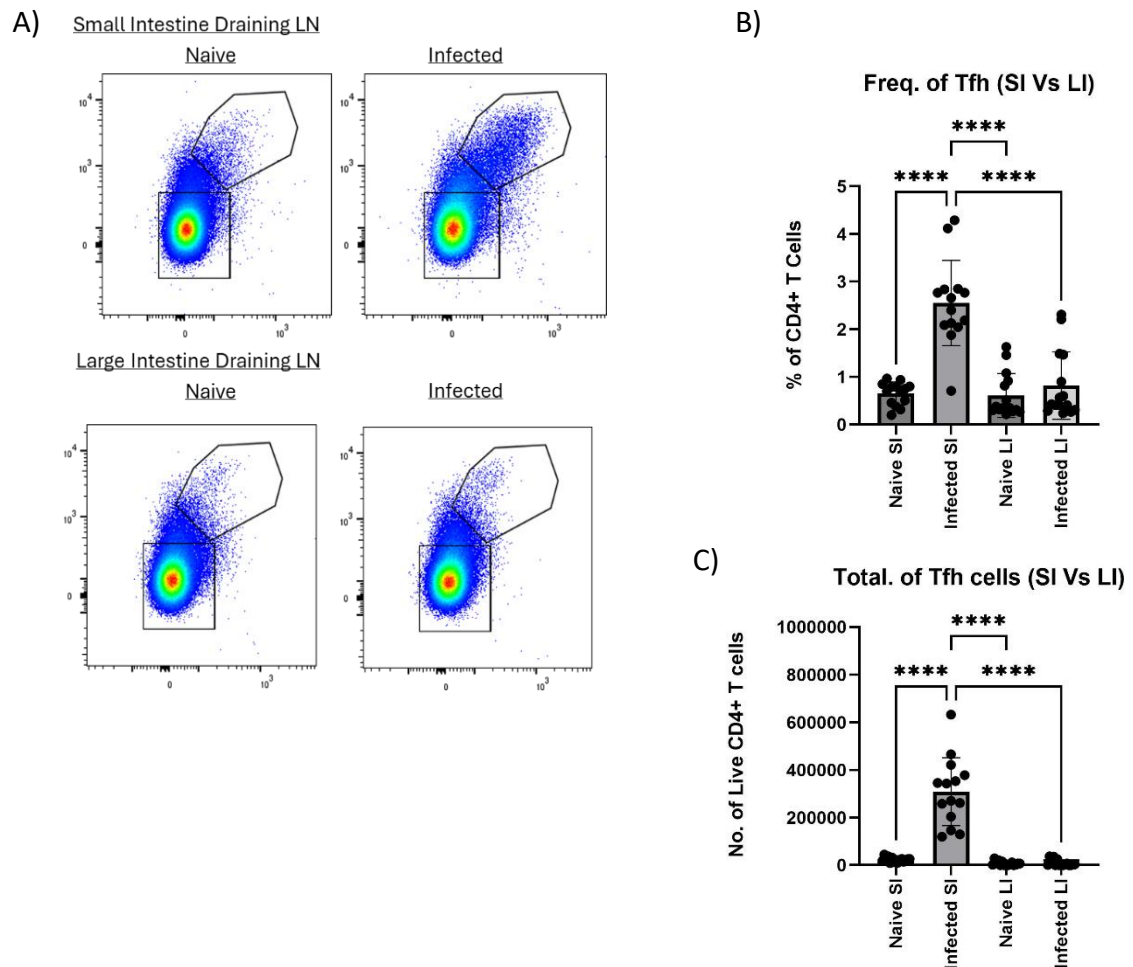


Figure 3.3. Tfh Cells Increase during *H. polygyrus* is concentrated in the MLNs draining the Small Intestine.

C57BL/6 mice were infected with 200 L3 *H. polygyrus* larvae, and 14 days post-infection, the mLNs were removed. A) Representative Flow scatter images of CXCR5 and PD-1 CD4+ Live T cells to highlight the increase in Tfh cells (CXCR5 high +PD-1 high) between naïve and infected mice in both small and large intestine draining mLN. B) Representative graph of the Frequency of Tfh cells. C) Representative graph of the absolute number of Tfh cells. Graphed data is from 1 experiment (n=5), shown with mean +/- SD. This data represents three repeated experiments with n = 5 mice per experiment. Statistical significance was determined using a one-way ANOVA with Tukey's post-test for multiple comparisons in groups where data were deemed normally distributed, as distinguished through the Shapiro-Wilk test. When data was not normally distributed, the Kruskal-Wallis with post hoc Dunn's Test was conducted between groups. Significance was denoted by * p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001.

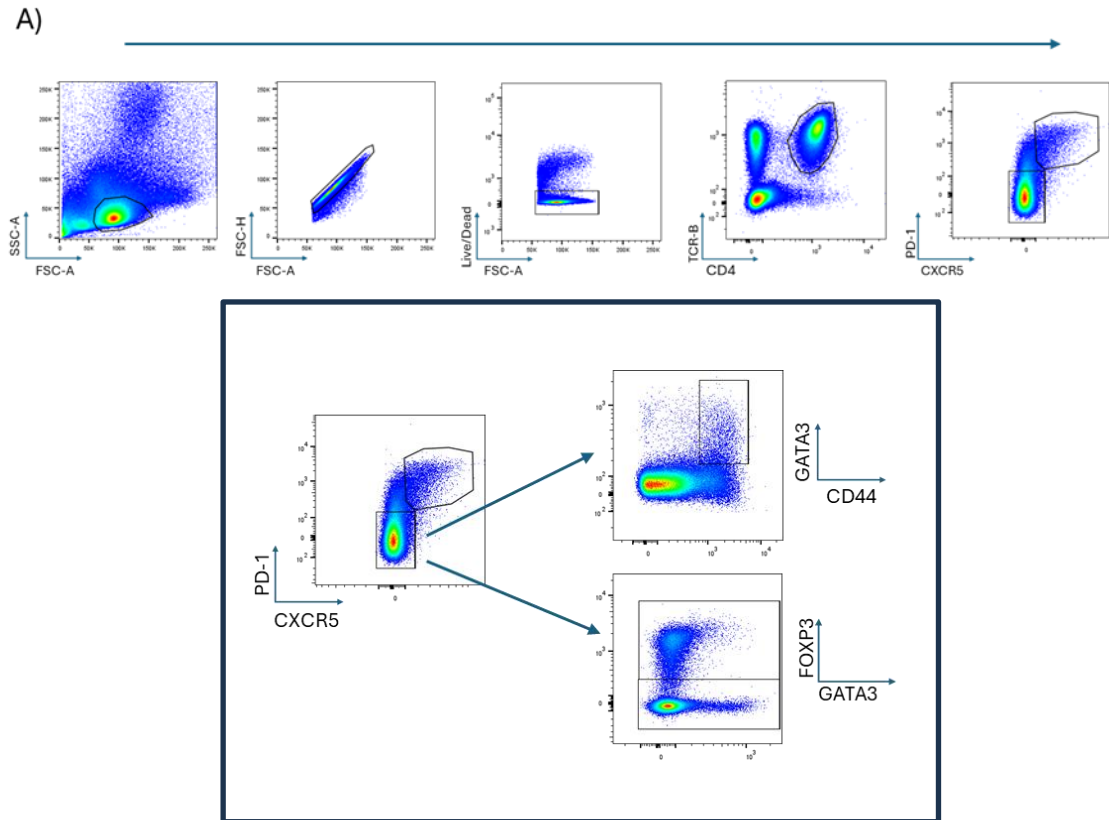


Figure 3.4; Gating Strategy used to identify Th2 and Tregs.

C57BL/6 mice were infected with 200 L3 *H. polygyrus* larvae, and 14 days post-infection, the mLNs were removed. Representative flow plots highlight the gating strategy to isolate CD4⁺ TCRβ⁺ non-Tfh T cells. Further gating results in two populations: CD44^{High} GATA3⁺ (Th2) cells and FOXP3⁺ (Treg) cells.

3.3.2 Tfh Cells Exhibit Reduced Proliferative Capacity Compared to Th2 Cells During *H. polygyrus* Infection

Previously, our lab suggested that Tfh cells were more proliferative and metabolically active compared to naïve and Th2 cells in the mLN of *H. polygyrus* infection (Heieis, 2019). However, this contrasts with previously defined beliefs about the restricted proliferative capacity of Tfh cells to drive selectivity in the GC, through restriction of Tfh cell help to B cells (Merkenschlager et al., 2021). Using the intracellular marker of proliferation, Ki-67, I compared the proliferative capacity of CD4⁺ T cells within the reactive LN. I found that while Tfh cells increase KI-67 expression compared to naïve T cells, Th2 cells present in the mLN exhibited the highest expression of Ki-67 and thus had

a significantly higher proliferative capacity than Tfh cells (Figure 3.5B). This is consistent with the restricted capacity to increase selectivity in the GC reaction.

The proliferative capacity of cells is intrinsically linked to activation and metabolic capacity. This link is mainly through mTOR signalling (Pearce, 2010), which also provides a strong indication of the metabolic phenotype. Tfh cells have been previously suggested to have lower metabolic requirements and proliferative capacity than effector Th1 cells (Ray et al., 2015) but how they compare to Th2 cells is not well-defined. Thus, to assess whether Tfh cells are metabolically quiescent, their mTOR signalling status in the type 2 immune response was investigated.

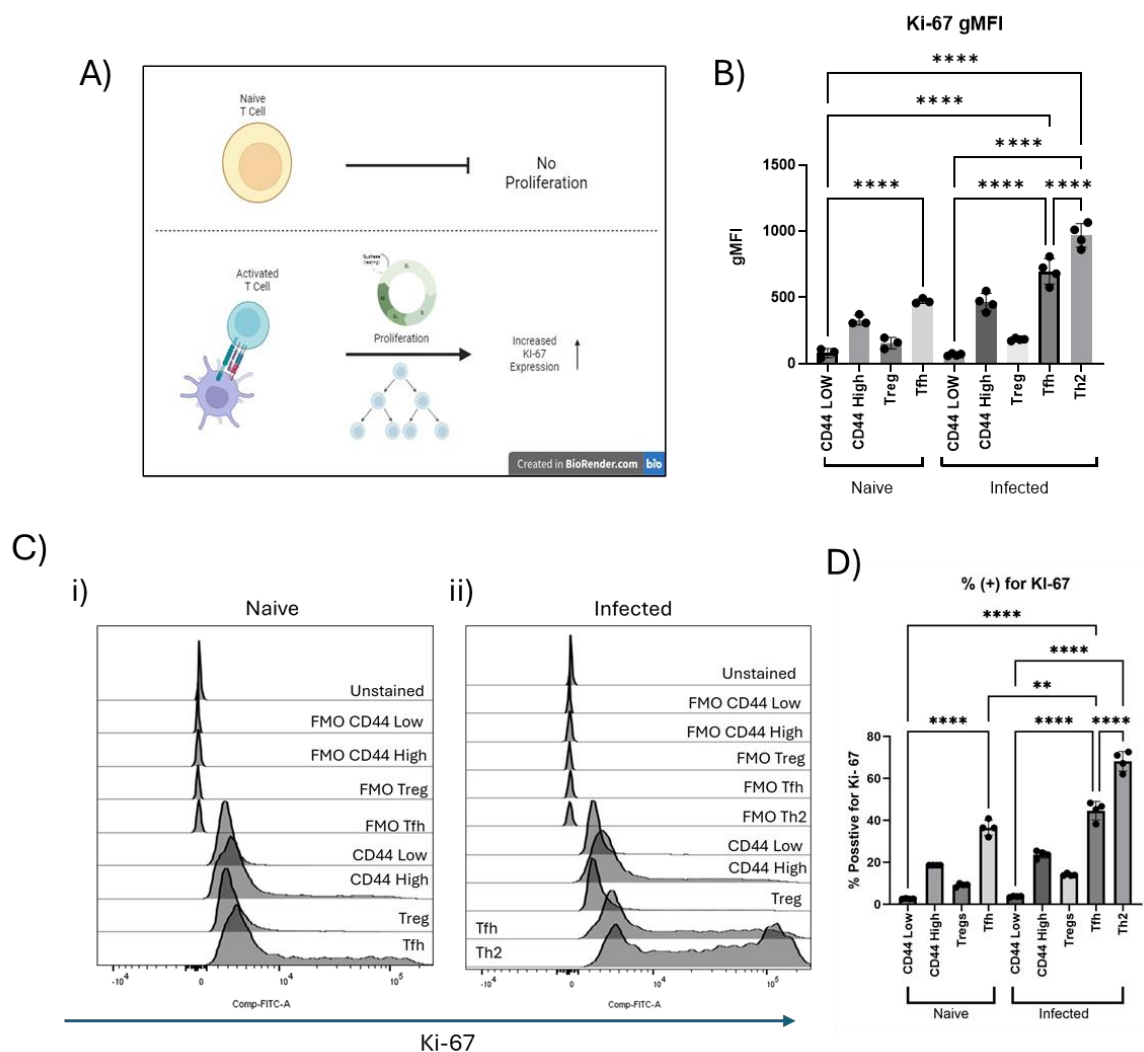


Figure 3.5. Tfh cells express lower levels of Ki-67 compared to other key effector CD4+ T cells in the mLN.

C57BL/6 mice were infected with 200 L3 *H. polygyrus* larvae, and 14 days post-infection, the mLNs were removed. A) Representative diagram of Ki-67 expression. B) Representative graph of Ki-67 gMFI explain/define across T cell subsets in both naïve and infected groups. C) Representative histograms of Ki-67 in both naïve (i) and infected groups (ii). D) Graph of percentage of positive staining for Ki-67 in each

population. Graphed data is from 1 experiment (n=5), shown with mean +/- SD. This data represents three repeated experiments with n = 5 mice per experiment. The experiment shown has n = 3 for naïve (uninfected) samples and n = 4 for *H. polygyrus-infected* samples. Statistical significance was determined using the one-way ANOVA with Tukey's post-test for multiple comparisons in groups where data were deemed normally distributed, as distinguished through the Shapiro-Wilk test. Significance was denoted by * p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001.

3.3.3 Tfh Cells Utilise mTOR Complex Activation During Differentiation

Tfh cells have previously been shown to depend on signalling via both mTOR complexes for their differentiation and function. Thus, it is well-characterised that activation of mTOR complex 1 promotes T cell proliferation, whilst mTOR complex 2 drives Tfh cell differentiation through AKT activation and increasing TCF1 expression. Loss of both complexes results in stunted differentiation (Yang et al., 2016a, Zeng et al., 2016).

Using surrogate markers of mTOR activation, phosphorylated S6 (Serine 235/236 RPS6) and AKT (Serine 473), the levels of mTOR activation in Tfh cells were assessed. The following targets of phosphorylation were chosen as they are widely used and provide a robust method to show pathway activation (Ray et al., 2015).

AKT is phosphorylated upstream of the mTOR complex and helps drive its activation, whilst S6 phosphorylation occurs downstream as a result of mTORC1 signalling via S6K1. Through concurrently analysing both upstream and downstream phosphorylation states of mTOR-related effectors, we can evaluate the activation status of the AKT-mTOR-S6 signalling axis in our cells (Annovazzi et al., 2009). It is the easiest way to measure mTOR and can be quantified using flow cytometry (Pollizzi et al., 2015). The phosphorylation of both proteins, relative to their total levels of expression, allows for an understanding of whether phosphorylation and/or protein abundance are being modulated. However, such Phos-flow analysis presented challenges, as conventional Phos-flow permeabilisation and fixation reagents disrupted CXCR5 detection. Therefore, several fixation/permeabilisation approaches were employed to optimise this approach. The FOXP3 permeabilisation and fixation kit allowed for maintenance of CXCR5 staining as well as the desired markers, pS6/S6 and pAKT/AKT.

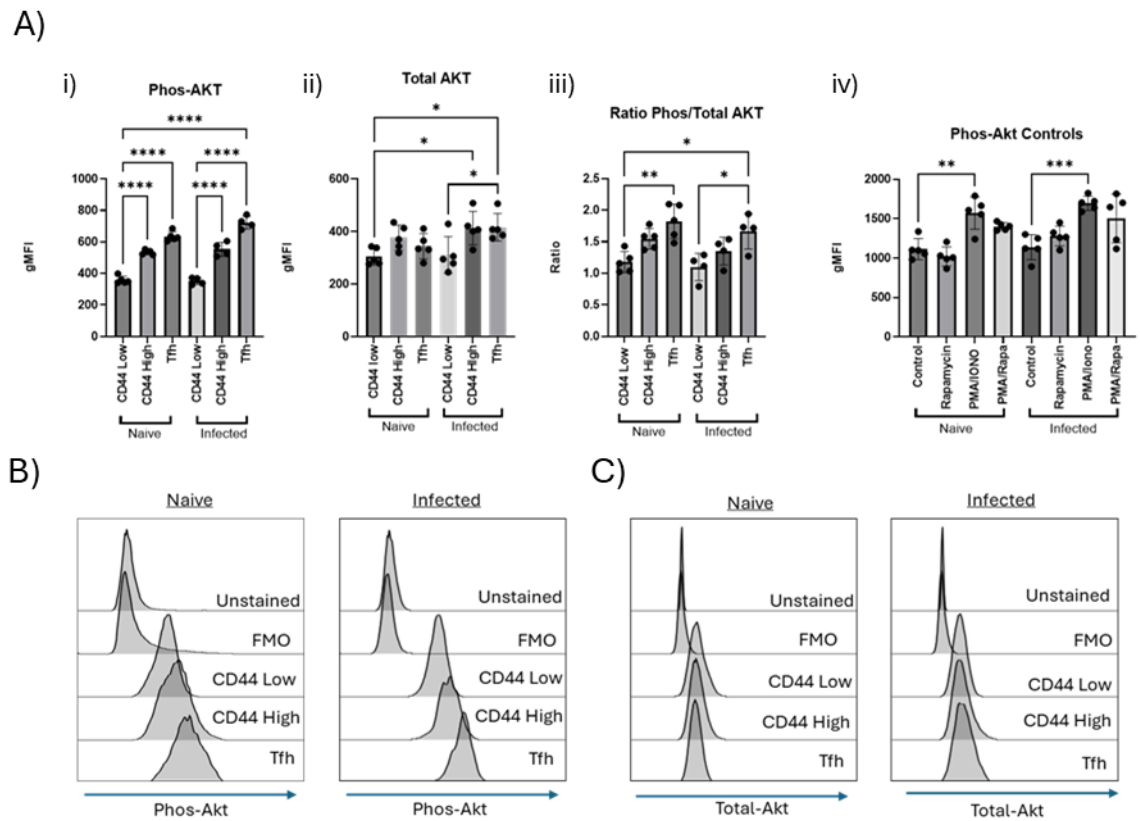


Figure 3.6. Tfh Cells Show Increased Phos-AKT Compared to Naive T cells in the mLN.

C57BL/6 mice were infected with 200 L3 *H. polygyrus* larvae, and 14 days post-infection, the mLN were removed. A) i) Representative graph of Phos-AKT MFI. A) ii) Representative graph of total AKT protein MFI. A) iii) Representative graph of the ratio of gMFI of Phos-Akt/Total Akt to show relative levels of activated AKT. A) iv) Representative graph of Phos-Akt gMFI compared with positive control (PMA/Ionomycin). B) Representative Histograms of Phos-Akt from both naïve and infected groups. C) Representative Histogram of Total Akt from Naïve and Infected groups. Graphed data is from 1 experiment (n=4/5) shown with mean $\pm$ SD. This data represents three repeated experiments with n = 4/5 mice per experiment. Statistical significance was determined using the one-way ANOVA with Tukey's post-test for multiple comparisons in groups where data were deemed normally distributed, as distinguished through the Shapiro-Wilk test. Significance was denoted by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

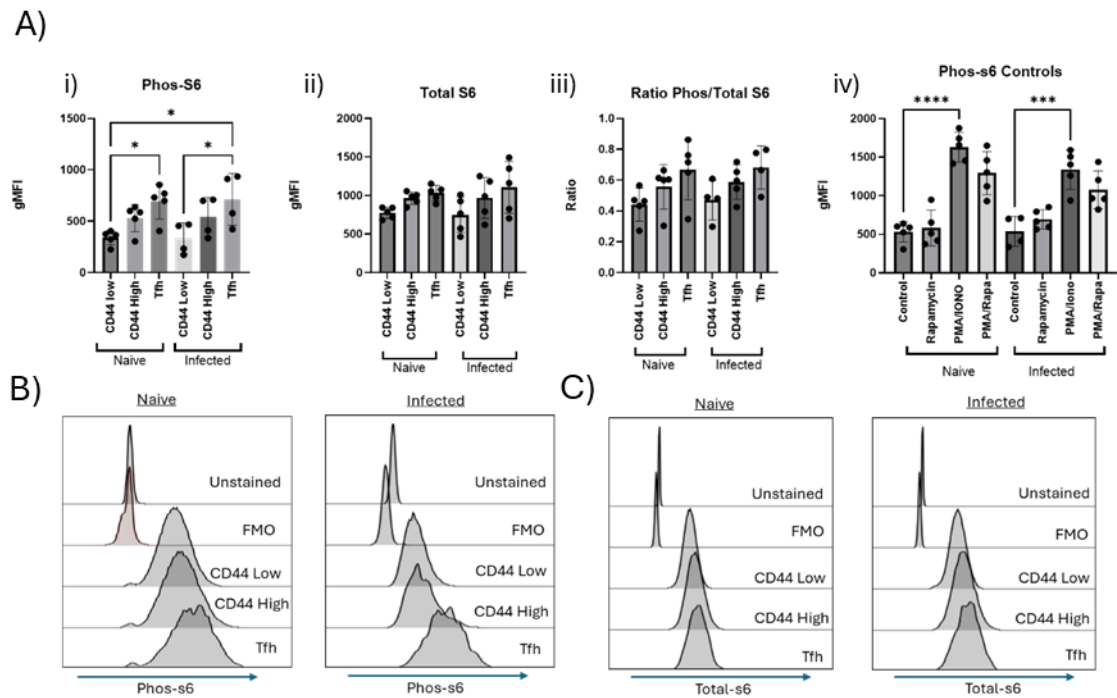


Figure 3.7. Tfh Cells Show increased Phos-S6 levels compared to Naive T cells in the mLN.

C57BL/6 mice were infected with 200 L3 *H. polygyrus* larvae, and 14 days post-infection, mLN were removed. A) i) Representative graph of Phos-S6 gMFI. A) ii) Representative graph of total S6 protein gMFI. A) iii) Representative graph of the ratio of gMFI of Phos-S6/Total S6. A) iv) Representative graph of Phos-S6 gMFI compared with positive (PMA/Ionomycin) and negative (Rapamycin) controls. B) Representative Histograms of Phos-S6 from both naïve and infected groups. C) Representative Histogram of Total Akt from Naïve and Infected Groups. Graphed data is from 1 experiment (n=4/5) shown with mean $\pm$ SD. This data represents three repeated experiments with n = 4/5 mice per experiment. Statistical significance was determined using a one-way ANOVA with Tukey's post-test for multiple comparisons in groups where data were deemed normally distributed, as distinguished through the Shapiro-Wilk test. Significance was denoted by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

As predicted, the Tfh cells exhibited increased phosphorylation of AKT and S6 (Figure 3.6 A (i) & Figure 3.7 A (i)) compared to CD44 low naïve T cells. Similar levels were observed in both the Tfh from uninfected mice and the *H. polygyrus* -infected Tfh groups. Tfh cells also increase the expression of both AKT and S6 protein in response to differentiation (Figure 3.6 A (ii) & Figure 3.7 A (ii)). To understand whether P-AKT and P-S6 were significantly elevated over the increased protein expression, the ratio of phosphorylated protein gMFI to that of protein gMFI was determined. This enables us to determine whether the levels of the phosphorylated (i.e., activated) protein are increased, giving us a more detailed understanding of mTOR activity. This highlighted that AKT

phosphorylation was significantly increased in Tfh cells compared to naïve CD4⁺ T cells (Figure 3.6 A (iii)), whilst pS6 was not significantly increased, although it trended towards higher expression in Tfh cells over both naïve and activated CD4⁺ T cells (Figure 3.7 A (iii)).

The use of Rapamycin and PMA/Ionomycin as positive and negative controls was to check that our staining was correct and specific for S6 and Akt. PMA/ionomycin activates T cells and drives intracellular signalling, whilst rapamycin is a selective inhibitor of mTORC1 and decreases pS6. However, it does not affect pAKT. The use of a PI3K inhibitor may have been better for pAKT; however, it was deemed unnecessary, as the positive control would highlight that the antibodies were specific if we saw an increase in phosphorylation levels (Salerno et al., 2017, Li et al., 2014).

3.3.4 Tfh Cells Exhibit Increased Nutrient Transporter Expression During Differentiation

Due to the apparent increase in mTOR activation, it was decided to investigate the functional consequences of this. For example, it has been widely reported that mTOR activation can directly induce the expression of various nutrient transporters, especially those involved in amino acid and glycolytic metabolism (Saxton and Sabatini, 2017). Coupled with activation and signalling through c-MYC, nutrient transport is essential for many cellular processes (Wang et al., 2011a). To understand the potential modulation of expression of nutrient transporters by Tfh cells during *H. polygyrus* infection, I focused on three primary transporters associated with T-cell activation: GLUT1, CD71 and CD98. GLUT1 is an essential glucose transporter, CD71 facilitates the uptake of iron, whilst CD98 is responsible for the uptake of essential amino acids, key nutrients required for proliferation, activation and effector functions (Feral et al., 2005, Ned et al., 2003, Nii et al., 2001).

Based on previous research on T cell activation (Macintyre et al., 2014, Voss et al., 2023, Ren et al., 2017). It has been hypothesised that all of these receptors would increase in expression; however, what is of interest here is the level of expression required by Tfh cells compared to effector cells. My results demonstrate that Tfh cells upregulate GLUT1 significantly during *H. polygyrus* infection compared to naïve T cells (Figure 3.8 B (i)). This was as predicted; however, there was no significant difference in GLUT1 expression in Tfh compared to Th2 cells. The results also suggest increased expression of CD71 and

CD98 in Tfh cells compared to their naïve counterparts during infection; however, the levels of expression did not exceed those of Th2 cells. Interestingly, CD98 expression was significantly elevated between Th2 and Tfh cells (Figure 3.8 B (ii & iii)).

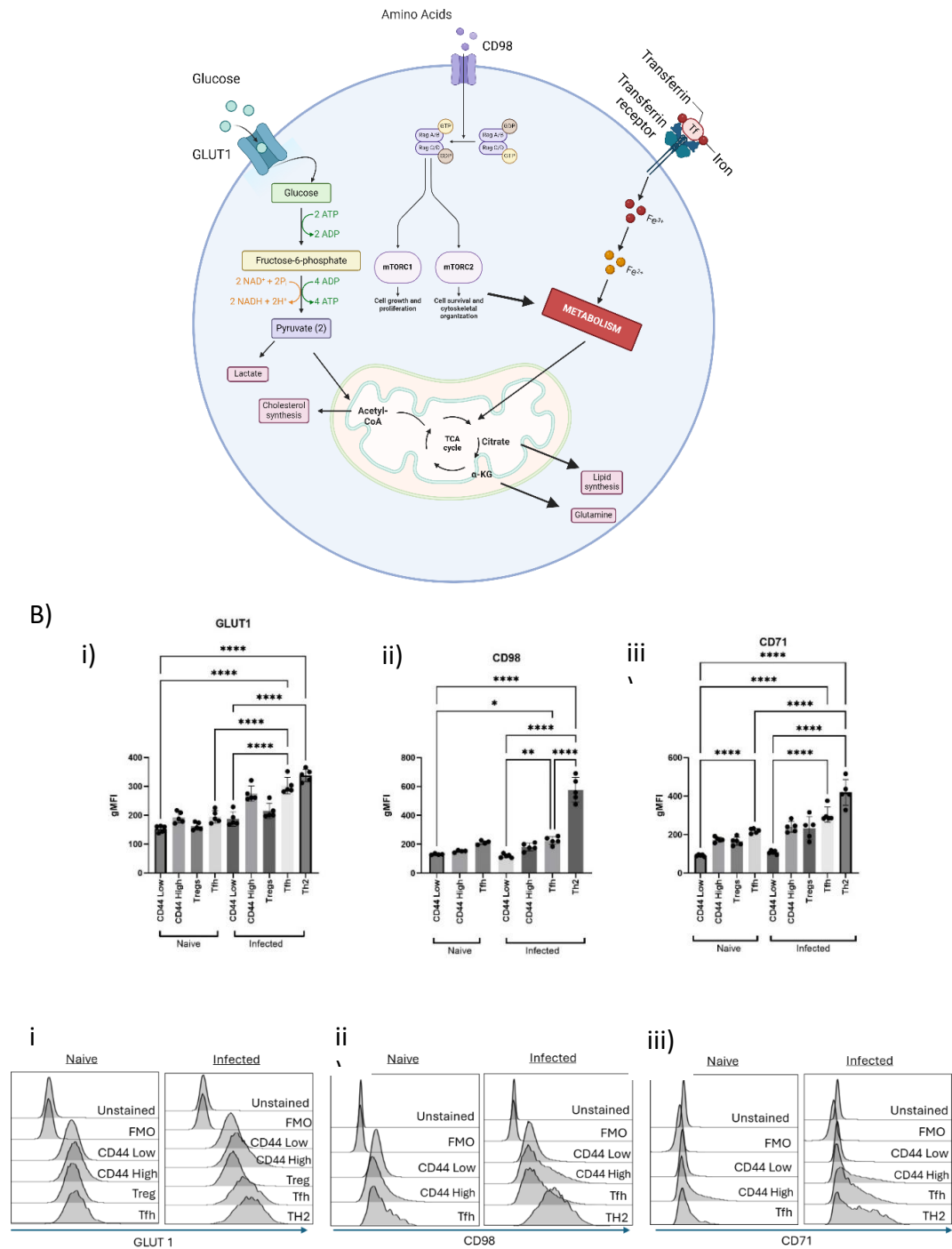
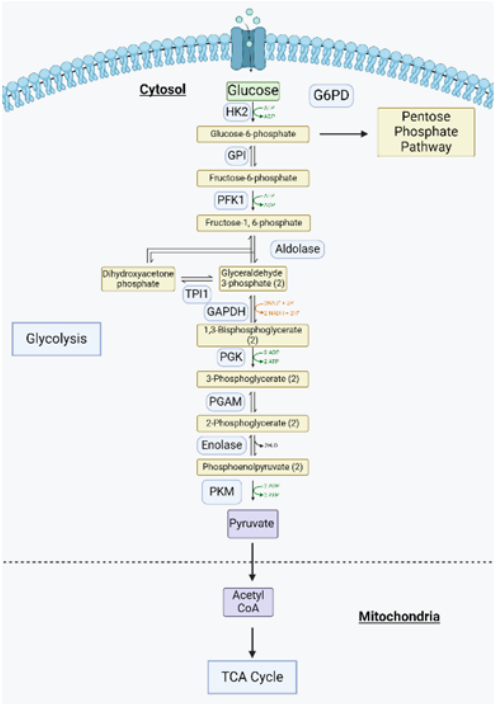


Figure 3.8. Tfh Cells Upregulate of Transporters Receptor Abundance Associated with Extracellular Nutrient Uptake Compared to Naïve T cells (Uninfected)

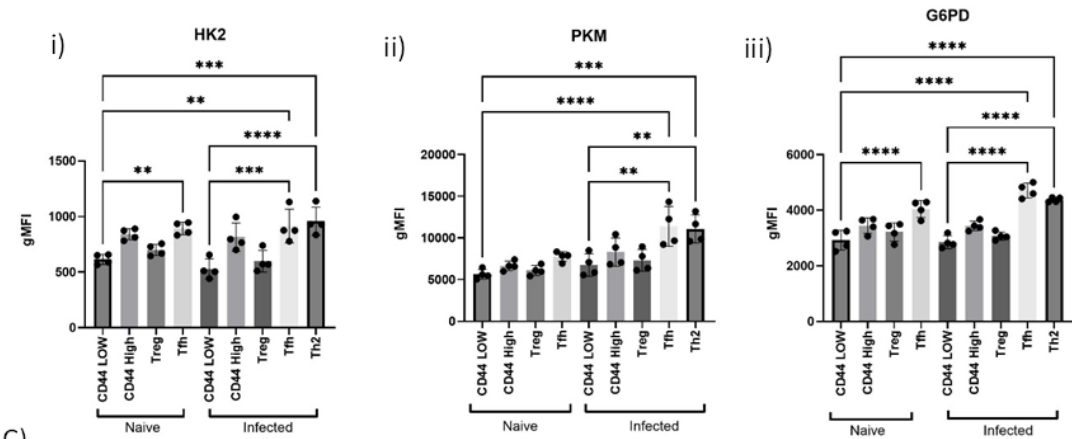
C57BL/6 mice were infected with 200 L3 *H. polygyrus* larvae, and 14 days post-infection, the mLN were removed. A) Representative graph of key transporter pathways involved in T cells. B) Representative graphs of i) Glut1, ii) CD98 and iii) CD71 gMFI. C) Representative GLUT1, CD98 and CD71 histograms in both naïve and infected groups. Graphed data is from 1 experiment (n=4/5) shown with mean +/- SD. This data represents three repeated experiments with n = 4/5 mice per experiment. Statistical significance was determined using the one-way ANOVA with Tukey's post-test for multiple comparisons in groups where

data was deemed normally distributed, as distinguished through the Shapiro-Wilk test. When data was not normally distributed, the Kruskal-Wallis with post hoc Dunn's Test was conducted between groups. Significance was denoted by * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

A)



B)



C)

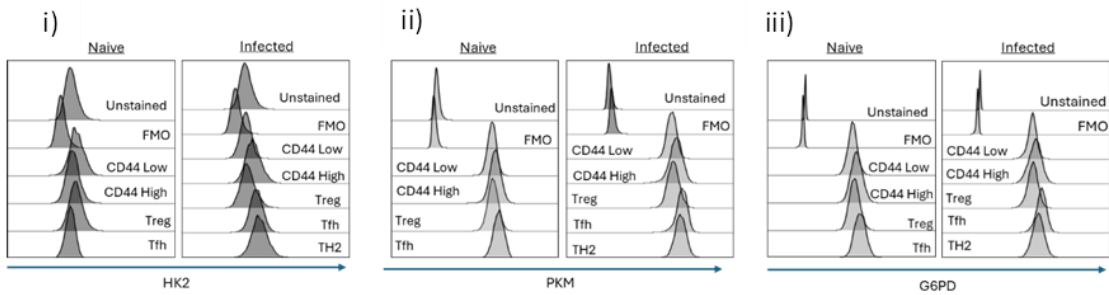


Figure 3.9. Tfh Cells Upregulate Expression of Enzymes Associated with Glycolysis and PPP During Differentiation Compared to Naïve T cells (Uninfected)

C57BL/6 mice were infected with 200 L3 *H. polygyrus* larvae, and 14 days post-infection, the mLN were removed. A) Representative graph of key enzymes associated with glycolytic and PPP pathways involved in T cells. B) Representative graphs of i) HK2, ii) PKM and iii) G6PD gMFI. C) Representative histograms of HK2, PKM and G6PD in both naïve and infected groups. Graphed data is from 1 experiment (n=4/5) shown with mean +/- SD. This data represents three repeated experiments with n = 4/5 mice per experiment. Statistical significance was determined using a one-way ANOVA with Tukey's post-test for multiple comparisons in groups where data was deemed normally distributed as distinguished through the Shapiro-Wilk test. When data was not normally distributed, the Kruskal-Wallis with post hoc Dunn's Test was conducted between groups. Significance was denoted by * p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001.

3.3.5 Tfh Cells Exhibit Similar Glycolytic Metabolic Phenotype as Th2 Cells

Based on the increased expression of GLUT1, the main glucose transporter, and previous work in our lab suggesting Tfh cells have high 2-NBDG uptake compared to Th2 cells, (Heieis, 2019), it was decided to assess the role of glycolysis in Tfh cells. To do this, METFLOW was utilised to determine if the expression of key glycolytic enzymes was higher in Tfh cells than other CD4 T cell subsets in the mLN (Ahl et al., 2020). I assessed the expression of HK2, which is a key enzyme that facilitates glucose uptake (Zur et al., 2025) and directs glucose metabolism into both glycolysis and the pentose phosphate pathway. PKM, a downstream glycolytic enzyme, catalyses the final step of glycolysis by converting phosphoenolpyruvate to pyruvate, and G6PD indicates pentose phosphate pathway activity (Liu et al., 2023a). The Metflow analysis revealed that Tfh and Th2 cells upregulate the expression of HK2, PKM, and G6PD compared to naïve T cells (Figure 3.9 B (i)-(iii)); however, no difference was observed between Tfh from the naïve uninfected and *H. polygyrus* -infected groups, or between Th2 and Tfh cells.

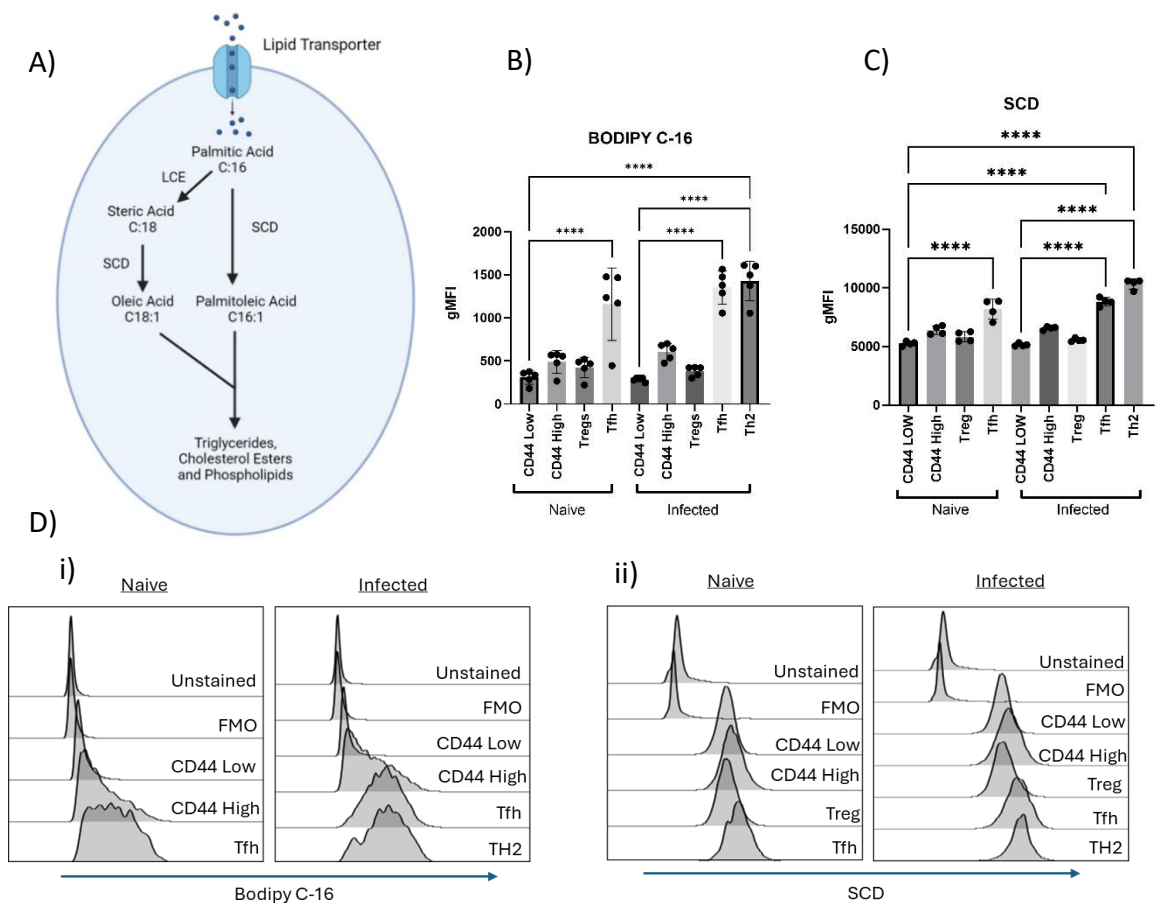


Figure 3.10; Tfh cells increase the Uptake and enhance the enzyme levels associated with processing Palmitic acid compared to Naïve T cells (Uninfected)

C57BL/6 mice were infected with 200 L3 *H. polygyrus* larvae, and 14 days post-infection, the mLN were removed. A) Representative diagram of fatty acid uptake in T cells. B) Representative graphs of BODIPY FL C-16 uptake gMFI. C) Representative graphs of SCD expression (gMFI). D) Representative histograms of BODIPY C-16 (i) and SCD expression (ii) in both naïve and infected groups. Graphed data is from 1 experiment (n=4/5) shown with mean $\pm$ SD. This data represents three repeated experiments with n = 4/5 mice per experiment. Statistical significance was determined using a one-way ANOVA with Tukey's post-test for multiple comparisons in groups where data was deemed normally distributed as distinguished through the Shapiro-Wilk test. When data was not normally distributed, the Kruskal-Wallis with post hoc Dunn's Test was conducted between groups. Significance was denoted by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.3.6 Th2 cells increase Neutral Lipid Accumulation compared to Tfh cells

Once it became clear that glycolytic enzymes were enriched in Tfh cells over naïve T cells, I turned my attention to exploring the link between glycolysis and fatty acid synthesis (FAS). In T cells, this connection is crucial for supporting proliferation, effector functions, and differentiation (Almeida et al., 2016). Previously, Heieis et al. (2019) showed that Tfh cells increase BODIPY FL C16 uptake compared to non-Tfh cells. This is important as it suggests that Tfh cells transport lipid to fuel their metabolic requirements. The increase in glycolysis and FAS may be derived from mTOR activation, which enhances both glycolysis and lipid synthesis (Waickman and Powell, 2012). The uptake of (C16-Bodipy) fatty acids into Tfh and Th2 cells was indeed significantly increased compared with naïve and T regulatory cells (Figure 3.10 B), however there was no significant difference in the uptake of lipid between Tfh and Th2 cells, possibly suggesting a similar requirement for lipid uptake in both cell types.

Understanding of such an increase in lipid uptake posed a key question: do Tfh cells use, store or oxidise the transported lipid? Each of these pathways can result in different effector functions, so it was of interest to determine which Tfh preferred in the context of *H. polygyrus* infection. To address this question, the expression of key enzymes associated with FAS-defined pathways was determined, even though this does not allow determination of flux through them. First, SCD-1, the enzyme responsible for processing C-16 fatty acids, involved in the breakdown of saturated fatty acids into monounsaturated fatty acids, for use by the cells and/or prevention of redox-induced ferroptosis or autophagy through BENCN1 and PIK3C3-dependent processes, was examined. BENCN1 and PIK3C3-dependent autophagy is a process whereby cells degrade and recycle their own components to maintain homeostasis (Bankaitis, 2015, Paton and Ntambi, 2009). The results highlight increased expression of SCD-1 in Tfh and Th2 cells compared with Treg and naïve T cells. However, there is no difference in expression between uninfected and *H. polygyrus* -infected Tfh cells (Figure 3.10A). These data suggest the importance of SCD to both of these effector CD4⁺ cell types and highlight the potential requirement of this enzyme in the processing of transported lipid. This finding is consistent with the BODIPY FL C-16 uptake data, which highlights the role of fatty acids in both Tfh and Th2 cells.

At this point, I have shown the importance of transport and elucidated the possible processing of lipids through SCD, but many other branches of fatty acid metabolism exist.

To understand whether the lipid was being processed or stored, the role of fatty acid storage, known as neutral lipid storage, was explored using the neutral lipid stain LipidTox. This stain, analysed through flow cytometry, allows for the assessment of lipid abundance at single-cell resolution.

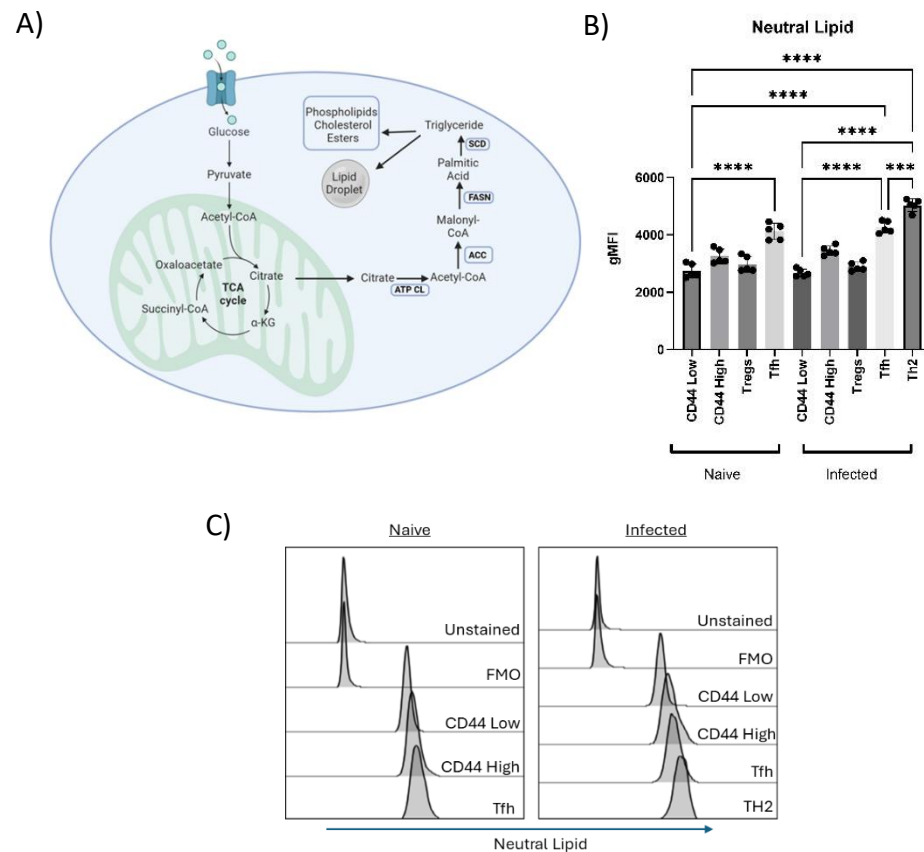


Figure 3.11. Tfh cells have Increased Storage of Neutral Lipids Compared to Naïve T cells (Uninfected)

C57BL/6 mice were infected with 200 L3 *H. polygyrus* larvae, and 14 days post-infection, the mLN were removed. A) Representative fatty acid metabolism/ neutral lipid storage diagram in T cells. B) Representative graphs of neutral lipid stain (LipidTox) gMFI. C) Representative histograms of neutral lipid abundance in both naïve and infected groups. Graphed data is from 1 experiment (n=4/5) shown with mean +/- SD. This data represents three repeated experiments with n = 4/5 mice per experiment. Statistical significance was determined using a one-way ANOVA with Tukey's post-test for multiple comparisons in groups where data was deemed normally distributed as distinguished through the Shapiro-Wilk test. When data was not normally distributed, the Kruskal-Wallis with post hoc Dunn's Test was conducted between groups. Significance was denoted by * p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001.

This analysis showed that Tfh and Th2 cells had increased neutral lipid accumulation compared to naïve T and Treg cells. This is consistent with previous findings in this thesis, which state that Tfh and Th2 cells increase lipid uptake through C-16 BODIPY (Figure 3.10 A&B) and the expression of the enzyme SCD-1. Interestingly, Th2 cells stored significantly more neutral lipids than Tfh cells (Figure 3.11 B), even though their uptake of saturated fatty acids was similar. This finding may result from many factors, such as alternative lipid accumulation (through uptake through lipoproteins (e.g., LDL, VLDL), uptake of free fatty acids bound to albumin or lipid-containing apoptotic bodies or microparticles) or increased transport of citrate into the fatty acid synthesis pathway in Th2 cells or increased breakdown of fatty acid through β -oxidation by Tfh cells.

To explore these possibilities, Metflow was employed to assess the expression of key enzymes associated with citrate transport out of the upper TCA cycle and into the cytosol for fatty acid synthesis, namely ATP citrate lyase (ATP CL), fatty acid synthase (FAS), and acetyl-CoA carboxylase (ACC). Interestingly, ATP CL was upregulated in both Tfh and Th2 cells compared to naïve T and T regulatory cells (Figure 3.12A). This presumably reflects that ATP CL is important in early T cell activation and results in the induction of IRF4, a key TF required for the differentiation of Tfh and Th2 cells (Vaughn and Haviland, 2021, Krishnamoorthy et al., 2017).

When compared to naïve T cells, Tfh and Th2 exhibit increased expression of Acetyl CoA carboxylase; however, Th2, unlike Tfh cells, also significantly increases the expression of FAS compared to naïve T cells (Figure 3.12 B&C). This aligns with the higher accumulation of neutral lipids observed in Th2 cells (Figure 3.11 B). This increase in FAS was not present in Tfh cells compared to naïve T cells, and it should also be noted that there was no significant difference in FAS abundance between Tfh and Th2 cells. Furthermore, enzymes involved in the breakdown of citrate through the TCA such as aspartate aminotransferase (ASA) and also for the breakdown of glutamate into the TCA, such as isocitrate dehydrogenase (IDH2) are present, highlighting the probable convergence of multiple different metabolic pathways at this juncture (Figure 3.13 B&C) that may be fuelling *de novo* lipogenesis.

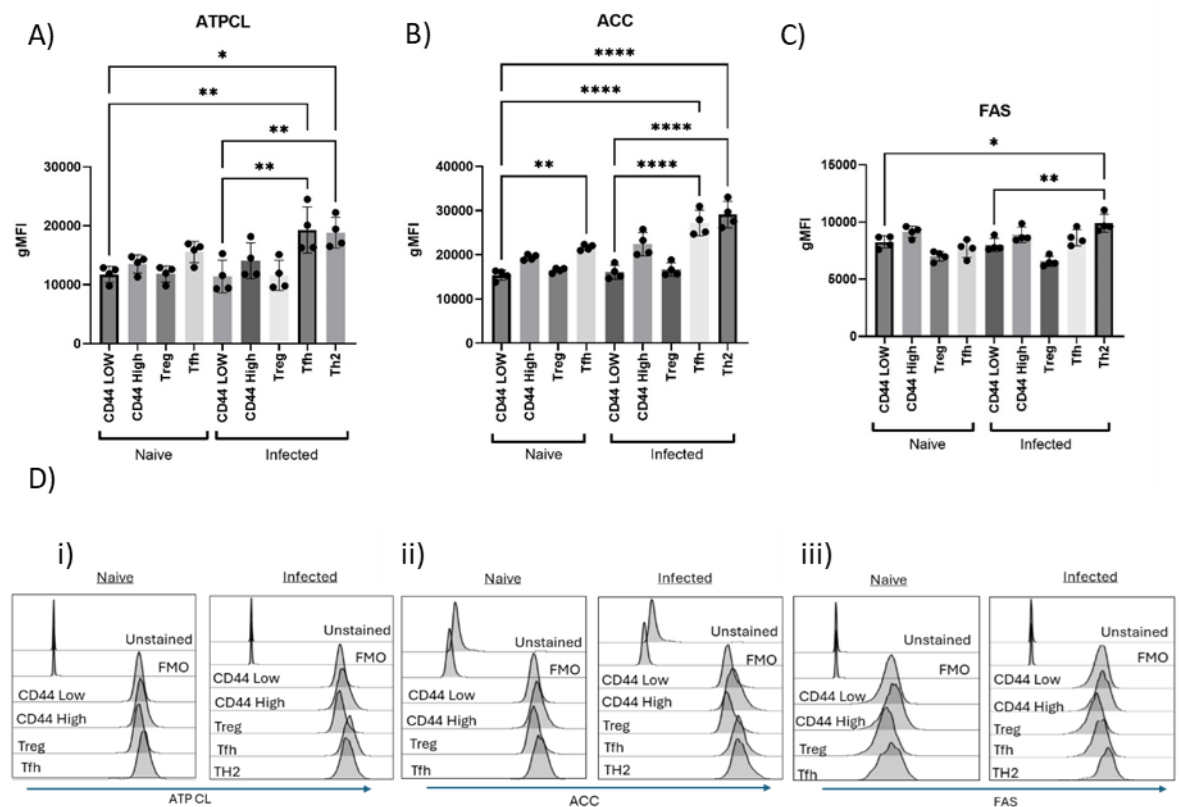


Figure 3.12. Tfh cells have increased expression of enzymes associated with the processing of glycolysis products to fuel *De novo* fatty Acid synthesis Compared to Naïve T cells (Uninfected)

C57BL/6 mice were infected with 200 L3 *H. polygyrus* larvae, and 14 days post-infection, the mLN were removed. A) Representative graphs of ATP Citrate Lyase gMFI. B) Representative graphs of Acetyl CoA carboxylase gMFI, C) Representative graphs of Fatty Acid Synthase. D) Representative histograms of ATP CL (i), ACC (ii), and FAS (iii) abundance in both naïve and infected groups. Graphed data is from 1 experiment (n=4/5) shown with mean $\pm$ SD. This data represents three repeated experiments with n = 4/5 mice per experiment. Statistical significance was determined using a one-way ANOVA with Tukey's post-test for multiple comparisons in groups where data was deemed normally distributed as distinguished through the Shapiro-Wilk test. When data was not normally distributed, the Kruskal-Wallis with post hoc Dunn's Test was conducted between groups. Significance was denoted by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.3.7 Tfh Cells Exhibit Similar FAO/Cytoplasmic Citrate Shuttling Metabolic Phenotype as Th2 cells

To further understand the difference in neutral lipid accumulation between Tfh and Th2, the expression of enzymes associated with the β -oxidation of fatty acids was examined, especially as it was previously reported that FAO is downregulated in Tfh in the spleen due to decreased levels of FA-conjugated carnitines (Gong et al., 2023). Thus, it was decided to test whether this was a location-dependent form of metabolism, given the high abundance of free fatty acids present within the mucosal setting (Wisniewski et al., 2019). Rather, the levels of CPT1A and ACADM were increased in Tfh compared to naïve T cells. These enzymes are both involved in the breakdown of fatty acids in the mitochondria, and interestingly, the levels of FAO enzymes were not increased in Tfh over Th2 cells. Thus, suggesting that higher levels of neutral lipid accumulation in Th2 cells may not be attributed to a decrease/lack of β -oxidation compared to Tfh cells (Figure 3.14 B & C). This may suggest an alternative metabolic phenotype active in Th2 cells compared to Tfh; however, further research is required to understand these differences, which will be explored in more detail in this thesis.

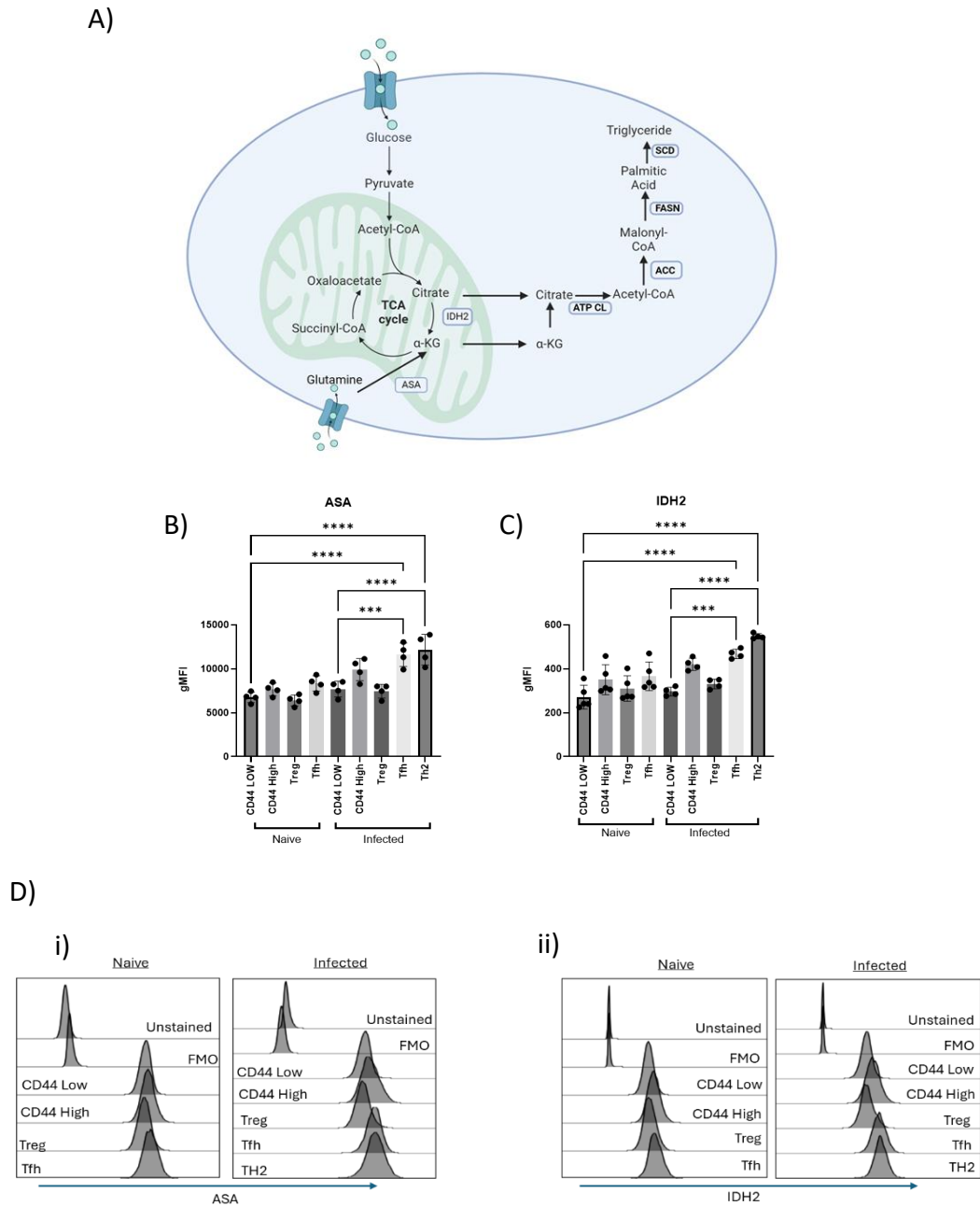


Figure 3.13. Tfh cells have increased levels of enzymes associated with the processing of Glutamine and Citrate into the citric acid shuttle Compared to Naïve T cells (Uninfected)

C57BL/6 mice were infected with 200 L3 *H. polygyrus* larvae, and 14 days post-infection, the mLN were removed. A) Representative diagram of ASA and IDH2 metabolic pathways converging within the TCA cycle. B) Representative graphs of ASA gMFI, C) IDH2 gMFI. D) Representative histograms of ASA (i) and IDH2 (ii) abundance in both naïve and infected groups. Graphed data is from 1 experiment (n=4/5) shown with mean $\pm$ SD. This data represents three repeated experiments with n = 4/5 mice per experiment. Statistical significance was determined using a one-way ANOVA with Tukey's post-test for multiple comparisons in groups where data was deemed normally distributed as distinguished through the Shapiro-Wilk test. Significance was denoted by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

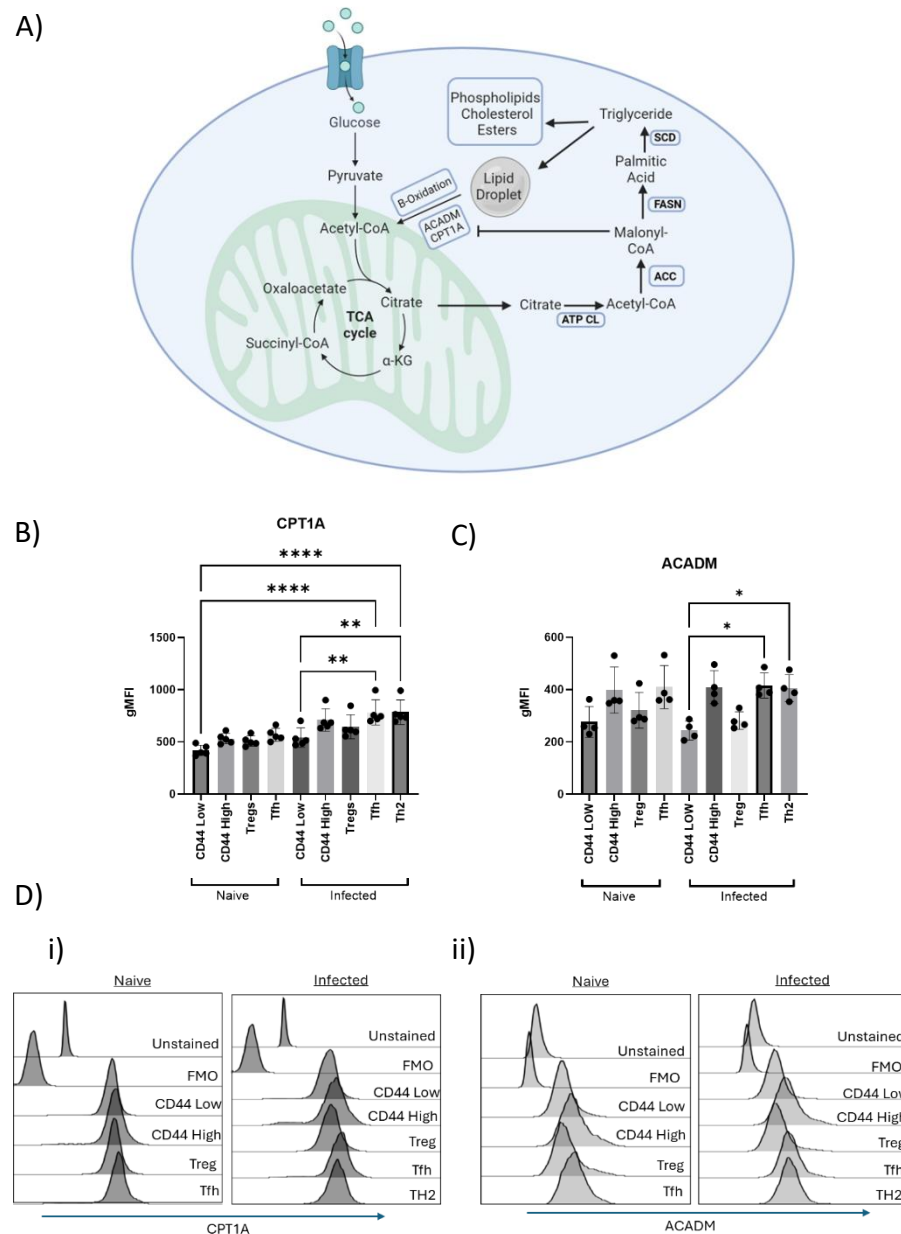


Figure 3.14: Tfh Cells increase the abundance of enzymes associated with the breakdown of lipids as a fuel source compared to Naïve T cells (Uninfected)

C57BL/6 mice were infected with 200 L3 *H. polygyrus* larvae, and 14 days post-infection, the mLN were removed. A) Representative diagram of ASA and IDH2 metabolic pathways converging within the TCA cycle. B) Representative graphs of CPT1A gMFI, C) Representative graphs of ACADM gMFI. D) Representative histograms of CPT1A (i) and ACADM (ii) abundance in both naïve and infected groups. Graphed data is from 1 experiment (n=4/5) shown with mean $\pm$ SD. This data represents three repeated experiments with n = 4/5 mice per experiment. Statistical significance was determined using a one-way ANOVA with Tukey's post-test for multiple comparisons in groups where data was deemed normally distributed, as distinguished through the Shapiro-Wilk test. When data was not normally distributed, the Kruskal-Wallis with post hoc Dunn's Test was conducted between groups. Significance was denoted by * p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001.

This chapter demonstrates that Tfh cell expansion occurs in the small intestine draining mLNs, where these cells exhibit active yet reserved proliferation compared to Th2 cells. While Tfh and Th2 cells exhibit several shared metabolic features, their requirements are not identical. The data reveal important distinctions in immunometabolism among T cell subsets. For example, variations in effector enzyme expression suggest that Tfh and Th2 cells may utilise different metabolic pathways compared to regulatory and naïve T cells. Furthermore, Th2 cells accumulate higher expression of CD98 and elevated levels of neutral lipids than Tfh cells, a difference that merits further investigation.

3.4 Discussion

The role of Tfh cells in *H. polygyrus* infection has been well-documented, although their immunometabolism is not yet fully understood. Initial research into Tfh cells suggested that they mainly produced IL-21 to instruct B cell responses and did not express genes associated with Th1 (IFN- γ or T-bet), Th2 (IL-4 or GATA3), due to the repression driven by Bcl-6 expression. They were also shown not to produce IL-4, IL-10, IL-17 or IFN- γ (Nurieva et al., 2008). This data aligned with results shown in the human Tfh cells, suggesting the same mechanism of action across the species (Kim et al., 2004, Chtanova et al., 2004). This theory has since been proven incorrect, as Tfh cytokine production depends not only on the specific environment but also on the type of stimulation. Thus, Tfh cells can take on a flavour of the relevant T helper cell response through expression of different TFs and production of IL-4, IL-10, IFN- γ and IL-17 to assist class-switching and the GC response (King and Mohrs, 2009, Olatunde et al., 2021).

This suggests that there are alternative transcriptional profiles for each class/function of Tfh cells, resulting in the differential expression of TFs that could alter cell metabolism by upregulating pathways linked to their expression. This is what was of interest, as modulation of these subtle differences may alter their function to boost or decrease their Tfh response in an environment-dependent manner, and potentially, this could allow shutting down of a specific branch of the Tfh cell response without blocking their function when required.

Research has highlighted differences in the ontogeny of Tfh cell differentiation, particularly in the Th2 setting. For example, it has been shown that Tfh cells differentiate from Th2 cells in response to helminth antigens and that Tfh cells in the helminth setting produce the most significant volumes of IL-4 (King and Mohrs, 2009, Glatman Zaretsky et al., 2009). The ability of Tfh cells to differentiate and produce cytokines is intrinsically linked to their metabolism and can be altered through manipulation. Specifically, Tfh cell differentiation and IL-21 production are inherently linked to glycolysis, and modulation of this metabolic pathway drives decreased responses (Dong et al., 2019).

The potential range of Tfh cell phenotypes may explain the contradictory data relating to our understanding of Tfh immunometabolism. Thus, whilst some studies suggest that oxidative phosphorylation is critical in Tfh differentiation (Ray et al., 2015), others report that glycolysis and lipid metabolism are crucial for their differentiation and function. This

can be controlled in part by hypoxia, which drives HIF-1 α -dependent induction of glycolytic genes, to increase/suppress Tfh cell differentiation (Huang et al., 2022). It is well-established that the metabolic environment surrounding CD4⁺ T cells can influence their phenotype and function; therefore, a core aim was to understand how type 2 inflammation affects this.

3.4.1 Tfh Cells Expansion occurs in the Small Intestine Draining Lymph Nodes During *H. polygyrus* Infection

Consistent with previous reports (King and Mohrs, 2009), a significant increase in the number and frequency of Tfh cells was found between infected and naïve uninfected mice (Figure 3.2). Interestingly, a population of Tfh cells appeared to exist in the absence of infection. These cells are believed to arise spontaneously and appear to be involved in maintaining the gut microbiome through supporting IgA secretion (Fagarasan et al., 2010). These would provide a good control for understanding Tfh immunometabolism during different activation mechanisms.

The effects of *H. polygyrus* driving the activation of T cells (Figure 3.1 B) and the redistribution of naïve T cells into the mLNs during infection (Figure 3.1D) are also congruent with our understanding of how *H. polygyrus* drives systemic changes in T cell populations. Previous research highlighted that during *H. polygyrus* infection leads to naïve T and B cells trafficking to the type 2-reactive small intestine, draining mLNs, and depleting populations in other peripheral LNs (King et al., 2017). This systemic redistribution is intriguing, as it could be predicted that with a large cellular influx during infection, nutrient availability may be altered to meet the different metabolic requirements of each T cell subset.

H. polygyrus infection drives systemic changes in the host (Mohrs et al., 2005) and this new data reveals that the Tfh cell expansion is localised to the small intestine, draining LNs within the mesenteric branch of LNs in C57BL/6 mice. This reflects that *H. polygyrus* infects the small intestine, and its secretory products, such as HES, may indirectly activate CD4⁺ T cells in proximal sites (McManus, 2022). Notably, there was no increase in the Tfh population in the large intestine draining LN.

Initial analysis of Tfh cells and their relationship to other T cell subsets in the mLN, focused on categorising all effector cells based on CD44 expression (CD4⁺, non-Tfh,

CD44^{High}), without segregation of subtypes (Th2, Treg, etc.). However, this proved too broad a group, which was felt may miss key metabolic features of different subsets or inadvertently skew the characterisation, based on one particular cell type. Thus, it was decided to analyse Th2, Tregs (Figure 3.3) and CD44^{low} and ^{high} (Figure 3.1) CD4 T cells. This was achieved by specifically gating Th2 cells to exclude Tfh markers (CXCR5^{high} and PD-1^{high}), as IL-4-producing Tfh cells were previously shown to be able to express GATA3, the critical regulator of Th2 differentiation (Glatman Zaretsky et al., 2009). Through gating in this manner, Th2 and Tfh cells can be more precisely distinguished for analysis.

3.4.2 Tfh Cells Favour Active mTOR Signalling

Although the pathways have not been delineated, it has been reported that the input of mTOR complex signalling is required for Tfh cell development. The role of both mTORC1&2 complexes was demonstrated to be vital for Tfh cell responses, as the depletion of the corresponding regulators, RAPTOR and RICTOR, impaired Tfh responses, with mTORC2 mediating Foxo1 inhibition to allow for Tfh differentiation. Moreover, mTOR links ICOS and GLUT1 to the metabolic phenotype of these cells (Zeng et al., 2016) This data highlights the importance of mTOR signalling in Tfh cell responses; however, to date, work has focused on the Peyer patches and spleen and not in the Th2 context, so a goal was to understand if the mTOR signature in this inflammatory environment was similar to that previously described (Zeng et al., 2016). Compared to naïve T cells, Phos-Akt and Phos-S6 are upregulated in Tfh cells; however, in the context of S6, the protein abundance also increases, hence highlighting that increased phosphorylation may be the result of enhanced protein availability and is not directly indicative of boosted mTOR activation.

This finding may reflect a critical stage in controlling Tfh differentiation *in vivo*, as decreased mTORC-1 expression is associated with the ability of HIF-1 α to control Tfh differentiation through suppressing mTORC-1 and MYC-related pathways and driving cycle arrest, which may affect S6 phosphorylation to a higher degree than that of AKT (Huang et al., 2022).

A possible limitation to my work was the choice of which residues for phosphorylation were chosen. For a more direct readout of mTOR1 activity, pS6K1 Thr389 or p4E-BP1 Thr37/46 is preferred over S6 Ser235/236 if possible. Particularly, p70 S6 kinase (S6K1)

Thr389, which is considered the most direct substrate of mTORC1 (Kim et al., 2013) . Phosphorylation here reliably indicates mTORC1 activity, however though when this assay was developed our question was more general activation of the mTOR complex as a whole rather than understanding specific differences between mTORC1 and mTORC2.

Further limitations of the experimental design here meant that I was only able to assess the mTOR activation regarding CD44^{high} CD4⁺ T cells (activated) rather than the distinct Th2 and Treg populations; however, previous research highlights the importance of mTOR2 in the differentiation of Th2 cells (Delgoffe et al., 2011) congruent with the current finding of high levels of Phos-AKT. Previously, it was shown that Th1 cells have higher mTOR activation than Tfh cells (Ray et al., 2015). Further work is needed to understand the level of mTOR activation between Tfh and Th2 cells during helminth infection, as no data exist comparing these two cell types in this context. However, due to the similar responses of Th2 and Tfh cells established here, a similar pattern of favouring mTORC2 over mTORC1 could be predicted (Glatman Zaretsky et al., 2009)

3.4.3 Tfh Cells are Less Proliferative and have Reduced Nutrient Transporter Expression compared to Th2 cells

Our lab and others reported that compared with non-Tfh cells, Tfh cells favoured higher Ki-67 expression and were more proliferative (Heieis, 2019, Luthje et al., 2012). These new results now show that whilst Tfh cells show higher Ki-67 expression than naïve T cells, they have significantly lower expression of this proliferation marker than Th2 cells. This could arise from response dynamics, where Tfh cells actively limit proliferation through stringent regulation to drive competition for selecting high-affinity B cells in the GC response (Crotty, 2019).

The microenvironment around a cell is essential for providing nutrients, whilst cellular transporters facilitate their uptake. During T-cell activation, mTOR signalling drives the increased expression of key transporters, CD71 and CD98 and the translocation of Glut1 to the cell surface (Waickman and Powell, 2012). Consistent with this, these new studies indicate higher expression of all these transporters in Tfh relative to naïve CD4 T cells. Interestingly, Th2 cells exhibited increased CD98 compared with Tfh cells, highlighting a relatively lower requirement for this amino acid transporter in Tfh cells. Interestingly, CD98 has also been implicated in regulating migration through β 1 integrin, and given the evident differences in migration needs of Tfh and Th2, this may explain some of the

observed differences. Specifically, CD98 acts as a molecular facilitator that enhances the activation of integrins, activation of Akt and Rac GTPase, which all contribute to survival and emigrational capacity (Feral et al., 2005, Warren et al., 2000, Cantor and Ginsberg, 2012).

3.4.4 Tfh Cells express similar levels of glycolytic enzymes compared to Th2 cells

Elevated proliferation is linked to anabolic metabolism. Consistent with this, previous findings involving key receptors and TFs associated with Tfh cells highlight their propensity to carry out glycolysis (Zeng et al., 2016, Oestreich et al., 2014, Lin et al., 2024). Reflecting this, the present data highlight increased levels of the key glycolytic enzymes, HK2 and PKM (PKM2), in Tfh and Th2 cells compared to naïve, CD44 high effector, and Treg cells, suggesting that glycolysis may be activated in these cells. Furthermore, this lab also previously reported that Tfh cells show increased 2-NBDG (a glucose analogue) uptake (Heieis, 2019), however emerging evidence suggests that the use of the 2-NBDG assay alone is unreliable for measuring glucose uptake (Sinclair et al., 2020), and thus further work should be carried out to determine the requirement of glycolysis during *H. polygyrus* infection for Tfh cells.

Interestingly, in the MRL/lpr mouse model of lupus, PKM2- a critical regulatory glycolytic enzyme – has been shown to regulate Tfh differentiation. It was reported that decreased PKM2 resulted in decreased Bcl-6 expression, stunting glycolysis, which ultimately led to a diminished Tfh cell response (Lin et al., 2024). Whether this is a Tfh cell response solely restricted to the lupus model or a general response has yet to be defined; however, it highlights the potential to modulate the Tfh cell response through immunometabolic manipulation. This could yield very promising therapeutic potential.

The relationship between Bcl-6 and glycolysis is complex and appears to depend on both environmental context and the transcriptional programs operating within the cells in which Bcl-6 is expressed. Previous studies have reported that Bcl-6 can repress glycolysis in *in vitro*–polarised Th1 cells. One plausible mechanism for this effect involves the modulation of Bcl-6 expression and its downstream impact on T-bet signalling. Bcl-6, a transcriptional repressor classically associated with the Tfh cell phenotype, has been shown to inhibit the expression of *Tbx21* (encoding T-bet) by directly binding its promoter region, thereby suppressing transcription (Oestreich et al., 2012). This repressive mechanism may extend

beyond Th1 cells. For instance, Lin et al. (2024) observed that decreased Bcl-6 levels in Tfh cells correlated with enhanced IL-2 signalling and increased glycolytic activity. This finding suggests that the elevated glycolysis typically observed in Th1 cells, compared to Tfh cells, may be partly driven by Bcl-6-mediated repression (Lin et al., 2024)

Earlier studies proposed that the divergence between Th1 and Tfh phenotypes is influenced by the IL-2–mTORC1 axis (Ray et al., 2015). IL-2 is a potent activator of mTORC1, a central metabolic regulator that promotes glycolysis in effector T cells. In contrast, Bcl-6 antagonizes mTORC1 signalling (Yi et al., 2017). Under conditions of limited IL-2, reduced STAT5 activation may further contribute to this metabolic shift. STAT5 is a key TF required for the expression of *Tbx21* and glycolysis-related genes such as *Glut1* and *Hk2* (Villarino et al., 2022).

In this context, even modest Bcl-6 expression may become functionally significant. Bcl-6 could repress glycolysis both directly, by inhibiting key glycolytic genes, and indirectly, through the suppression of T-bet expression. This suggests that low IL-2 signalling and increased Bcl-6 activity may act synergistically to enforce a glycolysis-repressed state, serving as a potential Bcl-6-dependent metabolic checkpoint during Th1 differentiation. This mechanism may facilitate the transition from a Th1 to a Tfh phenotype, as previously reported (Ray et al., 2015; Nakayamada et al., 2011).

Further supporting this theory, Tregs have been shown to reduce IL-2 availability at the T–B cell border (Leon et al., 2014), potentially creating a metabolic niche that favours Tfh cell differentiation through reduced IL-2–mTORC1 signalling and enhanced Bcl-6 function. Taken together with the findings of Ray et al. (2015) and others, this supports the idea that IL-2 availability acts as a key determinant in the balance between Bcl-6 and T-bet expression, modulating mTORC1 activity and glycolytic capacity in a feedback loop. Notably, our data are consistent with this model, as we observe increased mTORC2-associated phosphorylation relative to mTORC1, further suggesting a metabolic bias favouring Tfh differentiation. This would shed light on how there is a difference between glycolytic rates observed between Tfh cells and Th1, and how this balance between these two TFs may dictate the requirement of glycolysis in a particular cell type (T-bet^{high}/Bcl-6^{Low} = high glycolysis and T-bet^{low}/Bcl-6^{High} = lower glycolysis)

After understanding that Tfh exhibited increased expression of genes associated with glycolysis and the Pentose Phosphate Pathways relative to naïve T cells, it was decided to

ascertain whether pyruvate was shuttled into active TCA and if citrate was shuttled out of the mitochondria into the cytosol to take part in fatty acid synthesis pathways as previous reports have highlighted the importance of oxidative phosphorylation, fatty acid synthesis and active TCA cycle in Tfh cell metabolism (Mayberry et al., 2022). This study indicated some interesting features in the processing and uptake of lipids within Tfh and Th2 Cells.

3.4.5 Th2 cells have increased neutral lipid accumulation compared to Tfh cells

Thus, in Tfh cells, there is an increase in the expression of key enzymes associated with citrate transport from the mitochondria into fatty acid synthesis, the uptake of fatty acids (C-16 Bodipy), and the processing of these fatty acids into neutral lipids or to be broken down by β -oxidation. Interestingly, the increase in neutral lipid accumulation in Th2 compared to Tfh cells could be a direct consequence of Th2 cells' requirement for sterol metabolism for the production of pregnenolone by Th2 to restore immune homeostasis after infection, as they will require large quantities of sterol precursors for synthesis, which would be stored as neutral lipid to prevent lipid oxidation (Mahata et al., 2014).

Lipids play an essential role in Tfh and Th2 function (Fu et al., 2021). Reflecting this, in mice with hypercholesterolemia, Th2 differentiation is preferred over that of Th1 toward an exogenous antigen challenge (Robertson et al., 2004). Moreover, the use of statins, which block cholesterol metabolism, has been shown to suppress DC-mediated Th2 responses (Inagaki-Katashiba et al., 2019). Indeed, treating asthma, a Th2-hypersensitivity disease, with statins has been shown to drive anti-inflammatory effects through modulation of Th2 cells (Park et al., 2024). These findings suggest a potential metabolic pathway that could be targeted to modulate Th2 function, potentially using existing treatments such as simvastatin, which may aid in improving patients' health in asthma. This treatment has been shown to alleviate inflammation and enhance lung physiology (Zeki et al., 2009). Conversely, Tfh cells do not favour elevated cholesterol levels, and this can impact their expansion and IL-21 production, as cholesterol affects intracellular signalling (Li et al., 2020b). Interestingly, other areas of lipid metabolism are critical in Tfh differentiation and function, such as SCD and PE (Fu et al., 2021, Son et al., 2020)

An issue encountered in these experiments was understanding how these expression data related to metabolic flux. Ideally, *ex vivo* Tfh and Th2 cells would have been used in metabolic assays (Seahorse, etc.); however, it did not prove possible to achieve a sufficient

yield required to run this assay successfully per mouse (van der Windt et al., 2016). In line with the 3Rs of animal licensing in the United Kingdom (Lindsjo et al., 2016), and due to the stresses involved in sorting and processing the cells, we deemed this not to be the correct approach or even representative of the actual metabolism of Tfh cells *in vivo*. Other assays, such as the SCENITH, could have been utilised; however, they presented limitations, as the puromycin readout may not be an indicative measurement for activity in all metabolic pathways in cells (Arguello et al., 2020).

Despite the limitations of cell abundance, we were able to elucidate a critical difference between Tfh and Th2 cells. We found that Th2 cells store significantly higher amounts of neutral lipids than Tfh cells, highlighting that even though Th2 cells can be precursors of Tfh cells, there are different metabolic responses in these cells. The next step in this research would have been to test the functional relevance of the findings by using different metabolic inhibitors to assess the effects of blocking pathways of interest on the differentiation and function of *in vitro*-derived murine Tfh and Th2 cells. However, to date, unlike in the human system for Tfh, murine Tfh cell differentiation is not fully elucidated, and often requires APC to drive differentiation, and this would have led to discrepancies regarding whether our metabolic manipulation had a direct or indirect effect on the Tfh or the APC driving activation (Gao et al., 2020).

Another possible next step would be to explore the flux through pathways of interest to understand the requirements and usage of lipid and glucose by helminth-associated Tfh cells. This could be achieved through new experimental setups such as Click-chemistry, which have been widely used to measure metabolite uptake at the single-cell resolution using flow cytometry, using biorthogonal reactions that can label flux inside single cells *ex vivo* (Pelgrom et al., 2023). This information could guide the approach to *in vivo* blockade of specific metabolic pathways to understand the effect on the immune response towards *H. polygyrus*.

Nevertheless, METFLOW analysis allowed understanding of which pathways were likely involved in the responses of each cell type (Th2 increased uptake/synthesis of neutral lipid, or whether Tfh increased β -oxidation enzyme expression) and perhaps disappointingly but not surprising given their shared development, the results did not suggest critical differences in these pathways. Again, the limitation was that flux could not be measured with this approach. However, the novel findings of this chapter provided a platform to address mechanistically in the following chapters through the use of transcriptomics. By

indicating the key pathways upregulated or downregulated at the gene expression level in the different cell populations, it was hoped that this approach would provide a clearer understanding of the differential Tfh and Th2 immunometabolism during *H. polygyrus* infection.

3.5 Concluding Remarks

Emerging evidence supports that Tfh cells are not a single population but can take on a different flavour based on their progenitor cell in different settings (Schmitt et al., 2014a). At this point, the data have solely focused on the Th2-mediated environment in the gut-draining LNs. However, in subsequent chapters, the differential regulation of immunometabolism will be further addressed in this context (*H. polygyrus*) and the context of IAV infection (Th1-mediated) in the lung. This suggests that the data presented above indicate that, although Tfh and Th2 cells in *H. polygyrus* infection share a common precursor, further divergence and differentiation can alter the metabolic phenotype of both cells. For example, Th2 cells exhibit increased CD98 expression and neutral lipid accumulation compared to Tfh cells, suggesting that Tfh cells are less dependent on these metabolic mechanisms. This provides a clear basis for further unravelling the divergent metabolic signatures of Tfh compared to other T cell subsets in LNs.

Chapter 4: Metabolic and Transcriptional Divergence of Tfh During *Heligmosomoides polygyrus* Infection in the Murine mLNs

4.1 Introduction

In the previous chapter, I investigated the distinctive metabolic pathways of Tfh and Th2 cells on day 14 of *H. polygyrus* infection. This exploration revealed that while these cells share similar profiles for most enzymes in key metabolic pathways, they exhibit unique metabolic features. Notably, Th2 cells exhibit a significant increase in CD71/98 expression and neutral lipid accumulation compared to Tfh cells, which in turn show a marked increase relative to naïve T cells. The progressive differentiation from naive to Tfh to Th2 may be the driver of the shared characteristic of Tfh and Th2 metabolism (Glatman Zaretsky et al., 2009). However, the understanding of why Tfh cells employ different metabolic processes compared to Th2 cells remains ill-defined. In this chapter, I aim to understand possible mechanisms underlying these differences in Tfh and Th2 biology, while further developing our understanding of Tfh cell metabolism in active helminth infection through unbiased analysis of Bulk RNA-seq.

I employed bulk transcriptomics to elucidate the metabolic differences between Tfh cells and other T cells in the mLN of both uninfected and *H. polygyrus* polygyrus-infected mice. This method is known for its precision and comprehensive analysis; although it may not provide definitive functional data, it cannot be overlooked for the insights it provides. It can be used to drive progress in areas of research where limited cell numbers can hinder more comprehensive functional analysis. For example, this method allowed me to discern differences in metabolic gene expression and gain insights into the metabolic pathways that Tfh cells favour in the context of helminth infection compared to other T cell subsets in the mLN.

Previously, bulk transcriptomics has been used to understand the metabolic gene expression profile of Tfh cells in mice and humans. This research has primarily focused on human tonsillar-derived Tfh cells, or, in the murine setting, Tfh cells derived from reactive LNs in the autoimmune context of lupus (Rane, 2020, Gong et al., 2023, Choi et al., 2018). Whilst highlighting key metabolic pathways associated with Tfh cell biology (glycolysis,

OXPHOS) in these contexts, the interpretation of these findings in relation to Tfh cell metabolism during infection may differ, as recently highlighted by the Morel lab (Choi et al., 2018).

They showed that Tfh cells differentiated during lupus pathology are different to those derived in response to a pathogenic stimulus, as inhibition of glycolysis with 2-DG can selectively target autoreactive murine Tfh cells in lupus, whilst preserving exogenous antigen-specific Tfh (Choi et al., 2018). This highlights the unmet need to understand the transcriptional differences, especially metabolically, between Tfh cells from type 1 and type 2 infections, as limited resources exist and a gap remains in our knowledge of whether Tfh cells exhibit transcriptional differences in response to alternative stimuli or microenvironments.

More recently, the Zaph lab attempted to delineate the differences in the heterogeneity of Tfh cells in Th1 and Th2 settings using the nematode parasite infection *Trichuris muris* (*T.muris*). This whipworm parasite infects the cecum of mice and can induce different immunological phenotypes depending on the dosage (High Dosage = Th2, Low Dosage = Th1). This model demonstrated that Tfh cells derived from the low-dose challenge model exhibited cytokine and TF profiles similar to those of Th1 cells, characterised as T-bet⁺ and producing IFN- γ . Conversely, under challenge with high dosage, which drives a Th2-mediated response, Tfh cells display a phenotype more strongly associated with Th2 cells, namely by expressing GATA3 and IL-4 (Zaini et al., 2023). However, this study did not investigate the differential immunometabolism of such Tfh cells and limitations in this research and experimental design (n=2) suggest an unmet need for more robust datasets to delineate the differences between the two settings.

Surprisingly, there has been a dearth of research on the metabolic landscape of Tfh cells in the context of different immune challenges (Th1 vs Th2). This chapter aims to fill this gap by presenting my findings on the transcriptional and metabolic differences observed between naïve murine CD4⁺ T cells, Tfh, and Th2 from the mLNs of day 14 *H. polygyrus* infection. I will employ various forms of analysis to map the immunometabolism of Tfh cells during active helminth infection and compare this with that of Th2 and naïve T cells.

4.2 Aims

- To understand the metabolic transcriptional landscape of Tfh and Th2 cells, compared to naïve T cells, during *H. polygyrus* infection.
- To explore the metabolic differences between Tfh and Th2 cells to better understand the key pathways associated with each cell type.
- To explore the rationale for lower neutral lipid accumulation in Tfh compared to Th2 cells in this context.

4.3 Results

4.3.1 Delineating the Transcriptional Metabolic Profile for Tfh and Th2 Differentiation in the mLNs

To perform transcriptional analysis of Tfh cells in *H. polygyrus*, we isolated Tfh, Th2 and naïve cells from the mLN of infected & uninfected mice, and performed bulk sequencing on each population. To obtain sorted populations of pure Tfh, Th2 and CD44^{low} CD4⁺ T cells, we exploited the antibody panel used in Chapter 3 to identify and separate each cell type. Additionally, we used the B6-4get (IL-4⁺ eGFP⁺) reporter mice, previously discussed in Materials and Methods (Chapter 2.1.1), to isolate IL-4⁺ Th2 cells. Cells were gated based on the parameter shown in Figure 4.1A. CD4⁺ T cells were defined as Live CD4⁺ TCR-B⁺ lymphocytes; naïve T cells as CD4⁺ CXCR5^{Low} PD-1^{Low} IL-4⁻ CD44^{low}; Tfh cells as CD4⁺ CXCR5^{high} PD-1^{high}, and Th2 cells as CD4⁺ CXCR5^{Low} PD-1^{Low} CD44^{high} IL-4⁺ (Figure 4.1A). This gating strategy allowed me to exclude Tfh cells before gating on Th2 cells, as previously it was shown that Tfh cells inside the LN of *H. polygyrus*-infected mice produced the highest levels of IL-4 (King and Mohrs, 2009).

According to the material and methods, I harvested mLNs from naïve and *H. polygyrus*-infected (day 14) B6-4get mice and processed the samples into a single-cell suspension for subsequent flow cytometry sorting. Mouse LNs were carefully pooled to achieve the desired count of 100k cells per sorted population. I successfully sorted the Tfh, Th2, and naïve CD4⁺ (CD44^{low}) T cells from *H. polygyrus*-infected mice, as well as naïve CD4⁺ (CD44^{low}) T cells from uninfected mice, with high purity and the desired abundance. For the *H. polygyrus* samples, 3 mice were pooled to achieve the desired cell count, whilst for the naïve group, a total of 1 mouse per sample was used (Figure 4.1(B)).

Samples were then processed, and mRNA was sent for bulk transcriptomic sequencing. To understand the quality of the sequencing data through initial bioinformatic evaluation, first, I performed PCA component analysis, highlighting that the replicate samples within a particular cohort of T cells grouped and formed distinct clusters (Figure 4.1 (C)). This independent clustering revealed the presence of distinct cell types, and the close clustering of samples within the groups indicated that they shared strong similarities in gene expression profiles. It should be noted that one of the CD44^{low} samples from the uninfected mice presents as a possible outlier and clusters closer with the CD44^{low} samples from *H.*

polygyrus -infected mice. This could be attributed to many factors, and subsequent analysis will determine the effect that this has on the gene expression signature.

To confirm the identity of each sorted population, I tested the expression of markers associated with Tfh and Th2 biology to understand the transcriptional phenotype of each sorted population. This involved screening a panel of “signature” TFs and cytokines associated with both populations. For Tfh cells, I selected HIF-1 α , IL-21, Bcl-6, and IL-4 as signature genes. GATA3, IL-5, IL-13, plus IL-4 were used as signature genes of the Th2 population. This analysis showed that HIF-1 α , IL-21 and Bcl-6 expression were increased in the Tfh cell population compared to all other cells, and this data is consistent with previous findings (Figure 4.1 (D)) (Huang et al., 2022). In contrast, the Th2 cells showed increased expression of GATA3, IL-4, IL-5 and IL-13, as expected (Figure 4.1 (D)) (Fort et al., 2001). Tfh cells also expressed IL-4 and GATA3 (Figure 4.1 (D)), which highlighted the ability of our method to capture heterogeneity, which is consistent with published work reporting that Tfh cells produce the highest levels of IL-4 in the LN during *H. polygyrus* infection compared to other effector cell populations (Th2, etc.) (King and Mohrs, 2009, Zaini et al., 2023). This highlights that the cells sorted were the correct populations and that contamination was low.

The decision to include naïve CD4⁺ T cells from both the uninfected and infected groups enabled me to determine whether the microenvironment could drive the metabolic rewiring of naïve T cells. Previously, *H. polygyrus* infection has been shown to increase the recruitment of naïve CD4⁺ T cells to the mLNs (King et al., 2017). Therefore, it could be suggested that naïve T cells may require increased metabolism to migrate into the mLN.

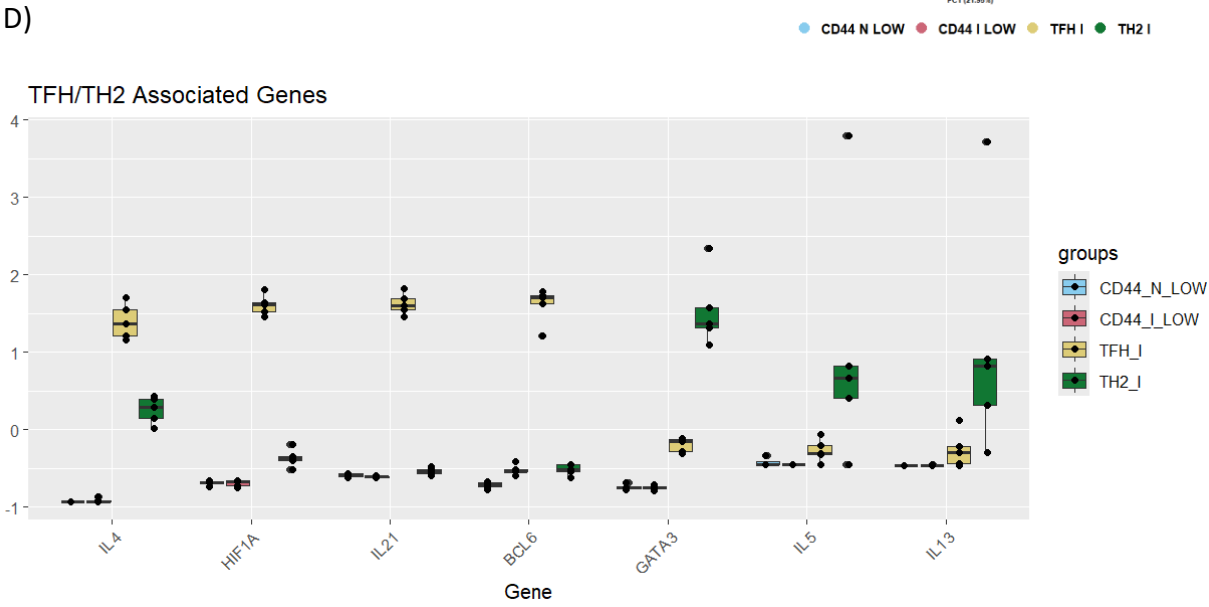
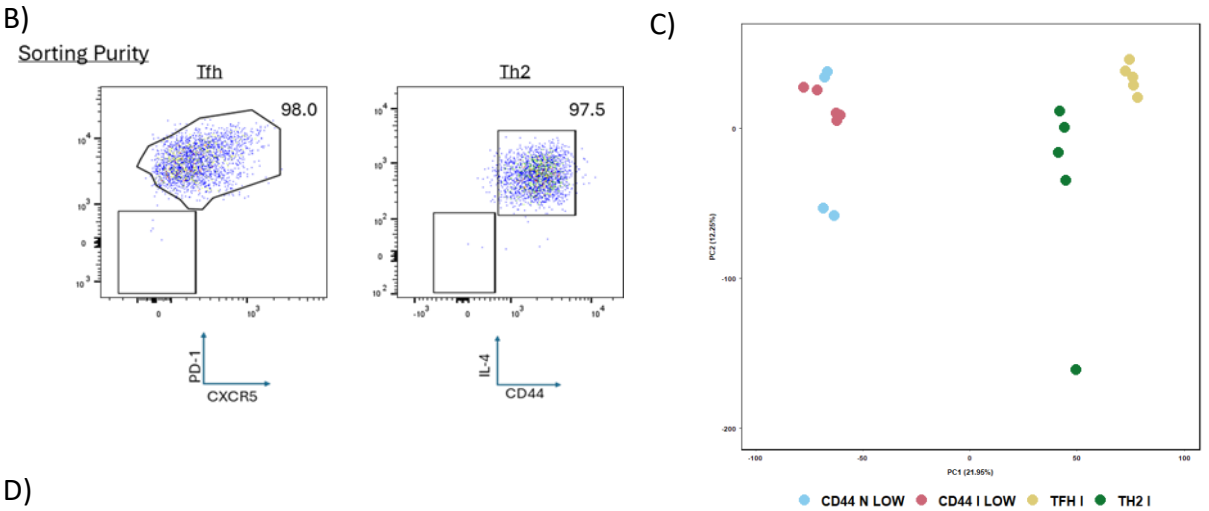
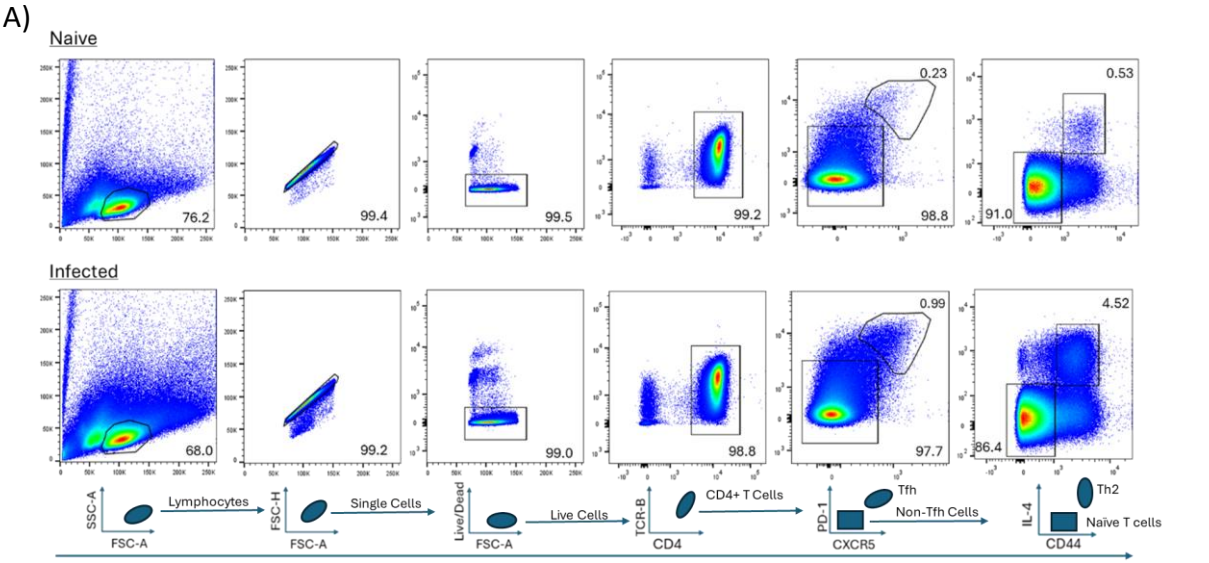


Figure 4.1. Tfh and Th2 populations isolated from the mLN of Uninfected and *H. polygyrus* -infected mice for Bulk RNA Seq.

B6/4-get mice were infected with 200 L3 *H. polygyrus* larvae, and 14 days post-infection, the mLN were removed. Tfh, Th2 and naïve T cells were sorted, and RNA was then extracted and sent for bulk RNA seq. (as detailed in methods). A) Representative gating strategy used to separate Tfh cells (Live CD4⁺ CXCR5^{High}, PD-1^{high} cells), Th2 cells (Live CD4⁺, non-Tfh, CD44^{High}, IL-4^{High}) and naïve CD44^{low} CD4⁺ T cells from mLN samples of uninfected and Infected mice. % highlight abundance of cells within T cell gate. B) Representative Flow scatter plots of sort purity for Tfh and Th2 cells from Infected mice. % highlights the purity of Tfh and Th2 in samples. C) PCA Scatterplot of each sample in the bulk RNAseq transcriptomic analysis. The percentage of total variation explained by each component is given on the x-axis and y-axis as appropriate. All gene expression values were scaled on a gene-by-gene basis using the z-score transformation before the PCA to control the representation of very highly expressed genes. D) Representative Boxplot of Key Tfh and Th2 genes. All significantly differentially expressed genes (p.adj < 0.05, absolute log2 fold > 0.5). Expression levels have been scaled per gene Z-score.

To test this hypothesis, I used SL2 (Searchlight2, a rapid analysis pipeline for bulk RNAseq (Cole et al., 2021) to analyse the CD44^{Low} T cells from the uninfected and *H. polygyrus*-infected mice (Day 14). My investigation revealed diverse transcription profiles among the two naïve T cell groups (Figure 4.2A), with 260 genes upregulated and 154 downregulated (Infected naïve vs uninfected naïve) (Figure 4.2C). I performed hierarchical clustering, which highlighted the diverse nature of gene expression in both cell types (Figure 4.2 (B)). To comprehend the changes observed, I first examined the top 10 up- and down-regulated genes to investigate key differences.

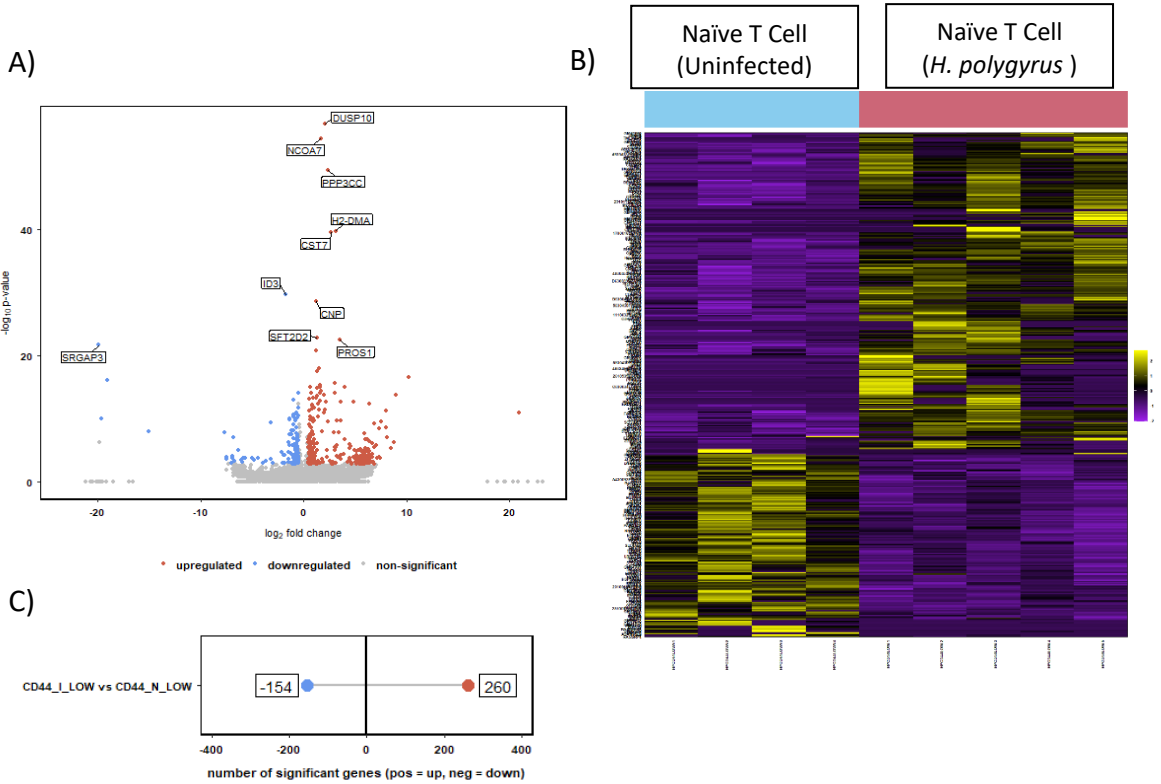
Results highlighted that genes involved in T cell regulation (DUSP10) (Zhang et al., 2004, Zhang et al., 2019), development (SLC39A6, CST7) (Zhao et al., 2019), activation (PPP3CC, PROS1) (Carrera Silva et al., 2013, Zhang et al., 2019) and in response to an inflammatory environment (CST7) were upregulated in naïve T cells from *H. polygyrus*-infected mice compared to naïve cells from uninfected mice (Figure 4.2 (D(i))).

Interestingly, CST7 can drive the differentiation of naïve T cells into Th2 cells, with the help of DUSP5, highlighting how naïve T cells may be primed for Th2 differentiation before receiving an antigenic stimulus (Ning et al., 2024). Conversely, the downregulated genes were mainly involved in T cell survival (ID3, CD5)(Shaw et al., 2022), activation (MPEG1,VSIR,CD5) (Domingues et al., 2016, Ebrahimnezhaddarzi et al., 2022, Li et al., 2017b) and infiltration/trafficking (VASP) (Figure 4.2 (D)(ii)) (Estin et al., 2017).

The decrease in T cell trafficking and migration genes that we observed in naïve T cells in infected mice, compared to those from uninfected mice, is congruent with findings from

King et al., 2017, as it suggests once the T cells have migrated into the mLNs, they decrease transcription associated with infiltration and trafficking. Of note is the decrease in PTPN6, the gene involved in SHP1 expression. A decrease in SHP1 favours increased IL-4 production and Th2 skewing (Johnson et al., 2013), perhaps suggesting that even as a naïve T cell, the microenvironment plays an important role in the skewing towards the type 2 immune response.

Finally, I utilised DAVID analysis to understand the metabolic differences between these two naïve T cell groups. DAVID is an online web server that provides functional enrichment and KEGG pathway analysis of gene lists created through DESeq2 analysis. This highlighted two enriched KEGG pathways associated with naïve T cells in (*H. polygyrus* -infected mice) compared to naïve T cells in (uninfected controls). These were butanoate and tryptophan metabolism (Figure 4.2 (E)).



D) Most Upregulated Genes (P-value)

Gene	log2fold	p	p.adj
DUSP10	2.08	1.530559e-57	2.433741e-53
NCOA7	1.70	2.890582e-55	2.298157e-51
PPP3CC	2.34	3.533828e-50	1.873046e-46
H2-DMA	3.13	1.541910e-40	6.129477e-37
CST7	2.69	2.408017e-40	7.657975e-37
CNP	1.22	2.254016e-29	5.120158e-26
SFT2D2	1.32	1.337034e-23	2.657522e-20
PROS1	3.53	3.048839e-23	5.386621e-20
SLC39A6	1.24	1.536520e-21	2.221110e-18
HS6ST1	1.55	8.932370e-19	1.183613e-15

E) Most Downregulated Genes (P-value)

Gene	log2fold	p	p.adj
ID3	-1.69	1.830390e-30	4.850839e-27
SRGAP3	-19.89	2.120149e-22	3.371250e-19
MPEG1	-19.04	9.118162e-17	9.665859e-14
RBM38	-0.51	7.963003e-15	5.216774e-12
TAGLN2	-0.92	1.230739e-13	5.930298e-11
VSIR	-0.45	4.116301e-13	1.678290e-10
VASP	-0.52	1.787168e-12	6.458581e-10
CD5	-0.67	1.166534e-11	3.637069e-09
RHOF	-0.79	1.680911e-11	5.043051e-09
PTPN6	-0.61	2.201420e-11	6.482366e-09

F) KEGG Pathway Analysis

Term	Count	%	P-Value
Butanoate Metabolism	3	1.2	2.8E(-2)
Tryptophan Metabolism	3	1.2	9.2E(-2)

Figure 4.2. Differential Expression Analysis Between CD44 Low T Cells in Uninfected and *H. polygyrus* Infection (Day 14):

B6/4-get mice were infected with 200 L3 *H. polygyrus* larvae, and 14 days post-infection, the mLNs were removed. Cells were processed and sorted, and the RNA was sent for bulk RNAseq. (as detailed in methods). A) A volcano plot showing differential gene expression between naïve T cells from uninfected animals (left) and naïve T cells from *H. polygyrus* -infected animals (right) ($p_{adj} < 0.05$, absolute $\log_2$ fold > 0.5) are shown in red (Upregulated (right) /blue (Downregulated(Left)) and non-significant genes in grey in the comparison. A positive fold change indicates higher expression in naïve T cells (*H. polygyrus*) than in naïve T cells (uninfected). B) Hierarchically clustered heatmap of the significantly differentially expressed genes

($p_{\text{adj}} < 0.05$, absolute $\log_2$ fold > 0.5) between CD44^{Low} (Naïve) and CD44^{Low} T cells (*H. polygyrus*), showing all replicate samples in the sample groups. Samples are on the x-axis, and genes are on the y-axis. Colour intensity represents expression level, with purple representing low expression and yellow representing high expression. Expression levels have been row-scaled into z-scores. The x- and Y-axis have been hierarchically clustered using Spearman distances, with UPMGA agglomeration and mean reordering. C) Schematic highlighting the number of upregulated and downregulated genes between CD44^{Low} (uninfected) and CD44^{Low} T cells (*H. polygyrus*). Table showing the ten most upregulated (D) and downregulated (E) genes by adjusted p-value in naïve T cells (*H. polygyrus*) than naïve T cells (uninfected). F) Table of KEGG pathway analysis of metabolic genes in naïve CD44^{Low} T cells (*H. polygyrus* vs uninfected). All significantly differentially expressed genes ($p_{\text{adj}} < 0.05$, absolute $\log_2$ fold > 0.5).

To understand the transcriptional changes associated with Tfh differentiation, I next compared the Tfh cells to the naïve T cells from uninfected mice due to the differences observed in Figure 4.2. The reason for comparing Tfh cells to naïve T cells from uninfected mice was that naïve T cells (*H. polygyrus*) already exhibit early signs of microenvironment priming. To better understand the specific changes that occur during differentiation into Tfh cells, I used naïve T cells (uninfected), as they are not influenced by microenvironmental priming caused by the immune response to *H. polygyrus*. This comparison is crucial in understanding the molecular mechanisms underlying Tfh cell differentiation. Initial analysis of the differentially expressed genes between Tfh and naïve T cells from uninfected mice was performed by SL2. The analysis highlighted the differential transcriptional profiles of both cell types (Figure 4.3A), with many upregulated (2353 genes) and downregulated genes (1609 genes) (Figure 4.3C).

To understand the gene differences between the two groups, I first examined the top 10 upregulated and downregulated genes to investigate any noticeable metabolic differences (Figure 4.3(B)). This approach also enabled me to verify that the analysis generated the expected gene signatures associated with the Tfh cell type. Reassuringly, we found ICOS-PDCD-1, HIF-1A, CXCR5, Bcl-6 and Maf were upregulated in Tfh compared to naïve T cells (Figure 4.3 (B)(i)). This is expected as these genes are key in Tfh cell biology and function (Wellford and Schwartzberg, 2024).

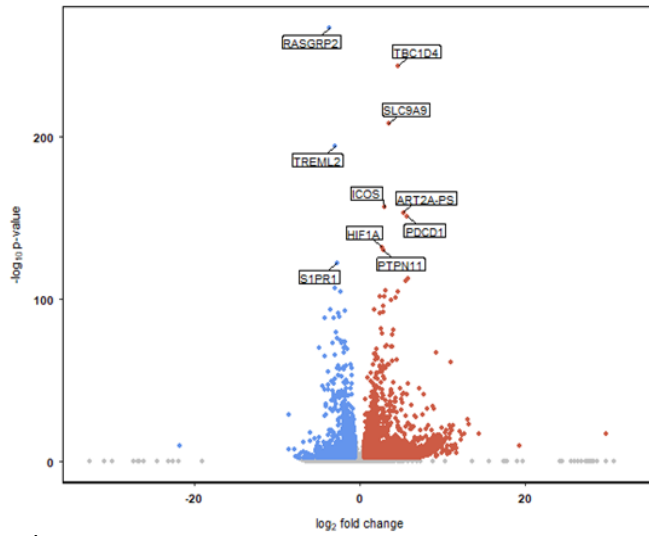
Interestingly, Tfh cells increased expression of SLC9A9 over naïve T cells (Figure 4.3 B(i)), a sodium/hydrogen exchanger essential in recycling transferrin receptors to the cell surface. This is consistent with data from Chapter 3 Results (Figure 3.8) that showed Tfh cells increased CD71 during differentiation from naïve T cells, as previously reported (Kondapalli et al., 2013).

Investigating the top 10 downregulated genes (Tfh vs Naïve Uninfected) presented a different angle of Tfh cell differentiation (Figure 4.3 (B)(ii)). My analysis highlighted gene changes associated with preventing Tfh cell migration. Our data show that CCR7 is one of the top 10 most downregulated genes in Tfh cells compared to naïve T cells. This is consistent with published work showing that decreased CCR7 is a hallmark of Tfh cells and is associated with increased CXCR5; this axis allows Tfh cells to migrate to the B cell follicle (Hardtke et al., 2005). Similarly, the downregulation of S1PR1, the sphingosine phosphate receptor 1, is a crucial step in Tfh cell differentiation and localisation in the GCs. This downregulation is necessary to retain cells within the LN, facilitating their interaction with B cells and promoting the GC reaction (Lee et al., 2015).

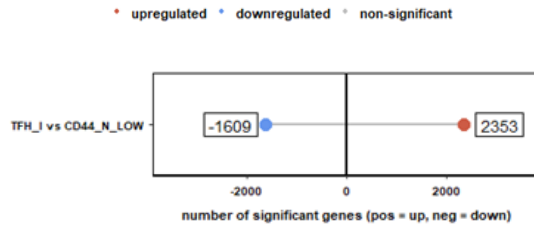
Contrary to the increased metabolic phenotype (transferrin recycling by SLC9A9 in Tfh cells compared to naïve T cells), genes associated with other metabolic signalling pathways were found to be downregulated, including MYC, SLC17A9, and ESYT1. These genes play important roles in T cell metabolism. MYC, for instance, is crucial for amino acid uptake by T cells (amongst other functions such as cell cycle), whilst SLC17A9 is essential for transporting ATP across membranes (Cao et al., 2014, Zhang et al., 2023a). ESYT1 is associated with lipid transport from the endoplasmic reticulum to the plasma membrane and internal storage of calcium (Janer et al., 2024).

I then performed STRING analysis of all differentially expressed genes to identify whether metabolic pathways were changed in the most enriched and downregulated gene lists. However, the output generated by the SL2 pipeline lacked depth and only highlighted pathways associated with the cell cycle, processing of different RNA forms, nuclear division, and mitosis (Figures 4.3 D, E, F). To understand the metabolic landscape of our cells, we utilised DAVID Analysis. This comprehensive tool provided the depth of analysis required to unpick the genes and pathways associated with Tfh cell metabolism, which the output of the SL2 platform lacked (Sherman et al., 2022).

A)



C)



B)

i)

Most Upregulated Genes (P-value)

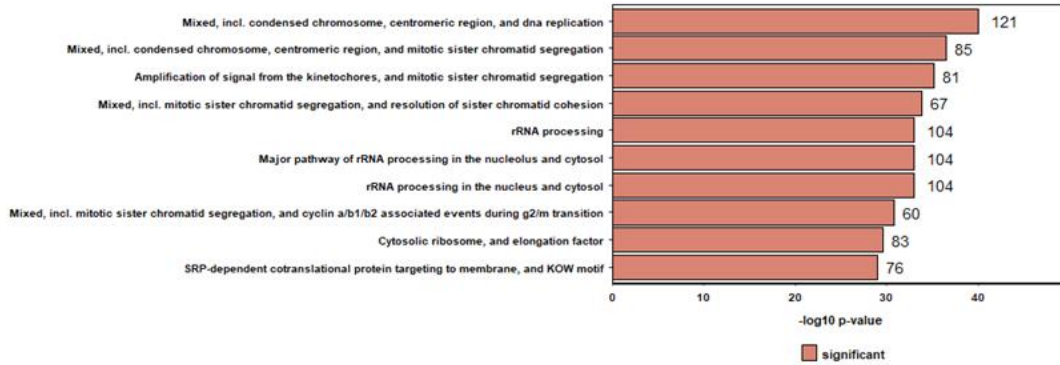
Gene	log2fold	p	p.adj
TBC1D4	4.68	4.330444e-244	3.666153e-240
SLC9A9	3.47	1.359673e-208	7.673994e-205
ICOS	2.95	6.178119e-157	2.092158e-153
ART2A-PS	5.25	1.057797e-153	2.985102e-150
PDCD1	5.74	2.916883e-151	7.055524e-148
HIF1A	2.65	1.668583e-132	3.531557e-129
PTPN11	2.80	1.031465e-130	1.940529e-127
CXCR5	7.18	4.363258e-120	6.716244e-117
BCL6	3.30	2.053716e-119	2.897793e-116
MAF	5.90	2.989997e-113	3.894356e-110

ii)

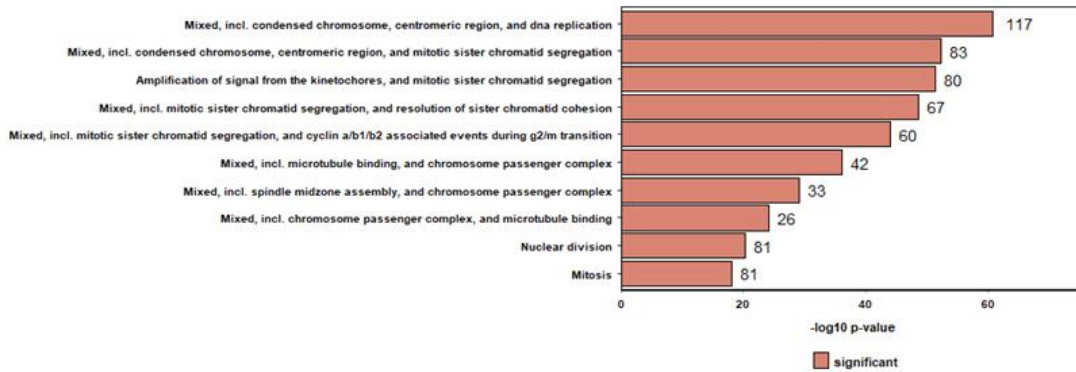
Most Downregulated Genes (P-value)

Gene	log2fold	p	p.adj
RASGRP2	-3.68	2.165326e-267	3.666330e-263
TREML2	-2.99	1.219728e-194	5.163110e-191
S1PR1	-2.79	5.834437e-123	9.878868e-120
PIK3R5	-3.06	2.277157e-107	2.570455e-104
MYC	-2.37	3.528287e-105	3.514174e-102
IL7R	-3.61	1.033960e-93	7.002805e-91
ACTN1	-1.79	5.269805e-93	3.431859e-90
SLC17A9	-2.61	1.256039e-91	7.333533e-89
ESYT1	-2.45	1.738593e-89	9.812619e-87
CCR7	-4.17	3.355097e-89	1.832532e-86

D) Most Enriched Amongst All Significant Genes



E) Most Enriched Amongst Upregulated Genes



F) Most Enriched Amongst Downregulated Genes

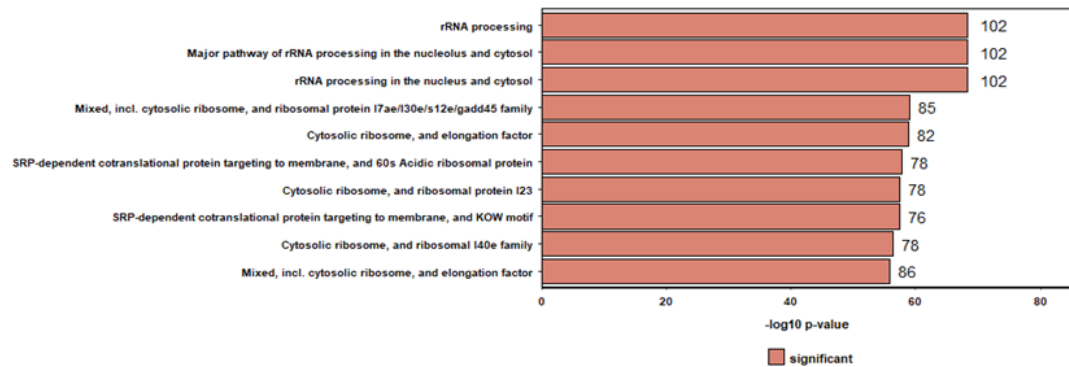


Figure 4.3. Differential Expression Analysis Between Tfh (*H. polygyrus*) and naïve CD4+ T Cells (Uninfected).

A) A volcano plot showing differential gene expression between naïve T cells from uninfected animals (left) and Tfh cells from *H. polygyrus*-infected animals (right) ($p_{\text{adj}} < 0.05$, absolute $\log_2$ fold > 0.5) are shown in red (Upregulated (right)/blue (Downregulated(Left))) and non-significant genes in grey in the comparison. A positive fold change indicates higher expression in Tfh cells (*H. polygyrus*) than in naïve T cells (Uninfected). B) Table showing the ten most upregulated B(i) and downregulated B(ii) genes by adjusted p-value in Tfh compared to naïve T cells (Uninfected). C) Schematic showing the number of upregulated and downregulated genes exhibited by Tfh relative to Naïve T cells (Uninfected). D) Bar chart of the most enriched pathways amongst all significant genes in Tfh cells vs Naïve T cells (Uninfected). E) Bar chart of the most enriched pathways amongst all upregulated pathways in Tfh cells vs Naïve T cells (Uninfected). F) Bar chart of the most downregulated pathways amongst the differentially expressed genes in Tfh cells vs Naïve T cells (Uninfected). Enrichment analysis was performed using Hypergeometric Gene Set Enrichment on the gene set databases STRING11.5.

Analysing the metabolic genes included in the differentially expressed genes from Tfh (*H. polygyrus*) compared to naïve T cells from the uninfected model highlighted the upregulation of genes involved in Lipid/Fatty acid and glycolytic metabolism. Several essential lipid and fatty acid metabolism genes were upregulated in Tfh cells compared to naïve T cells; these included SCD2, which controls monounsaturated fatty acid metabolism, and ACOT7, which plays roles in fatty acid metabolism in the mLN in response to dietary intake (Figure 4.4 B). Interestingly, genetic deletion of SCD2 can activate type 1 interferon signalling, highlighting how metabolism can control effector function in Tfh cells (Ohtomo et al., 2013, Kanno et al., 2022).

Key glycolytic genes were also upregulated in Tfh cells compared to naïve T cells. This is consistent with previous findings from the Morel lab (Gong et al., 2023). I observed upregulation of genes such as ALDOC, PFKL, TP1, PKM, and HK1. While critical in the

glycolysis pathway, these genes can also facilitate other cellular functions. For instance, PFKL can control cytokine production (Toledano Zur et al., 2024). Crucially, however, it should be noted that there is a lack of understanding as to whether these other functions are entirely independent of glycolysis or whether they are a direct consequence of altered glycolytic metabolism.

Congruent with increased transporter expression of CD71 in Tfh compared to naïve T cells (Infected) in chapter 3, DAVID analysis highlighted the importance of iron in Tfh cell biology, as Heme biosynthetic pathways were upregulated in Tfh cells (*H. polygyrus*) compared to naïve T cells (Uninfected) (Figure 4.4 A & E). I observed the increased expression of key genes involved in Heme biosynthesis, such as SRRD, ALAD and SLC25A39. This suggests that Heme synthesis is active, as SRRD, a Heme-dependent inducible protein, was increased (Adachi et al., 2017).

In addition to the analysis of individual genes through DAVID, I also used KEGG pathway analysis to look at metabolic pathways, which highlighted an increase in Galactose, fructose, and mannose metabolism in Tfh cells (*H. polygyrus*) vs Naïve T cells (Uninfected) (Figure 4.4 (F)). This analysis highlights the diverse metabolic requirements of Tfh cells, and subsequent analysis will reveal how these relate to other effector T cells, such as Th2 cells.

G)

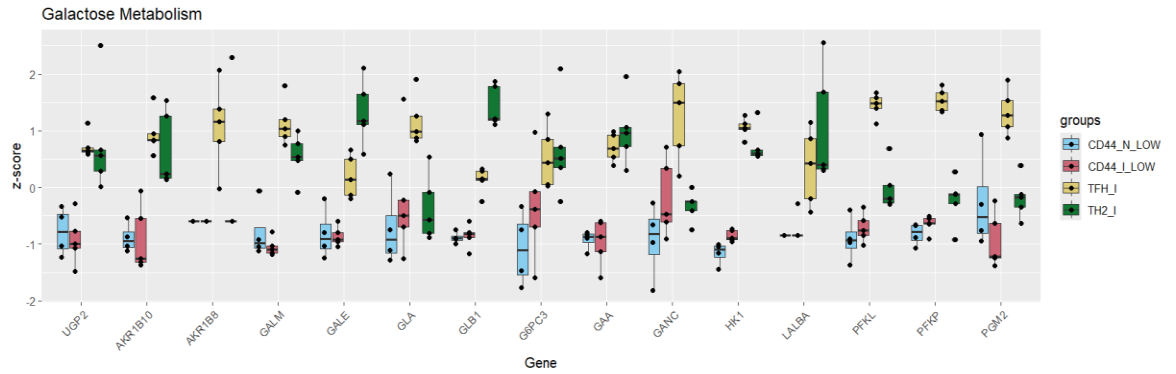


Figure 4.4. Tfh Cells upregulate Pathways associated with Lipid, Iron and Glycolysis during differentiation in *H. polygyrus* Infection.

David Analysis was used to understand upregulated pathways using functional and KEGG pathway analysis. Data from upregulated genes from Tfh Cells (*H. polygyrus*) vs Naïve T cells (Uninfected) was used, whereby all significantly differentially expressed genes ($p_{\text{adj}} < 0.05$, absolute $\log_2$ fold > 0.5). A) Representative Table of upregulated metabolic pathways in Tfh Cells (*H. polygyrus*) vs Naïve T cells (Uninfected). B) Representative Heatmap of upregulated genes in the fatty acid metabolism pathway in Tfh Cells (*H. polygyrus*) vs Naïve T cells (Uninfected) based on significantly upregulated genes ($p_{\text{adj}} < 0.05$, absolute $\log_2$ fold > 0.5). C) Representative Heatmap of upregulated genes in the lipid degradation pathway in Tfh (*H. polygyrus*) vs Naïve CD4⁺ T cells (Uninfected). D) Representative boxplot of glycolysis genes upregulated in Tfh cells (*H. polygyrus*) vs naïve T cells (Uninfected). E) Representative Boxplot of upregulated Heme biosynthesis genes in Tfh cells (*H. polygyrus*) vs naïve CD4⁺ T cells (Uninfected). F) Table of KEGG pathway analysis of metabolic genes in Tfh cells (*H. polygyrus*) vs naïve CD4⁺ T cells (Uninfected). G) Representative boxplot of genes associated with KEGG pathway signature for upregulated differentially expressed genes in Tfh (*H. polygyrus*) vs naïve T cells (Uninfected). All significantly differentially expressed genes ($p_{\text{adj}} < 0.05$, absolute $\log_2$ fold > 0.5). Expression levels have been scaled per gene Z-score.

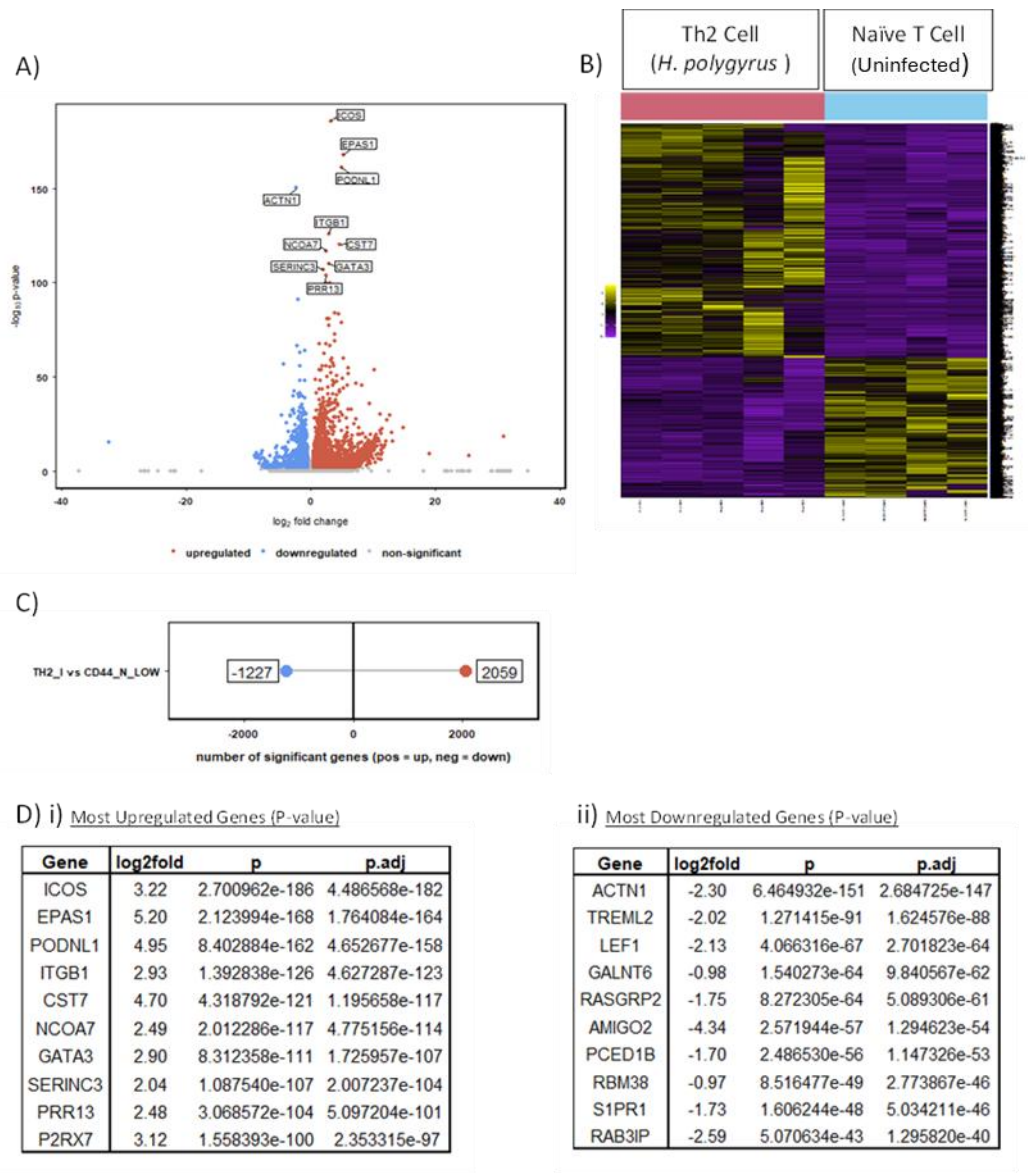


Figure 4.5. Differential Expression Analysis Between Th2 and Naïve T Cells (Uninfected) in *H. polygyrus* Infection:

A) Volcano plot showing differential gene expression between Naïve T (Left) and Th2 cells (*H. polygyrus*) (right) ($p_{adj} < 0.05$, absolute $\log_2$ fold > 0.5) are shown in red (upregulated) /blue (downregulated) and non-significant genes in grey. A positive fold change indicates higher expression in Th2 than in naïve T cells (Uninfected). B) Hierarchically clustered heatmap of the significantly differentially expressed genes for the comparisons Naïve T (uninfected) and Th2 (*H. polygyrus*). Samples are on the x-axis, and genes are on the y-axis. Colour intensity represents expression level, with purple representing low expression and yellow representing high expression. Expression levels have been row-scaled into z-scores. The x-axis and y-axis have been hierarchically clustered using Spearman distances, with UPMGA agglomeration and mean reordering. C) Schematic showing the number of upregulated and downregulated genes exhibited by Th2 relative to Naïve T cells (Uninfected). D) Table showing the ten most upregulated D(i) and downregulated D(ii) genes by adjusted p-value in Th2 than naïve CD4⁺ T cells (Uninfected). All significantly differentially expressed genes ($p_{adj} < 0.05$, absolute $\log_2$ fold > 0.5).

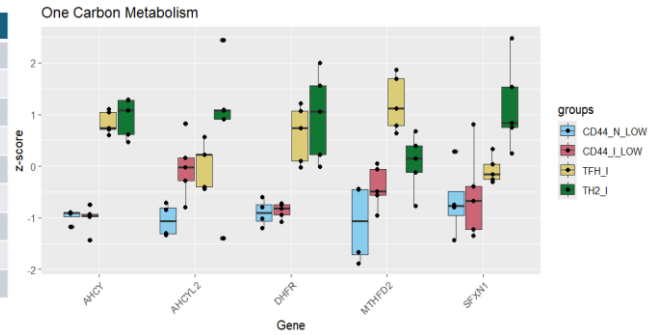
To understand Tfh metabolism and its relationship to Th2 metabolism, I first needed to understand the transcriptional profile of Th2 cells compared to naïve T cells. I analysed the transcriptional profile of Th2 (*H. polygyrus*) cells relative to naïve T cells (Uninfected). This comparison enabled me to identify gene expression changes associated with general T cell activation, as by analysing another activated CD4⁺ T cell subset, I could distinguish features common to activation from those unique to the Tfh lineage. Characterisation of Th2 metabolism formed the basis of a previous PhD student's thesis in the Perona-Wright laboratory (Heieis, 2019). This PhD thesis highlighted that Th2 cells in infected tissues are metabolically characterised by conversion to fatty acid uptake and oxidation in the tissue, which drives ST2 expression and decreases the requirement for glycolysis (Heieis, 2019).

SL2 analysis of the differential gene expression between Th2 and naïve T cells (Uninfected) highlighted a diverse gene signature between these two populations (Figure 4.5 A&B). Analysis highlighted 2059 upregulated and 1227 downregulated genes between Th2 (*H. polygyrus*) and naïve T cells (uninfected) (Figure 4.5 C). To comprehend the changes observed, as a first step, the top 10 up- and down-regulated genes were examined to investigate any noticeable metabolic differences. This approach also allowed me to check that the analysis displayed the predicted correct gene signature associated with the Th2 cell type. Notably, I found that ICOS and GATA3 were upregulated in Th2 cells compared to naïve T cells, as expected (Figure 4.5D (i)). Further to this, the analysis highlighted that, like Tfh cells, Th2 cells also downregulate S1PR1 (Figure 4.5D (ii)) ; this is possibly to assist in migration out of the LNs to the tissue to carry out effector functions (Xiong et al., 2019).

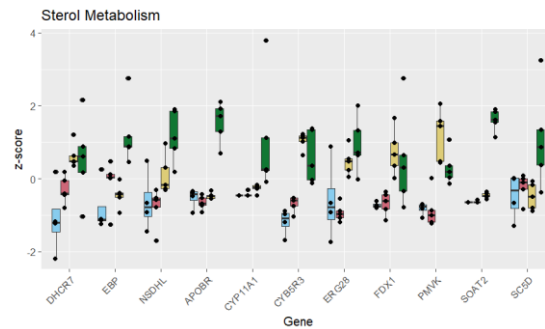
A) Functional Analysis

Term	Gene Count	%	P-value
Lipid Biosynthesis	28	1.4	4.10E-03
Lipid Metabolism	88	4.5	4.70E-03
Fatty Acid Metabolism	25	1.3	9.70E-03
Sterol Biosynthesis	7	0.4	3.90E-02
Lipid Transport	22	1.1	4.60E-02
Lipid Degradation	15	0.8	6.00E-02
Sugar Transport	8	0.4	6.20E-02
One-Carbon Metabolism	5	0.3	7.70E-02
Sterol Metabolism	11	0.6	9.90E-02

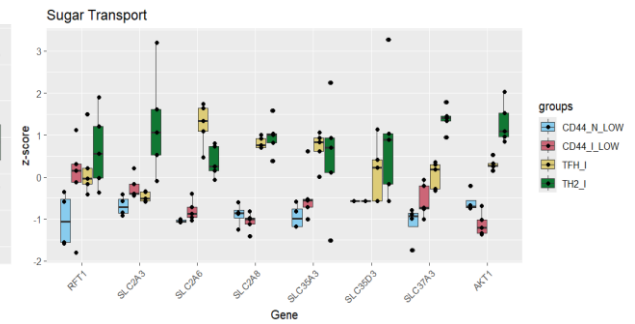
B)



C)



D)



E) KEGG Pathway Analysis

Metabolic Pathway	Gene Count	%	P-value
Metabolic Pathways	190	9.7	1.10E-03
Glycerolipid Metabolism	15	0.8	1.90E-03
Fructose and Mannose Metabolism	10	0.5	5.20E-03
Amino Sugar and Nucleotide Sugar Metabolism	11	0.6	2.20E-02
Fatty Acid Metabolism	12	0.6	2.90E-02
Steroid Biosynthesis	6	0.3	3.50E-02
Biosynthesis of Nucleotide Sugars	8	0.4	6.20E-02
Glycerophospholipid Metabolism	15	0.8	7.60E-02
One Carbon Pool by Folate	5	0.3	9.70E-02

Figure 4.6. Th2 Cells upregulate Pathways associated with Lipid, Sterol, sugar, and one-carbon metabolism during differentiation in *H. polygyrus* Infection.

Using functional and KEGG pathway analysis, data were processed through SL2 and David analysis to identify upregulated pathways. Data from upregulated genes from Th2 Cells (*H. polygyrus*) vs naïve T cells (uninfected) was used, whereby all significantly differentially expressed genes were defined as; ($p_{adj} < 0.05$, absolute $\log_2$ fold > 0.5). A) Representative Table of upregulated metabolic pathways in Th2 cells (*H. polygyrus*) compared to Naïve T Cells (Uninfected). B) Boxplot of one-carbon metabolism genes upregulated in Th2 cells (*H. polygyrus*) compared to Naïve T Cells (Uninfected) based on significantly upregulated genes ($p_{adj} < 0.05$, absolute $\log_2$ fold > 0.5) highlighted in DAVID analysis of Th2 (*H. polygyrus*) vs Naïve T cells. C) Boxplot of upregulated Sterol biosynthesis genes in Th2 cells (*H. polygyrus*) compared to Naïve T Cells (Uninfected). D) Boxplot of upregulated Sugar Transport genes in Th2 cells (*H. polygyrus*) compared to Naïve T Cells (Uninfected). E) Table of KEGG pathway analysis of metabolic genes Th2 cells (*H. polygyrus*) compared to Naïve T Cells (Uninfected). All significantly differentially

expressed genes ($p_{\text{adj}} < 0.05$, absolute $\log_2$ fold > 0.5). Expression levels have been scaled per gene Z-score.

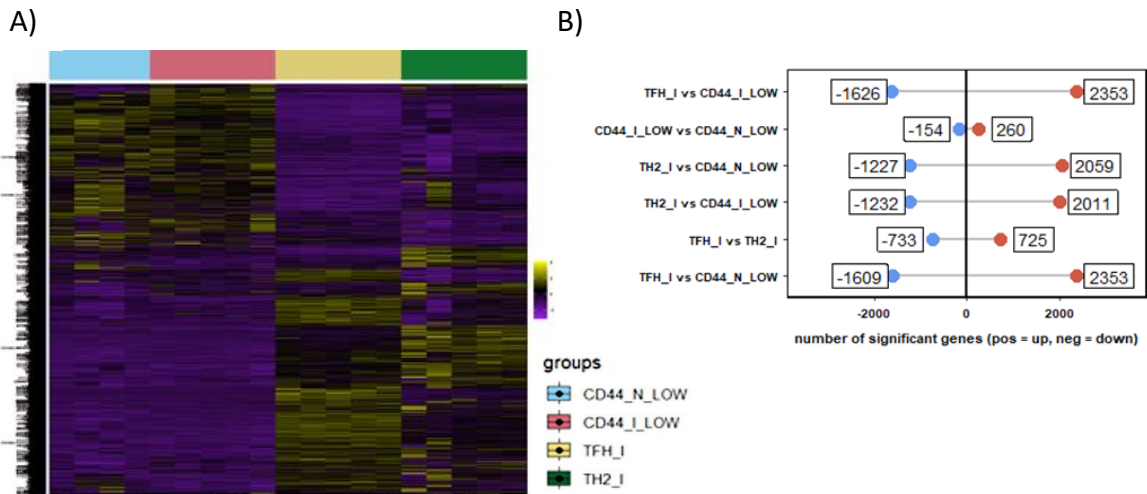
To understand the transcriptional metabolic landscape of Th2 compared to naïve T cells, I again utilised DAVID analysis (Figure 4.6). My analysis showed that the differential expression (DE) genes associated with Th2 cells were similarly enriched in the same metabolic pathways associated with lipid and fatty acid metabolism identified when Tfh cells (*H. polygyrus*) were compared to naïve T cells (Uninfected) (Figure 4.6 A). Interestingly, Th2 analysis highlighted the requirement for sugar transport over glycolysis, underscoring differences in Th2 metabolism compared to Tfh cells and their naïve counterparts. The prominence of sugar transport was not seen when comparing Tfh cells to naïve cells, illustrating potential differences between Tfh and Th2 metabolism, possibly alluding to the transport of sugars into the cell that are not broken down or utilised by a different metabolic pathway (Figure 4.4 (D)).

The analysis also highlighted that genes involved in the regulation of sterol and one-carbon metabolism in Th2 cells were upregulated compared to naïve T cells (uninfected) (Figure 4.6 (B & C)). KEGG pathway analysis further confirmed that different metabolic pathways are likely utilised between Th2 and naïve T cells (Figure 4.6E). Th2 cells have an enrichment for pathways such as fructose and mannose metabolism; this highlights similarly to that previously identified in Tfh cells (Figure 4.4). Th2 cells also exhibited enrichment of pathways associated with amino acid, steroid, and one-carbon metabolism when compared to naïve T cells (Figure 4.6E).

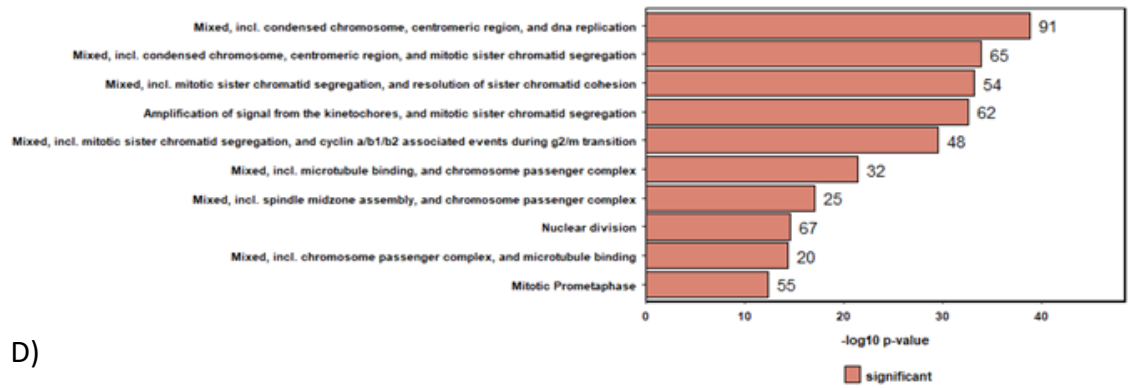
4.3.2 Delineating the metabolic differences between Tfh and Th2 cells in the mLN during active *H. polygyrus* Infection

To further investigate the potential differential metabolic profile of Tfh and Th2 cells, using the SL2 pipeline, multiple pairwise differential expression (MDE) analysis of our data were performed, explicitly investigating the genes significantly modulated across all the cell types, namely naïve T cells (uninfected/Infected), along with Tfh and Th2 samples from *H. polygyrus* -infected mice (Figure 4.7 A & B).). This analysis demonstrated that the transcriptional profiles of these sorted cell types were diverse (Figure 4.7A and B). This MDE analysis highlighted that, in all genes expressed more highly in Tfh cells than in the other T cell types, I found increased gene expression associated with positive regulation of cell growth and activation (Figure 4.7 C-E).

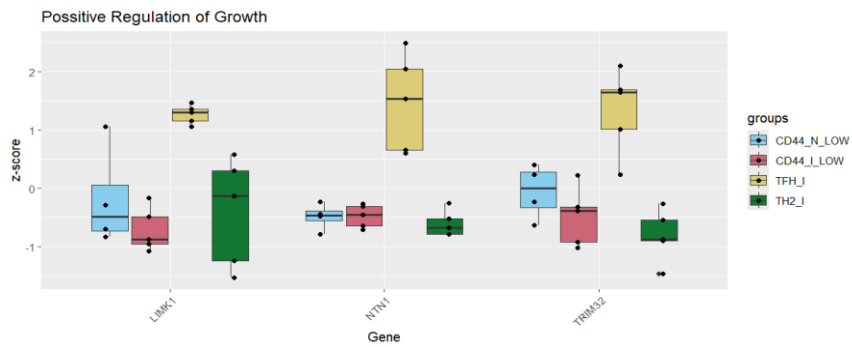
Using the analysis, I then delved deeper to understand the genes expressed more highly in Tfh cells than in other T cell types, such as CCR6 and CXCR4 (Figure 4.7E). These chemokine receptors are crucial for T cell migration and localisation within the GC. CXCR4, like CXCR5, is critical for Tfh cells to localise to the GC LZ (Bastow et al., 2022). Genes such as CLBL are also critical for fine-tuning Tfh cell responses, as a deficiency of CLBL ubiquitin ligases leads to hyper-Tfh responses in lupus through reduction of Bcl-6 degradation (Li et al., 2024). Together, these results highlight increased expression of key genes involved in Tfh cell biology. However, they fail to address any specific metabolic pathways differentially expressed by Tfh cells compared to the other T cells in this analysis, as the analysis was limited to the top 10 upregulated genes.



C) Most Enriched Amongst All Significant Genes



D)



E)

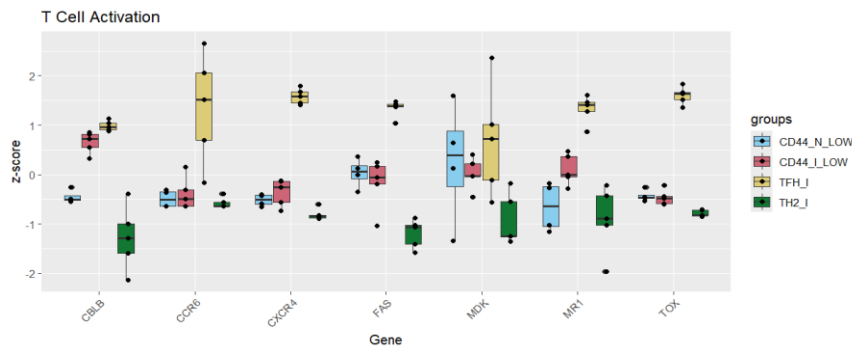


Figure 4.7. Differential Analysis Across all cell types of highlights genes associated with the Activation and Growth of T cells:

A) Hierarchically clustered heatmap of the significantly differentially expressed genes for the comparisons (naïve T cells (uninfected and Infected), Tfh and Th2. Samples are on the x-axis, and genes are on the y-axis. Colour intensity represents expression level, with purple representing low expression and yellow representing high expression. Expression levels have been row-scaled into z-scores. The y-axis (both plots) and x-axis (right plot) have been hierarchically clustered using Spearman distances, with UPMGA agglomeration and mean reordering. B) Bar chart showing the number of significantly differentially expressed genes for the comparisons. The comparison is given on the x-axis, and the number of significant genes is on the y-axis. C) Summary bar charts of the ten most enriched gene sets for the top differential expression signature. For each plot, the $-\log_{10}$ enrichment p-value is given on the x-axis, and the gene set name is on the y-axis. Data labels represent the number of significantly differentially expressed genes within each gene set. Enrichment analysis was performed using Hypergeometric Gene Set Enrichment on the gene set databases STRING11.5. Significantly enriched gene sets ($p_{\text{adj}} < 0.05$, absolute $\log_2$ fold > 0.5) are red and non-significant black. D) Representative boxplot of positive regulation of growth-upregulated genes across all cell types. E) Representative boxplot of T cell activation upregulated genes across all cell types. All significantly differentially expressed genes ($p_{\text{adj}} < 0.05$, absolute $\log_2$ fold > 0.5). Expression levels have been scaled per gene Z-score.

To further delineate the differences between Tfh and Th2 biology, SL2 was again used to unravel each T cell subset's different transcriptional profiles (Figure 4.8A) revealing 725 upregulated and 733 downregulated genes in Tfh compared to Th2 cells (*H. polygyrus*) (Figure 4.8 (C)). To comprehend the changes observed, as a first step, the top 10 up- and down-regulated genes were examined to investigate any noticeable metabolic differences. The most upregulated genes in Tfh, relative to Th2 cells, are shown in Figure 4.8 (B(i)). This revealed canonical markers of Tfh cell biology to be upregulated PDCD1 (PD-1), Bcl-6, HIF-1 alpha, TOX, TNFSF8 (CD153). CD27, the co-stimulatory molecule, was also more highly expressed by Tfh relative to Th2 cells.

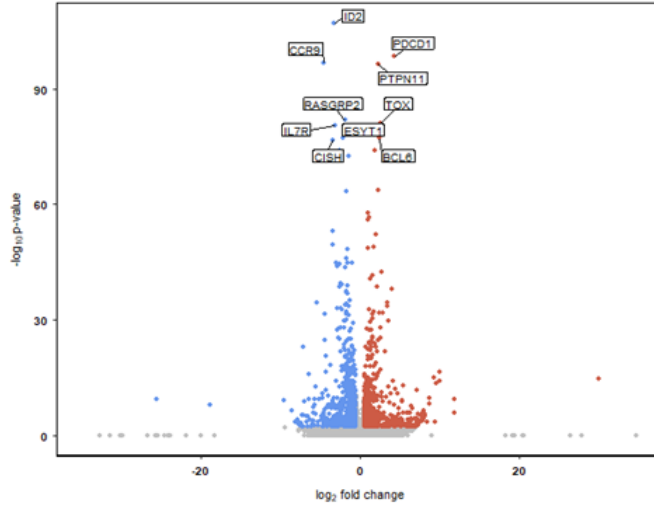
Diacylglycerol Kinase 2 (DGK2) was also upregulated in Tfh compared to Th2 cells; this belongs to a family of enzymes responsible for controlling the level of diacylglycerol (DAG) and phosphatidic acid (PA) in cells (Figure 4.8 B(i)). DGK2 has been shown to play an important role in self-tolerance, T cell development and the generation of the immune synapse (Chen et al., 2016). The upregulation of this gene possibly reflects the mechanism used to prevent hyperactivation of a T cell through sustained TCR stimulation (Krishna and Zhong, 2013), a common feature resulting from Tfh-B cell interaction (Ulutekin et al., 2024).

Interestingly, I also found the upregulation of TBC1D4, which prevents GLUT4 translocation to the cell's surface (Stockli et al., 2008), in Tfh cells compared to Th2. This is consistent with Tfh cells using an alternative mechanism for glucose uptake, such as the GLUT1 transporter, as was highlighted in Chapter 3 (Figure 3.6) or possibly there might also be sufficient GLUT4 on the surface already.

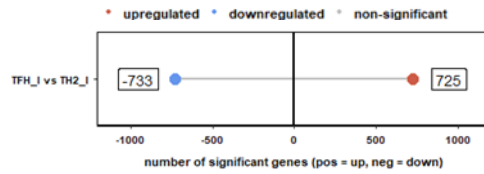
I also looked at the top 10 downregulated genes in Tfh relative to Th2 cells. These downregulated genes in Tfh are those previously shown to be upregulated in Th2 cells, relative to naïve cells (Figure 4.7 B (ii)). Thus, compared to Tfh cells, Th2 cells exhibit increased expression of ID2, a critical regulator of the Th1/Th2 balance (Kusunoki et al., 2003) as well as CCR9, the gut-homing chemokine (Cosorich et al., 2018). IL-7 was also upregulated, which, unlike the situation for Tfh cells, is critical to Th2 immune responses (Huang et al., 2021a). Relative to Tfh cells, there is also an increase in ESY1T (lipid transporter) and CISH (glucogenesis regulator), potentially suggesting some metabolic differences. The other genes upregulated in Th2 are associated with migration (ITGB3 and SELPLG) and Th2 cytokine production (SATB1) (Tinoco et al., 2021, Sunkara et al., 2018, Zhu et al., 2019a).

Utilising STRING analysis carried out by the SL2 program, I was able to compare the enrichment of pathways associated with the upregulated and downregulated genes in Tfh cells (compared to Th2 cells) to address whether any metabolic pathways were differentially utilised. The most upregulated pathways were those associated with abnormal interferon and IL-2 secretion alongside T-cell activation and regulation (Figure 4.8 (D)). The downregulated pathways were associated with G-protein-coupled receptor signalling and expression (Figure 4.8 (E/F)). Since no prominent metabolic pathway was enriched in the top 10 upregulated/downregulated gene lists or the STRING pathway analysis, DAVID analysis was utilised again to characterise the metabolic landscape.

A)



C)



B)

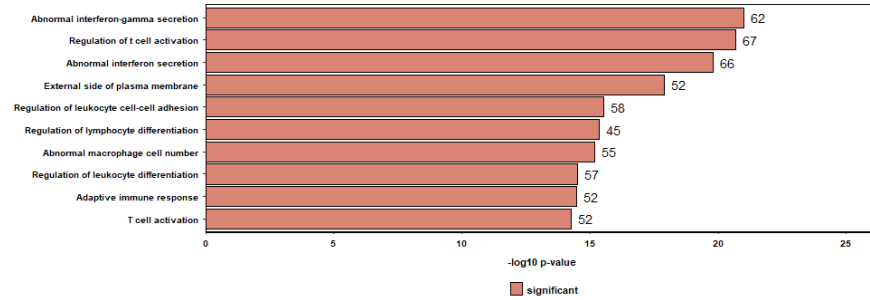
i) Most Upregulated Genes (P-Value)

Gene	log2fold	p	p.adj
PDCD1	4.30	4.421503e-99	3.595566e-95
PTPN11	2.27	4.393225e-97	1.786285e-93
TOX	2.60	6.476581e-82	1.755585e-78
BCL6	2.48	8.133991e-78	1.469903e-74
HIF1A	1.85	1.042905e-74	1.541982e-71
TNFSF8	2.24	2.103758e-64	2.443966e-61
DGKZ	0.92	1.601192e-58	1.627612e-55
CD27	1.14	2.431855e-57	2.326570e-54
FYN	0.98	1.378447e-56	1.245503e-53
TBC1D4	1.99	6.778551e-53	5.512318e-50

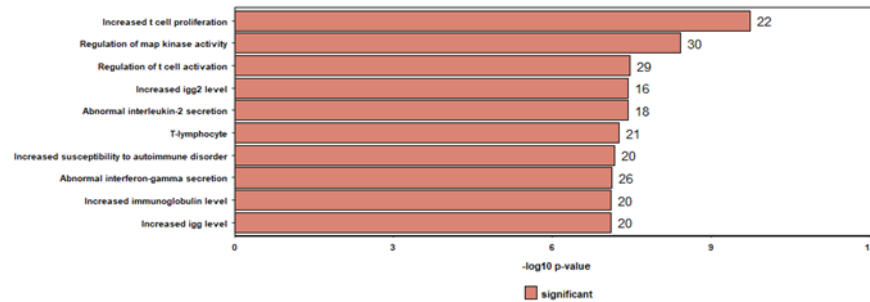
ii) Most Downregulated Genes (P-Value)

Gene	log2fold	p	p.adj
ID2	-3.23	9.963845e-108	1.620520e-103
CCR9	-4.61	1.542940e-97	8.364795e-94
RASGRP2	-1.92	1.070898e-82	3.483417e-79
IL7R	-3.17	3.232071e-81	7.509486e-78
ESYT1	-2.16	4.509994e-78	9.168817e-75
CISH	-3.38	2.365572e-77	3.847367e-74
PADI2	-2.65	1.221090e-74	1.654984e-71
SELPLG	-1.45	3.451661e-73	4.318293e-70
SATB1	-1.75	5.358224e-64	5.809743e-61
ITGB3	-3.46	9.346728e-54	8.000799e-51

D) Most Enriched Amongst All Significant Genes



E) Most Enriched Amongst All Upregulated Genes



F) Most Downregulated Amongst All Significant Genes

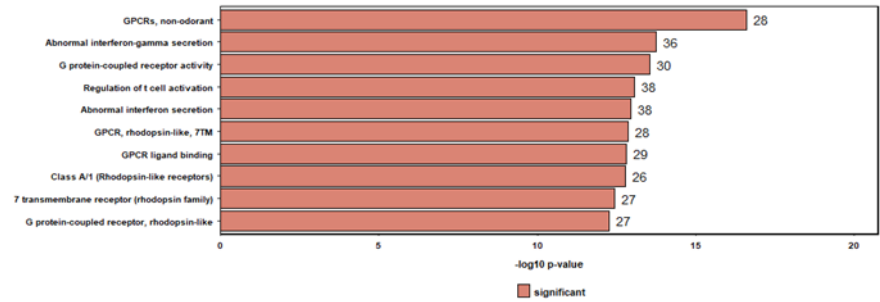


Figure 4.8. Differential Expression Between Tfh and Th2 Cells.

A) Volcano plot showing differential gene expression between Th2 (Left) and Tfh (right) (*H. polygyrus*) (p.adj < 0.05, absolute log₂ fold > 0.5) are shown in red (Upregulated) and blue (downregulated) whilst non-significant genes are in grey. A positive fold change indicates higher expression in Tfh than in Th2. B) i) Table showing the ten most upregulated ii) and downregulated iii) genes by adjusted p-value. C) Representative Schematic showing differential count for upregulated and downregulated genes between Tfh and Th2 cells. D) Bar chart of the most enriched pathways amongst all significant genes in Tfh cells vs Th2 cells. E) Bar chart of the most enriched pathways amongst all upregulated Tfh cells vs Th2 cells. F) Bar chart of the most downregulated pathways amongst all significant genes in Tfh cells vs Th2 cells. Enrichment analysis was performed using Hypergeometric Gene Set Enrichment on the gene set databases STRING11.5.

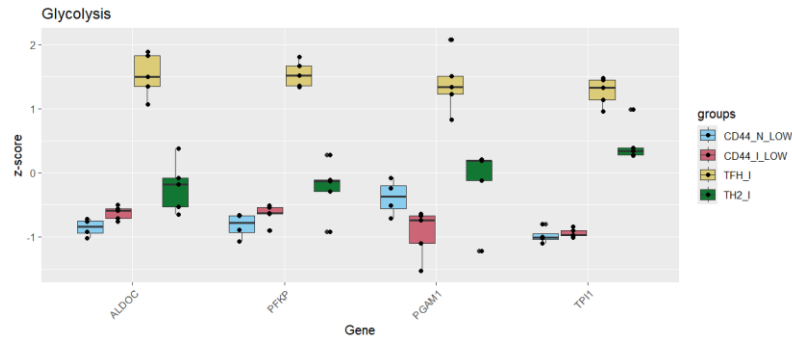
DAVID analysis of the significantly upregulated genes between Tfh and Th2 cells revealed that Th2 cells and Tfh cells show striking metabolic similarities (Figure 4.9 (A)). This analysis highlighted that of the 725 genes upregulated between Tfh and Th2 cells, only 4 correlated with a metabolic pathway, and these were associated with glycolysis, namely ALDOC, PFKP, PGAM1 and TPI1 (Figure 4.9 (B)). This highlights a possible increased glycolytic demand in Tfh cells compared to Th2 cells.

Furthermore, KEGG analysis (Fig. 4.9 (C)) suggested additional metabolic pathways that were preferentially associated with Tfh cells, with central carbon (Fig.4.9 (D)), choline, glucose, and pentose phosphate metabolism highlighted in this analysis (Figure 4.9 (C)). This analysis corroborates the role of glycolysis and convergence with the pentose phosphate pathway in Tfh cells relative to Th2 cells. These findings provide a comprehensive understanding of Tfh cell metabolism and inspire further research into the potential implications of these metabolic pathways on Tfh cell function and immune response.

A) Functional Analysis

Term	Gene Count	%	P-value
Glycolysis	4	0.6	0.074

B)



C) KEGG Pathway Analysis

Pathway	Count	%	P-value
Central Carbon Metabolism in Cancer	9	1.3	9.74E-4
Choline Metabolism In Cancer	9	1.3	8.7E-3
Glycolysis/Glucogenesis	7	1.0	1.48E-2
Pentose Phosphate Pathway	4	0.6	8.0E-2
Fructose and Mannose Metabolism	4	0.6	9.1E-2

D)

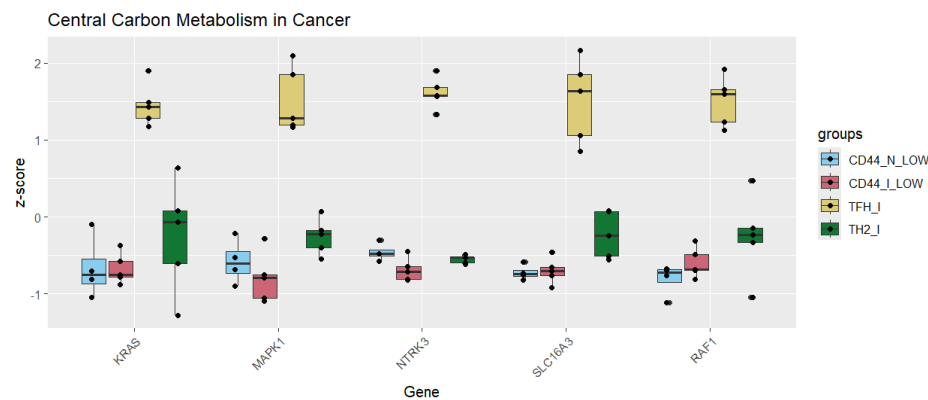


Figure 4.9. Tfh Increases Expression of Key Glycolysis Genes Compared to Th2 Cells.

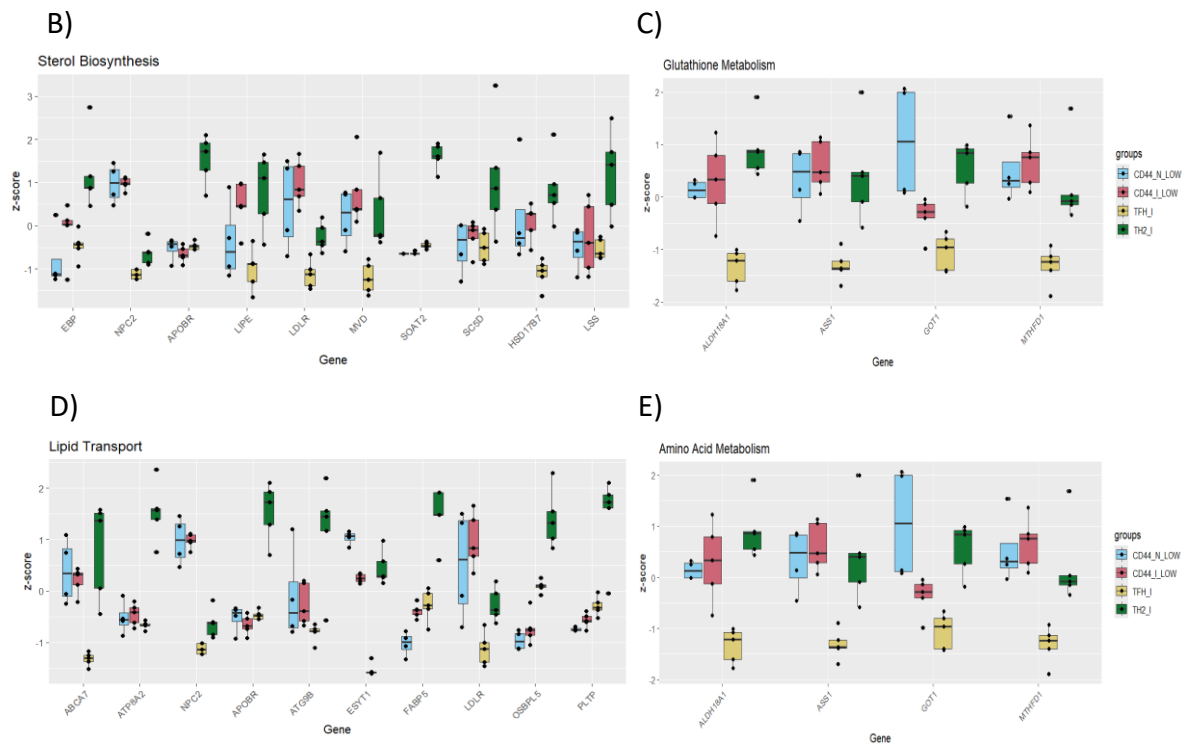
Using functional and KEGG pathway analysis, the data were processed through SL2 and DAVID analysis to understand the upregulated pathways. Data from upregulated genes from Tfh Cells vs Th2 cells (*H. polygyrus*) was used, whereby all significantly differentially expressed genes were denoted by ($p_{\text{adj}} < 0.05$, absolute $\log_2$ fold > 0.5). A) Table of upregulated metabolic pathways in Tfh cells compared to Th2 Cells. B) Boxplot of glycolysis genes upregulated in Tfh cells vs Th2 cells. C) Table of upregulated KEGG metabolic pathways in Tfh cells compared to Th2 Cells. D) Boxplot of Central Carbon Metabolism in Cancer KEGG Pathway in Tfh cells vs Th2 cells. All significantly differentially expressed genes ($p_{\text{adj}} < 0.05$, absolute $\log_2$ fold > 0.5). Expression levels have been scaled per gene Z-score.

When I looked at the downregulated genes between Tfh and Th2 (upregulated Th2 vs Tfh), the DAVID analysis further highlighted differential metabolism between Th2 and Tfh cells. Specifically, Th2 cells have increased sterol, cholesterol, glutathione, lipid, and amino acid metabolism-related gene expression profiles (Figure 4.10 (A)).

This analysis highlighted significantly increased expression of lipid metabolism-related genes such as APOBR and SOAT2 (Figure 4.10 (B)). The role of SOAT2 in T cells is well understood, as it catalyses the conversion of cholesterol into cholesteryl esters, which are stored as lipid droplets within the cell. This role in neutral lipid conversion aligns with the findings presented in Chapter 3. It underscores key gene expression distinctions between Th2 and Tfh cells —namely, the higher accumulation of neutral lipids in Th2 cells compared to Tfh cells (Figure 4.11) (Brenes et al., 2021, Zhang et al., 2025b). Lipid transport genes such as PLTP, OSBPL5, FABP5 and ATG9B were also significantly upregulated in Th2 over Tfh cells (Figure 4.10 (D)). Of Interest, therefore, FABP5 has been reported to regulate the function and lipid metabolism of Tfh cells through the uptake and oxidation of fatty acids (Jin et al., 2023).

A) Functional Analysis

Term	Count	%	P-Value
Sterol metabolism	8	1.1	5.20E-03
Cholesterol metabolism	7	1	1.20E-02
Glutathione biosynthesis	3	0.4	1.80E-02
Steroid metabolism	8	1.1	3.20E-02
Steroid biosynthesis	5	0.7	4.60E-02
Lipid transport	10	1.4	5.00E-02
Amino-acid biosynthesis	4	0.6	5.40E-02
Lipid biosynthesis	10	1.4	8.50E-02
Lipid metabolism	30	4.3	8.60E-02



F) KEGG Pathway Analysis

Pathway	Count	%	P-Value
Steroid Biosynthesis	6	0.9	6.10E-04
Metabolic Pathways	74	10.5	3.40E-02
Cholesterol Metabolism	6	0.9	3.40E-02
Arginine and Proline Metabolism	6	0.9	4.50E-02
Biosynthesis of Amino Acids	7	1	6.70E-02
Fatty Acid Metabolism	6	0.9	7.40E-02
Nucleotide Metabolism	7	1	8.50E-02
Fatty Acid Elongation	4	0.6	8.70E-02

Figure 4.10. Th2 Cells are metabolically distinct from Tfh cells.

Using functional and KEGG pathway analysis, the significantly upregulated gene list (Th2 vs Tfh) was created by SL2 and subsequently processed through DAVID analysis to understand the upregulated metabolic pathways between Th2 cells and Tfh cells (*H. polygyrus*), where all significantly differentially expressed genes ($p_{adj} < 0.05$, absolute $\log_2$ fold change > 0.5) were identified. A) Table of upregulated metabolic pathways in Th2 cells compared to Tfh Cells. B) Boxplot of Sterol biosynthesis genes upregulated in Th2 cells vs Tfh cells. C) Boxplot of Glutathione Metabolism genes upregulated in Th2 cells vs Tfh cells.

D) Boxplot of Lipid transport genes upregulated in Th2 cells vs Tfh cells. E) Boxplot of Amino Acid Metabolism genes upregulated in Th2 cells vs Tfh cells. F) Table of upregulated KEGG metabolic pathways in Tfh cells compared to Th2 Cells. All significantly differentially expressed genes ($p_{\text{adj}} < 0.05$, absolute $\log_2$ fold > 0.5). Expression levels have been scaled per gene Z-score.

Interestingly, the metabolic pathways associated with Glutathione and Amino acid transport appear to be downregulated in Tfh cells compared to all T cell groups (Figure 4.10 (C/E)). This highlights the potential inhibition of specific metabolic pathways in Tfh cells at the level of gene expression, possibly to control cellular proliferation or to control energy demands (Mak et al., 2017). This intriguing finding prompts further investigation into the downregulated metabolic pathways in Tfh cells, to enhance our understanding of Tfh cell metabolism.

To understand the potential implications of the downregulated genes in Tfh cells compared to naïve T cells (Uninfected), I used the DAVID platform to analyse the gene list created through DESeq2. This analysis highlighted two main trends: the decreased expression of genes relating to amino acid metabolism and protein biosynthesis in Tfh cells (Figure 4.11). These findings may support the data presented in the previous chapter, which suggests that Tfh cells are not as proliferative as Th2 cells (Figure 3.5), as they would require fewer amino acids and protein biosynthesis. Collectively, therefore, these data support the published report that cell division is not a priority in Tfh cell biology (Hosios et al., 2016).

In particular, the analysis highlighted decreased Purine, Amino Acid, protein, methionine, nucleotide, leukotriene and pyrimidine metabolism/biosynthesis (Figure 4.11 (A)). Lipid transport was also decreased, suggesting that Tfh favours *de novo* lipid biosynthesis rather than uptake (Figure 4.11 (A)). Interestingly, we see a downregulation of leukotriene biosynthetic pathways (Figure 4.11 (A)), which may highlight the requirement of leukotrienes' transport to support their function rather than biosynthesis (Nagaya et al., 2017).

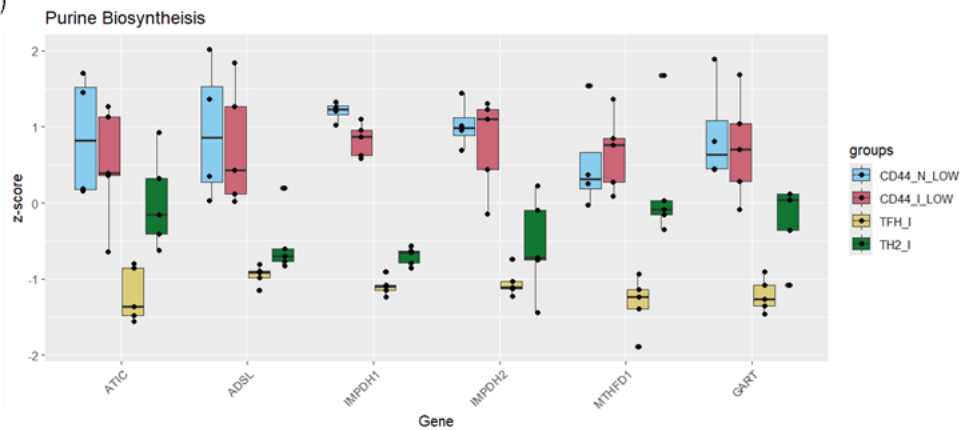
The downregulation of nucleotide metabolism in Tfh cell compared to naïve T cells (Uninfected) further strengthens the argument that Tfh cells do not favour proliferation, as purines and pyrimidine-derived nucleotides regulate the cell cycle and control the transition from G(1) to S phase and the progression throughout the S phase of cell division

(Figure 4.11 A and B) (Quemeneur et al., 2003). KEGG pathway analysis also highlighted a similar response to the functional analysis performed by DAVID analysis (Figure 4.11D), showing enrichment of pathways associated with protein metabolism associated with the downregulated genes.

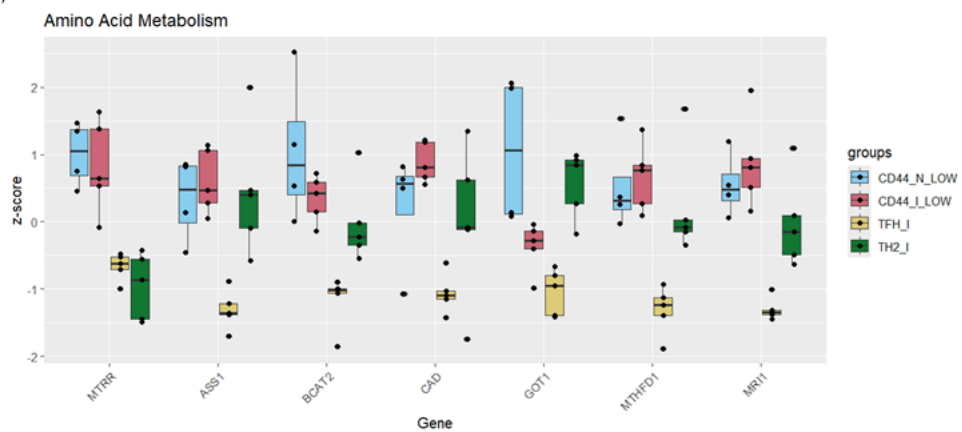
A) Functional Analysis

Term	Count	%	P-Value
Purine Biosynthesis	6	0.4	9.3E-4
Amino Acid Biosynthesis	7	0.5	1.7E-3
Lipid Transport	20	1.3	5.3E-3
Protein Biosynthesis	18	1.2	8.9E-3
Methionine Biosynthesis	4	0.3	1.2E-2
Nucleotide metabolism	5	0.3	6.6E-2
Leukotriene Biosynthesis	3	0.2	7.1E-2
Pyrimidine Biosynthesis	3	0.2	9.0E-2

B)



C)



D) Functional Analysis

Term	Count	%	P-Value
Purine Metabolism	18	1.2	1.3E-2
Central Carbon Metabolism in Cancer	11	0.7	2.2E-2
Cysteine and Methionine Metabolism	9	0.6	3.8E-2
Biosynthesis of Amino Acids	11	0.7	5.0E-2
Arginine and Proline Metabolism	8	0.5	8.3E-2
Carbon Metabolism	14	0.9	8.8E-2
mTOR Signalling Pathway	17	1.1	9.0E-2

Figure 4.11. Tfh Cells Decrease Expression of Genes Associated with Amino Acid, Purine and Lipid Mediator/Transporters during Differentiation.

The significantly upregulated gene list (naïve T cells (uninfected) vs Tfh) was created by SL2 and subsequently processed through DAVID analysis to understand the upregulated metabolic pathways between naïve T cells (Uninfected) and Tfh cells (*H. polygyrus*), where all significantly differentially expressed genes ($p_{\text{adj}} < 0.05$, absolute $\log_2$ fold change > 0.5) were identified. A) Table of upregulated metabolic pathways in naïve T cells compared to Tfh Cells. B) Boxplot of Purine Biosynthesis genes upregulated in naïve T cells vs Tfh cells. C) Boxplot of Amino Acid Biosynthesis genes upregulated in naïve T cells vs Tfh cells. D) Representative Table of KEGG Pathway enrichment in naïve T cells vs Tfh cells. All significantly differentially expressed genes ($p_{\text{adj}} < 0.05$, absolute $\log_2$ fold > 0.5). Expression levels have been scaled per gene Z-score.

The findings of Chapter 3 revealed that Th2 cells accumulate high levels of neutral lipids compared to other T cells in the mLN during *H. polygyrus* infection, raising a key question: why and how does this occur? To address this, I used Venn diagram analysis to identify the upregulated metabolic genes specific to Th2 cells. This Th2-specific metabolic profile was obtained by comparing the significantly upregulated genes in the Th2 vs naïve and Th2 vs Tfh cell analyses. DAVID software was then used to identify the genes and pathways preferentially associated with Th2 metabolism, as indicated by the overlapping genes in the centre of the Venn diagram (Figure 4.12 (A)).

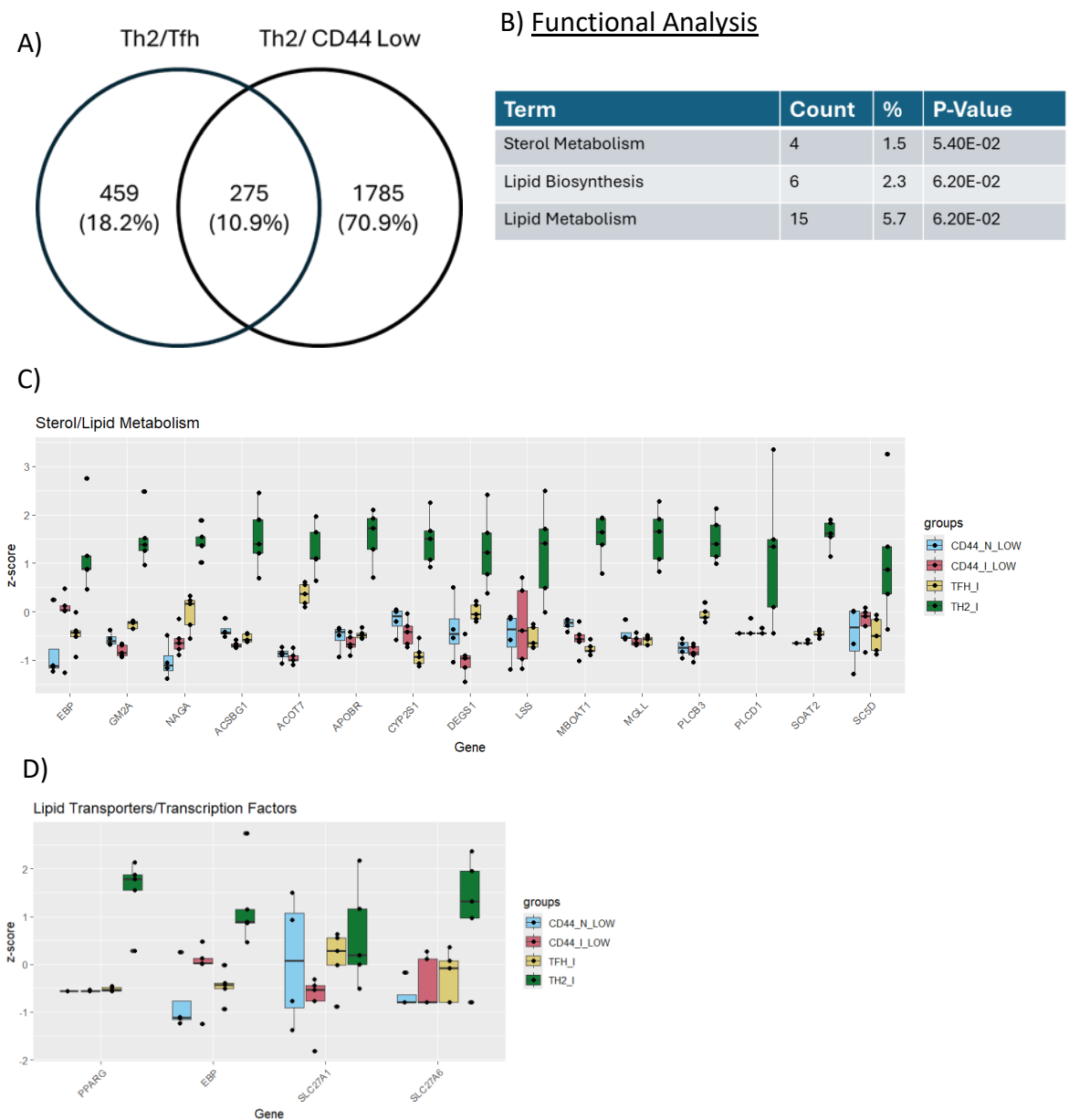


Figure 4.12. Th2 cells upregulate distinct Sterol Metabolism associated with Very Long-chain Fatty Acids.

The significantly upregulated gene list (Tfh vs Th2 and Th2 vs CD44 Low) was created by SL2. Using Venny, I was able to identify the genes that overlapped or were specific to Th2 cells. This approach enabled me to generate a significantly upregulated gene list for Th2 and Tfh cells. This gene list was then subsequently processed through DAVID analysis to understand the upregulated metabolic pathways between naïve T cells (Uninfected) and Tfh cells (*H. polygyrus*), where all significantly differentially expressed genes ($p_{adj} < 0.05$, absolute $\log_2$ fold change > 0.5) were identified. A) Venn diagram of the upregulated genes between Tfh/ Th2 and CD44 Low Naïve cells, the overlapping segment of the Venn Diagram is genes specific to Th2 and used in subsequent analysis. B) Table of upregulated metabolic pathways in Th2 cells compared to Tfh and naïve Cells, whereby all genes are significantly differentially expressed genes ($p_{adj} < 0.05$, absolute $\log_2$ fold > 0.5) in each comparison. C) Boxplot of 15 upregulated genes highlighted through a thorough analysis specific to Th2 Cell metabolism. D) Boxplot of Lipid Transporters (SLC27s) and upstream

TFs associated with the distinct metabolic phenotype across all cell types. All significantly differentially expressed genes ($p_{\text{adj}} < 0.05$, absolute $\log_2$ fold > 0.5). Expression levels have been scaled per gene Z-score.

Through DAVID analysis of the 275 overlapping genes (Figure 4.12 (A)), I identified 15 metabolic genes that were upregulated in a Th2-specific manner. These genes were associated with lipid and sterol metabolism. The robustness of my method to identify these genes is highlighted in Figure 4.12 (C), which shows the upregulation of all these genes in comparison to all other T cells (CD44 Low (Naïve/*H. polygyrus*) and Tfh cells (*H. polygyrus*)).

From these genes, I predicted that Th2 cells would utilise PPAR- γ and C/EBP, regulators associated with increased cholesterol and lipid metabolism, to drive this increase in neutral lipid accumulation. Indeed, I found that Th2 cells, compared to other T cells, expressed the highest levels of PPAR- γ and C/EBP transcriptionally (Figure 4.12 (D)). This upregulation of PPAR- γ and C/EBP being restricted to Th2 cells, further underscoring the specificity of the metabolic pathways in these cells, as these factors play a crucial role in regulating lipid metabolism and storage (Lee et al., 2013),

The lack of increase in expression of genes associated with *de novo* fatty acid synthesis pathways in Th2 cells over Tfh cells suggests that Th2 cells may be acquiring or sequestering lipids from the external microenvironment (Figure 4.12(A)). Previous reports suggest a role for PPAR- γ in activating the SLC27 family of receptors (Anderson and Stahl, 2013). I hypothesised, therefore, that Th2 cells may have increased expression of this transporter family. Therefore, to understand further how Th2 cells facilitate their increase in neutral lipids, I explored the family of lipid transporters related to SLC27A (1-6) to see if Th2 cells differentially utilise the uptake mechanism of fatty acids compared to other T cell populations (Figure 4.12 (D)).

DAVID analysis highlighted that both Tfh and Th2 exhibited increased expression of SLC27A1, also known as FATP1, compared to naïve T cells (Figure 4.12 (D)). This receptor is involved in the uptake of long-chain fatty acids, such as palmitic and monocarboxylic acid transport and glycerophospholipid biosynthesis (Anderson and Stahl, 2013). This finding is consistent with data presented in Chapter 3, where Bodipy C-16 uptake (fluorescent analogue of palmitic acid—C-16 long-chain fatty acid) was increased in Tfh and Th2 cells compared to naïve T cells (Figure 3.10 (B)).

Further analysis revealed that Th2 cells upregulate the expression of SLC27A6, which is involved in the uptake of very-long-chain fatty acids such as arachidonic acid (Figure 4.9 (D)) (Anderson and Stahl, 2013). This increase in expression was only observed in Th2 cells, independent of Tfh and naïve T cells. Previous studies have linked increased SLC27A6 in Nasopharyngeal Carcinoma (NPC) cells to higher intracellular accumulation of triglyceride and total cholesterol, which contributes to lipid biosynthesis and increased metastatic potential (Zhong et al., 2021). Collectively, this suggests that the unique upregulation of SLC27A6 in Th2 cells may contribute to their distinctly lipid-rich metabolic phenotype. Previous research suggests that the increase in lipid accumulation may also be a direct result of increased iron uptake to prevent oxidative stress by packaging iron clusters inside lipid droplets, which may provide a functional and rational explanation for this desired increase in neutral lipid accumulation. Packing iron inside lipid droplets prevents oxidative stress, especially ROS, which iron can catalyse the formation of (Rockfield et al., 2018). This finding is also consistent with the observed increased CD71 expression in Chapter 3 (Figure 3.8 B(iii)).

Collectively, this chapter presents novel findings related to glycolytic and lipid metabolic pathways associated with Tfh and Th2 biology. Specifically, the data demonstrate that Tfh cells are remarkably similar to Th2 cells in terms of metabolic pathway modelling, except for their increased expression of some key glycolytic enzymes. In contrast, Th2 cells exhibit significantly higher levels of genes associated with neutral lipid accumulation, with 15 genes showing significantly elevated levels of expression compared to Tfh and Naïve T cells. These findings could have implications for our understanding of immune response and potential therapeutic strategies, underscoring the significance of this research.

4.4 Discussion

Characterising the metabolic phenotype associated with the differentiation of Tfh and Th2 cells *in vivo* presents several challenges. These include distinguishing between correlation and causality, as well as accounting for the sensitivity of enzyme activity and metabolic flux. Conventional metabolic approaches are often unsuitable due to the limited number of cells that can be isolated from the mLN of mice *ex vivo*, hence limiting the number of approaches that can be used to try to delineate these differences (Vasilakou et al., 2016). Using methods such as METFLOW, as reported in Chapter 3, I identified key differences in the metabolic phenotypes associated with Naïve, Tfh, and Th2 cells, specifically in the expression of enzymes involved in glycolysis, iron metabolism, and fatty acid synthesis. In addition, there appeared to be distinct expression profiles for nutrient transporters, such as CD71 (transferrin receptor) and CD98 (amino acid transporter), and differences in the storage of lipids between Tfh and Th2 cells.

To further understand how these differential responses are programmed during Tfh and Th2 differentiation, I have evaluated the transcriptional and metabolic profiles of these cells using bulk RNA-Seq analysis in this chapter. Although this transcriptomic approach has limitations, specifically in not accounting for metabolic flux, protein translation and stability, or enzyme activation, it does provide valuable and novel insights into the programmed nature of the metabolic environment. However, caution should be taken not to over-interpret these findings. Nevertheless, bulk transcriptomics allows for the development of a comprehensive analysis of a broad range of immune and metabolic pathways from a sorted population of cells and is more sensitive and has greater depth than platforms such as single-cell RNA-Seq. (Li and Wang, 2021).

Platforms such as Searchlight2 and DAVID, allowing analysis of the differential gene expression data, were exploited to address the understanding of the key differential metabolic pathways associated with the development of the particular effector T cell populations. Previously, bulk RNAseq analysis was used to understand the metabolism of Tfh cells from Lupus-prone TC mice, and this showed that Tfh cells had increased amino acid and solute transporter expression compared to their C57BL/6 control equivalents (Gong et al., 2023).

4.4.1 Tfh cells favour increased gene expression associated with glycolytic, lipid and Iron metabolism in the mLN during *H. polygyrus* Infection

In the current analysis, it was observed that Tfh cells exhibit increased expression of genes associated with glycolytic, lipid, and heme metabolism (Figure 4.4) compared to their naïve counterparts. Notably, Tfh cells showed elevated expression of key enzymes involved in lipid metabolism, particularly stearoyl-CoA desaturase 2 (SCD2) (Figure 4.4B), linked to Bcl-6 expression. Previous studies have similarly reported increased SCD2 expression in murine Tfh cells compared to non-Tfh CD4⁺ T cells following IAV X31 immunisation *in vivo*, whilst Bcl-6 knockout significantly reduced SCD2 expression under *in vitro* Tfh-polarising conditions (Son et al., 2020). SCD2 plays a critical role in lipid processing by catalysing the conversion of saturated fatty acids into monounsaturated fatty acids (MUFA). This process is particularly important for protecting cells from ferroptosis, an iron-dependent form of cell death characterised by the accumulation of lipid peroxides. Tfh cells, due to their heightened metabolic activity and substantial iron utilisation during the immune response, are especially vulnerable to ferroptosis (Yao et al., 2021, Lee et al., 2021). Excess iron can catalyse the formation of ROS, leading to lipid peroxidation and cell death (Zhang et al., 2022). Therefore, the activity of SCD isoforms, such as SCD2, in generating MUFAs is crucial for mitigating the risk of ferroptosis. Recent studies have further demonstrated that SCD activity serves as a protective mechanism against ferroptosis in Tfh cells (Sen et al., 2023).

My results suggest increased iron utilisation in Tfh cells; as in Chapter 3, CD71 expression was increased in Tfh cells compared to naïve T cells (Figure 3.8). DAVID analysis of Tfh cells also suggests the active use of iron by Tfh cells, as heme biosynthetic pathways were enriched in the upregulated gene group (Figure 4.4). Iron uptake has been shown to be critical in Tfh cells, as iron accumulation in CD4⁺ T cells in patients with Systemic lupus erythematosus (SLE) promotes Tfh differentiation (Scindia et al., 2022). Iron overload in lupus-prone mice drives Tfh cell expansion, increased pro-inflammatory cytokines, and generation of autoantibodies, whilst iron chelation inhibits Tfh differentiation (Gao et al., 2022).

Iron biosynthesis requires the assistance of lipid metabolism for the correct storage of heme. My results from Chapter 3 suggest that Th2 cells increase the expression of CD71

on their surface compared to Tfh cells (Figure 3.8). It has been proposed that neutral lipid droplets are utilised as sites for excess iron storage and may contribute to the regulation of iron homeostasis in immune cells (Rockfield et al., 2018). In both Tfh and Th2 cells, where CD71 expression is increased, we also see an increase in the accumulation of neutral lipids (Figure 3.6/3.8). This may suggest that Th2 and Tfh cells utilise lipid droplets as a storage mechanism for excess iron to prevent oxidative stress (Rockfield et al., 2018).

My results also highlight increased glutathione metabolism in Th2 cells compared to Tfh cells (Figure 4.10 (A)). This could represent an alternative mechanism underlying the increased lipid droplet accumulation in Th2 cells relative to Tfh cells, potentially driven by elevated expression of the mitochondrial glutathione transporter SLC25A39. Enhanced SLC25A39 expression may increase mitochondrial glutathione import, thereby supporting redox homeostasis and reducing oxidative stress (Wang et al., 2021b). This, in turn, could promote lipid biosynthesis and storage by preserving mitochondrial function and enabling reductive biosynthetic pathways essential for lipid droplet formation. Glutathione is implicated in several cellular functions, including proliferation and the regulation of glucose, lipid, and amino acid metabolism. Hence, Tfh may utilise an alternative mechanism that prevents increased lipid droplet accumulation compared to Th2 (Crooks et al., 2018).

This observation is noteworthy, as several glutathione metabolism-related genes, including ALDH18A1, ASS1, GOT1, and MFHFD1, are downregulated in Tfh cells compared to Th2 cells (Figure 4.10 (A)). The genes upregulated in Th2 vs Tfh cells in the functional analysis pertain to downstream products of glutathione metabolism and are associated with the biosynthesis of amino acids and other metabolites (arginine (ASS1), Proline and ornithine (ALDH18A1), Serine (GOT1) and thymidylate (MTHFD1)) (Ohshima et al., 2017, Hu et al., 1999, Xu et al., 2023, Field et al., 2016).

Subsequent analysis highlighted that Tfh cells downregulate genes associated with amino acid and protein biosynthesis compared to naïve T cells (Figure 4.8). This may suggest that Tfh cells do not favour cell biosynthetic pathways required for cell division, which are highly energy-consuming (Raynor and Chi, 2024). The same could be said for pathways that feed into amino acid biosynthesis, such as methionine, nucleotide, purine and pyrimidine metabolism. Purine and pyrimidine are involved in nucleotide synthesis and methionine metabolism, which supplies formate for purine/pyrimidine synthesis (Boss, 1985, Moffatt and Ashihara, 2002). This observation is interesting, as you would suspect

Tfh cells to require these key pathways to assist their function, particularly protein biosynthesis, as Tfh cells are required to make large amounts of cytokines.

However, regarding the literature, it is not surprising that naïve T cells favour increased expression of these pathways over Tfh cells. Research has shown that during differentiation, Bcl-6 downregulates genes associated with protein translation and biosynthesis, particularly MYC (Ci et al., 2009), which we have also demonstrated to be decreased in Tfh cells compared to naïve T cells. This suggests that elevated levels of MYC expression in T cells correspond with an increased favouring of protein biosynthesis pathways (Pourdehnad et al., 2013). Regarding the microenvironment, the GC is also a nutrient-limited area of the LN, so it could be speculated that Tfh cells favour function over capacity for growth and proliferation (Arulraj et al., 2021). It should also be noted that naïve T cells are metabolically primed to proliferate rapidly upon activation (Xu and Powell, 2018). Tfh, on the contrary, have already undergone this rapid burst of proliferation at activation and are now more focused on effector functions and limiting proliferation to drive selection pressures inside the GC reaction (Crotty, 2014)

Butanone metabolism may have been expected due to the proximity of the mLN to the intestine, whereby butyrate, a bacterial metabolite, is produced. Butyrate can reprogram the metabolism of naïve T cells to favour oxidative phosphorylation (He et al., 2024). Interestingly, I also observed an increase in tryptophan metabolism in naïve T cells during *H. polygyrus* infection compared with uninfected counterparts. Tryptophan is a key amino acid that plays a crucial role in maintaining gut lumen homeostasis (Roth et al., 2021). It has been shown that tryptophan metabolism can affect naïve T cells in many ways, favouring the differentiation into Th17 or Treg cells (Stone and Williams, 2023), possibly indicating that the increase in tryptophan metabolism may be aiding in the development of the Treg response in *H. polygyrus* infection (White et al., 2020).

4.4.2 Tfh cells have higher Glycolytic Gene Expression than Th2 cells in the mLNs during *H. polygyrus* Infection

My analysis also highlighted the increased glycolytic gene signature in Tfh cells compared to naïve and Th2 cells (Figure 4.4 & 4.9). The relationship for increased glycolysis due to iron uptake is well-documented, and studies show that in colon tumour cells, iron could increase glycolysis by inhibiting the PDH complex (Liu et al., 2023b). Similarly, iron was shown to enhance glycolysis to drive a more pro-inflammatory phenotype in T cells (Lai et

al., 2022). This could reflect an iron-dependent mechanism utilised by Tfh cells to drive increased expression of glycolysis genes compared to naïve T cells. Hence, excessive iron in Th2 cells may be acting to dampen down glycolytic gene expression through the PDH complex, almost in a Goldilocks fashion, whereby if a cell has too much iron, it reprograms glycolysis to prevent oxidative stress from occurring.

In a similar vein, the GC microenvironment may foster increased glycolytic activity in Tfh cells over Th2 cells. Hypoxia, characterised by reduced oxygen levels, is a significant factor that stabilises and activates the TF HIF-1 α (hypoxia-inducible factor 1-alpha). HIF-1 α not only promotes glycolysis but also inhibits oxidative phosphorylation (OXPHOS), playing a vital role in Tfh cell differentiation and functioning (Cho et al., 2019). More specifically, HIF-1 α modulates the expression of CD40L and affects cytokine production in Tfh cells (McGettrick and O'Neill, 2020). Furthermore, HIF-1 α can diminish mitochondrial respiration by increasing levels of pyruvate dehydrogenase kinase 1 (PDK1), which prevents pyruvate from entering the TCA cycle (Kim et al., 2006). Considering that Bcl-6—a crucial TF for Tfh cells—downregulates glycolysis, it can be suggested that the hypoxia-driven increase in glycolysis is essential for developing the appropriate metabolic profile for Tfh cell maturation function (Oestreich et al., 2014).

Furthermore, I observed increased levels of the glycolysis gene, PKM, in Tfh cells compared to naïve T cells. Recently, the Yang lab showed that PKM expression was vital in controlling Tfh cell glycolysis through modulation of the tetramerisation of PKM2. They showed the link between glycolysis, BCL-6 expression, and Tfh cell differentiation, thus showing the critical requirement of glycolysis in Tfh cell biology (Lin et al., 2024). Compared to Th2 cells, my data highlights the significantly increased expression of 4 key glycolytic genes: ALDOC, PFKP, PGAM-1, and TPI1. These enzymes are critical in this pathway. Previous research has shown that Tfh is highly susceptible to glycolytic manipulation, and this can drive decreased differentiation and function, whether this is through 2-DG or PKM2 inhibition (Choi et al., 2018, Lin et al., 2024).

Interestingly, I showed the downregulation of ASS1 (Figure 4.11 (C)). Previous research has highlighted that the downregulation of ASS1 is associated with higher intracellular glutamine storage, particularly in cancer cells, as downregulation of ASS1 enables these cells in acidic environments to depend less on external sources of glutamine (Silberman et al., 2019). This possibly suggests that the GC could be a highly acidic environment, but this currently remains unknown and previous research has been inconclusive on the role of

glutamine metabolism in Tfh biology, especially *in vitro*. However, Tfh context-dependent lack of reliance on glutamine may be a result of ASS1 downregulation, as more extended cultures may drive the formation of a highly acidic environment, which would decrease Tfh cell reliance on glutamine (Prochazkova et al., 2024).

The downregulation of 1-carbon metabolic enzymes MTHFD1 and GOT1 highlights the importance of controlling serine and folate metabolism in Tfh cells (Figure 4.11). This situation suggests the development of a folate acid pool similar to that observed in cancer (Elizabeth, 2021), as evidenced by higher levels of MTHFD2 in Tfh cells compared to other T cell subtypes. This folate trap, therefore, would restrict DNA biosynthesis through MTHFD1 to conserve methionine for vital methylation reactions (Crider et al., 2012). This again links back to the differences observed in Tfh cells compared to naïve T cells, whereby they downregulate pathway elements associated with protein translation; further emphasising the desire of Tfh cells to promote functional rather than biosynthetic pathways required for cell division.

4.4.3 Tfh cells exhibit decreased Sterol Metabolism Gene Expression relative to Th2 cells in the mLNs during *H. polygyrus* Infection

To understand the metabolic pathways preferentially upregulated in Th2 cells, we evaluated the distribution (using Venn diagram analysis) of the significantly upregulated genes between two groups (Naïve T cell (Uninfected) vs Th2 and Tfh vs Th2). This analysis revealed that 15 genes associated with lipid and sterol metabolism were selectively upregulated in Th2 cells. These genes are likely induced by upregulating C/EBP and PPAR- γ TFs, which are known to drive lipid metabolism (Stark et al., 2019). These 15 identified genes associated with sterol and lipid metabolism highlight several intriguing avenues that warrant further exploration in our findings.

Firstly, the upregulation of SC5D, which facilitates the breakdown of lathosterol in the cholesterol synthesis pathway, is consistent with the reported requirement of cholesterol metabolism by Th2 cells (Skubic et al., 2024). The observed upregulation of LSS, which is responsible for lanosterol synthesis, further supports this requirement for cholesterol synthesis in Th2 cells (Romano et al., 2018).

Additionally, SOAT2 upregulation provides further evidence of the requirement for cholesterol, as it facilitates the formation of cholesterol esters from long-chain fatty acids and acyl-CoA. This observation aligns with the upregulation of the long-chain fatty acid transporter SLC27A6 (Figure 4.10), highlighting possible flux through this system. Previous findings from Chapter 3 highlighted the increase in neutral lipid accumulation in Th2 cells, which is consistent with the upregulation of SOAT2, which is involved in the processing of cholesterol in lipid droplets (Sun et al., 2021). It could be suggested that this increase in the expression of genes associated with lipid synthesis may be able to drive lipid droplet formation (Pramfalk et al., 2022).

Consistent with my findings above, Th2 cells favour (relative to Tfh cells) increased neutral lipid accumulation and expression of lipid biosynthetic pathways. Cholesterol has been demonstrated to influence a Th2 immune response, as in severe hypercholesterolemia, T cells can transition from Th1 to Th2 cells, driving the production of Th2 cytokines in atherosclerotic plaques (Zhou et al., 1998). Moreover, statin treatment modulates cholesterol metabolism and can facilitate a reduction in the expression of OX40L and CCL17 on Th2 cells, which are critical for the induction of a Type 2 immune response (Inagaki-Katashiba et al., 2019).

The increased expression of SLC27A6 in Th2 cells compared to all other T cell subsets analysed (Figure 4.12 (D)) suggests the possible uptake of long-chain fatty acids through the receptor. To utilise these long-chain fatty acids, T cells are required to facilitate lysosomal processing of these fatty acids into more minor, usable moieties, such as diacylglycerol and acyl-CoA. This processing appears to be a central theme of Th2 metabolism, driving the formation and transformation of lipid precursors into phospholipids or biologically active molecules, such as ceramides and eicosanoids. The enhanced requirement of phospholipids by Th2 cells may be linked to the enhanced proliferation and cytokine signalling by Th2 cells, as membrane expansion is required for this (Meiri, 2005).

Notably, there is an elevation in GM2A, a crucial lipid transport protein involved in the enzymatic processes related to gangliosides (Hsieh et al., 2022). Interestingly, it has been shown that GM2A can drive T-cell activation through lipid presentation, alluding to an alternative activation mechanism in Th2 cells (Wright et al., 2004).

Additionally, I observed increased NAGA expression, which is involved in the lysosome breakdown of glycoproteins into glycolipids (Garman et al., 2002), whilst APOBR is also involved in the uptake of dietary fatty acids and lipid-soluble vitamins (Sun et al., 2018) (Figure 4.12). These 3 genes are all associated with lipid uptake and processing, highlighting the lipid-centric nature of Th2 cells. Furthermore, Th2 cells selectively upregulate ACSBG1 (acyl-CoA synthase) and catalyse acyl-CoA formation from long-chain fatty acids (Ye et al., 2024). Similarly to ACSBG1, MGLL contributes to the hydrolysis of triglycerides into glycerol (Tardelli, 2020). This highlights the active role of managing and moving lipids through Th2 cells, and thus, a deeper understanding of the roles of acyl-CoA and glycerol may yield therapeutic targets to modulate Th2 cell function.

The observed increased expression of DEGS1 (Figure 4.12) is essential in synthesising ceramides and is influential in maintaining the integrity of mitochondrial membranes. Ceramide synthesis is derived from the condensation of palmitic acid and serine (Planas-Serra et al., 2023). In Chapter 3, I showed that Th2 cells increase their uptake of C-16 Bodipy, a surrogate for palmitic acid uptake. This suggests that palmitic acid may be involved in ceramide synthesis in Th2 cells, a proposal consistent with the observed increased levels of the palmitic acid transporter, SLC27A1 (Figure 4.12).

The role of ceramides in the Th2 response has been explored previously. A study using the OVA-aerosol challenge in mice with a genetic deletion of Ceramide Synthase 2 (CERS2) showed a lower Th2 response than the wild-type (Shin et al., 2021). It has been observed that Th2 cytokines can decrease ceramide production in atopic dermatitis, suggesting a complex relationship between ceramides and Th2 responses.

Furthermore, arachidonic acid metabolic pathways may also be of interest in Th2 metabolism and biology. The upregulation of ACOT7 has many functions inside the cell, specifically, it regulates cell cycle, proliferation, and glucose metabolism through arachidonic acid-mediated upregulation of glucose transporters, Glut1 and Glut4. This gene is responsible for the hydrolysis of arachidonoyl-CoA to arachidonic acid and CoA and is the precursor to eicosanoid synthesis (Jung et al., 2017, Nugent et al., 2001). Significantly, the transfer of acyl-CoA facilitated through increased MBOAT1 expression is essential for phospholipid synthesis, thereby providing insight into how these pathways intertwine with cellular proliferation by generating new phospholipids for membrane development (Gijon et al., 2008).

4.4.4 Limitations and Future Directions

Currently, there is an increasing demand for a comprehensive understanding of the transcriptional expression of key metabolic genes in rare and functionally distinct immune cell populations. This necessity has led to the development of single-cell approaches, such as single-cell RNA sequencing (scRNA-seq), which enable detailed resolution of cellular heterogeneity. However, these technologies come with significant financial and technical constraints, making them inaccessible to many laboratories. Consequently, pooling samples for bulk RNA sequencing remains a more feasible alternative for many researchers (Artyomov and Van den Bossche, 2020)

Despite these challenges, compromises are often necessary when designing experiments in immunometabolism, particularly when working with low-input cell populations. I hypothesised that gene expression changes, although sometimes more subtle than shifts in metabolite abundance or flux, could still provide valuable insights into cellular metabolic states. I therefore prioritised the development of an optimised protocol for RNA extraction and bulk RNA sequencing from low cell numbers. Bulk sequencing offers considerable depth and sensitivity, and importantly, allows for the confident interpretation of absence-of-expression data, where an undetected transcript is more likely to be truly absent rather than obscured by technical limitations, as can occur in metabolomics (Artyomov and Van den Bossche, 2020). My approach proved effective, yielding over 20,000 genes per sample and providing the necessary resolution to interrogate the transcriptional landscape of our targeted cell populations.

A limitation to my approach was that it does not account for flux through pathways, nor does it account for protein expression, however techniques such as Seahorse XF assays and also metabolomics require several additional processing steps that introduce potential metabolic alterations—most notably, fluorescence-activated cell sorting (FACS) has been shown to alter the redox state and metabolome of cells, potentially confounding downstream metabolic analyses. However, changes in transcription are less affected by FACS sorting of cells (Llufrio et al., 2018).

Further experiments based on this work would allow me to investigate the impact of the differences I have observed. An example of an experiment that could further our understanding is to block the four upregulated glycolytic enzymes independently in murine *in vitro* cultures of T_{fh} and Th2 cells, to see if this affects their differentiation and/or

function. Furthermore, I would also like to explore the requirement of sterol metabolism, as shown to be upregulated in Th2 cells, and to understand the role of neutral lipid accumulation in these cells compared to all other T cells in the mLN, through inhibition of key enzymes associated with these pathways. The use of click-chemistry/carbon tracing to understand whether SLC27A6 is important in the uptake of lipids in Th2 cells could also provide depth to our understanding. It can be manipulated using C57BL/6NJ-Slc27a6^{em1} (IMPC)/Mmjax (Jackson Laboratory), a CRISPR-generated knock-out mutant. This model could then be used to understand the effects of this solute carrier *in vitro* and *in vivo* by driving a Th2-mediated response, such as that observed in *H. polygyrus*. A more robust model system, however, would be to cross the C57BL/6Smoc-Slc27a6^{em1(flox)Smoc} (Shanghai Model Organism Custom) with a B6.Cg-Tg(Cd4-cre)1Cwi/BfluJ mouse (CD4-cre mouse) (Lee et al., 2001), to generate the knockout of this receptor, SLC26A6, in the CD4 compartment of cells (will also affect CD8⁺ T cells due to the nature of the system). This would then allow for the specific effect of this receptor on CD4⁺ T cells to be understood.

4.5 Concluding Remarks

In summary, this chapter highlights several new findings, including the metabolic transcriptional signatures of Tfh and Th2 cells. This chapter explores and provides a rationale for the observed metabolic differences between Tfh and Th2 metabolism, as discussed in Chapter 3 (Increased CD71 and Neutral Lipid Accumulation). I have highlighted the key differences in metabolic gene signatures between Tfh and Th2, with Tfh cells preferentially increasing gene expression associated with glycolysis. Moreover, I explored the mechanisms underlying the increased neutral lipid accumulation in Th2 cells and how it could be linked to the uptake and storage of long-chain fatty acids via SLC26A7 or excess iron accumulation. This analysis suggests that the potential manipulation of glycolysis could provide a targeted approach to alter Tfh cell function and differentiation over Th2 cells selectively. Targeting the manipulation of cholesterol metabolism in Th2 may be an exciting therapeutic avenue (Madanayake et al., 2013, Wang et al., 2021a).

Chapter 5 - Transcriptional Analysis of Tfh Cell Metabolism During IAV Infection

5.1 Introduction

The previous chapter focused on type 2 immune response and the characterisation of metabolism in Tfh and Th2 cells in the mLN draining the small intestine during challenges with the helminth parasite *H. polygyrus*. However, I wanted to understand whether the Tfh metabolic profile was consistent across immune responses that are differently polarised and/or resulting from different infections. In this chapter, I explored the immunometabolism of Tfh cells from the mdLNs draining the lung during acute infection with IAV. This immunological stimulus drives a predominantly Th1 cell response.

Sublethal doses of IAV infection in C57BL/6 mice drive high viral titres in the lung from day 2 to 8 post-infection, with the clearance and resolution phase occurring from day 10 to 14 (Jelley-Gibbs et al., 2005). Tfh cells are crucial in this response to IAV infection, as they facilitate the development of GCs into a strong, high-affinity antibody response against the pathogen, as well as the generation of memory B cells, which protect against reinfection. Antibodies are vital in the adaptive response to IAV (Ahmadivand et al., 2025).

Previous research has highlighted the role of glycolytic and oxidative phosphorylation metabolism in the Tfh cell response to IAV infection, with glycolysis being critical for such Tfh differentiation when blocked with 2-DG (glycolysis) and succinate-mediated inhibition (OXPHOS) of metabolic pathways (Dong et al., 2019). It has also been shown that metabolic manipulation can alter the Tfh cell response to IAV infection. The Son lab highlighted that blocking lipid metabolism through SCD-1, a key enzyme responsible for fatty acid synthesis, decreased the Tfh cell response in IAV X31 immunisation through early contraction of the GC response: although this could suggest that SCD inhibition primarily targeted B cells, causing the GC to contract with the Tfh changes a consequence of the GC collapse, this was never explored fully in the paper (Son et al., 2020).

Recent research has highlighted the transcriptional differences between Tfh1 (Th1-like Tfh) and Tfh2 cells (Th2-like Tfh) (Zaini et al., 2023). However, the role of these cells' immunometabolism was not investigated in the analysis. Thus, I decided to investigate,

through bulk transcriptomics, which metabolic pathways in Tfh cells are upregulated in the Th1 setting and to compare these signatures with the previous data generated from the Th2 and *H. polygyrus* -stimulated datasets. I also compare my findings to a publicly available dataset from the Colby Zaph lab, where they used *T. muris* to drive type 1 and type 2 inflammatory contexts using the same pathogen (Zaini et al., 2023). This data allowed me to investigate whether Tfh cell metabolism differs based on the inflammatory environment, regardless of anatomical location.

This chapter, therefore, aims to understand the transcriptional metabolic profiles of Tfh, Th1, and naïve T cells during acute IAV infection. However, my interest predominantly focuses on using IAV infection as a means to generate Tfh cells in a predominantly Th1-mediated response, to understand whether the metabolic transcriptional signature observed in Tfh cells in Chapter 4 during a Th2-driven immune response is consistent/distinct from that of Tfh cells in different contexts, such as IAV infection.

5.2 Aims

- To investigate the metabolic pathways that are transcriptionally active in Tfh and Th1 cells during IAV infection
- To explore the difference in metabolic requirement of Tfh cells in Th1- and Th2-mediated settings.
- To understand the distinct metabolic profiles of naïve T cells during different immune responses, focusing on whether specific metabolic rewiring occurs in different anatomical sites (mLN and mdLN) under diverse immunological conditions (*H. polygyrus* and IAV infection)

5.3 Results

5.3.1 Delineating the metabolic profiles associated with for Tfh and Th1 differentiation in the mdLNs

To understand the metabolic transcriptional profile of Tfh cells compared to naïve and Th1 cells during IAV infection, I first had to isolate and sort pure populations of Tfh, Th1, and naïve CD4⁺ T cells from the local draining LNs, namely the lung-draining mdLNs. These LNs were harvested from uninfected and IAV-infected (day 10 post-infection) female C57BL/6 mice, and single-cell suspensions were generated for subsequent flow cytometric sorting. LN were pooled from four individual mice per group to achieve the 100,000 cells per sorted population required for bulk mRNA-seq. To obtain Tfh and naïve T cells, I utilised the antibody panel described in Chapter 3 (Figure 3.1). Additionally, Th1 cells were identified by their high expression of CXCR3 and CD44. Thus, cells were gated based on the parameters highlighted in Figure 5.1A to identify each population, whereby CD4⁺ T cells were defined as Live CD4⁺ TCR-b⁺ lymphocytes. Naïve T cells were deemed CD4⁺ CXCR5^{Low} PD-1^{Low} CXCR3^{Low} CD44^{low}, with Tfh cells denoted as CD4⁺ CXCR5^{High} PD-1^{High} and Th1 cells as CD4⁺ CXCR5^{Low} PD-1^{Low} CD44^{High} CXCR3^{High}.

CXCR5 and PD1 were used to identify Tfh cells, consistent with previous chapters, whilst CXCR3 was used to identify Th1 cells. These populations were sorted only from IAV-infected animals. Naïve T cells were sorted from both uninfected and infected mice and were identified as CD4⁺ CD44^{Low} cells. The antibody staining and gating strategies are shown in Figure 5.1A, with high purity obtained (Figure 5.1B) of the desired abundance. mRNA was then extracted from samples and sent to Novogene for commercial sequencing to obtain bulk transcriptomic datasets for each cell type, analysed below via the SL2 pipeline.

Firstly, to understand the degree of similarity between samples, I performed PCA component analysis, which highlighted that the replicate samples within a particular cohort of T cells grouped together and formed distinct clusters separate from the other T cell types (Figure 5.1C). To validate whether each cluster was functionally relevant, a biased approach was used to investigate the expression of markers associated with Tfh and Th1 biology, thereby confirming the predicted transcriptional phenotype of each population. This involved screening a panel of “Signature” TFs and cytokines: HIF-1 alpha, IL-21,

Bcl-6 and IL-4 as signature genes for Tfh cells (Huang et al., 2022, Liu et al., 2013), and TBX21 (T-bet) as a marker of Th1 cells. FOXP3 was also included to observe the purity of sorted populations (Figure 5.1D)

This analysis showed that HIF-1 alpha, IL-21 and Bcl-6 expression were increased in the Tfh cell population, data consistent with previous findings (Huang et al., 2022).

Interestingly, the “Tfh” cells also expressed IL-4 and TBX21 (T-bet), suggesting that our purification method captured some Tfh heterogeneity. This data is also consistent with previous findings that Tfh cells produce the highest levels of IL-4 in the LN during *H. polygyrus* infection (King and Mohrs, 2009, Zaini et al., 2023) and suggests that Tfh cells may also transcribe IL-4 during an active response to IAV infection. The results also highlight T-bet expression in Tfh, albeit at a lower level than that associated with the Th1 phenotype.

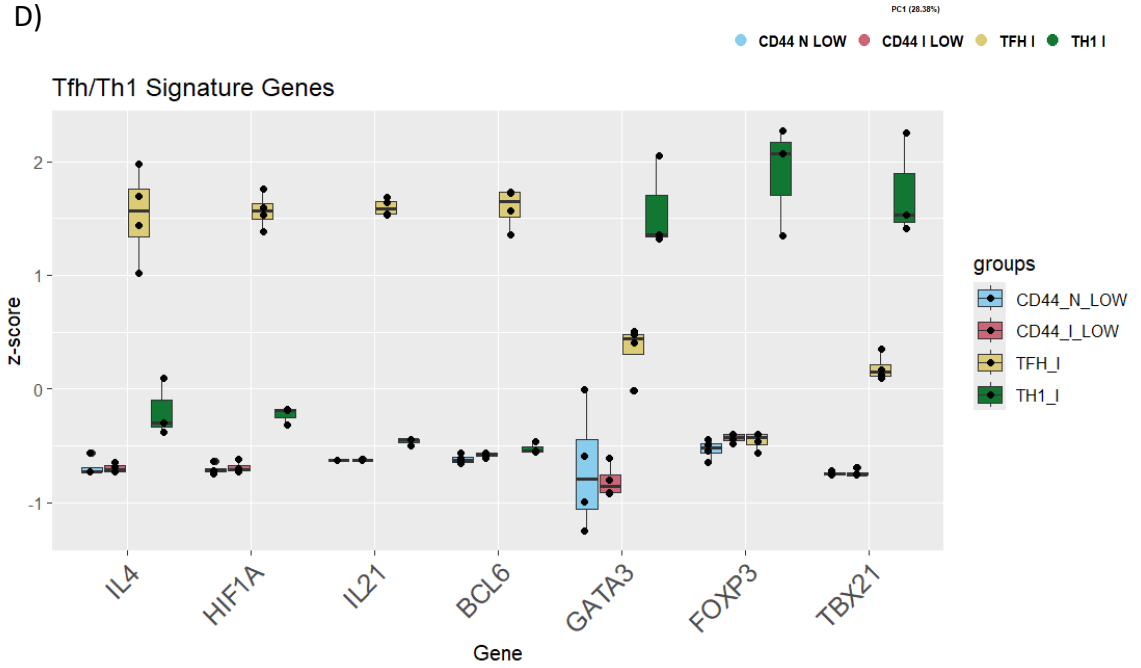
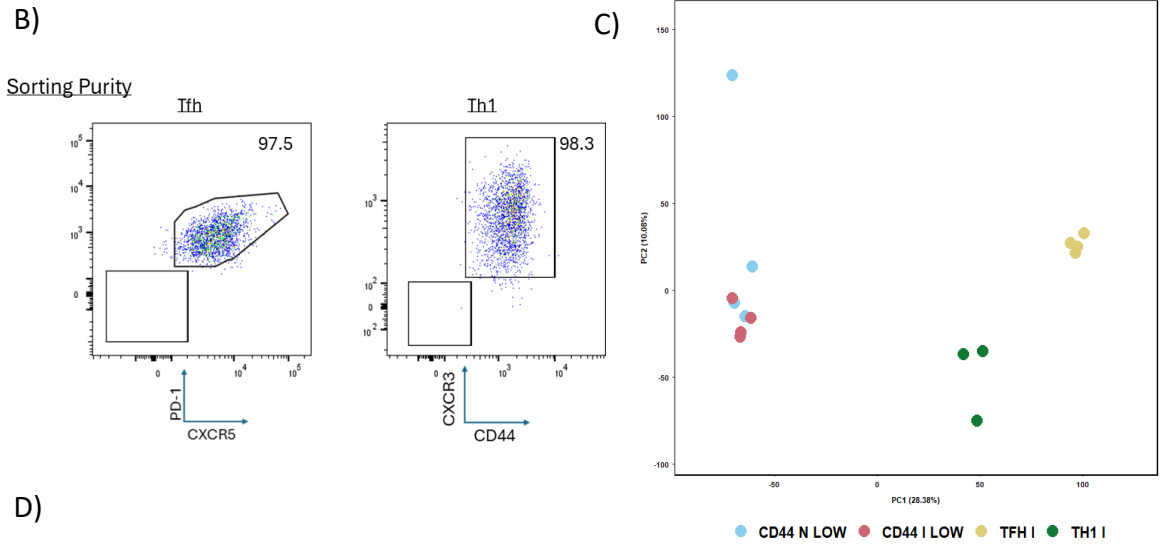
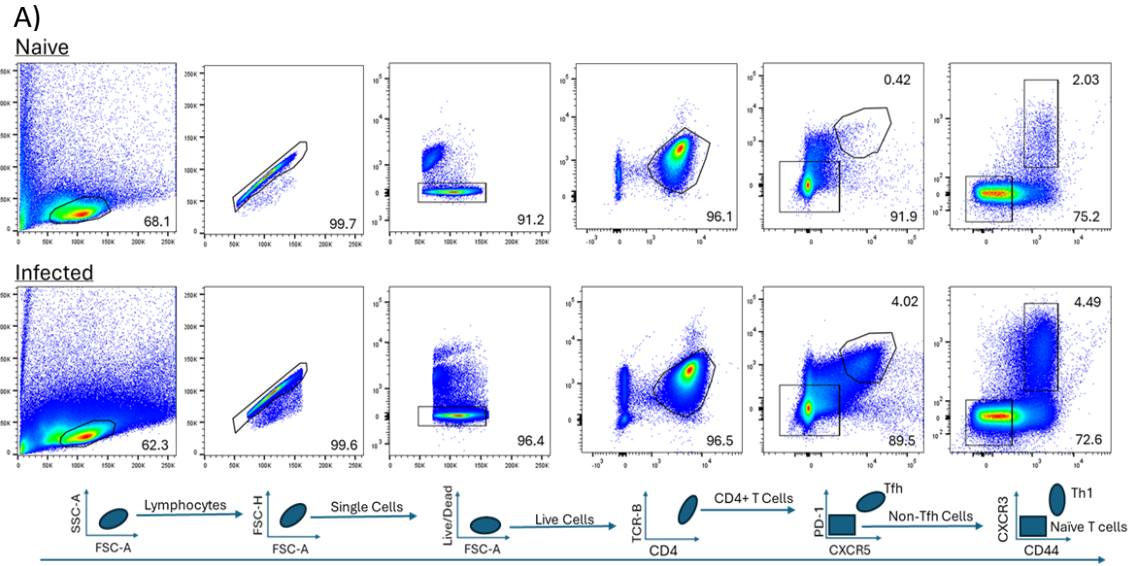


Figure 5.1. Purification of Tfh, Th1 and Naïve T populations from the mdLN of uninfected and IAV-infected mice for Transcriptional analysis.

C57/B6 mice were infected with 110 PFU of IAV PR8 (H1N1 Puerto Rico), and 10 days post-infection, the mdLN were removed. A) gating strategy of Tfh cells (Live CD4⁺ CXCR5 High, PD-1 high cells) and Th1 cells (Live CD4⁺, non-Tfh, CD44 High, CXCR3 High) from mdLN samples of uninfected and Infected mice. B) Flow scatter plots showing sort purity for Tfh and Th1 cells from Infected mice. C) PCA Scatterplot of each sample in the bulk transcriptomic analysis. The percentage of total variation explained by each component is given on the x-axis (PCA-1) and y-axis (PCA-2) as appropriate. All gene expression values were scaled on a gene-by-gene basis using the z-score transformation before performing PCA to control for the representation of highly expressed genes. D) Boxplot of Key Tfh and Th1 genes. Graphed data is shown with mean $\pm$ SD with n= 3/4 for each experiment. All significantly differentially expressed genes are determined by $p_{\text{adj}} < 0.05$, absolute $\log_2$ fold > 0.5 . Expression levels have been scaled as per gene Z-score.

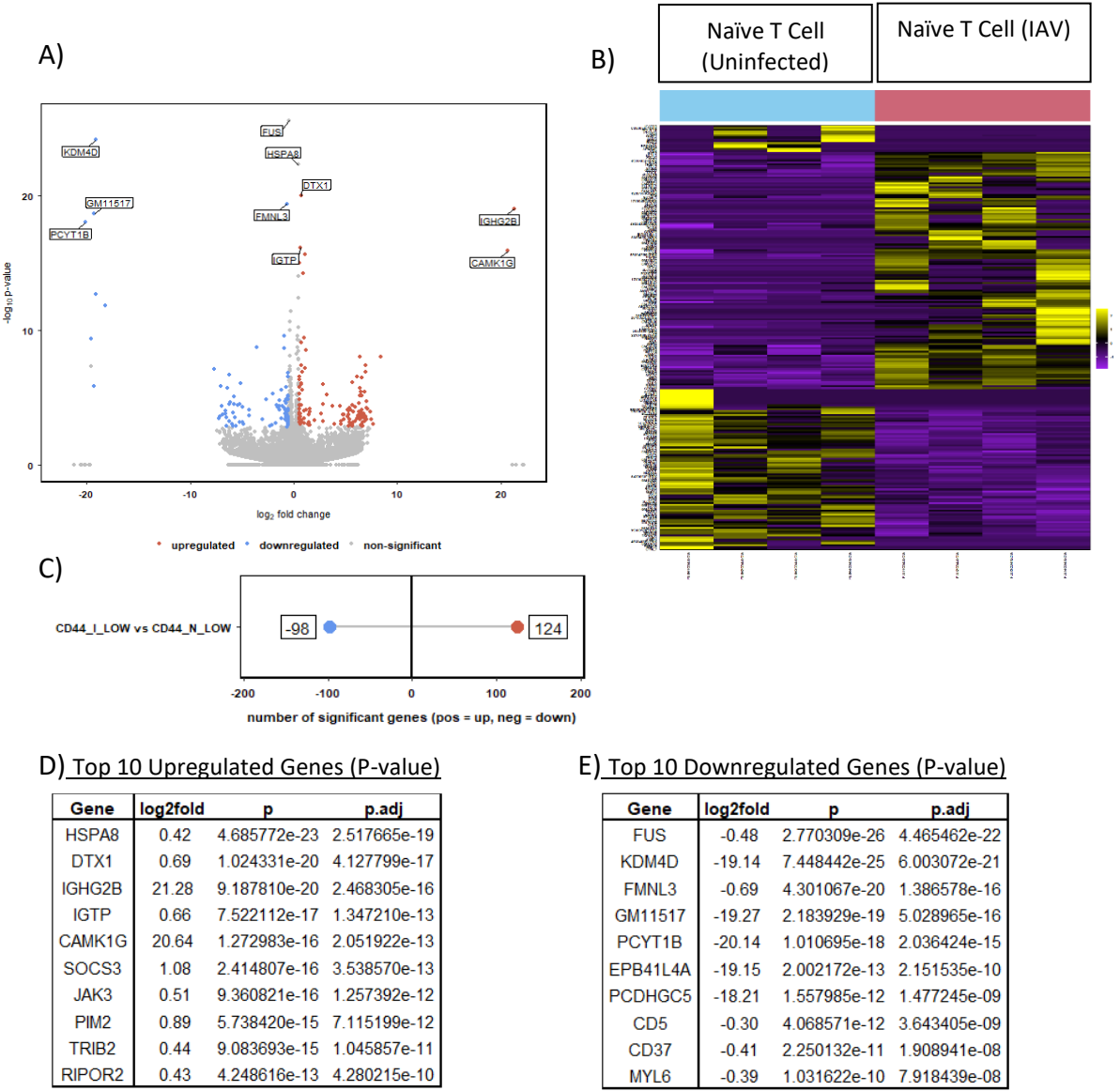


Figure 5.2. Differential Expression Analysis of Naïve T Cells from Uninfected and IAV Infected mice:

A) Volcano plot showing the differential gene expression of Naïve T cells from uninfected and IAV infected mice where significantly ($p_{adj} < 0.05$, absolute $\log_2$ fold > 0.5) downregulated and upregulated genes are shown in blue or red, respectively, and non-significantly regulated genes are denoted in grey. A positive fold change indicates higher expression in naïve T cells from IAV-infected mice compared to naïve T cells in uninfected mice. B) Hierarchically clustered heatmap of the significantly differentially expressed genes ($p_{adj} < 0.05$, absolute $\log_2$ fold > 0.5) between naïve T cells from IAV-infected mice and naïve T cells in uninfected mice for all mice in each sample groups. Samples are on the x-axis, and genes are on the y-axis. Colour intensity represents expression level, with purple representing low expression and yellow representing high expression. Expression levels have been row-scaled as z-scores. The y-axis (both plots) and x-axis (right plot) have been hierarchically clustered using Spearman distances, with UPMGA (Unweighted Pair-group method with Arithmetic Mean) agglomeration and mean reordering. C) Schematic highlights the number of upregulated genes in both naïve T cells from IAV-infected mice and naïve T cells in uninfected mice. Tables showing the ten most upregulated D) and downregulated E) genes as determined by adjusted p-

value in naïve T cells from IAV-infected mice compared to naïve T cells in uninfected mice, whereby significance was defined as $p_{\text{adj}} < 0.05$, absolute $\log_2$ fold > 0.5 .

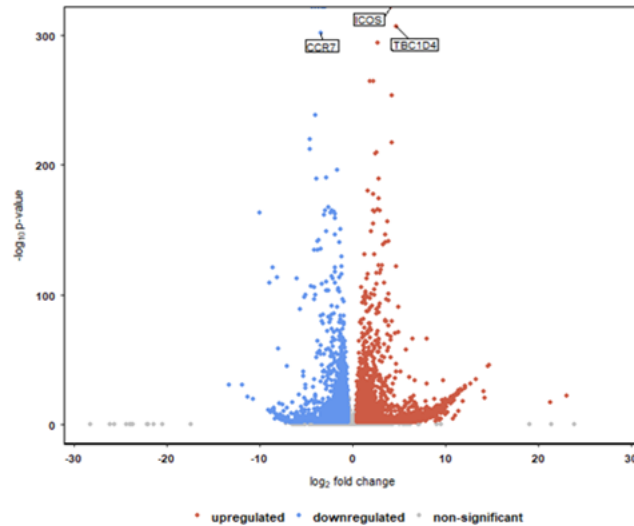
A comparison of naïve CD4⁺ T cells from both the uninfected and infected groups was undertaken to understand whether the microenvironment in a Th1-type inflamed LN can drive metabolic rewiring of “naïve” T cells. This comparison was achieved by separating the differences in DE gene expression observed between the microenvironment and immunological stimulus by analysing the naïve T cells from the uninfected/IAV-infected mice (Day 10). This highlighted, perhaps surprisingly, diverse transcriptional profiles between the naïve T cell groups (Figure 5.2A). Hierarchical clustering highlighted the diverse nature of gene expression in both cell types (Figure 5.2B), with 124 significantly upregulated and 98 significantly downregulated genes (Figure 5.2C). As a first step to understand the biology underpinning these changes, the “top 10” (by significance) up- and down-regulated genes were examined to investigate any key patterns.

In the top 10 significantly upregulated genes between Naïve CD4⁺ T cells in infected animals and the same population in uninfected mice, I found that the genes upregulated were involved in a host of cellular processes, such as protein folding/integrity (HSPA8 (Bonam et al., 2019)), activation of T cells (DTZ1, CAMK1G (Hsiao et al., 2009, Lin et al., 2005)), cytokine production and signalling (IGTP, SOC3, JAK3 (Collazo et al., 2002, Carow and Rottenberg, 2014, Ghoreschi et al., 2009)), proliferation and differentiation (RIPOR2, PIM2 (Daenthanasanmak et al., 2018)) and cellular homeostasis through inhibition of Akt required for proliferation (TRIB2 (Cao et al., 2023, Shimada et al., 2018)).

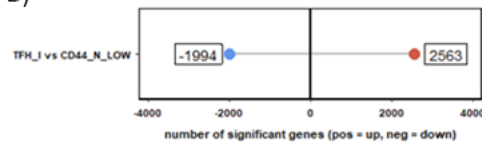
As for the top 10 downregulated genes, I also witnessed that many of the genes were involved in key cellular processes involved in the maintenance of T cells, such as migration and activation (FUS, KDM4, CD5, FMNL3, MYL6 (Qu et al., 2023, Ward et al., 2014, Matson et al., 2020, Thompson et al., 2020, Li et al., 2020a). It has been shown that inhibition of KDM4 can lead to increased transcription of CXCL10, which enhances T cell migration, whilst downregulation of CD5 is believed to be a strategy by T cells to increase their sensitivity to TCR/p:MHC interactions (Dorothee et al., 2005). I also found that genes associated with proliferation were downregulated, namely CD37. CD37 deficiency can lead to hyperproliferation and increased IL-2 production, suggesting that these T cells may

be in the early stages of upregulating IL-2 production and possibly proliferation (van Sriel et al., 2004). The microenvironment around these T cells could be conditioning them in anticipation of activation. Overall, it appears that naive T cells in infected mice exhibit higher gene expression associated with activation and proliferation compared to naive T cells in uninfected mice.

A)



B)



C)

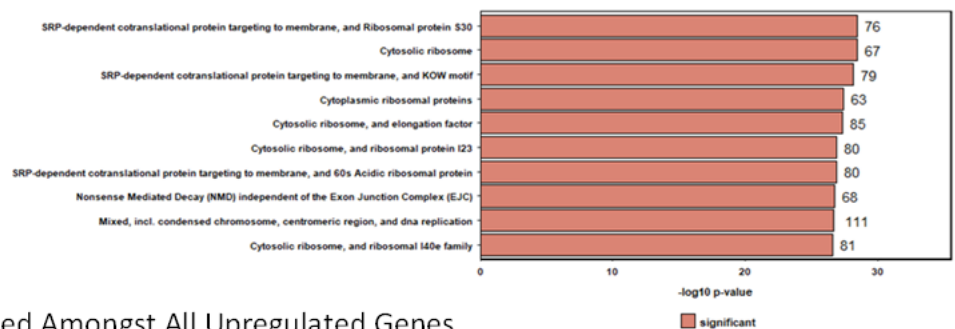
i) Most Upregulated Genes (P-Value)

Gene	log2fold	p	p.adj
ICOS	4.14	0.000000e+00	0.000000e+00
TBC1D4	4.66	1.090786e-307	2.079158e-304
IL2RB	2.68	2.754085e-295	4.295120e-292
ITGAL	1.82	2.525985e-265	3.611107e-262
ASAP1	2.26	3.464459e-265	4.571754e-262
ITGB1	4.22	1.133393e-254	1.388811e-251
IKZF2	4.26	1.242556e-217	1.253885e-214
SLAMF6	2.57	1.324306e-210	1.195709e-207
IZUMO1R	2.47	6.777691e-210	5.813565e-207
HIF1A	2.79	1.020683e-189	7.295760e-187

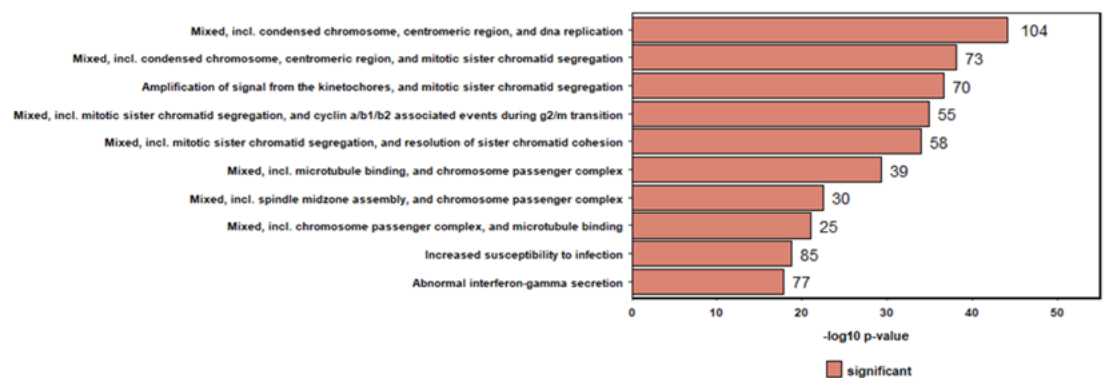
ii) Most Downregulated Genes (P-value)

Gene	log2fold	p	p.adj
ALS2CL	-2.90	0.000000e+00	0.000000e+00
ARL4C	-4.01	0.000000e+00	0.000000e+00
SATB1	-3.13	0.000000e+00	0.000000e+00
RASGRP2	-3.05	0.000000e+00	0.000000e+00
IL7R	-3.32	0.000000e+00	0.000000e+00
VIPR1	-3.68	0.000000e+00	0.000000e+00
SMAD7	-4.30	0.000000e+00	0.000000e+00
CCR7	-3.36	9.239644e-303	1.585061e-299
SCML4	-4.05	5.235560e-239	5.987735e-236
ST8SIA6	-4.63	3.341183e-220	3.582375e-217

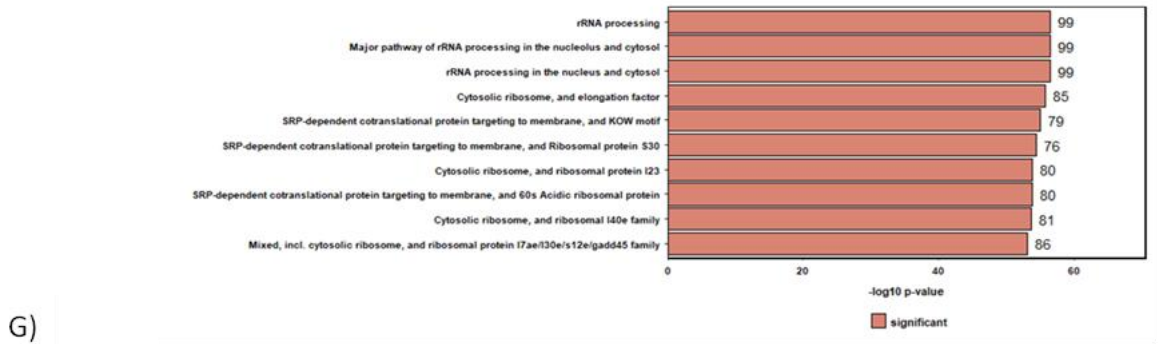
D) Most Enriched Amongst All Significant Genes



E) Most Enriched Amongst All Upregulated Genes



F) Most Downregulated Amongst All Significant Genes



G)

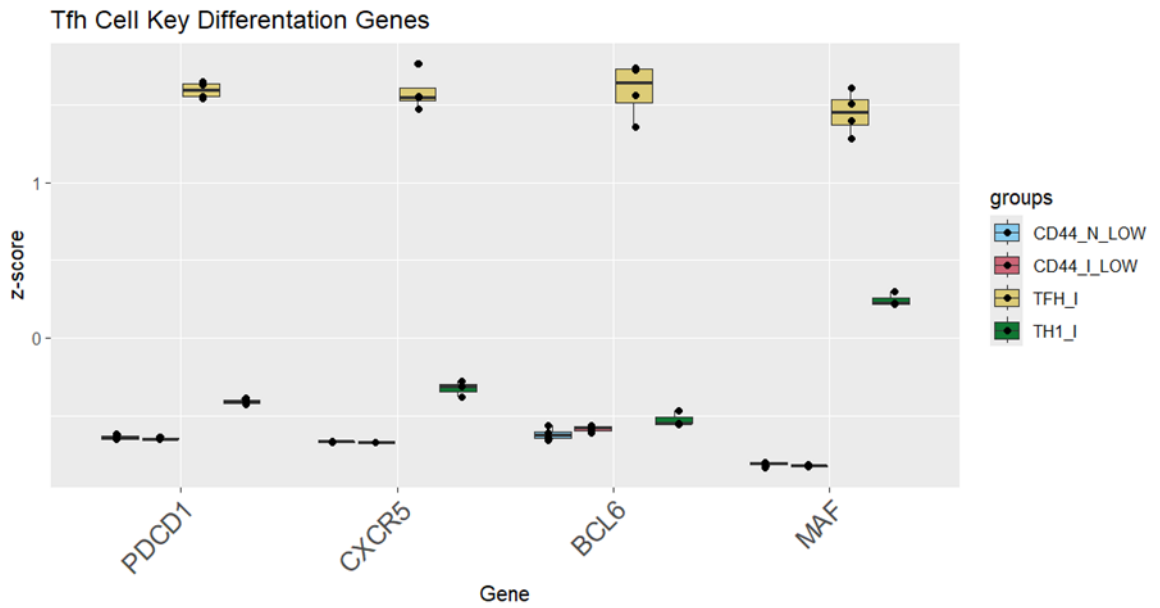


Figure 5.3. Differential Expression Analysis of Tfh and Naïve T Cells from Uninfected and IAV Infected mice:

C57/B6 mice were infected with 110 PFU of IAV PR8 (H1N1 Puerto Rico), and 10 days post-infection, the mdLN were removed. A) Volcano plot for the comparison between gene expression in Tfh (IAV) and Naïve T cells (Uninfected). Significantly differentially expressed genes ($p_{adj} < 0.05$, absolute $\log_2$ fold change > 0.5) are shown in red, significantly downregulated genes in blue, and non-significant genes in grey. A positive fold change indicates higher expression in Tfh (IAV) than in Naïve T cells from uninfected mice. B) Schematic showing differential count for upregulated genes for Tfh (IAV) and Naïve T cells from uninfected mice. C) Table showing the ten most upregulated ii) and downregulated ii) genes by adjusted p-value. Upregulated genes are higher, and downregulated genes are lower, in Tfh (IAV) than in Naïve T cells from uninfected mice. D) Bar chart of the most enriched pathways amongst all significant genes in Tfh cells (IAV) vs Naïve T cells from uninfected mice. E) Bar chart of the most enriched pathways amongst all upregulated pathways in Tfh cells (IAV) vs Naïve T cells from uninfected mice. F) Bar chart of the most downregulated pathways amongst all significant genes in Tfh cells (IAV) vs Naïve T cells from uninfected mice. G) Boxplot of Key Tfh genes. Enrichment analysis was performed using the Hypergeometric Gene Set Enrichment tool on the gene set databases STRING 11.5. Significantly enriched gene sets ($p_{adj} < 0.05$, absolute $\log_2$ fold > 0.0) are red and non-significant black in each bar graph (Fig. 5.3 D-F).

After analysing both naïve T cell populations, I aimed to understand the transcriptional profile of Tfh cells compared to naïve T cells from uninfected mice, to identify which genes were up- and downregulated during activation and differentiation. I decided to compare the Tfh cells to the naïve T cells from uninfected mice, as previously I showed that naïve T cells from IAV-infected mice appeared to be conditionally primed as a result of inflammation in reactive LN due to the ongoing response towards IAV infection. To understand this transcriptional diversity, the DE gene expression profiles of the bulk RNA-Seq data from Tfh cells compared to naïve T cells in uninfected mice were determined (Figure 5.3A). This highlighted 2563 significantly upregulated and 1994 significantly downregulated genes (Figure 5.3B).

To discern the mechanisms underpinning these gene changes, the first step was to examine the top 10 up- and down-regulated genes to determine if they indicated any key metabolic differences (Figure 5.3C). This analysis also provided corroborative data for the enrichment of Tfh cells, as the analysis displayed gene signatures associated with Tfh cell differentiation (Wellford and Schwartzberg, 2024), namely increased expression of ICOS and HIF-1 α (Figure 5.3C (i)), genes which are commonly upregulated in Tfh cells compared to naïve T cells (Dong et al., 2019).

Further examination of the top 10 genes upregulated in Tfh cells highlighted TBC1D4 as differentially upregulated in Tfh cells compared to naïve T cells and involved in glucose transport into the cell (Andreatta et al., 2021). Likewise, ITGAL, also known as CD11a/LFA-1, is upregulated in Tfh cells compared to naïve T cells and is known to be crucial for the function and differentiation (Meli et al., 2016). Interestingly, two of the top 10 upregulated genes have been shown to drive the induction of the key TF Bcl-6, which is instrumental in the development of Tfh cells. Thus, ITGB1 and LFA-1 can promote Bcl-6 gene expression, and crucially, it has been shown that LFA-1 deletion can abolish Tfh cell formation during intestinal helminth infection (Meli et al., 2016).

Similarly, IKZF2, which exhibits significantly increased expression in Tfh cells compared to naïve T cells, has been shown to drive the expression of ICOS and is implicated as critical for the function of Th cells during *S. japonicum* infection. IKZF2 encodes the TF Helios, which is critical to the control of Tfh expansion, as deletion results in increased Tfh and GC formation (Hetemaki et al., 2025). Furthermore, SLAMF6 is also upregulated compared to naïve T cells in Tfh, and is a key receptor involved in the formation of the GC response (Crotty, 2014).

I also examined the top 10 most downregulated genes in Tfh cells vs naïve T cells from uninfected mice, which provided additional information relating to Tfh cell differentiation (Figure 5.3C (ii)). Similarly to what was found with Tfh differentiation in the Th2 environment (Chapter 4), there was decreased expression of CCR7 in Tfh cells, compared to naïve cells, in the type 1 inflammatory setting. Decreased CCR7 is associated with the upregulation of CXCR5, which allows for the migration of the Tfh cell to the B cell follicle for the development of the GC response (Hardtke et al., 2005). This is expected as Tfh migration via CXCR5 is fundamental to the GC response, and collectively, these data provide strong evidence that we successfully isolated Tfh cells during our protocol.

Consistent with previous findings, there is also a downregulation of SATB1 in Tfh cells compared to naïve T cells from uninfected mice. This gene is responsible for maintaining the naïve T cell program while also serving as a restraint against PD-1 expression. This highlights a potential key mechanism for the induction of PD-1 expression in Tfh cells. SATB1 deficiency has previously been shown to allow for Tfh cell differentiation in published studies (Chaurio et al., 2022, Nussing et al., 2022, Stephen et al., 2017), and a similar downregulation was observed in Tfh cells in the *H. polygyrus* infection study (Chapter 4), as shown here in the IAV infection (Figure 5.3C (ii)).

Such decreased expression of markers associated with naïve T cell phenotype was common throughout the top 10 downregulated gene list. For example, decreased expression of IL-7R was exhibited by Tfh cells compared to naïve T cells (uninfected mice). This aligns with previous reports that Tfh cells exhibit reduced IL-7 expression in IAV infection, especially in the later stages, as IL-7 represses Bcl-6 and the genes required for Tfh cell differentiation and function through STAT5 activation (McDonald et al., 2016). Another key downregulated gene was VIPR1, which has previously been shown to decrease significantly after TCR stimulation, suggesting that it is required to maintain resting naïve T cells (Rosenberg and Singh, 2019).

Interestingly, SMAD7, a negative regulator of TGF- β signalling, is downregulated in Tfh cells compared to naïve cells. TGF- β is crucial for Tfh cell differentiation and can control SATB1 expression, as TGF- β can silence SMAD7, a crucial genomic organiser. This, in turn, drives increased ICOS expression, which is crucial for the function of Tfh cells within the GC. TGF- β signalling can protect pre-Tfh cells from IL-2-induced mTOR activation, thereby promoting their differentiation (Ma and Nakayamada, 2021, Chaurio et al., 2022).

To understand the relationship between increased and decreased gene expression and how this links into pathways involved in cellular function in Tfh cells compared to naïve T cells, I next performed STRING analysis of the differentially expressed (DE) genes between Tfh and naïve T cells (Uninfected) using the SL2 platform. This was to understand whether elements of the metabolic pathways of interest were present in the most upregulated or downregulated gene lists. However, as this software only showcases the top 10 pathways, this approach did not provide the depth of analysis required: pathways involved in cell cycle, rRNA processing, protein biology, and mitosis (Figure 3.3 D, E, F) were highlighted as being the most enriched upregulated and downregulated pathways in Tfh cells compared to naïve T cells, and no metabolic changes were identified. I then used DAVID analysis (Sherman et al., 2022) to address this lack of depth in the pipeline.

5.3.2 Analysis of metabolic pathways enriched in differentially expressed genes in Tfh compared to naïve T cells

We employed DAVID analysis to analyse the genes and associated pathways upregulated in Tfh cells from IAV-infected animals compared to naïve T cells (uninfected mice), in search of metabolic differences. This approach enabled a deeper analysis, as it highlights the upregulated genes and the pathways they are associated with, allowing me to understand and answer the research question posed: What are the metabolic transcriptional differences observed between Tfh (IAV-infected) and naïve T cells (Uninfected)?

Such analysis indicated the enrichment of pathways associated with the regulation of glycolysis, fatty acid metabolism, lipid transport, and metabolism in Tfh cells, albeit not all of these networks were significantly enriched (Figure 5.4A). These pathways exhibit commonalities with those upregulated in Tfh cells compared to naïve T cells during *H. polygyrus* infection.

Indeed, key glycolytic genes were upregulated in Tfh cells compared to naïve T cells in IAV infection, including Hexokinase (HK) 1 and 2, similarly to the elevation of HK2 expression in Tfh cells over naïve T cells in the *H. polygyrus* model (Figure 3.9B(i)). The increase in DE glycolytic gene expression highlights that transcriptionally, Tfh cells are increasing their reliance on glycolysis to fuel their function. This suggests a rapid requirement for ATP to fuel their metabolic demands and the need to synthesise key

biosynthetic molecules that can be utilised in other pathways, such as lipid and fatty acid metabolism (Mayberry et al., 2022).

There was also an enrichment, albeit non-significantly, in lipid and fatty acid metabolic pathways in Tfh cells compared to naïve cells. Relating to this, DECR1 expression was significantly elevated in the Tfh compared to the naïve T cells, and this gene is involved in the regulation of lipid homeostasis and the β -oxidation pathway (Zhou et al., 2022). Interestingly, this gene is also involved in protecting cells from ferroptosis (Nassar et al., 2020), and this may be vital for Tfh cells, as this is a form of cell death that Tfh cells are susceptible to (Yao et al., 2021). Another key mediator of lipid processing, LPIN2, was also significantly upregulated in both *H. polygyrus* and IAV models, consistent with suggestions that lipid processing is a key trait of Tfh cell biology that helps regulate cell inflammatory profiles (Valdearcos et al., 2012).

Genes involved in lipid transport were upregulated in Tfh cells compared to naïve T cells. These include genes such as SLC43A3, which are responsible for transporting purines and free fatty acids (Hasbargen et al., 2020). VMP-1 is also involved in lipid secretion and formation. Interestingly, it has previously been shown that VMP-1 helps to form lipid droplets (Morishita et al., 2019). This finding is consistent with my previous results, where neutral lipid accumulation was increased in Tfh cells compared to naïve T cells under Th2 conditions of *H. polygyrus* infection (Chapter 3, Figure 3.11).

In conjunction with the functional analysis by DAVID, which highlights both genes and pathways enriched in the dataset for Tfh vs. naïve T cells from uninfected mice, KEGG pathway analysis (Figure 5.4(E)) also showed that there was enrichment in pathways associated with amino acid, nucleotide sugar, fructose/Mannose, and galactose metabolism. This emphasises the broad nature of Tfh metabolism and that divergent metabolic pathways could be employed to facilitate their energetic and biosynthetic requirements.

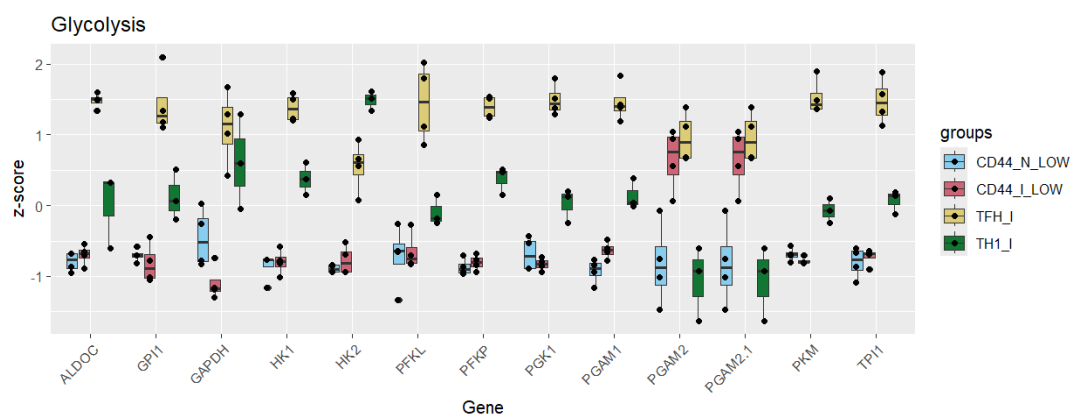
These results also underscore a direct comparison between activated T cells (Tfh) and resting naïve T cells (uninfected). Activation will inevitably lead to the broad activation of multiple metabolic programs. To understand the differences between activation and Tfh cell differentiation on the metabolic landscape of effector CD4⁺ T cells, I decided to look at Th1 cells (IAV-infection) versus naïve T cells (Uninfected) to see how the immunometabolism of other activated effector T cell populations are transcriptionally

controlled during activation, and subsequent differentiation to understand if Tfh cell immunometabolism is distinct or whether activation drives a similar metabolic rewiring to that of Th1 cells.

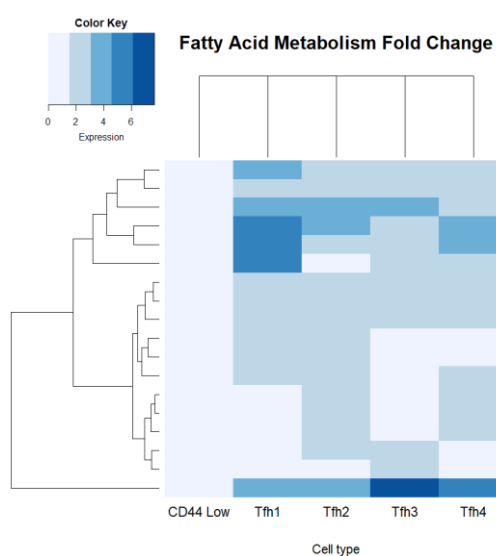
A) Functional Analysis

Term	Gene Count	%	P-value
Glycolysis	12	0.5	0.0056
Fatty Acid Metabolism	25	1	0.07
Lipid Transport	24	1	0.082
Lipid Metabolism	92	3.8	0.099

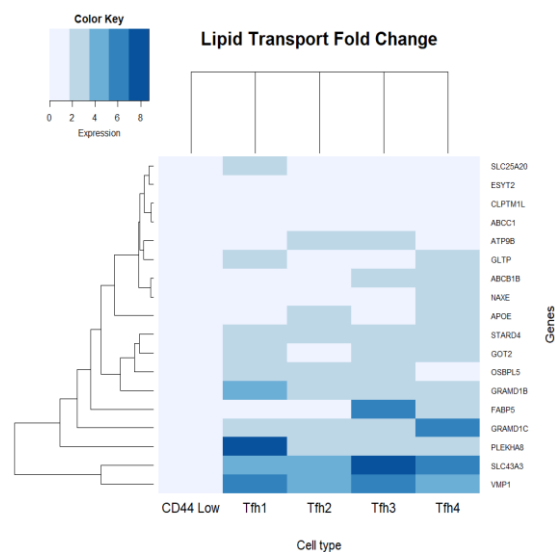
B)



C)



D)



E) KEGG Pathway Analysis

Pathway	Genes	%	P-Value
Amino Sugar and Nucleotide Sugar Metabolism	16	0.7	2.9E-4
Fructose and Mannose Metabolism	12	0.5	1.3E-3
Galactose Metabolism	11	0.5	1.8E-3
Nucleotide Metabolism	18	0.7	9.9E-3
Pyrimidine Metabolism	12	0.5	4.2E-2
Glycerolipid Metabolism	13	0.5	4.2E-2
Choline Metabolism in Cancer	17	0.7	7.3E-2
Carbon Metabolism	20	0.8	7.5E-2
Pentose Phosphate Metabolism	8	0.3	7.6E-2
Metabolic Pathways	198	8.2	8.1E-2
Nicotinate and Nicotinamide Metabolism	9	0.4	8.8E-2

Figure 5.4. Tfh Cells increase metabolic gene expression associated with Glycolysis and Lipid Metabolism during differentiation in IAV Infection.

C57/B6 mice were infected with 110 PFU of IAVPPR8 (H1N1 Puerto Rico), and 10 days post-infection, the mdLN were removed. Data from upregulated genes from Tfh Cells (IAV) compared to naïve T cells from uninfected mice was used, whereby significantly differentially expressed genes were determined by ($p_{adj} < 0.05$, absolute $\log_2$ fold > 0.5). A) Table of upregulated metabolic pathways in Tfh cells compared to naïve T cells from uninfected mice. B) Boxplot of upregulated genes in glycolysis metabolism pathway in Tfh cells vs naïve T cells from uninfected mice. Graphed data is shown with mean $\pm$ SD with $n=3-5$ for each experiment. Expression levels have been scaled per gene Z-score. C) Heatmap of upregulated genes in the fatty acid metabolism pathway in Tfh cells vs naïve T cells from uninfected mice. D) Heatmap of upregulated genes in the lipid transport pathway in Tfh cells vs naïve T cells from uninfected mice. E) Table of KEGG pathway analysis of metabolic genes in Tfh cells vs naïve T cells from uninfected mice.

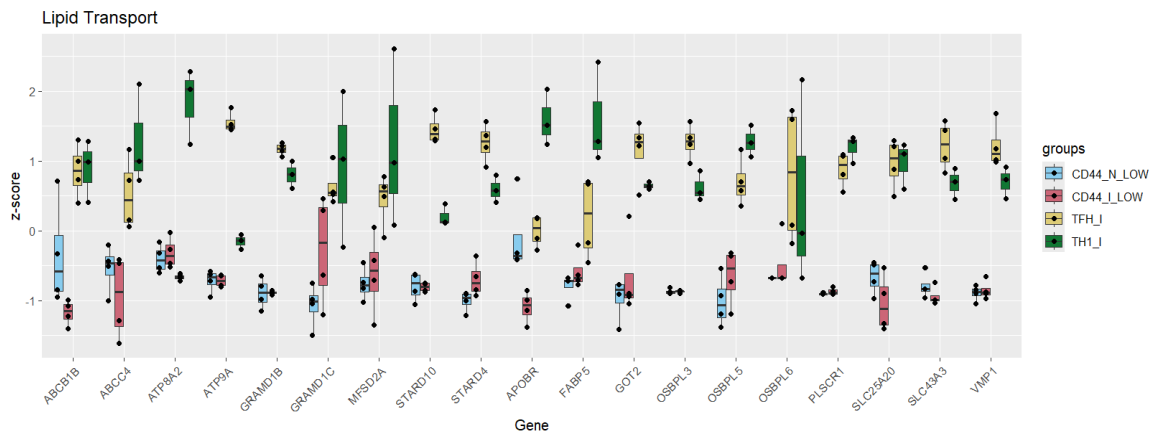
5.3.3 Analysis of Metabolism Pathways enriched in upregulated genes in Th1 in the mdLNs during active IAV Infection with naïve T cells from uninfected mice

Using DAVID analysis, I identified the genes upregulated in Th1 cells and their associated metabolic pathway enrichment compared to naïve T cells from uninfected mice (Figure 5.5). This analysis highlighted the increased expression of genes involved in lipid transport and degradation in Th1 cells (Figure 5.5B & C). Enrichment of these metabolic pathways was unexpected, as previous reports suggested that Th1 cells rely predominantly on glycolysis and glutaminolysis to support their function. Indeed, glucose uptake and aerobic glycolysis appear to be required for IFN- γ production by Th1 cells (Montes de Oca et al., 2020). KEGG pathway analysis further emphasised the metabolic differences between the effector Th1 cells and the naïve T cells, suggesting enrichment of pathways involved in pyrimidine, nucleotide and amino acid sugar metabolism (Figure 5.5D). Overall, this highlights to us that the Th1 cells inside the mdLN favour the transcriptional profile associated with lipid uptake and degradation compared to naïve T cells from uninfected mice. This suggests that the main fuel source used by these cells involves the breakdown of lipids, which is a highly efficient method to generate ATP (Chandel, 2021).

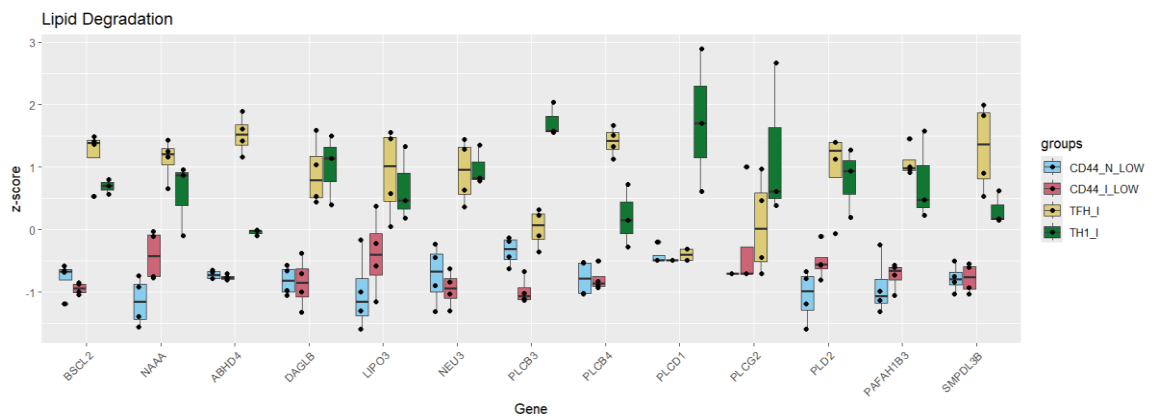
A) Functional Analysis

Term	Count	%	P-Value
Lipid transport	19	1.1	7.60E-02
Lipid degradation	13	0.7	9.20E-02

B)



C



D) KEGG Pathway Analysis

Metabolic Pathway	Count	%	P-Value
Nucleotide Metabolism	15	0.9	6.60E-03
Pyrimidine Metabolism	11	0.6	1.30E-02
Amino sugar and nucleotide sugar metabolism	9	0.5	5.40E-02

Figure 5.5. Th1 cells increase metabolic gene expression associated with lipid transport and degradation during differentiation in IAV Infection.

C57/B6 mice were infected with 110 PFU of IAV PR8 (H1N1 Puerto Rico), and 10 days post-infection, the mdLN were removed. Data from upregulated genes in Th1 Cells (IAV) compared to naïve T cells from uninfected mice were used, where all significantly differentially expressed genes ($p_{adj} < 0.05$, absolute $\log_2$

fold change > 0.5) were included. A) Table of upregulated metabolic pathways in Th1 cells compared to Naïve T cells from uninfected mice . B) Boxplot of upregulated genes in the Lipid transport pathway in Th1 cells vs Naïve T cells from uninfected mice . C) Boxplot of upregulated genes in the Lipid Degradation pathway in Th1 cells vs Naïve T cells from uninfected mice . D) Table of KEGG pathway analysis of metabolic genes in Tfh cells vs naïve T cells from uninfected mice . Graphed data is shown with mean $\pm$ SD with n=3-5 for each experiment. Expression levels have been scaled per gene Z-score.

5.3.4 Delineating the differential metabolic profiles of Tfh and Th1 cells in the mdLN during active IAV infection

So far, we have shown that Tfh cells upregulate pathways associated with glycolysis, lipid and fatty acid metabolism whilst Th1 cells favour lipid transport and degradation compared to naïve T cell (uninfected mice), so to understand further the potential differential metabolic profile of Tfh and Th1 cells, multiple differential analyses were performed using the data obtained for the Tfh and Th1 groups from the IAV-infected mice. I analysed the transcriptional differences between the Tfh and Th1/Treg effector groups using the SL2 platform. This DE analysis revealed diverse transcriptional differences between these two subsets, with 591 upregulated and 752 downregulated genes in Tfh cells compared to Th1, isolated from IAV-infected mice (Figure 5.6C). To understand these changes, the first step was to examine the top 10 upregulated and downregulated genes to investigate any noticeable metabolic differences. The most upregulated genes in Tfh, relative to Th1/Treg cells, are shown in Figure 5.6 B (i).

The most upregulated genes in Tfh cells were canonical markers associated with this cell type, such as BCL-6, CEBPA, TOX and HIF-1 alpha (Choi et al., 2020, Cho et al., 2019, Tanaka et al., 2014, Xu et al., 2019). Interestingly, C/EBP was the most highly enriched gene in Tfh cells compared to Th1 cells. Tanaka et al. 2014 showed that C/EBP negatively regulates IFN- γ expression in Tfh cells, consistent with the presence of IFNGR1 in the 10 most downregulated genes in Tfh cells compared with the Th1/Treg cohort (Figure 3.6Bii). Moreover, it has been recently shown that IFN- γ derived from T cells can suppress Tfh cell differentiation during LCMV, possibly explaining why Tfh cells regulate IFN- γ signalling. However, the suppression is not direct and may involve other cells, like DCS (Sala et al., 2025).

Upregulated genes such as MALT1, a paracaspase, are important in the development of Tfh cells, as MALT1 ^{-/-} mice have fewer Tfh cells and spontaneous GCs (Bornancin et al., 2015), whilst TNFSF8 is the gene that encodes CD153, which is a marker for Tfh cell biology, and CD153 blockade can reduce ICOS expression on antigen-specific Tfh cells, ultimately resulting in diminished antibody responses (Thomas et al., 2023). Moreover, PTPN11 regulates the differentiation of Tfh cells through SHP2 (Vinay S Mahajan, 2021).

The most downregulated genes between Tfh cells and the Th1 cells were associated with adhesion, trafficking and infiltration of cells (ARL4C, RASGRP2, ADGRE5, SELPLG), which are required by Th1/Treg cells to migrate to tissues; however, they are less fundamental for Tfh cell migration (Crotty, 2014). Downregulation of SMAD7 in Tfh cells is expected, as SMAD7 can suppress TGF- β signalling, which is essential for Tfh cell function (Yan et al., 2009b, Schmitt et al., 2014b). Likewise, downregulation of ANXA6, involved in IL-2 signalling, is expected also, as IL-2 is inhibitory to the Tfh phenotype (Cornely et al., 2016, Ballesteros-Tato et al., 2012). Ultimately, in the top 10 downregulated genes, we see regulators of factors that prevent Tfh cell differentiation or function, for example, ID2, which reinforces Th1 differentiation and inhibits E2A, which can suppress Tfh cell differentiation (Shaw et al., 2016). This is further reinforced through the downregulation of SELPLG, which is required for Tfh cell migration to the GC, whilst IL7R is downregulated as signalling via this cytokine receptor can repress BCL-6 and the Tfh cell gene profile (Choi and Crotty, 2021, McDonald et al., 2016). These results further corroborate the effective isolation of Tfh cells by FAC sorting.

Next to address which gene signatures were upregulated in Tfh cells compared to Th1 cells in IAV infection and vice versa (Th1 vs Tfh), I used STRING analysis performed by the SL2 package (Figures 5.6 D, E, F) to reveal pathways enriched in the upregulated gene signatures, which were involved in abnormal IFN- γ responses, acute inflammatory responses, and defence responses to the virus. Likewise, the most enriched pathways associated with the downregulated genes were mainly involved in the effector response towards viruses, such as IAV infection, and in the response towards IFN- γ , which is expected, as we have highlighted above, and is required for Th1 responses, rather than Tfh cells.

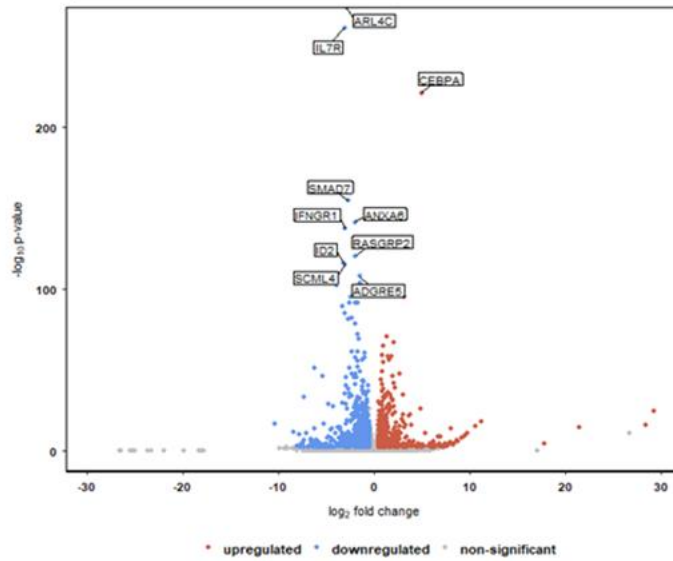
Further pathway analysis, utilising DAVID analysis, provided insight into the metabolic differences between Tfh and Th1 cells during IAV infection (Figure 5.7). This analysis showed the enrichment of glycolytic, heme & lipid biosynthesis pathways, as well as lipid

metabolic profiles. However, not all were significantly enriched in Tfh cells compared to Th1 in IAV-infected mice. Furthermore, KEGG analysis (Figure 5.7 E) also highlighted several additional pathways associated with Tfh cells compared to the Th1 group. These included glycolysis, amino acid, fatty acid, glycine, serine, and threonine metabolism. These findings highlight potential metabolic differences between Tfh cells and other T cells in the mdLN during active IAV infection, which could be exploited for therapeutic gain.

Interestingly, the Tfh and Th1 groups show commonality in the upregulation of genes SLC25A20 and SLC43A3, which are associated with the transport of key molecules required for lipid and purine metabolism. SLC25A20 is involved in transporting acylcarnitines into the mitochondria for FAO (Yuan et al., 2021), whilst SLC43A3 is involved in purine salvage (Furukawa et al., 2015) highlighting two key pathways possibly overlooked in Tfh cell metabolism that have not yet been defined.

Interestingly, in Chapter 3, we demonstrated that Tfh cells exhibit increased levels of enzymes associated with β -Oxidation in the *H. polygyrus* model compared to naïve T cells. This further strengthens our understanding of the possible requirement of Tfh to recycle and oxidise lipid. Furthermore, Figure 5.5C shows that between the Tfh and the “Th1” group, a similar expression of genes involved in lipid degradation is observed, emphasising that, compared to naïve T cells, Tfh cells exhibit increased gene expression associated with lipid breakdown. Previous reports have suggested a role for β -oxidation in Tfh cells function, whilst contradictory evidence has also been presented (Gong et al., 2023) This may suggest a context-dependent activation of this metabolic pathway. However, I observe this signature in both *H. polygyrus* and IAV infection in mice.

A)



B)

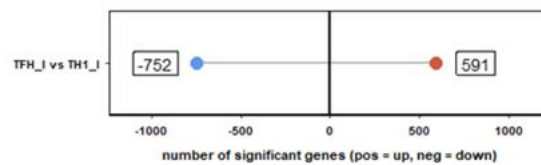
i) Most Upregulated Genes (P-Value)

Gene	log2fold	p	p.adj
CEBPA	4.98	6.679701e-222	3.511519e-218
BCL6	3.12	1.103412e-95	1.242994e-92
MALT1	1.34	1.668973e-71	1.096724e-68
TNFSF8	2.09	1.178150e-67	7.146387e-65
TCF7	0.96	2.574202e-65	1.503620e-62
NSG2	0.90	1.065725e-59	5.421788e-57
RNF19A	1.45	4.088141e-59	2.014815e-56
PTPN11	1.76	1.040319e-58	4.971781e-56
HIF1A	1.62	6.167740e-57	2.701984e-54
TOX	1.56	8.299750e-57	3.444615e-54

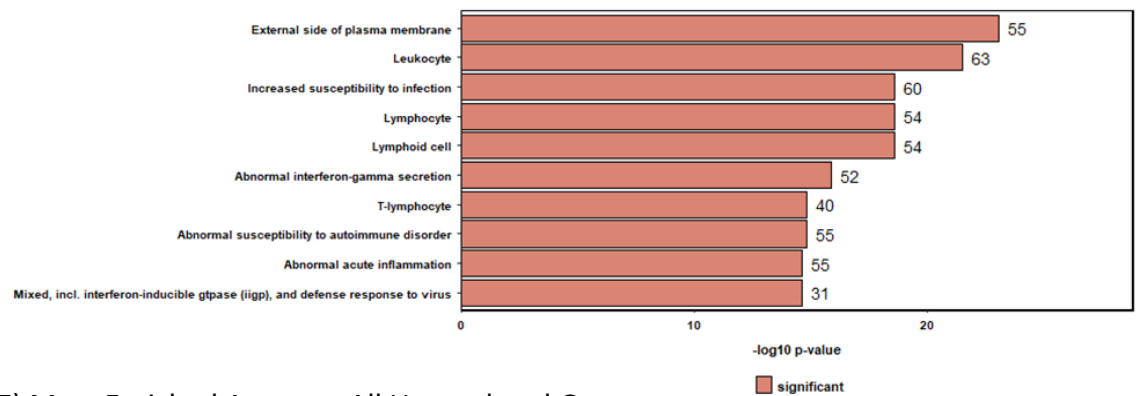
ii) Most Downregulated Genes (P-value)

Gene	log2fold	p	p.adj
ARL4C	-2.97	0.000000e+00	0.000000e+00
IL7R	-3.09	4.620980e-262	3.643874e-258
SMAD7	-2.71	3.586366e-155	1.414015e-151
ANXA6	-1.96	3.663569e-142	1.155563e-138
IFNGR1	-3.06	4.258618e-138	1.119378e-134
RASGRP2	-1.92	3.655840e-121	8.236608e-118
ID2	-3.16	1.945712e-116	3.835727e-113
SCML4	-3.03	3.308964e-116	5.798409e-113
ADGRE5	-1.53	1.476750e-108	2.328982e-105
SELPLG	-1.44	1.113424e-104	1.596346e-101

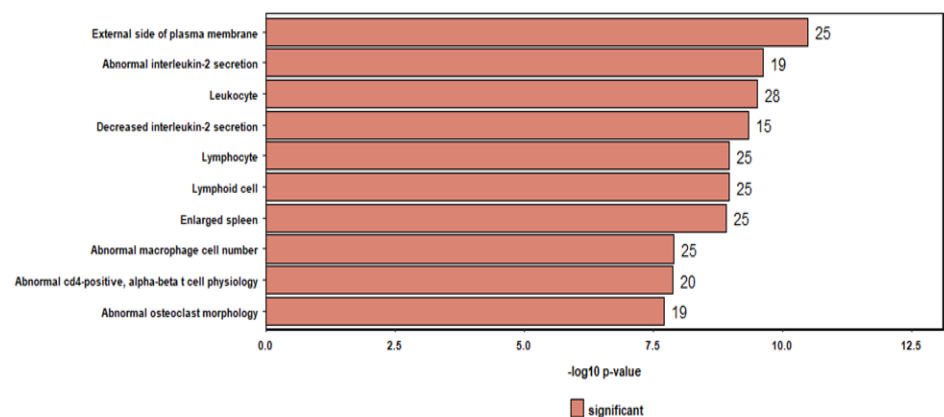
C)



D) Most Enriched Amongst All Significant Genes



E) Most Enriched Amongst All Upregulated Genes



F) Most Downregulated Amongst All Significant

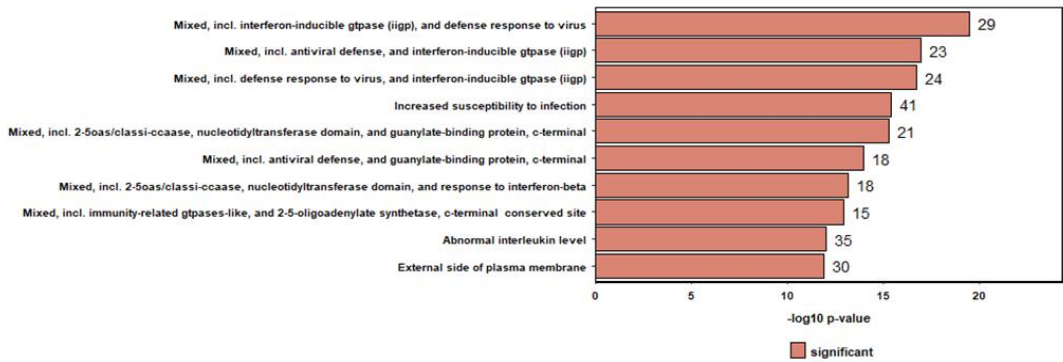
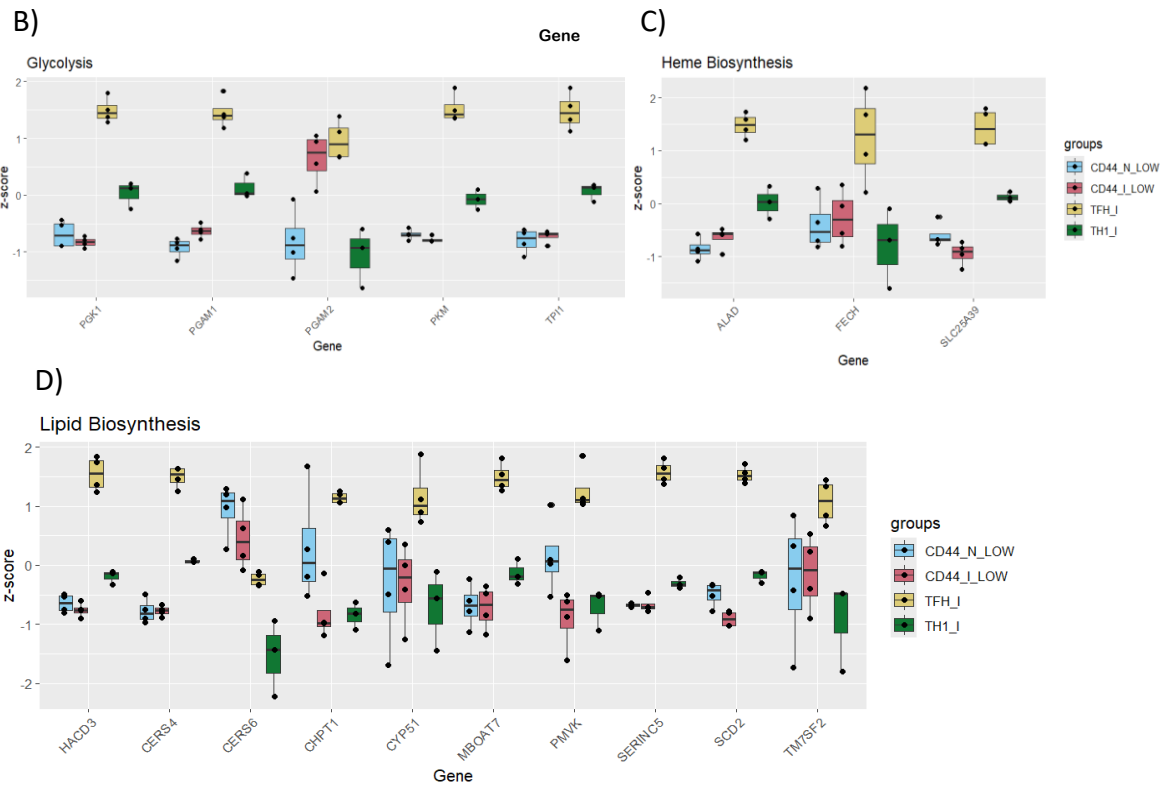


Figure 5.6. Differential Expression Analysis of Tfh and Th1 during IAV-infected mice.

C57B/6 mice were infected with 110 PFU of IAV PR8 (H1N1 Puerto Rico), and 10 days post-infection, the mdLN were removed. A) Volcano plot comparing Tfh and Th1 cells (IAV Infection). Significantly differentially expressed genes are those in which $p_{adj} < 0.05$, absolute $\log_2$ fold > 0.5 . Downregulated and upregulated genes are shown in blue or red, respectively, and non-significantly regulated genes are denoted in grey. A positive fold change indicates higher expression in Tfh than in Th1. B) Tables showing the ten most (i) upregulated and (ii) downregulated genes by adjusted p-value. C) Schematic showing differential gene count for upregulated and downregulated genes between Tfh and Th1 cells. D) Bar chart of the most enriched pathways amongst all significant genes in Tfh cells vs Th1 cells. E) Bar chart of the most enriched pathways amongst all upregulated Tfh cells vs Th1 cells. F) Bar chart of the most downregulated pathways amongst all significant genes in Tfh cells vs Th1 cells. Enrichment analysis was performed using Hypergeometric Gene Set Enrichment on the gene set database STRING 11.5. Significantly differentially expressed genes are those in which $p_{adj} < 0.05$, absolute $\log_2$ fold > 0.5 .

A) Functional Analysis

Term	Count	%	P-Value
Glycolysis	5	0.9	0.011
Lipid Metabolism	29	5.1	0.029
Lipid Biosynthesis	10	1.8	0.04
Heme Biosynthesis	3	0.5	0.066



E) KEGG Pathway Analysis

Pathway	Count	%	P-Value
Glycolysis / Gluconeogenesis	7	1.2	9.6E-3
Central Carbon Metabolism in Cancer	7	1.2	1.1E-2
Steroid Biosynthesis	4	0.7	1.6E-2
Glycine, Serine and Threonine metabolism	5	0.9	2.3E-2
Metabolic pathways	58	10.2	2.3E-2
Choline Metabolism in Cancer	7	1.2	5.1E-2
Biosynthesis of amino acids	6	1.1	6.4E-2
Fatty Acid Metabolism	5	0.9	8.8E-2

Figure 5.7. Tfh cells increase glycolytic, Lipid and Heme gene expression compared with Th1 cells during IAV infection.

Data from upregulated genes in Tfh vs Th1 Cells (IAV), where all significantly differentially expressed genes ($p_{adj} < 0.05$, absolute $\log_2$ fold change > 0.5) were included. A) Table of upregulated metabolic pathways in Tfh cells compared to Th1 cells in IAV infection. B) Boxplot of glycolysis genes upregulated in Tfh cells vs Th1 cells. C) boxplot of Heme Biosynthesis genes upregulated in Tfh cells vs Th1 cells. D) boxplot of Lipid Biosynthesis genes upregulated in Tfh cells vs Th1 cells. Graphed data is shown with mean

+/- SD with n=3-4 for each experiment. Expression levels have been scaled per gene Z-score. E) Table of upregulated KEGG metabolic pathways in Tfh cells compared to Th1 Cells.

5.3.5 Delineating the differential metabolic profiles amongst all cell types sorted from the mdLN during active IAV infection and Uninfected mice

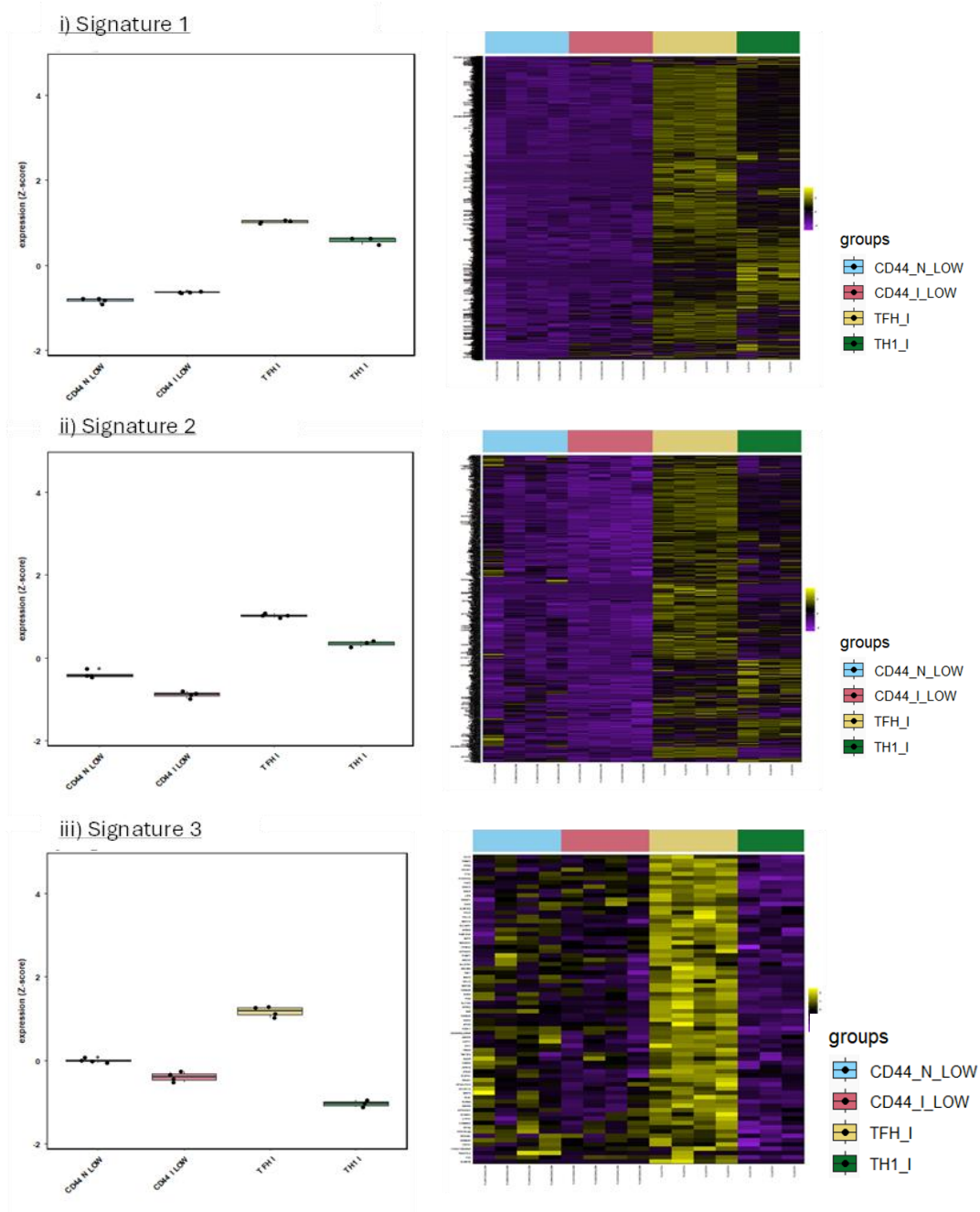
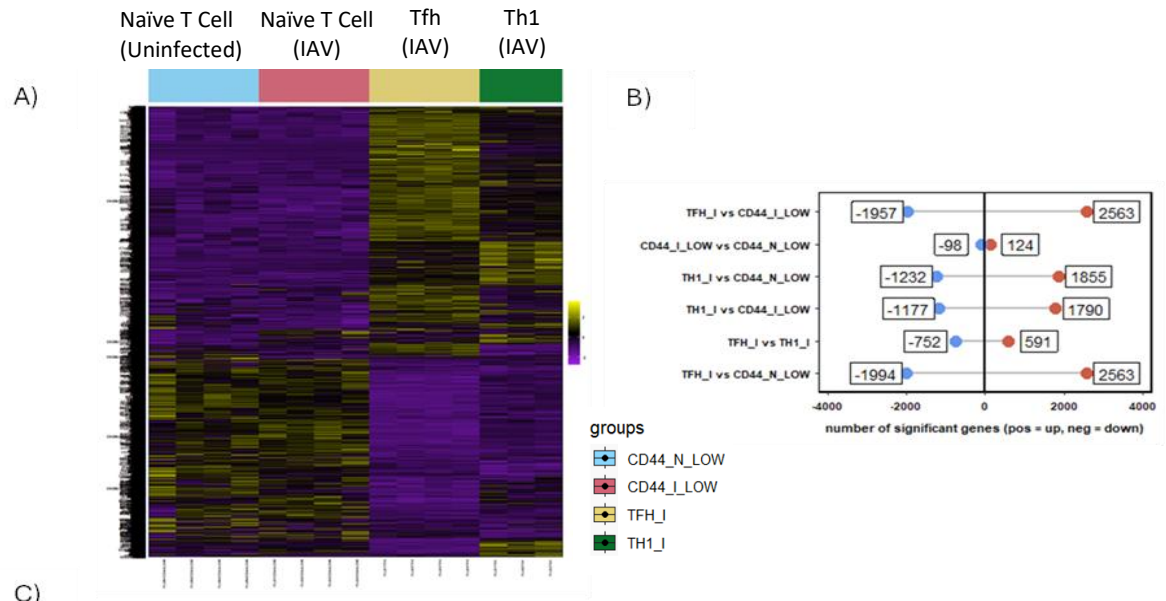
To investigate the key metabolic differences between all the groups (Naïve T cells from uninfected and IAV Infection, Tfh and Th1/Treg (IAV Infection)), I once again utilised the SL2 pipeline. Using the MDE analysis, which provides multiple differential expression analyses of all samples within the dataset. I found that the transcriptional profiles across all cell types were diverse (Figure 5.8 (A, B)). For this analysis, I focused on the signatures where genes are most upregulated in Tfh cells (Figure 5.8C). MDE splits the analysis into cohorts based on the highest genes that are highly upregulated within a sample group. For my analysis, I chose the signatures (cohorts) in which Tfh cells were most differentially expressed and had the highest expression level compared to the other groups.

This analysis highlighted many enriched pathways associated with Tfh cell biology; however, the enriched metabolic pathways mainly focused on the electron transport chain, glycoprotein, steroid/sterol metabolic, and biosynthetic pathways (Figure 5.8D (i-iii)). Positive regulation of lipid metabolism was enriched, but not significantly, supporting the lipid-centric nature of Tfh cells compared to other cell types in this dataset.

In addition, this SLC2 pipeline also identified the genes enriched in each pathway and their relative expression across all groups. The genes enriched in the electron transport chain primarily belonged to three gene families: the ATP-associated, COX, and NDUFA families. The genes involved include ATP5C1, COX19, NDUFA4, ETFB, and many others associated with these classes (Figures 5.9A and 5.9 B). The ATP5C1 gene encodes the mitochondrial subunit of ATP synthase, highlighting possible engagement of OXPHOS in Tfh cells (Ledderose et al., 2014).

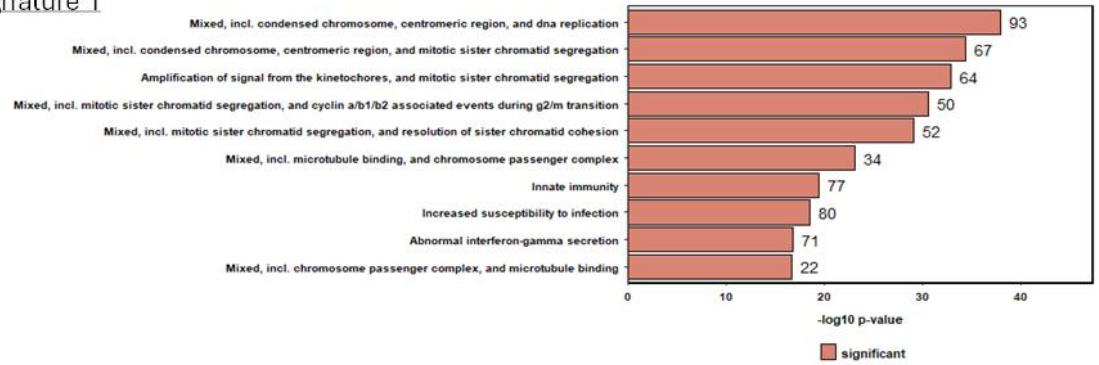
The analysis also highlighted 6 genes of interest in sterol metabolism: APOE, CYP51, FECH, PMVK, TM7SF2 and LIPA (Figure 5.9 C). APOE is a glycoprotein that is critical for the function of Tfh cells (Clement et al., 2015) as APOE-deficient mice have defective Tfh cell expansion, specifically driven by blocking the conversion of Tregs into pro-atherogenic Tfh cells, which normally occurs during atherogenesis, highlighting the

essential role of APOE in the generation of Tfh in certain disease contexts (Gaddis et al., 2018). Moreover, CYP51, a key enzyme in sterol synthesis, plays a crucial role in the proliferation and survival of T cells (Yin et al., 2023). Overall, this shows that Tfh cells, compared to the other T cells present (Naïve T cells (uninfected/IAV-infection) as well as Th1 cells have a distinct transcriptional metabolic program inside the LN during IAV infection.

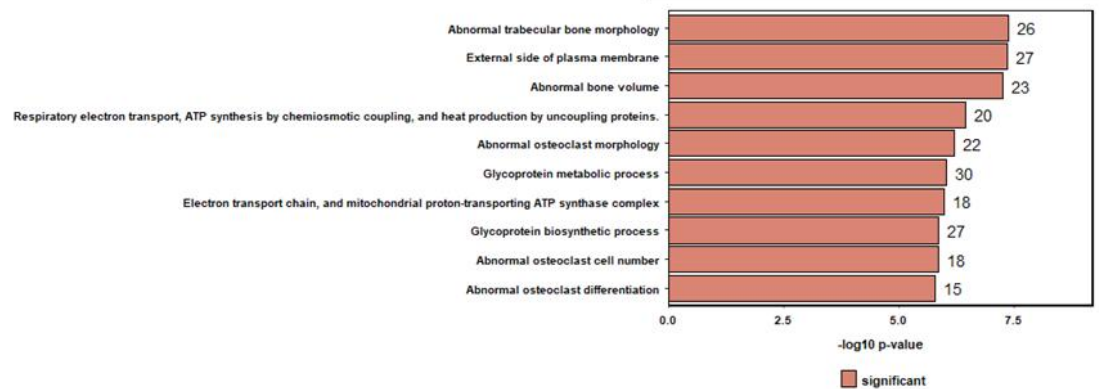


D) i) Most Upregulated Pathways In Tfh

Signature 1



ii) Signature 2



iii) Signature 3

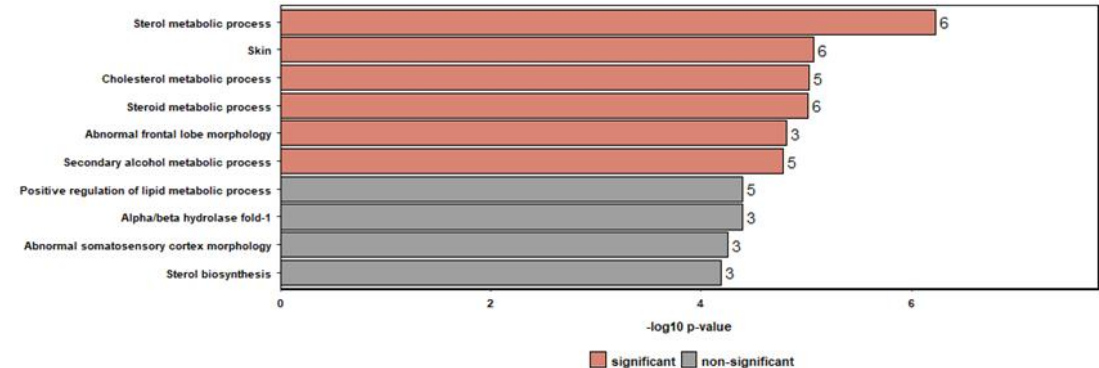


Figure 5.8. STRING Analysis of all cell types during IAV(PR8) infection in the mdLN highlights an Increased Metabolic Phenotype in Tfh cells.

Data from upregulated genes from Tfh, Th1 Cells (IAV) and Naïve T Cells from IAV-infected and uninfected mice were used, whereby all significantly differentially expressed genes ($p_{adj} < 0.05$, absolute $\log_2$ fold > 0.5). Data was processed through Searchlight Analysis to understand the association and upregulated pathways. A) Hierarchically clustered heatmap of the significantly differentially expressed genes for the comparisons of Tfh, Th1 Cells (IAV) and Naïve T Cells from IAV-infected and uninfected mice. Samples are on the x-axis, and genes are on the y-axis. Colour intensity represents the expression level, with

blue indicating low expression and red indicating high expression. Expression levels have been row-scaled into z-scores. The y-axis (both plots) and x-axis (right plot) have been hierarchically clustered using Spearman distances, with UPMGA agglomeration and mean reordering. B) Bar chart showing the number of significantly differentially expressed genes for the comparisons: Tfh vs CD44 n low (Naïve T cells from uninfected mice), Tfh vs Th1, Th1 vs CD44 n low (Naïve T cells from uninfected mice), Th1 vs CD44 I low (Naïve T cells from IAV infected mice), CD44 n low (Naïve T cells from uninfected mice) vs CD44 I low (IAV infected mice) and Tfh vs CD44 I low (IAV infected mice). The comparison is given on the x-axis, and the number of significant genes is on the y-axis. C) Heatmap and boxplot showing selection of Tfh cell groups that had enrichment for increased gene expression associated with each signature shown (i,ii, iii). D) Summary bar charts of the ten most enriched gene sets for each differential expression signature. For each plot, the $-\log_{10}$ enrichment p-value is given on the x-axis, and the gene set name is on the y-axis. Data labels represent the number of significantly differentially expressed genes within each gene set. Enrichment analysis was performed using the Hypergeometric Gene Set Enrichment tool on the STRING 11.5 gene set database. Significantly enriched gene sets ($p_{\text{adj}} < 0.05$, absolute $\log_2$ fold change > 0.5) are shown in red, and non-significant gene sets are shown in black for the bar charts. Graphed data is shown with mean $\pm$ SD with $n=3-4$ for each experiment. All significantly differentially expressed genes ($p_{\text{adj}} < 0.05$, absolute $\log_2$ fold > 0.5). Expression levels have been scaled per gene Z-score

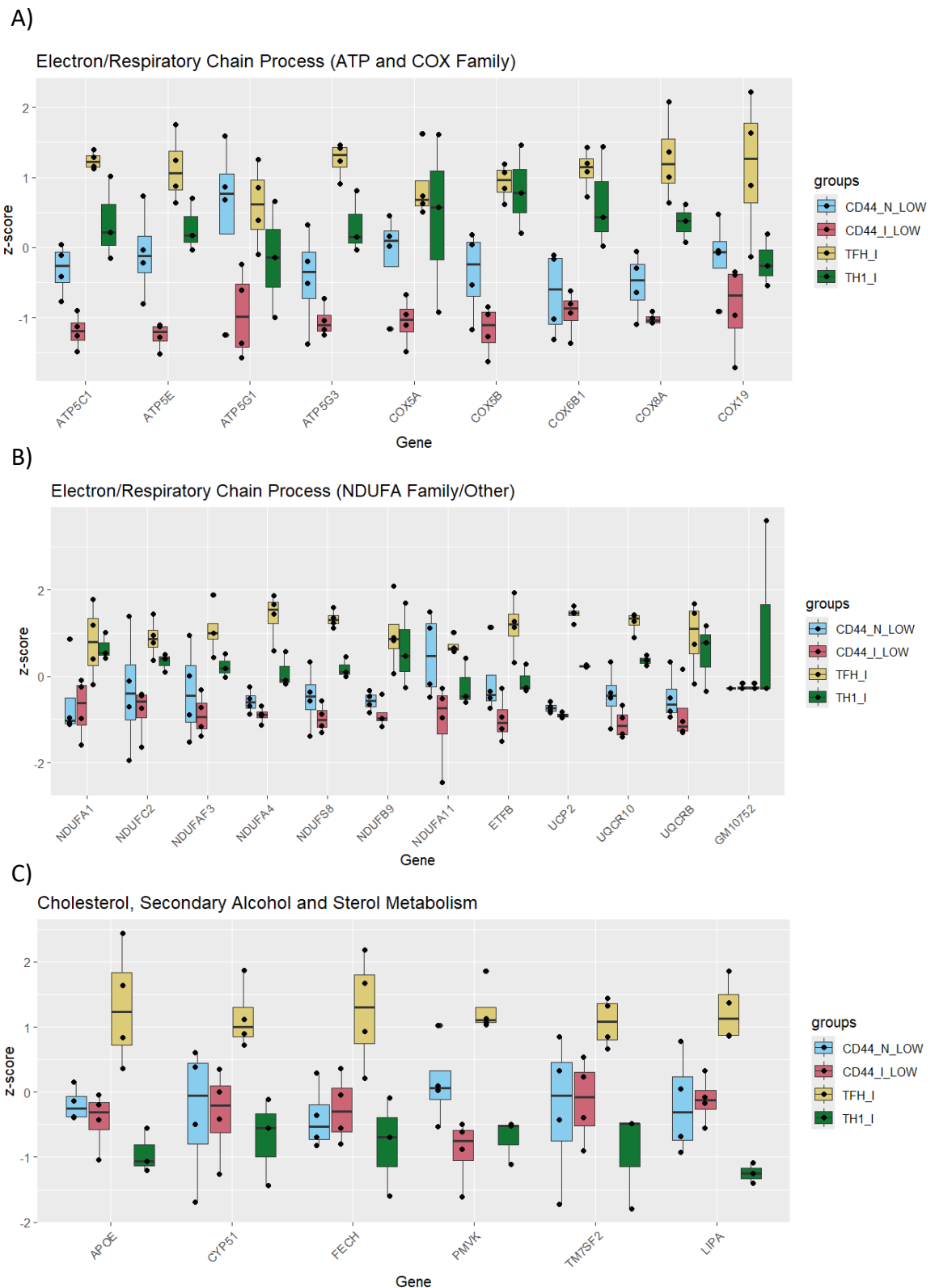


Figure 5.9. Significantly Upregulated Metabolic Pathways Associated with Tfh during IAV Infection Compared to all other groups.

Data from upregulated genes in Tfh Cells (IAV) compared to Th1 and naïve T cells from uninfected and IAV-infected mice were used, where all significantly differentially expressed genes ($p_{\text{adj}} < 0.05$, absolute $\log_2$ fold change > 0.5) were included. A) Boxplot of Electron/respiratory chain Process (ATP/COX family) – genes are selected based on Tfh-mediated signature. B) Boxplot of Electron/respiratory chain Process (NDUFA family/other) – genes are selected based on Tfh-mediated signature. C) Boxplot of cholesterol,

secondary alcohol, and sterol metabolism genes upregulated- genes are selected based on the Tfh-mediated signature. Graphed data is shown with mean $\pm$ SD with $n=3-4$ for each experiment. Expression levels have been scaled per gene Z-score.

5.3.6 Identifying the metabolic transcriptome profiles between Tfh in Type 1 and Type 2 Immune Responses across Different Anatomical Locations

So far, I have demonstrated the upregulation of genes associated with glycolysis and lipid metabolic pathways in Tfh cell biology during both *H. polygyrus* and IAV infection. From the analysis, I could identify common characteristics of Tfh cells' transcriptional immunometabolism; however, to properly understand this, a comparison of the genes upregulated in both Tfh cells and naïve T cells from uninfected mice must be carried out. A direct comparison of the two Tfh cell datasets was not possible, as they were from different experiments and infection models; therefore could not be integrated due to differences in the expression values obtained from bulk RNA-seq.

Recent research suggests that Tfh cells in polarised immune responses are unique, often referred to as “Tfh1” and “Tfh2” (Zaini et al., 2023) which leads me to ask: Are they metabolically divergent? To address this question directly, I utilised the significantly upregulated DE gene lists from Tfh vs naïve T cells (from uninfected mice) in both the *H. polygyrus* and IAV infection Bulk RNA-Seq data, generated from Chapters 4 and 5. The curated gene list was then uploaded to Venny 2.1 (<https://bioinfogp.cnb.csic.es/tools/venny/>). Venny 2.1 was used, as it allows a gene list to be uploaded from up to 4 different comparisons and creates a Venn Diagram to show divergent and overlapping genes between the groups presented. This method enables me to identify genes specifically upregulated by Tfh in both infections compared to naïve T cells, and to determine which genes are commonly upregulated and which are distinct (Figure 3.9A).

The analysis revealed that 689 genes were unique in Tfh cells from *H. polygyrus* compared to those generated by IAV. Conversely, 899 genes were specific to Tfh from IAV infection, whilst 1664 overlapped. Interestingly, this data highlights that while these cells show strong overlap in the genes expressed, they also exhibit diversity. DAVID analysis was used to assess whether metabolic pathways featured in the gene expression differences between the two sets of Tfh cells (Figure 5.10A). The functional analysis carried out by

DAVID analysis highlighted that Tfh cells derived from mLN from mice infected with *H. polygyrus* exhibit significantly increased expression of genes associated with lipid and cholesterol metabolism compared to Tfh cells from the mdLN of IAV infection (Figure 5.10 B). Using DAVID, we saw no distinct set of genes associated with any particular metabolic pathways in the genes specific for Tfh (IAV-infection), suggesting that the environment or possible Tfh2 skewing may be driving this altered, increased metabolic gene expression.

KEGG analysis of both cohorts highlighted that the Tfh cell gene list specifically upregulated in IAV infection was associated with pathways involved in amino acid, nicotinate/nicotinamide, and nucleotide metabolism. In contrast, Tfh cells from *H. polygyrus* favoured the upregulation of KEGG pathways involved in galactose and glycerophospholipid metabolism. This difference observed between DAVID functional analysis and KEGG pathway enrichment analyses is often a result of variations in the way they define and annotate data, such as organising results into specific gene sets, the information provided by their databases, and the statistical methods employed.

To strengthen the validity of these findings, I employed two additional approaches to test whether the observed differences were accurate. To assess the impact of cell activation, I analysed naïve T cells from *H. polygyrus* and IAV-infected mice, each compared to naïve T cells from uninfected mice. This allowed me to check that no obvious metabolic rewiring was present in the two anatomical locations, irrespective of cell activation, using a similar Venn diagram approach as used for Tfh in Th2 and Th1 conditions (Figure 5.10). Naïve T cells in *H. polygyrus* samples showed 253 unique genes, whilst the IAV samples had 117 distinct genes (Figure 5.11A). Pathway analysis showed that none of the metabolic pathways associated with the Tfh cells were enriched in these naïve cell lists (Figure 5.11 B, C). These data suggest that naïve T cells present in different infections were not differentially metabolically primed before their differentiation into Tfh cells.

Nevertheless, using the unique gene set from naïve T cells (*H. polygyrus*), I performed KEGG pathway analysis, which showed that naïve T cells from *H. polygyrus*-infected mice exhibited enrichment in butanoate metabolism. However, upon inspection of the expression profile of associated genes (Figure 5.11D), the increased expression of OXCT1 and ACAT2 observed is suppressed in Tfh cells, suggesting that downregulation of these genes may facilitate the development and activation of Th cells, as downregulation is observed in each Th1, Tfh and Th2 cells in both infection models.

Following this, I sought to gain a deeper understanding of the differences between Tfh cells in type 1 and type 2 immune contexts. To do this, I utilised a bulk transcriptomic dataset from the Colby Zaph lab, in which they used *T.muris* infection to drive type 1 and type 2 inflammatory contexts using the same pathogen (Zaini et al., 2023). Low dose *T.muris* drives type 1 inflammation, whilst the high dose drives type 2 inflammatory response (Klementowicz et al., 2012, Yousefi et al., 2021). This data allowed me to directly ask if Tfh cell metabolism differs based on the inflammatory environment, irrespective of anatomical location. The Tfh cells in this paper were isolated from the mLNs during *T.muris* enteric infection, whether acute or chronic. They were defined by the same markers used here, including CXCR5 and PD-1, while also adding GATA3 and T-bet, depending on the type of infection (chronic vs. acute), which highlights their functional heterogeneity. The paper also indicates that FACS-sorted populations were highly pure (>95%) for each isolated population.

Using their Bulk RNA-Seq data, SL2 analysis curated gene lists of significantly up- and downregulated genes in both Tfh cell groups. Once this was complete, DAVID analysis addressed whether any metabolic pathways were enriched. Initially, the SL2 analysis of “High-dose Tfh” vs “Low-dose Tfh” DE genes showed that the Tfh cells from type 1 and type 2 inflammation in this model exhibit distinct transcriptional profiles (Figure 5.12A), with 1395 upregulated genes in the high-dose Tfh and 925 genes in the low-dose Tfh cells.

However, subsequent DAVID Analysis of these DE genes highlighted similarities between this dataset and that of the Th2-polarised *H. polygyrus* cohort, in that Tfh cells subjected to a Th2-mediated environment, irrespective of the pathogen, exhibited enrichment of pathways associated with lipid metabolism (Figure 5.12 C). However, Tfh cells from the high-dose *T. muris* model also exhibited an overrepresentation of pathways associated with sugar transport, leukotriene biosynthesis, iron transport, and lipid degradation (Figure 5.12 C). Overall, these data from the Zaph lab, in conjunction with the *H. polygyrus* data, show that Tfh2 cells (Tfh cells from the Th2 setting) compared to Tfh1 cells (Tfh cells from a predominantly Th1 setting) exhibit increased Lipid metabolism, highlighting that Tfh cells in different inflammatory environments have altered metabolic profiles.

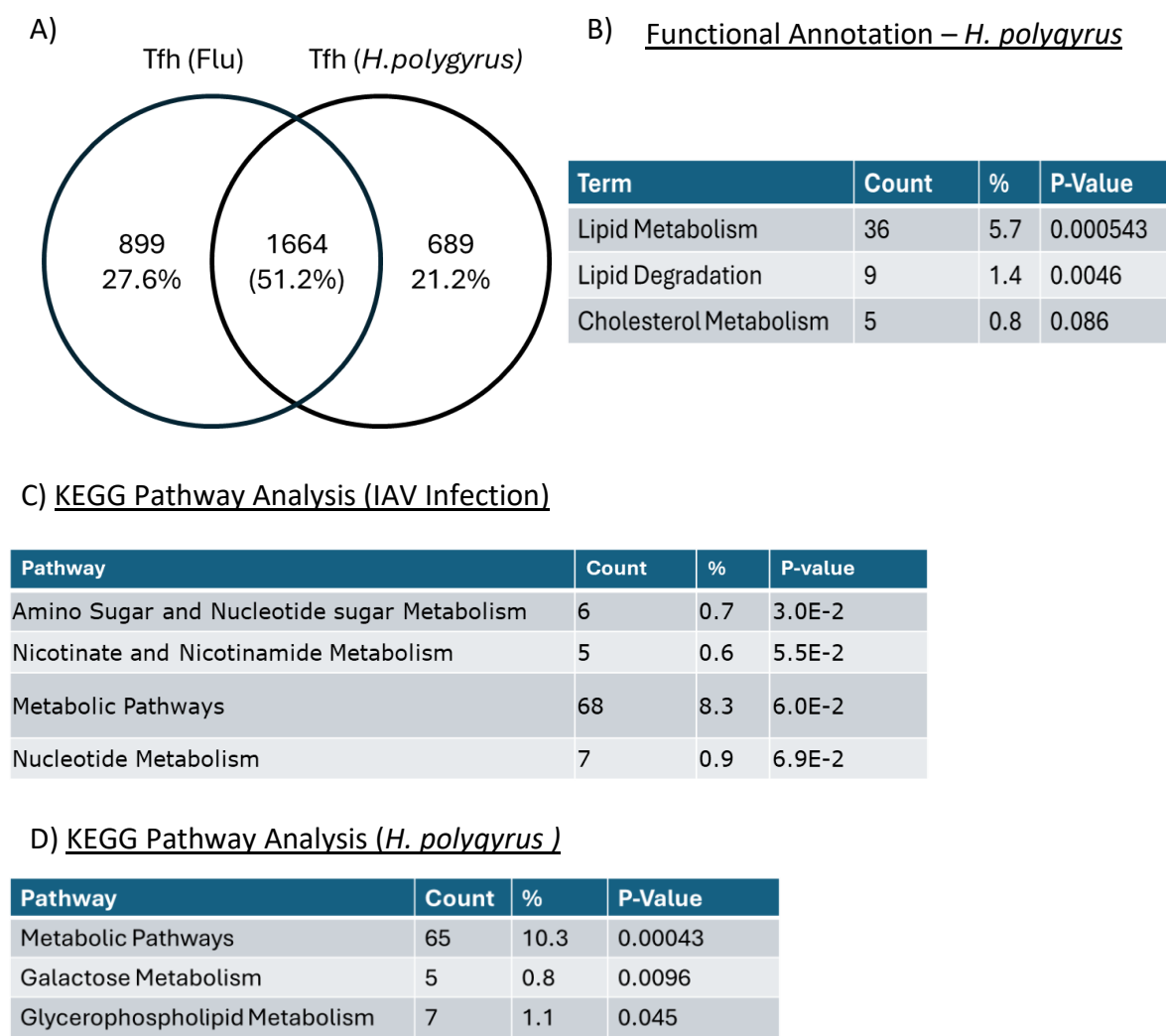


Figure 5.10. Tfh cells from *H. polygyrus* mLN show increased gene expression associated with Lipid and Cholesterol Metabolism compared to Tfh cells derived from mdLN during IAV infection.

Data from significantly differentially expressed genes ($p_{\text{adj}} < 0.05$, absolute $\log_2$ fold > 0.5). A) Venn diagram of the upregulated genes between Tfh (*H. polygyrus*) compared to naïve T cells from uninfected mice vs Tfh (IAV (Flu)) compared to naïve T cells from uninfected mice. B) Table of upregulated metabolic genes in Tfh cells specific to *H. polygyrus*. C) Table of upregulated KEGG metabolic pathways in Tfh cells specific to IAV infection. D) Table of upregulated KEGG metabolic pathways in Tfh cells specific to *H. polygyrus*.

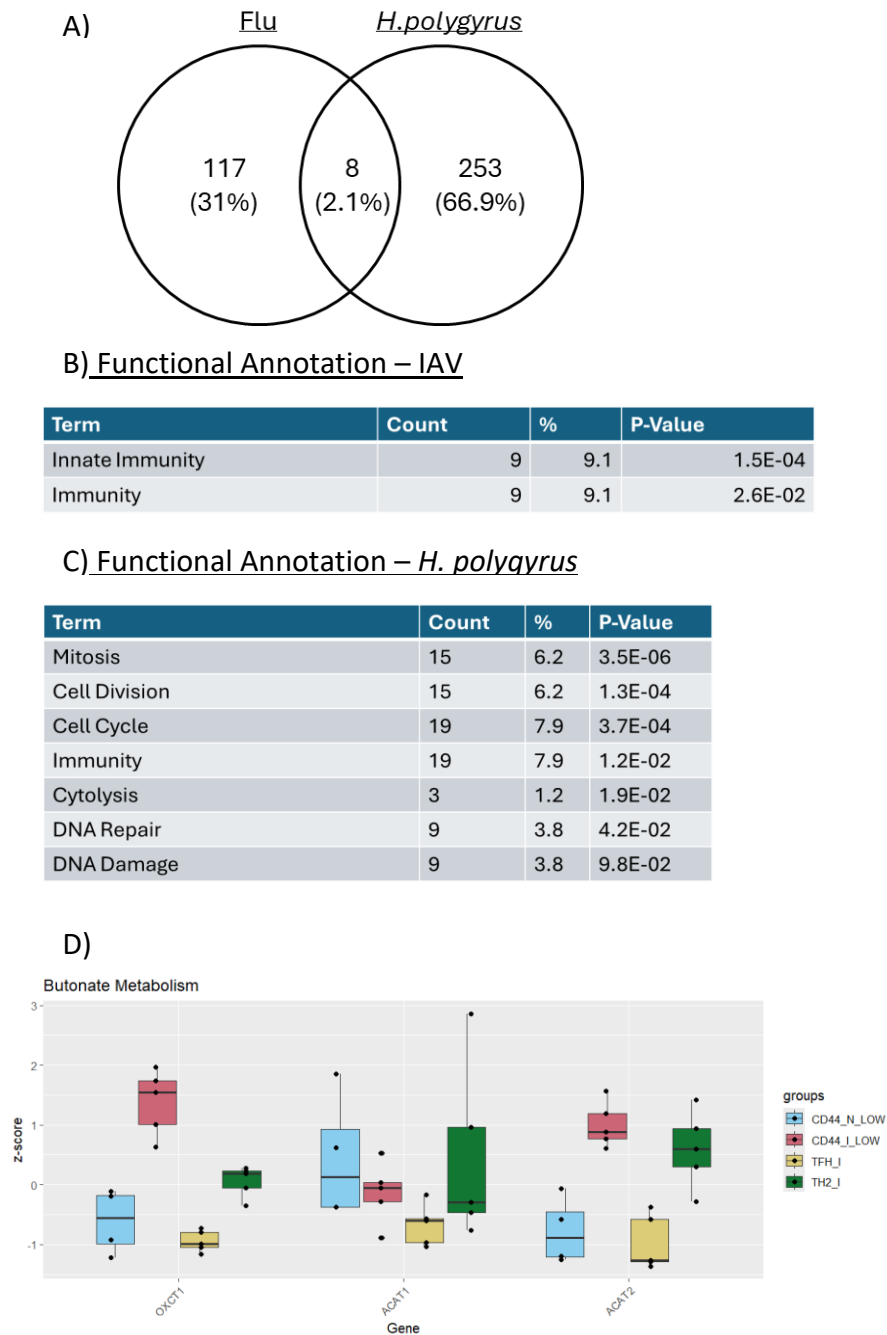
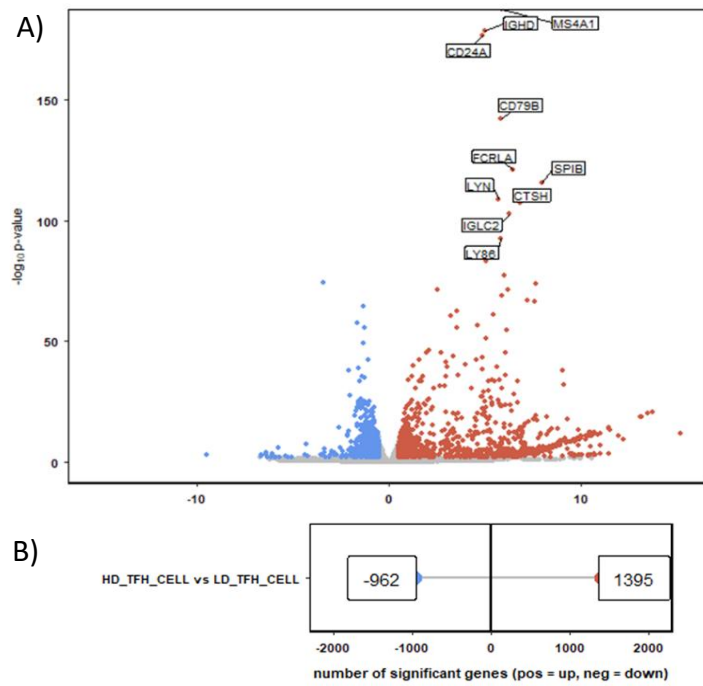


Figure 5.11. Comparing how *H. polygyrus* mLN and IAV mdLN reactive environments shape Naïve T cells' responses During Infection.

Data from significantly differentially expressed genes ($p_{\text{adj}} < 0.05$, absolute $\log_2$ fold > 0.5). A) Venn diagram of the upregulated genes between naïve T cells from *H. polygyrus* vs naïve mice / naïve T cells from (IAV infection) vs naïve mice. B) Table of upregulated metabolic genes in naïve T cells specific to IAV infection. C) Table of upregulated metabolic genes in naïve T cells specific to *H. polygyrus* infection. C) Boxplot of only upregulated KEGG metabolic pathways in naïve T cells specific to *H. polygyrus* infection. All significantly differentially expressed genes ($p_{\text{adj}} < 0.05$, absolute $\log_2$ fold > 0.5). Expression levels have been scaled per gene Z-score.



C) Functional Analysis (High Dose *T.Muris* Th2)

Term	Count	%	P-Value
Lipid Metabolism	60	4.4	2.0E-2
Sugar Transport	7	0.5	3.3E-2
Leukotriene Biosynthesis	3	0.2	6.6E-2
Iron Transport	4	0.3	8.6E-2
Lipid Degradation	11	0.8	9.9E-2

Figure 5.12. Tfh cells derived from a Th2-mediated environment have increased Lipid Metabolic Gene Expression compared to those in a Th1-mediated setting yet share a Common Lipid/Fatty Acid Profile across different Immunological Stimuli.

Transcriptional analysis of Tfh1 and Tfh2 from FIR (FOXP3-IRES-MRFP) mice on a C57BL/6 background were infected with high and low doses of *T.muris*. The mLN's were harvested on day 21 post-infection, and Tfh cells were defined as CD4⁺ CD44^{high} FOXP3⁻ Ly6C⁻ CD162⁻ PD-1⁺ CXCR5⁺. Cells were sorted and purified for RNAseq. Analysis (Zaini et al., 2023). Significantly differential genes (p.adj < 0.05, absolute log2 fold > 0.5). A) Volcano plot showing differential gene expression between Tfh1 (Left- chronic) and Tfh2 (right- acute) (p.adj < 0.05, absolute log2 fold > 0.5) are shown in red and non-significant genes in black. A positive fold change indicates higher expression in Tfh2 vs Tfh1. B) Schematic showing differential count for upregulated and downregulated genes between Tfh2 and Tfh1 cells. C) Table of metabolic pathways enriched in upregulated genes in Tfh2 vs Tfh1 cell comparison. All significantly differentially expressed genes (p.adj < 0.05, absolute log2 fold > 0.5).

To summarise, this chapter highlights the key Tfh metabolic pathways that are reprogrammed transcriptionally in the context of their differentiation during IAV infection. Specifically, these data showed how genes associated with the regulation of glycolysis and lipid metabolic pathways are upregulated following their differentiation from naïve cells. This data set also allowed for comparison of the reprogramming of key metabolic pathways associated with Tfh cells in a type 1 and type 2-mediated immunological environment, which identified that Tfh cells derived in the *H. polygyrus* reactive mLN favour increased expression of genes associated with lipid and sterol metabolism relative to those in IAV-associated mdLNs. Finally, these datasets were compared to a publicly available dataset of Th1- and Th2-associated Tfh cells generated by high and low dose infection with a parasitic worm in the same anatomic location that supported the idea that upregulation of pathways associated with regulation of lipid metabolism generally occurs in Tfh derived from a Th2 stimulus but not a Th1 environment. Collectively, these findings advance our understanding of the metabolic processes underlying diverse immune responses and, consequently, may ultimately lead to novel therapeutic targets in Tfh cell biology.

5.4 Discussion

This chapter facilitated a deeper understanding of the key metabolic pathways required by Tfh cells transcriptionally in response to various immunological stimuli. This data highlights the similarities and differences between Tfh cells and naïve and effector Th populations within the reactive LN of IAV infection, while exploring the differences observed in Tfh cell metabolism during type 1 and type 2 infections. These novel findings will advance our knowledge of Tfh cell immunometabolism and shed light on the need for classification of Tfh based on the flavour of the immune environment.

Bulk sequencing allowed us to gain a deep insight into Tfh cells and their transcriptional programs. Based on correct Tfh cell isolation and depth, our sequencing for this dataset proved successful, as we averaged 20,000 genes sequenced per sample. This, combined with the Chapter 4 dataset on *H. polygyrus*, will provide a crucial resource for understanding Tfh cell function and differentiation and will help advance the field. To date, this is the first large-scale dataset generated that allows comparison of Tfh cells in *H. polygyrus* and IAV infection.

5.4.1 Purity of CXCR3⁺ Th1 cells

However, one limitation of this IAV data set may be Treg contamination in the Th1 group of non-Tfh CD4⁺ CXCR3⁺ CD44^{High} cells, as suggested by the higher expression of FOXP3 observed in the Th1 cell group compared to all other samples (Figure 5.1D). It should also be noted, however, that Treg contamination may not be present in the Th1 samples, as during IAV infection, it has been shown that Th1 cells can express low levels of FOXP3 (Elfaki et al., 2021). Conversely, CD4⁺ FOXP3⁺ T cells in the mdLN of IAV infection also express high levels of CXCR3, whilst also expressing T-bet (Bedoya et al., 2013). Interestingly, it has been shown that CXCR3⁺ Tregs are more activated than CXCR3⁻ Tregs and have heightened effector function (Moreno Ayala et al., 2023). It should also be noted that in IAV infection, the majority of CXCR3⁺ CD4⁺ T cells in the mdLN are likely to be Th1 cells, rather than Tregs (Betts et al., 2012, Bedoya et al., 2013) and that IAV infection may not induce the maximal Th1 imprint in CD4 T cells, as seen in other intracellular infections, and this may be reflected in the transcriptional profile of our Th1 group (Finn and McKinstry, 2024).

It is also possible that Treg contamination may be present within the Th1 population, as indicated by the higher Maf gene expression highlighted in Figure 5.1 compared to all other groups. This is surprising, as Maf is associated with Th2/Treg cells, not Th1 cells (Ho et al., 1998). Its presence is often associated with the suppression of IFN- γ and potential promotion of IL-10 secretion in Th1 cells; however, this is not to say that the analysis does not capture a transitional state of Th1 during IAV infection at day 10 rather than Treg contamination (Saraiva et al., 2009).

Interestingly, in the above context, compared to Tfh cells, the Th1 cell group exhibited enrichment of lipid transport and degradation pathways (Figure 5.7), findings consistent with those proposed for Treg metabolism (Howie et al., 2017a). Moreover, Tregs favour using β -oxidation and free fatty acid transport into their cytoplasm (Field et al., 2020). FOXP3 drives increased lipid oxidation to fuel oxidative phosphorylation, which provides protection from fatty acid-induced cell death and contributes to Treg stability in lipid-rich environments (Howie et al., 2017a). Nevertheless, it should be noted that the role of Tregs has been documented as crucial in Tfh cell responses. It has been reported that the presence of Tregs can limit IL-2 availability through high expression of CD25 to scavenge IL-2 from the interfollicular areas of the LN, which favours Tfh cell development as IL-2 can inhibit Tfh cell differentiation through expression of BLIMP-1, which can repress Bcl-6 (Leon et al., 2014, Choi et al., 2013).

Importantly, it should be noted that our Th1 group also shows upregulation of genes associated with Th1 cells, such as high T-bet expression (Figure 3.1); however, the high expression of IKZF2 (Helios) that was induced in Th1 (infected) compared to naïve T cell groups (Uninfected) further aids in highlighting possible contamination as Helios is a key TF for Treg cells (Thornton et al., 2019).

The potential contamination by Tregs was due to technical limitations, which could perhaps be resolved by gaining access to IFN- γ reporter mice, such as GREAT Mice (Reinhardt et al., 2009). That would have allowed isolation of Th1 cells based on the subset markers and cytokine production. The use of magnetic bead separation exploiting the Treg surface marker CD25 could not have been employed, as it would have also removed Th1 and activated T cells, which also express CD25 (Yan et al., 2009a). The best practice would have been to cross IFN- γ and FOXP3 reporter mice to create a dual reporter mouse. The use of sole FOXP3 reporter mice could also have been used with CXCR3 staining of CD4⁺ T cells; however, CXCR3 can also be present on other Th cells (Th17,

etc.), which can develop during IAV infection, so the use of IFN- γ reporter would allow me to distinguish between Th17 and Th1 based on their expression of IFN- γ (Fadel et al., 2008). The use of the T-bet-Zs-green mouse could also have been employed if prior gating to exclude Tfh cells had been performed (Zhu et al., 2012), as Tfh cells can also express T-bet during IAV infection (Arroyo-Diaz et al., 2023)

5.4.2 Tfh cells from type 2-mediated Infection have an Increased Lipid Gene Expression Signature compared to Type 1-derived Tfh cells

Interestingly, Tfh cell metabolic reprogramming in response to IAV infection (Figure 5.4) exhibited similar enrichment of metabolic pathways associated with upregulated genes to that observed in Tfh generated during *H. polygyrus* infection (Figure 4.4), namely enrichment of glycolytic, fatty acid and lipid metabolic pathways. However, comparison of the data generated under these two conditions suggested that Tfh cells derived from the mLN, exposed to a Th2-type environment, have increased upregulated expression of genes associated with lipid and sterol metabolism compared to Tfh generated in the IAV-reactive mdLN. This highlights diversity in the transcriptional metabolic profile of Tfh cells; however, I was unsure if this was due to differences in the transcriptional profile of naïve T cells in different anatomical locations or the differentiation environment for the Tfh cells.

To investigate how different anatomical locations and microenvironments influence naïve T cells' metabolic adaptation without activation, naïve T cells from *H. polygyrus* and IAV infection microenvironments were compared with their respective cohorts from naïve mice. Naïve T cells subjected to an *H. polygyrus* infection environment exhibited increased expression of elements associated with butanoate metabolism relative to naïve cells from control animals, which was lost upon Tfh differentiation. Interestingly, this was not observed in naïve T cells exposed to an IAV-reactive microenvironment, which showed no functional upregulation associated with any metabolic pathways (Figure 5.11). This difference could be attributed to a myriad of different variables and warrants further investigation.

To further address this question, a published dataset associated with Tfh cells derived from the mLNs during high- and low-dose *T. muris* infection was analysed. This model enables the direct comparison of both Th1- and Th2-mediated immune responses towards the same pathogen within the same anatomical location, thereby addressing the understanding of

whether reprogramming to lipid metabolic pathways is preferentially associated with the differentiation of Tfh cells derived from a Th2 environment, relative to a Th1 environment, rather than their anatomical location. A caveat of this study was that each group only contained 2 animals, so statistical analysis could not be performed on the dataset. However, it provided a first step to understanding and appreciating the differences observed in metabolic gene expression between the two Tfh cell types generated in response to *T. muris* infection. Indeed, this dataset, supporting our data, highlighted that Tfh cells derived in a Th2 microenvironment exhibited enrichment of pathways associated with lipid metabolism (Figure 5.12 C) (Zaini et al., 2023).

5.4.3 Future Directions /Limitations of Research

Further work is needed to establish whether this proposal of preferential lipid metabolism in Th2-associated Tfh cells is correct. A possible route to explore this difference in “Tfh1” and “Tfh2” cells would be, for example, to utilise published single-cell sequencing data on Tfh cells extracted from the popliteal LN, following type 1 and type 2 immunisation-induced inflammation in a footpad model (Kumar et al., 2024). This would enable the analysis of the similarities and differences between these published signatures and the new *H. polygyrus* and IAV infection data presented in this thesis, particularly concerning whether Tfh2 cells are preferentially associated with lipid metabolic pathways, compared to those of Tfh1 cells (Kumar et al., 2024). This approach would also allow the role of anatomical location to be addressed; however, this analysis will not directly indicate whether the pathway is upregulated. However, the overrepresentation of genes associated with this pathway suggests its importance in the differentiation process of Tfh cells.

To understand whether Tfh cells from a Th2 context are more lipid-dependent compared to Tfh cells from a predominantly Th1 setting, defining the mechanisms that drive this altered immunometabolism would be critical to our understanding. To achieve this, further analysis of the approach used in Figures 5.10 and 5.11 is required to understand the specific genes upregulated in Tfh2 cells compared to Tfh1 cells in both infection contexts. Also, understanding the role of different cytokines in this response may yield an answer to this unknown. For instance, IL-4 has been shown to drive lipid metabolism, through promoting lipid uptake, storage and utilisation for energy (Lin et al., 2021). To understand this further, using an IL-4R/CXCR5 Cre-lox model system, we could prevent Tfh cells being able to use IL-4 through deletion of IL4R on the surface of cells that express CXCR5

and determine whether this alters their metabolic phenotype to be less lipid dependent compared to the WT.

Another key aspect is whether lipid metabolism directly affects differentiation or the functional capacity of Tfh cells, and this cannot be addressed using our bulk RNA-seq. data. Rather, this would require *in vitro* and possibly *in vivo* manipulation of metabolic pathways associated with Tfh cells to understand the direct and systematic effects of altering metabolism on Tfh cell differentiation and function and preliminary data addressing this is presented in the next Chapter.

5.4.4 Concluding Remarks

In conclusion, this chapter signposts the key metabolic pathways associated with Tfh generated during IAV infection. This chapter also examines the similarities and differences observed between Tfh and Th1 populations in the mLNs (mdLN). Tfh cells favour glycolytic and lipid metabolism, whilst the Th1 cohort appears favours lipid transport and degradation. Likewise, this data allowed investigation of whether Tfh cells derived during different (Th1 vs Th2) immune stimuli express distinct metabolic signatures and hence discussion of the relation of these findings to previously described Tfh1 and Tfh2 cells (Zaini et al., 2023) was also assessed. I found that Tfh2 cells are more lipid-dependent than Tfh1 cells across all of these datasets ((*H. polygyrus* /IAV infection) and (*T.muris*)). These findings also explored the role of immunological phenotype and anatomical location where the expression profiles of “naïve” T cells in various anatomical and inflammatory contexts, in terms of their distinct metabolic pathways, were also examined to determine whether any such programming facilitated differential Tfh differentiation. Overall, these findings shed some preliminary light on possible therapeutic avenues that could be explored to selectively prevent Th2-mediated Tfh cell responses while preserving the Th1 Tfh cell immune response.

5.5 Schematic depicting Tfh1 and Tfh2 Metabolism

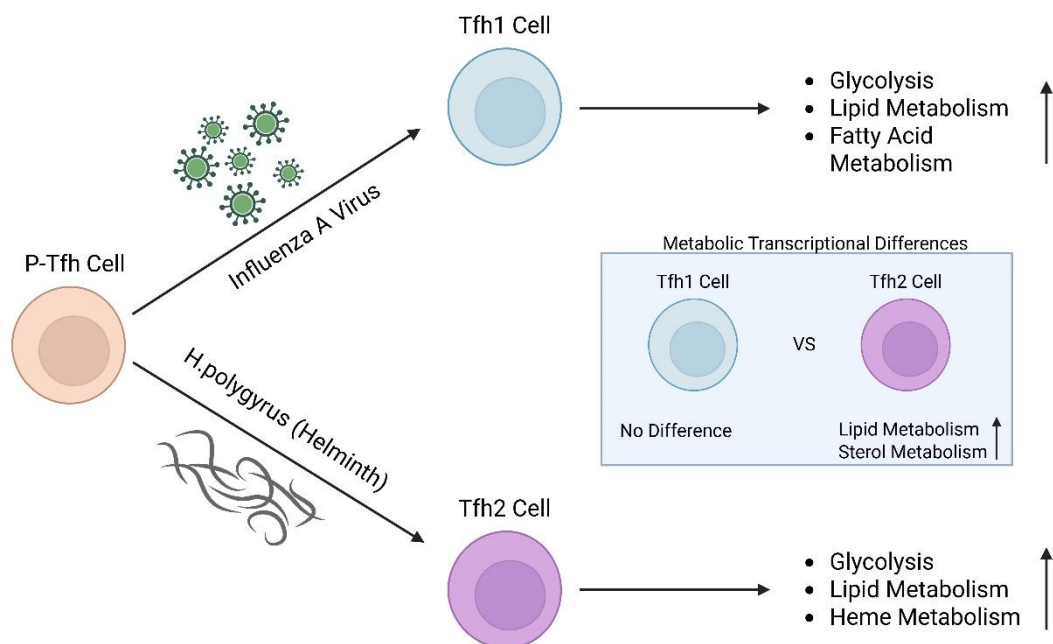


Figure 5.13. Summary of Tfh1 and Tfh2 Immunometabolism.

Diagram highlighting the key transcriptional metabolic profiles of Tfh1 and Tfh2 during IAV and *H. polygyrus* infection in their respective reactive draining LNs. Abbreviations: P-Tfh – precursor Tfh cell. Diagram created in BioRender and adapted from (Chi et al., 2022).

Chapter 6: The Role of Stearoyl CoA Desaturase 1 in the Differentiation and Maintenance of *In vitro* Derived Human T Follicular Helper Cells

6.1 Introduction

In previous chapters, the focus has been on characterising the immunometabolism of Tfh cells in model murine systems of type 1 and type 2 immune responses. However, understanding whether targeting the immunometabolism of Tfh cells has therapeutic translational application in the clinic has not yet been fully explored. Many immunotherapies have been shown to attenuate the Tfh cell response in mouse disease models. Immunotherapies like abatacept and belatacept, which are the first and second generations of monoclonal immunotherapies against the fusion protein of CTLA-4 (anti-CTLA-4), have been shown to decrease the frequency of Tfh cells (spleen) and suppress antibody generation towards a skin graft murine model (Kim et al., 2016). Anti-CTLA-4 also decreases circulatory Tfh cells in a murine heart transplantation model (Oh et al., 2016). However, indications from early human clinical trials suggest this approach may not be translational to humans, as treatment with belatacept in kidney transplantation patients only partially inhibited Tfh activation. The pool of remaining, activated Tfh cells was able to interact with and aid B cells, and drive rejection (de Graav et al., 2017).

Tfh cell biology is complex, and therapeutic manipulation often yields poor results; for example, the class of conventional immunosuppressants (cyclosporin A, tacrolimus, sirolimus, and steroids) can affect Tfh cell biology, but it has been shown that they cannot block Tfh cell activity completely and IL-21 can still be produced to assist B cell differentiation and autoantibody production (Wallin et al., 2018). The therapies can exhibit partial inhibition; however, they are not considered a viable treatment option to prevent Tfh cell-mediated pathology in many autoimmune and transplantation conditions (Yan et al., 2017).

Recently, it was shown that Ustekinumab, a monoclonal antibody against the p40 subunit of IL-12 and IL-23, can inhibit Tfh cell differentiation in patients with Crohn's disease, resulting in diminished GC activity (Globig et al., 2021). However, whilst blocking IL-12 effectively blocks Tfh cell differentiation, more tailored approaches may yield better

results (Elsner and Shlomchik, 2019). Recently, low-dose IL-2 therapy was trialled in patients with SLE to prevent Tfh differentiation, as IL-2 drives Blimp1 activation in Tfh cells, which is inhibitory to Bcl-6; however, clinical trials are still in the early stages for IL-2 treatment in autoimmune diseases in the context of Tfh cells (Santana et al., 2024, Raeber et al., 2023).

Other key cytokines, such as IL-6 and IL-21, could also be exploited therapeutically to prevent Tfh cell generation (Nian et al., 2021). To date, IL-21 monoclonal antibody therapies are still in development (Ignatenko et al., 2016), whilst the IL-6 monoclonal therapy, tocilizumab, has been shown to reduce circulatory Tfh cells and IL-21 production (Liu et al., 2021). However, these drugs are not specific to Tfh cells and would shut down other peripheral branches of the immune response. To date, we have not found a specific drug candidate to target Tfh cells solely (Yan et al., 2017).

Working with my industrial partner, Sitryx Therapeutics Ltd., in this chapter, I wanted to investigate the impact of blocking lipid biosynthesis to see if that could inhibit Tfh differentiation or function. To achieve this, it was first necessary to establish an *in vitro* system for differentiating Tfh cells from naïve CD4⁺ T cells isolated from human blood cones and set up a robust phenotyping flow cytometry panel to measure key Tfh cell markers, including CXCR5, PD-1, BCL-6, and ICOS, as well as develop a restimulation assay to measure the production of the key Tfh cell cytokine, IL-21.

Developing these assays would enable me to explore the role of stearoyl CoA desaturase 1 (SCD-1), a key enzyme in *de novo* lipid biosynthesis. In previous chapters (Chapter 3), I showed that Tfh cells increased SCD-1 expression as well as uptake of c-16 palmitic acid, a key molecule in this pathway, compared to naïve T cells from both infected (*H. polygyrus*) and uninfected mice. Previously, the role of SCD-1 was characterised in murine Tfh cells, a study identifying that SCD-1 is upregulated in both murine Tfh and human tonsillar-derived Tfh cells and that overexpression of SCD-1 in murine OT-II cells has been shown to increase Tfh frequency, whilst inhibition was shown to accelerate the contraction of the Tfh response towards the IAV X31 immunisation in mice. It was also reported that SCD-1 inhibition can induce ER stress in murine Tfh cells, leading to apoptosis, although the study did not address the effect of SCD-1 inhibition on human Tfh cells (Son et al., 2020). Thus, to understand the effect of SCD-1 inhibition on human Tfh cells, it was planned to use commercially available SCD-1 inhibitors to investigate the impact of blocking this enzyme on the differentiation and function of Tfh cells.

6.2 Aims

- To set up Tfh, Th2 and activated Th0 cell cultures *in vitro*, optimise flow cytometry assays to phenotype, and measure activation and cytokine profiles.
- To assess the expression of SCD-1 in human *In vitro* derived Tfh, Th2 and Th0 cells
- To block SCD-1 in Tfh cells *in vitro* to understand if inhibition alters Tfh cell phenotype and function.

6.3 Results

6.3.1 Development of Human *In vitro* Tfh cells Assays

To obtain T cells from human blood, I utilised commercially available blood cones, a byproduct of healthy plasma donors to the NHS, which are compliant with the 2004 Human Tissues Act and associated codes of practice, ensuring the donor's identity remains anonymous. Naïve T Cells ($CD4^+ CD45RA^+ CD45RO^-$) (Figure 6.1 (A)) were isolated as per Materials and Methods (Chapter 2.2.1) using negative selection of naïve $CD4^+$ T cells, and subsequently frozen at $-180\text{ }^\circ\text{C}$ for storage until required. Before storage, this process yielded high purity of naïve T cells ($>95\%$) (Figure 6.1 (B)). To understand the viability of the cells after storage, the cells were thawed and processed with RPMI media to dilute FBS/DMSO (90%/10%) from storage (cRPMI contains 10% FBS); this achieved high viability for all cells thawed and used (Figure 6.1 (C)).

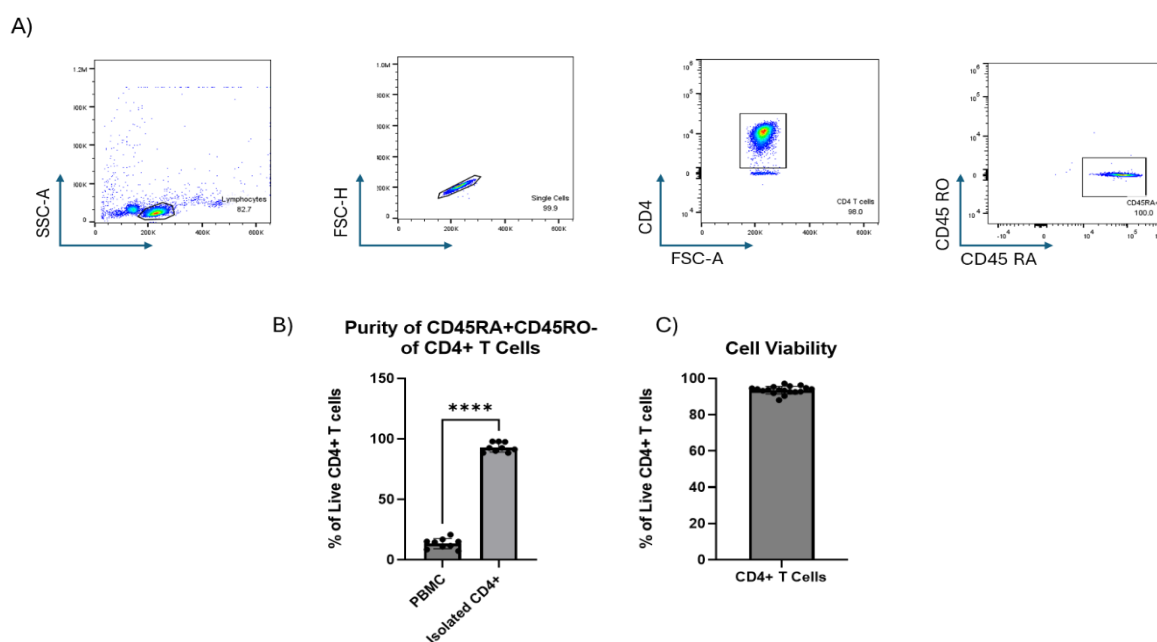


Figure 6.1. High Purity Isolation of Naïve $CD4^+$ T cells from Blood Cones and High viability after Storage at $-180\text{ }^\circ\text{C}$.

$CD4^+$ T cells were isolated from human blood cones (previously described in the materials and methods (section 2.2.1) and then stored at $-180\text{ }^\circ\text{C}$ until required. A) Representative gating strategy is used to identify and check the purity of samples before storage. B) Representative graph of $CD4^+$ T cell purity comparing bulk PBMC to isolated T cells. C) Representative graph of the viability of $CD4^+$ T cells after thawing. Graphed data is shown with mean $\pm$ SD. In Figure 6.1B, this data is from 9 separate blood cones from 9 separate donors that were purified for Naïve $CD4^+$ T cells. Figure 6.1 (A) and (B) are from 1 isolation experiment. Figure 6.1 C was from multiple experiments (>5). Statistical significance was determined using

the Unpaired Student T-test when data was normally distributed through the Shapiro-Wilk Test. Significance was denoted by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Once I had a reliable system to achieve high viability naïve T cells, the next stage was to create a robust Tfh cell differentiation assay. Dr Ryan Kyle, Principal Scientist at Sitryx Therapeutics and industrial principal investigator for this PhD, carried out preliminary optimisation of the assay at Sitryx following discussion of several published protocols reported to achieve Tfh cell differentiation. Dr Kyle tested these conditions in the laboratory at Sitryx, and we decided to follow up on two assays that showed the most promise. I then joined Sitryx for a 3-month research placement. Once there, I explored both assay formats and found the optimal conditions for *in vitro* differentiation of human Tfh cells: culture in the presence of human IL-21, IL-12, IL-6, TGF- β , and anti-IL-2, with stimulation provided through CD3/28.

The culture system was optimised for a 3-day incubation of cells with cytokines, rather than a longer period, to maintain cell viability and minimise alterations in metabolism as it has previously been demonstrated that prolonged cell culture methods with media changes can induce increased glycolytic activity when glucose is abundant (Golikov et al., 2022). Under these conditions, minimal colour change was observed in the media, consistent with a healthy culture system, with high viability at day 3 (~90% live). The optimised gating strategy to identify Tfh cells is shown in Figure 6.2 (A). The markers CXCR5 and PD-1 were used to identify Tfh cells, which significantly increase and are maintained on the surface of Tfh cells after differentiation (Deenick and Ma, 2011). These two markers were used throughout the thesis to identify Tfh cells and ensure consistency concerning the findings. A CD4 marker was not added to the gating strategy, as I previously highlighted that the naïve CD4⁺ T cells were isolated with high purity (Figure 6.1).

The Tfh cell assay was validated by two pre-existing T cell assays optimised by Sitryx to discriminate Th0 and Th2 cells. These results highlighted that Tfh cells, compared to Th0 and Th2 cells, exhibited the highest induction of PD-1 and CXCR5 expression, consistent with the Tfh cell phenotype (Figure 6.2B). By contrast, GATA3 was highest in Th2 cells, whilst T-bet was not significantly induced in any of the systems, compared to the relevant FMO control (Figure 6.3 (A)). Since no human Th1 assay had been developed and validated by Sitryx before/during my visit, there was no positive control for Th1 cells and T-bet staining.

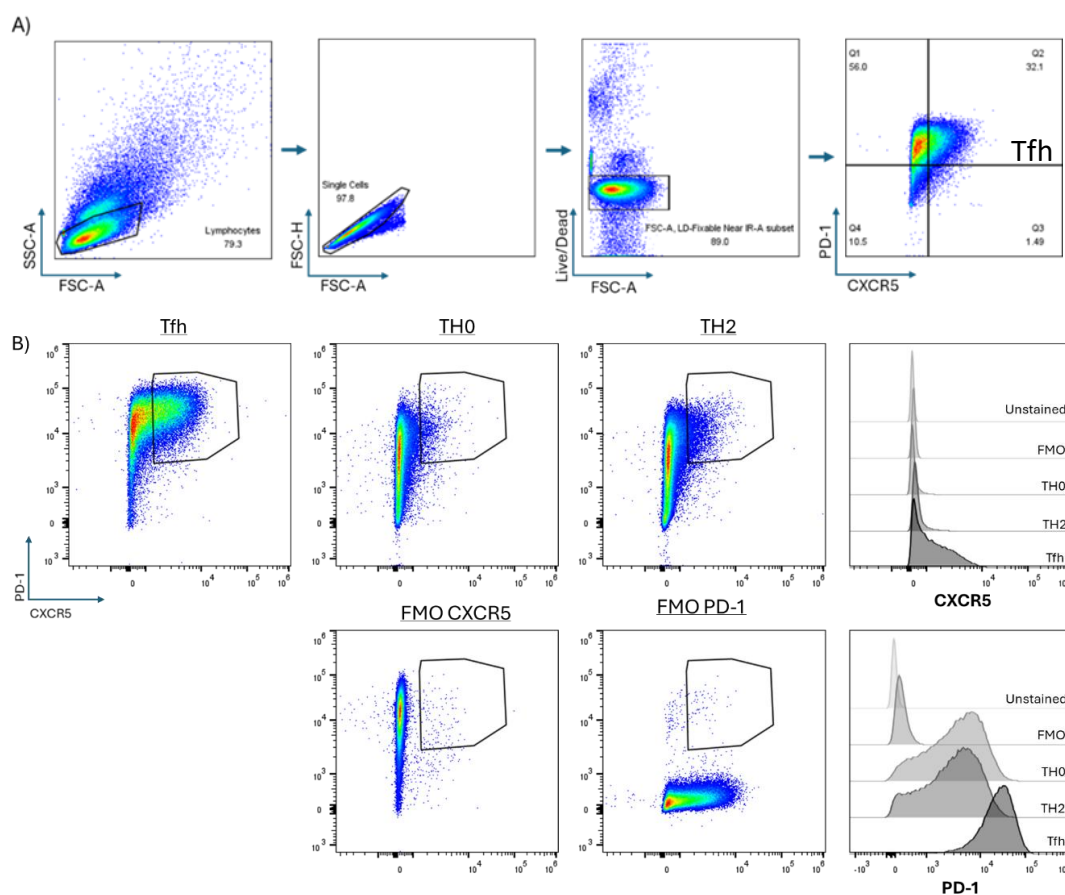


Figure 6.2. Generation of *in vitro* human Tfh cells from thawed naive CD4⁺ T cells isolated from human blood cones.

Naïve CD4⁺ T cells were subjected to a 3-day culture (previously described in methods) to generate CD4⁺ Tfh, Th2 and Th0 cells. A) A representative gating strategy is used to identify live Tfh cells (CXCR5 High, PD-1 High). B) Representative gating strategy of PD-1 vs CXCR5 for each cell population (Tfh, Th0 and Th2), as well as a representative histogram to highlight the induction of CXCR5 and PD-1 in Tfh cells compared to Th2 and Th0 cells. FMOs staining also highlights confirmation of positive staining.

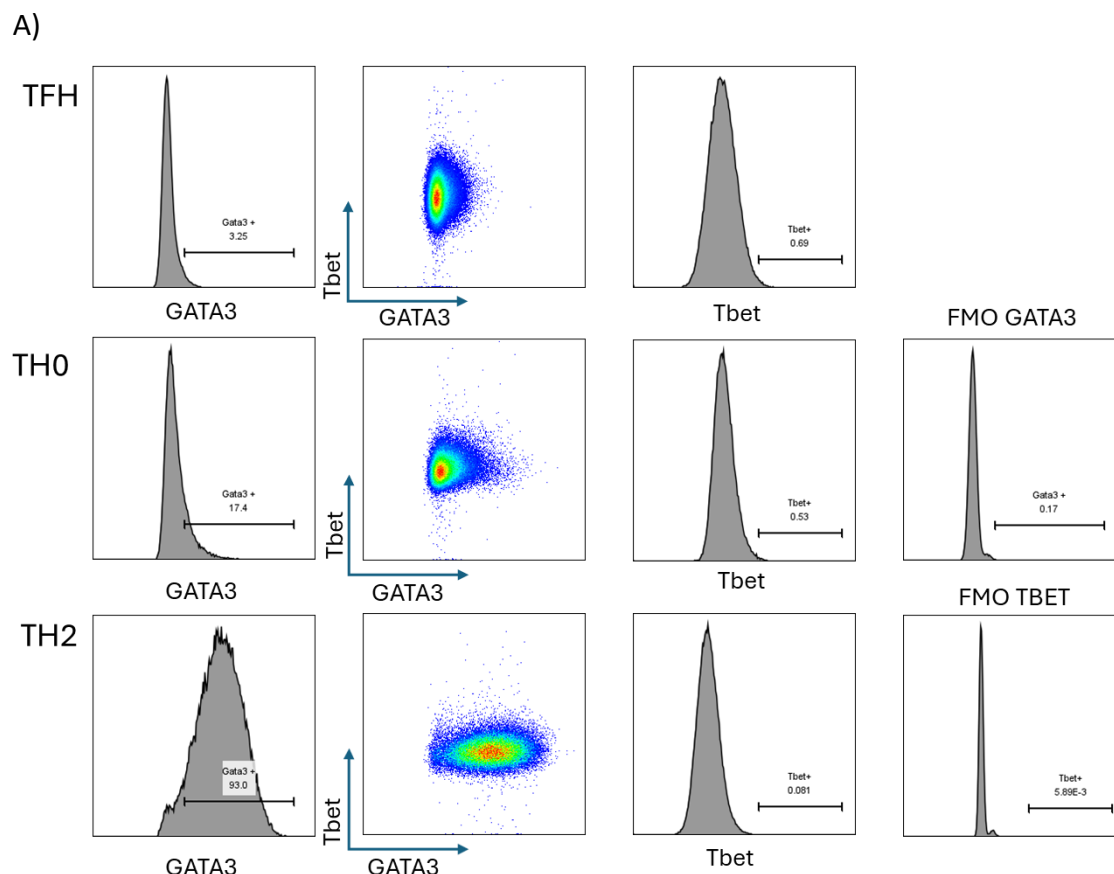


Figure 6.3. Generation of *in vitro* human Th2 and Th0 cells from thawed naive CD4+ T cells isolated from human blood cones.

Naïve CD4+ T cells were subjected to a 3-day culture (previously described in methods) to generate CD4+ Tfh, Th2 and Th0 cells. A) Representative flow plot and histograms to highlight the induction of GATA3 in Th2 Cells and the lack of induction of T-bet. Figure A also highlights the lack of induction of GATA3 and/or T-bet in Tfh and Th0 cells.

To assess the function of the Tfh cells, IL-21 production in response to stimulation with PMA/ionomycin, in the presence of a protein transport inhibitor for 4 hours, was measured (Figure 6.4). This assay could then be used to measure the functional output of Tfh cell biology (IL-21) by flow cytometry, which would enable the assessment of the therapeutic potential of the inhibitors for targeting Tfh cell biology. IL-21, however, can be produced by both Tfh and Th2 cells, and thus it was necessary to determine whether the system was robust enough to measure the differences observed between these two cell types (Wurster et al., 2002). Tfh cells are the primary producers of IL-21, which is critical for the GC response (Spolski and Leonard, 2010).

The results indeed showed that the gated Tfh cells produced the highest levels of IL-21 after restimulation (Figure 6.4 (A)), compared to Th0 and Th2 cells. To understand the effect of depleted media on IL-21 levels, restimulation was tested under two different conditions: media from day 0 (Depleted media (DM)) and also with a change of media before restimulation (new media (NM)), however, there was no significant change in IL-21 production between Tfh cell in these environments, however IL-21 levels were higher in the new media control.

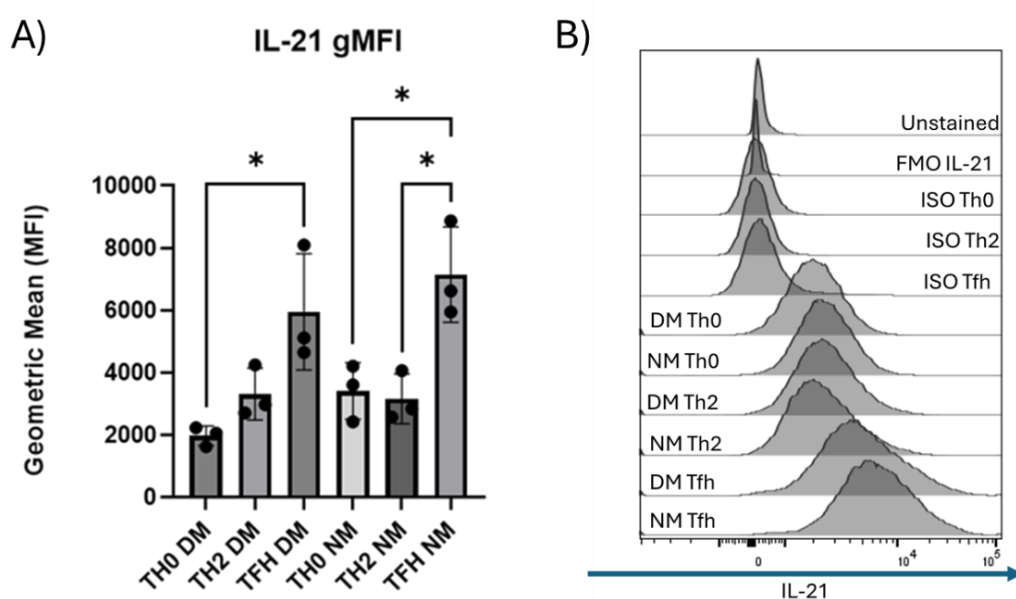


Figure 6.4. Robust IL-21 Intracellular Staining Assay for Tfh Cells.

Naïve CD4⁺ T cells were subjected to a 3-day culture (previously described in methods) to generate CD4⁺ Tfh, Th2 and Th0 cells. Cells were then incubated for 4 hrs with PMA/Ionomycin with protein transport inhibitors and stained for intracellular cytokines. A) Representative boxplot of IL-21 gMFI from Th0, Th2 and Tfh when reactivated with PMA/IONO in new media (NM) or depleted Media (DM). A) Representative histogram of IL-21 expression in unstained, IL-21 isotype staining, DM, and NM Tfh cells. Graphed data is shown with mean $\pm$ SD. The graph shows the mean data from 3 donors (3 replicates per donor to create a mean data point). Each experiment was repeated twice, and one representative graph of these two experiments is shown. Statistical significance was determined using a one-way ANOVA with Tukey's post-test for multiple comparisons in groups where data were deemed normally distributed, as distinguished through the Shapiro-Wilk test. When the data were not normally distributed, the Kruskal-Wallis with post hoc Dunn's Test was conducted to compare groups. Significance was denoted by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Previous chapters explored the upregulation of genes involved in *de novo* lipogenesis in Tfh cell biology. Previous research on *de novo* lipogenesis in Tfh cells has primarily found that SCD-1, an enzyme responsible for converting saturated fatty acids into

monounsaturated fatty acids, can regulate the longevity of murine Tfh cells in the GC response. However, this paper failed to address whether findings regarding SCD-1 inhibition could be translated to human Tfh cells (Son et al., 2020).

Thus, firstly, the aim was to assess the relative levels of SCD-1 protein in the cell cultures by Western blot analysis of lysates from Tfh, Th2, and Th0 cultures. However, as this system did not yield a culture of 100% pure Tfh cells, this must be considered when interpreting the results, as T cells of non-Tfh phenotypes will also be present. SCD-1 has previously been shown to be expressed more strongly in Tfh cells than in non-Tfh cells all other CD4⁺ T cells from human tonsils (Son et al., 2020) and consistent with this, the cells in the Tfh cultures expressed the highest levels of SCD-1 compared to Th0 and Th2 cells (Figure 6.5 (A, B); expression normalised to total protein stain (Figure 6.5 (C))). Thus, confirmation that Tfh cells expressed SCD-1 facilitated the question: How do SCD-1 inhibitors affect Tfh cell differentiation *in vitro*?

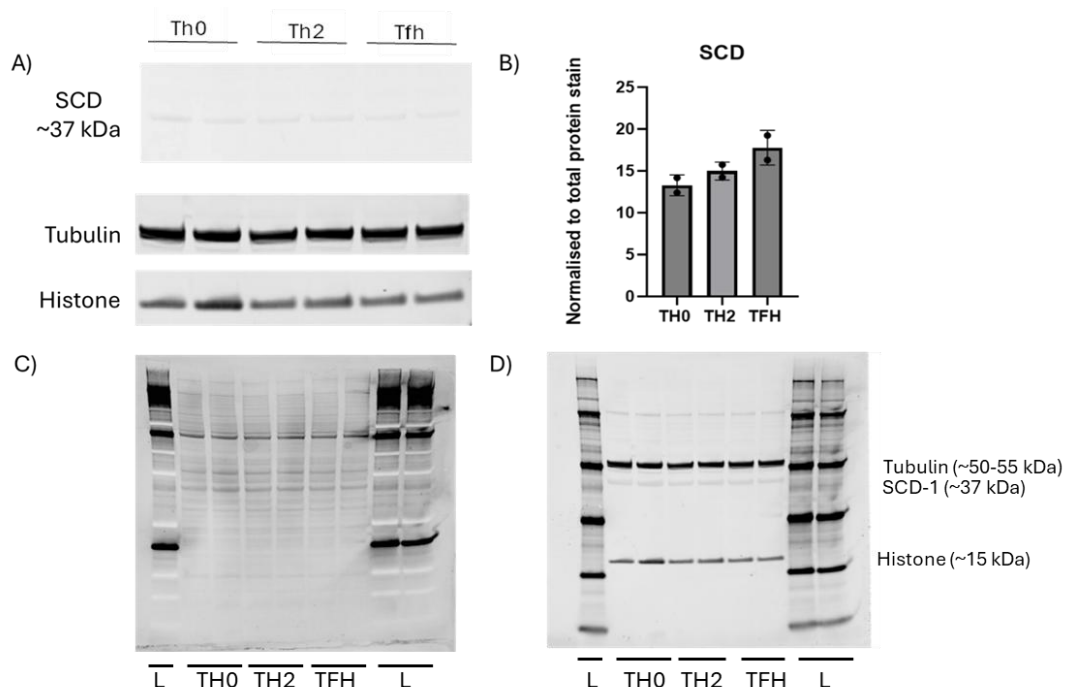


Figure 6.5. *In vitro*-derived Tfh cells have higher SCD-1 expression when compared with other CD4+ T effector cells.

CD4+ T cells were isolated from human blood cones (previously described in methods) and then stored at -180°C until required. Once the Tfh, Th2 and Th0 cells were cultured on Day 3, protein lysates were extracted and analysed by western blot. A) Representative protein expression western blot of SCD-1 for Tfh, Th2 and Th0 cells. B) Representative graph of SCD expression for Tfh, Th2 and Th0 cells. C) Representative image of a total protein stain with a protein ladder – labelled as L. D) Representative image of a western blot for SCD-1 with Protein ladder (L). Graphed data is shown with mean $\pm$ SD. Each well represents a donor; there were two donors per experiment, and a total of two repeats of this experiment were carried out, resulting in a total of four donors overall. The findings presented represent data from one of the two experiments, which involved two donors. Each donor was created using three replicates and then pooled to form a single cohesive sample from that donor.

6.3.2 Inhibition of SCD-1 in human *in vitro* Tfh cells

To answer this question, I utilised two commercially available SCD-1 inhibitors, A939572 and CAY10566, to study their effects on the differentiation of *in vitro*-derived Tfh cells. CAY10566 from Caymen Chemical Ltd. was used due to its highly potent and selective capacity as an SCD-1 inhibitor. CAY10566 was shown to have an IC_{50} of 4.5 nanomolar (nM) $\pm$ 4.0 (murine) and 26 nm $\pm$ 9.0 (Human); it also possesses excellent cellular activity at blocking the conversion of long-chain fatty acid CoAs (LCFA-CoAs) to monounsaturated LCFA-CoAs in HepG2 cells. CAY10566 had good mouse and rat

microsomal stability, reduced IV clearance, and high oral availability, suggesting good drug candidacy (Liu et al., 2007).

In research presented by Lui *et al.* (2007), a high-throughput screen was employed to assess the compound's cross-reactivity, a high-throughput screening assay utilised in early-stage drug discovery. It involves rapidly testing a library of compounds against a target enzyme (SCD-1) to identify potential drug candidates and assess their selectivity using the CEREP screen. The compound was found to have minimal cross-reactivity with 75 different receptors, transporters, and ion channels, with the only major interaction being in the human dopamine and 5-HT transporters. CAY10566 was also tested for co-enzyme reactivity as SCD-1 required the use of cytochrome B5/B5R for function; however, no interaction was found, and the compound was found to target SCD-1 directly (Liu et al., 2007).

Like CAY10566, A939572 is also a highly potent and selective inhibitor of SCD-1, with IC₅₀ values of 37 nM (Human) and 4 nM (murine) *in vitro*. Unlike CAY10566, this compound exhibits limited or no cross-reactivity and indirect effects on Cytochrome B5/B5R, making it an excellent choice for *in vitro* assessment of SCD-1 function in Tfh cell biology. In the development of this compound, *in vivo* testing showed that in OB/OB mice, using a 5-day efficacy study, A939572 reduced lipid profiles in all categories (Cholesterol, DAG, TAG, etc.) and caused a dose-dependent decrease in triglyceride. Together, these data suggest that both compounds make excellent tools to study the effects of SCD-1 inhibition on Tfh cell differentiation and function (Xin et al., 2008)

To understand the role of SCD-1 in Tfh biology, a dose-response assay was performed, using concentrations ranging from 0.5 to 500 nM of both compounds, to determine the optimal therapeutic index of each inhibitor. This index needs to reflect highly effective inhibition of SCD-1 function (e.g., high efficacy) while maintaining low toxicity profiles (e.g., no increase in cell death). Inhibitors were added at Day 0 and Day 2 of the 3-day culture to enable understanding, both of how SCD inhibition affects the differentiation of Tfh cells and how acute treatment with SCD inhibitors impacts Tfh cell function and phenotype.

Interestingly, with the addition of the SCD-1 inhibitor on Day 0, the percentage of Tfh (CXCR5 high, PD-1 High) cells decreased in a dose-dependent manner with both Cay10566 and A939572; however, the decrease was not statistically significant (Figure

6.6B). By contrast, with the Day 2 addition of the SCD-1 inhibitors, I found a significant decrease in the percentage of Tfh cells in a dose-dependent manner with both compounds (Figure 6.6 (C)). Furthermore, I observed no difference in the cell count (Figure 6.7(A)) or frequency of live cells (Figure 6.8(B)) in each of the culture systems, suggesting that Tfh cell death was not a factor in the decrease in frequency. Upon closer inspection, the decrease in Tfh cell populations could be attributed to decreased CXCR5 expression, whilst PD-1 expression remains unchanged (Figure 6.6 (A) and Figure 6.8).

To test this hypothesis, the gMFI of PD-1 and CXCR5 was analysed in both culture settings. All antibodies were used at optimal concentration (previously titrated), so they were appropriate for measuring MFIs. This approach revealed a dose-dependent decrease (by both SCD-1 inhibitors) in CXCR5 expression (Figure 6.8 (A)) and no change in PD-1 expression (Figure 6.8 (B)). Although the decrease in CXCR5 did not reach significance, the trend observed with CXCR5 was absent with respect to PD-1 expression, suggesting that surface expression of CXCR5 on Tfh cells decreases with acute SCD-1 inhibition.

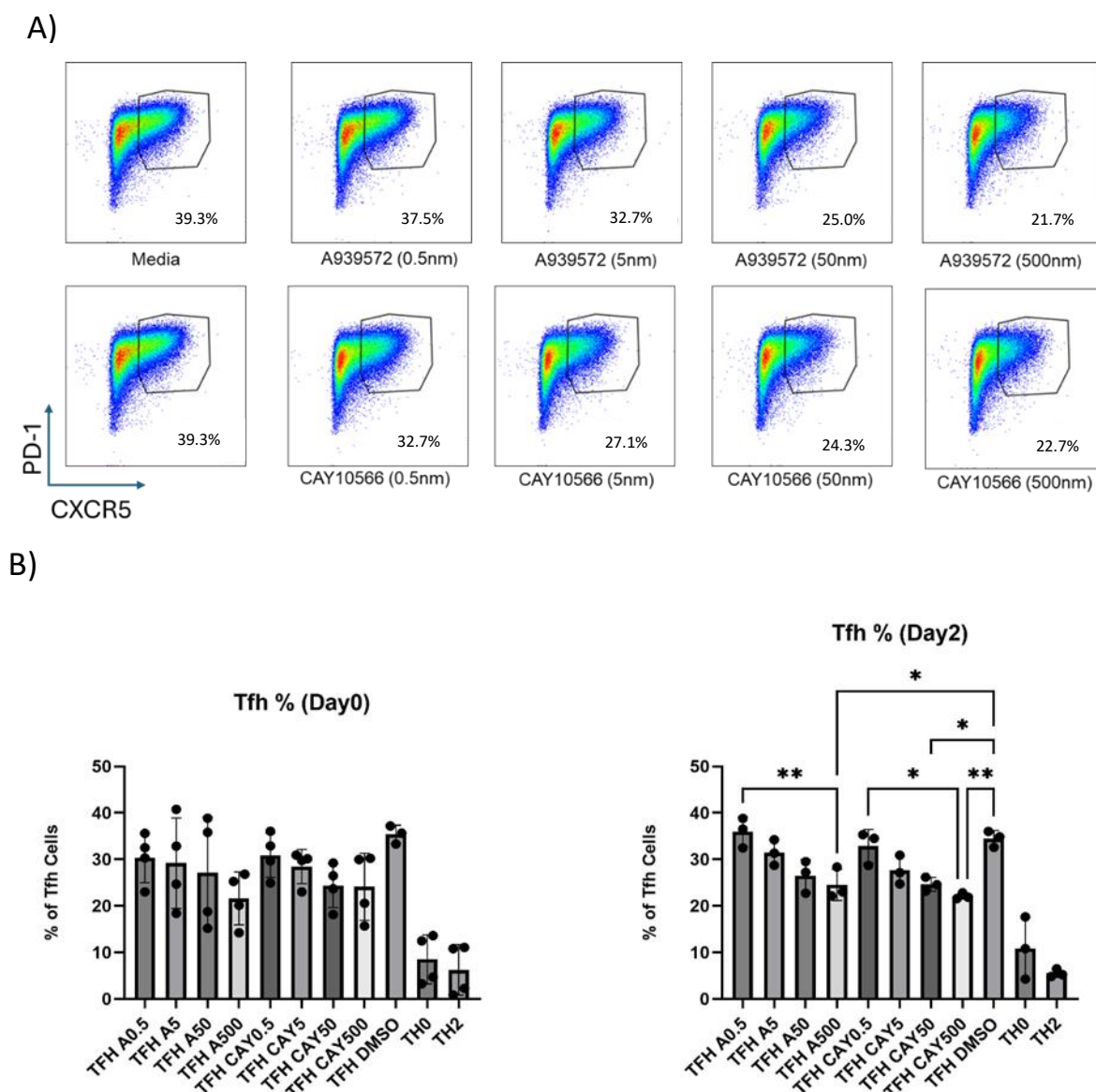


Figure 6.6. Inhibitors of SCD-1 decrease the levels of Tfh Cells *In vitro* in a dose-dependent manner.

In vitro derived Tfh cells were cultured for 1 (Day 2 addition) or 3 days (Day 0 Addition) in the presence of SCD-1 inhibitors A939572 (A0.5, A5, A50, A500 refers to concentration of A939572 in nanomoles) and Cay10566 (Cay0.5, Cay5, Cay50, Cay500 refers to concentration of Cay10566 in nanomoles) in complete RPMI media with skewing cytokines and cultured at 37°C .A) Representative gating for Tfh cells under different SCD inhibitors (A939572 and CAY10566) or in RPMI media (Day 2). B) Representative graph of % of Tfh Cells cultured presence of SCD-1 inhibitors A939572/Cay10566 at 10-fold dilution (500 nm-0.5nm) to create a dosage curve to plot therapeutic window of efficacy (Day 0) and C) Representative graph of % of Tfh Cells (Day 2) under Dosage Curve of SCD inhibitors (0.5 nm- 500nm). Graphed data is shown with mean $\pm$ SD. A total of 4 donors were used for the Day 0 assay, whilst 3 donors were used for Day 2. A total of three repeats per donor were carried out, and the mean from each was determined to provide one data point. Each experiment was repeated twice. Statistical significance was determined using one-way ANOVA

with multiple comparisons when data were normally distributed through the Shapiro-Wilk Test. Significance was denoted by * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

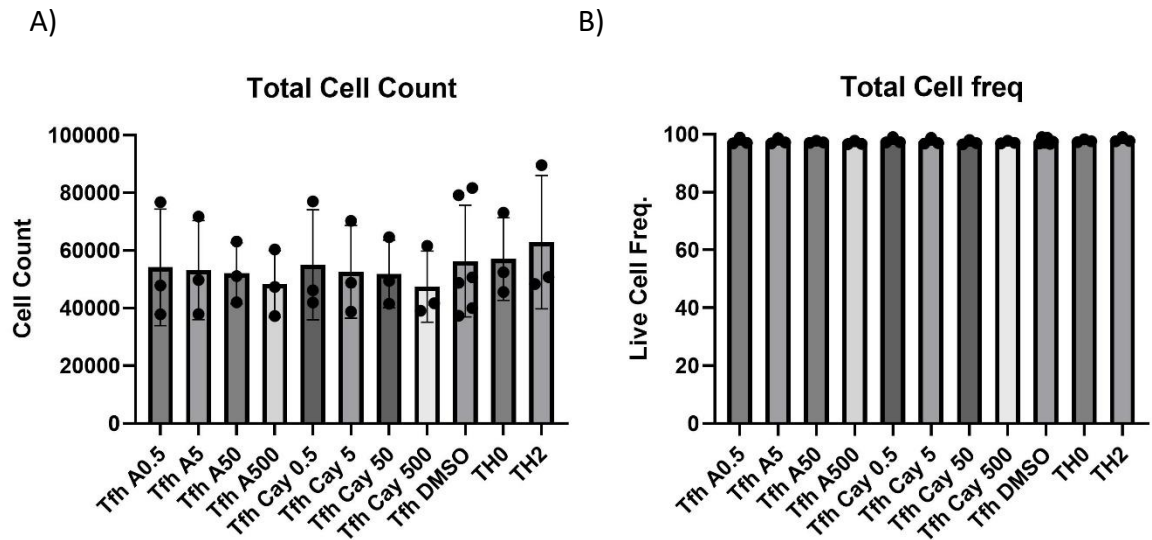


Figure 6.7. SCD inhibition does not increase cell death in Tfh Cells *In vitro*.

In vitro derived Tfh cells were cultured for 2 days before the addition of SCD-1 inhibitors A939572 and Cay10566, 1 day prior experimental endpoint (Day3). A0.5, A5, A50, A500 refers to concentration of A939572 in nanomole and CAY10566; Cay0.5, Cay5, Cay50, Cay500 refers to concentration of Cay10566 in nanomoles in complete RPMI media cultured at 37°C. Using Viability dye (L/D NIR reactive dye-Invitrogen), live single-cell lymphocytes were assessed using flow cytometry. A) Representative graph: Total Cell Count under different SCD inhibitors (A939572 and CAY10566) or in RPMI media. B) Representative graph of Total Cell Freq. Under different SCD inhibitors (A939572 and CAY10566) or in RPMI media. Graphed data is shown with mean $\pm$ SD, with 3 Donors used for each experiment, with 3 replicates per donor. The mean of the 3 replicates was then determined and plotted as 1 point on each graph. The experiment was repeated twice. Statistical significance was determined using one-way ANOVA with multiple comparisons when data were normally distributed through the Shapiro-Wilk Test. Significance was denoted by * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

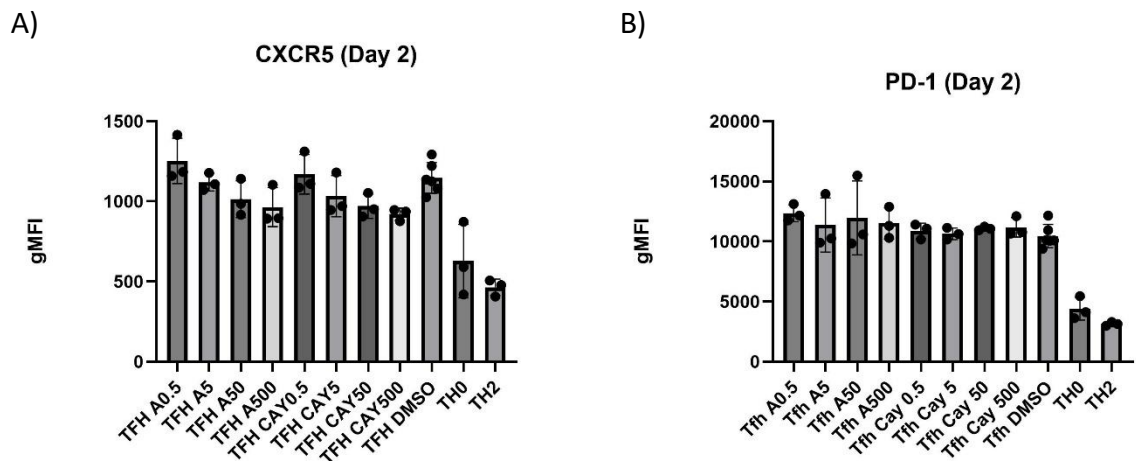


Figure 6.8. SCD-1 inhibition decreases CXCR5 on the surface of Tfh cells; however, it does not affect PD-1 expression.

In vitro derived Tfh cells were cultured for 2 days before the addition of SCD-1 inhibitors A939572 and Cay10566, 1 day prior experimental endpoint (Day3). A0.5, A5, A50, A500 refers to concentration of A939572 in nanomole and CAY10566; Cay0.5, Cay5, Cay50, Cay500 refers to concentration of Cay10566 in nanomoles in complete RPMI media with Tfh-skewing cytokines and cultured at 37°C. A) Representative graph of CXCR5 expression (gMFI) under different SCD inhibitors (A939572 and CAY10566) or in RPMI media. B) Representative graph of PD-1 expression (gMFI) under different SCD inhibitors (A939572 and CAY10566) or in RPMI media. Graphed data is shown with mean $\pm$ SD, with 3 Donors used for each experiment, with 3 replicates per donor. The mean of the 3 replicates was then determined and plotted as 1 point on each graph. The experiment was repeated twice. Statistical significance was determined using one-way ANOVA with multiple comparisons when data were normally distributed through the Shapiro-Wilk Test. Significance was denoted by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

To evaluate the impact of the SCD-1 inhibitors on the functional output of the Tfh cells, the previously validated (Figure 6.4) PMA plus Ionomycin (in the presence of a protein transport blocker) stimulation assay was used to measure the production of IL-21 by flow cytometry. This demonstrated that SCD inhibition did not affect IL-21 production, neither on acute nor extended exposure to the inhibitor (Figure 6.9 A and B). The effect on key markers associated with Tfh cell biology was also measured. For example, ICOS is essential for Tfh cell differentiation and maintenance through the regulation of Klf2, and blocking ICOS can result in Tfh cells reverting to non-Tfh cell phenotypes (Weber et al., 2015). Similarly, CCR7 is involved in the differentiation and migration of Tfh cells (Li et al., 2022) whilst CD40L is involved in the activation of Tfh cells (Laurent et al., 2010) and BCL-6 is the key TF associated with Tfh cell lineage, differentiation, and function (Choi et al., 2020).

Interestingly, our data suggest that SCD-1 inhibition may affect CCR7 (Figure 6.10(A)); however, further work with a larger number of donors would be necessary to confirm this. I also found that neither acute nor extended SCD-1 inhibition affected the expression of CD40L (Figure 6.10 (B)), BCL-6 (Figure 6.10 (C)), or ICOS (Figure 6.10 (D)). Taken together with my earlier data, these results suggest that SCD-1 inhibition directly affects CXCR5 expression on the surface of Tfh cells. Targeting SCD-1 may yield an interesting treatment to prevent Tfh cell migration (Kim et al., 2015) and hence GC formation.

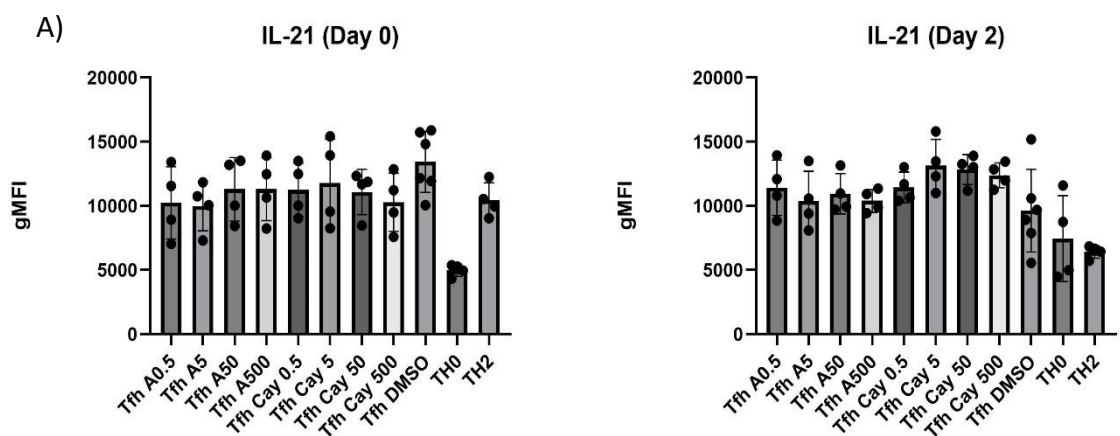


Figure 6.9. SCD inhibition does not affect IL-21 production by Tfh Cells.

CD4⁺ T cells were cultured using skewing cytokine cocktails into Tfh, Th2 and Th0. Tfh cells were then treated with different SCD inhibitors (A939572 and CAY10566) in RPMI media for either the full duration of the culture (Day 0) or 24 hours before the endpoint of the culture (Day 2). Following this, cells were subjected to 4 hrs of incubation with PMA/ionomycin and Protein Transport Inhibitor, and IL-21 production was measured through flow cytometry. A) Representative graph of IL-21 production (gMFI) under different SCD inhibitors (A939572 and CAY10566) or in RPMI media when added at Day 0 of the 3-day culture. B) Representative graph of IL-21 production (gMFI) under different SCD inhibitors (A939572 and CAY10566) or in RPMI media when added on Day 2 of the 3-day culture. Graphed data is shown with mean $\pm$ SD, with 3 Donors used for each experiment, with 3 replicates per donor. The mean of the 3 replicates was then determined and plotted as 1 point on each graph. The experiment was repeated twice. Statistical significance was determined using one-way ANOVA with multiple comparisons when data were normally distributed through the Shapiro-Wilk Test. Significance was denoted by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

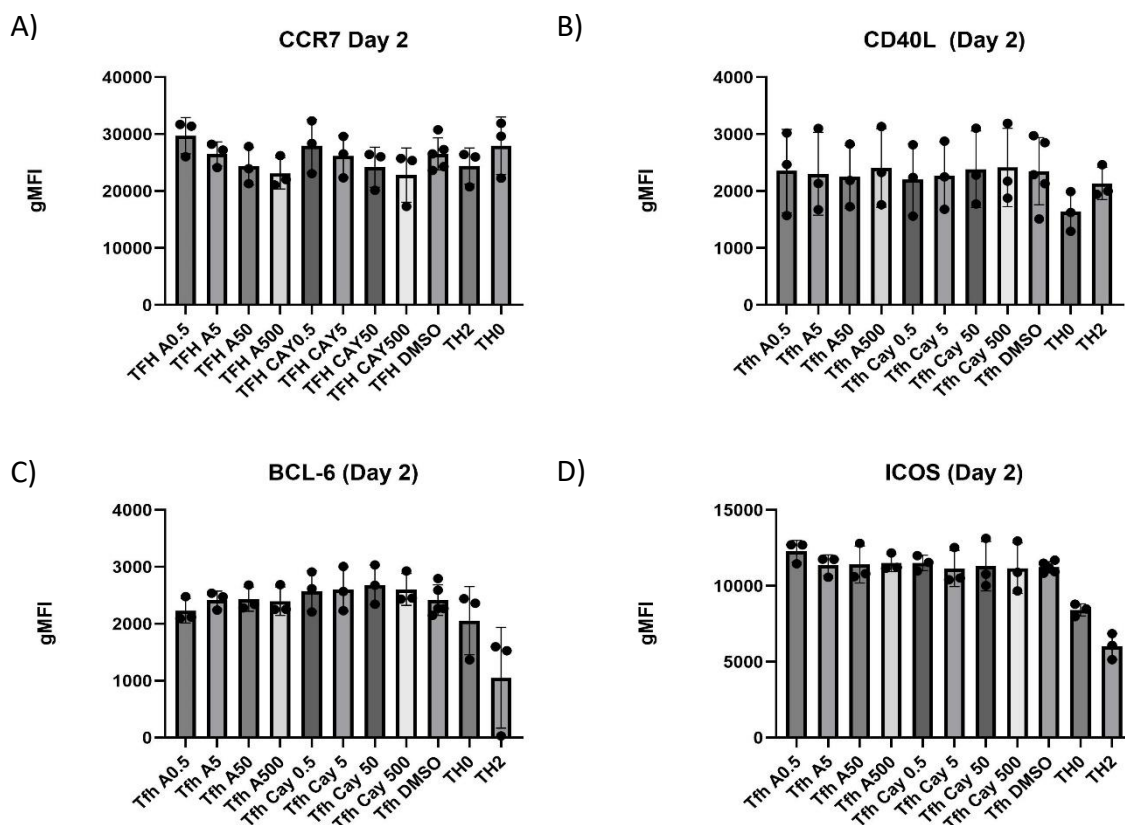


Figure 6.10. Acute SCD-1 inhibition does not significantly affect CCR7, CD40L, BCL-6 or ICOS expression by Tfh cells.

CD4⁺ T cells were cultured using skewing cytokine cocktails into Tfh, Th2 and Th0. Tfh cells were then treated with different SCD inhibitors (A939572 and CAY10566) in RPMI media for either the full duration of the culture (Day 0) or 24 hours before the endpoint of the culture (Day 2). SCD inhibitors were added to the cytokine-skewing cell culture environment to assess their effect on differentiation and acute inhibition of Tfh cells. A) Representative graph of CCR7 expression (gMFI) under different SCD inhibitors (A939572 and CAY10566) or in RPMI media. B) Representative graph of CD40L expression (gMFI) under different SCD inhibitors (A939572 and CAY10566) or in RPMI media. C) Representative graph of BCL-6 expression (gMFI) under different SCD inhibitors (A939572 and CAY10566) or in RPMI media. D) Representative graph of ICOS expression (gMFI) under different SCD inhibitors (A939572 and CAY10566) or in RPMI media. Graphed data is shown with mean $\pm$ SD, with 3 Donors used for each experiment, with 3 replicates per donor. The mean of the 3 replicates was then determined and plotted as 1 point on each graph. The experiment was repeated twice. Statistical significance was determined using one-way ANOVA with multiple comparisons when data was normally distributed through the Shapiro-Wilk Test. Significance was denoted by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Overall, this chapter has demonstrated the successful establishment of an *in vitro* human Tfh, Th2, and Th0 culture system and validation of an IL-21 restimulation assay to assess the function of such Tfh cells. Using these systems, the data suggest that acute SCD inhibition reduces CXCR5 expression on human Tfh cells *in vitro*. By contrast, they also highlighted that SCD does not affect BCL-6, CD40L and ICOS expression or IL-21 production. Developing a robust method to screen compounds against Tfh cell biology will allow Sitryx to continue exploring therapeutics against Tfh immunometabolism and may yield the first Tfh-specific therapy.

6.4 Discussion

Tfh cells, unlike other T cell subsets, are difficult to study as they are exclusively located within the lymphatic tissues (Crotty, 2019). Some studies have utilised tonsils to extract Tfh cells (Rane, 2020), though access to these tissues can be limited, and samples often come from inflamed tissue with abnormal Tfh cells (Geissler et al., 2020). A recent study found that people with recurrent tonsillitis caused by group A streptococcus have atypical Tfh cells, which can kill B cells (Baessler and Hale, 2019). Other studies have focused on longitudinal fine-needle sampling of draining LNs to understand Tfh cells *in vivo*; however, logistical challenges hinder the widespread use of this form of tissue procurement (Schattgen et al., 2024, Borchering et al., 2024). Many human studies focus on circulatory Tfh cells (cTfh); however, it is arguable that the role that these cells play compared to Tfh residing in the LN is distinct. For example, cTfh cells have low expression of BCL-6, whereas LN-Tfh cells have very high expression of this transcriptional repressor, so the environment may drive alternative functions and metabolic signatures (Vella et al., 2019).

This has left most researchers with two options: to utilise murine Tfh cells to understand pathways associated with different immune responses, or to create an *in vitro* culture of human Tfh cells from naïve T cells isolated from human blood (Podesta et al., 2023). In this thesis, murine *in vivo*-derived Tfh cells have predominantly been investigated to understand the physiologically relevant profiles of immunometabolism of Tfh cells. However, to complement and confirm the findings in mice with those in humans, it was decided to approach the question of whether manipulating Tfh cell metabolism alters their phenotype or function in the human-derived *in vitro* Tfh differentiation assay developed on site at Sitryx Therapeutics.

Both murine and human Tfh cells are similar in Bcl-6 expression, surface receptor, and gene expression profile, indicative of the evolutionary robust conservation biology of Tfh cells (Crotty, 2014). However, the role of cytokines in Tfh cell differentiation is different with IL-6 being a potent inducer of IL-21 in murine Tfh cells, whereas its role in the induction of IL-21 in human Tfh cells is less pronounced (Schmitt et al., 2014b). Whilst human and murine Tfh cells share similarities, distinctions are also present. An example of this would be the role of STAT3 and STAT4 signalling, as human Tfh cells rely heavily on both signal transducers, whilst murine Tfh cells show dominance for STAT3 signalling and are less reliant on STAT4 (Crotty, 2014). Also, it has been shown that TGF- β is critical for

Human Tfh cell differentiation and is not required to the same degree for murine Tfh differentiation (Schmitt et al., 2014b).

However, research suggests that the key transcriptional profiles between human and murine Tfh cells are conserved, indicating that the findings from the murine Tfh cells regarding their metabolic gene expression profiles may be robust enough to apply to the human system (Crotty, 2014). Specific targets may yield different outcomes based on subtle differences in differentiation and signalling; however, key themes such as the requirement for glycolysis and lipid metabolism are likely to be maintained through transcriptional programs directed by Bcl-6 induction (Schmitt et al., 2014b)

To assess if metabolic manipulation could merit therapeutic potential in targeting Tfh cells, the focus was on the key pathways upregulated in Tfh cells from Chapters 3, 4 and 5. These data highlighted *de novo* lipogenesis as a major theme of Tfh cell metabolism, with evidence for a role for SCD-1. To support this hypothesis, review of the current literature shows that SCD-1 expression is increased in both mouse Tfh and human tonsillar-derived Tfh cells compared to non-Tfh CD4⁺ T cells; however, these studies (Son et al., 2020) did not assess the role of SCD-1 in whether blocking SCD-1 can impact human *in vitro* Tfh differentiation/function or explore SCD as a therapeutic target .

In validating the Tfh culture system, Th2 and Th0 skewing conditions were also included as comparisons to assess the correct induction of the Tfh phenotype and the specific staining of PD-1 and CXCR5. An oversight was not to include a positive control of T-bet staining (for example, using Th1 cells); however, no Th1 assay had been developed at Sitryx at that point, and it was decided that it would hinder progress to spend time optimising this system too. Rather, a commercially available T-bet antibody that had been validated to be specific against T-bet (Huanosta-Murillo et al., 2021, Read et al., 2023) was used as a control. Overall, the Tfh assay provided a robust system for assessing the therapeutic manipulation of Tfh cells *in vitro*, similarly to other published research (Locci et al., 2016). It could be suggested, however, that the induction of CXCR5 and PD-1 in the Sitryx Tfh assay was more robust.

Ultimately, though, the classical 2D-plastic *in vitro* system does not account for factors associated with the GC (e.g. tissue architecture, cell interactions) and other microenvironment conditions (e.g. hypoxia, fluid dynamics) but modern techniques aiming to improve this through LN-on-a-chip technology, which factors in many of these

variables, are still in their infancy (Wang et al., 2024). To date, *in vitro* systems remain the most effective method for understanding Tfh cell biology *ex vivo*; however, caution should be exercised when interpreting the results. Nevertheless, new research has supported the validity of *in vitro* human Tfh cell generation, as it has been shown that such *in vitro* Tfh cells upregulate the same core transcriptome compared to *in vivo* Tfh cells derived from Human tonsils. Although this *in vitro* system differs slightly from the Sitryx system, these data emphasise that key Tfh cell characteristics can be achieved *in vitro* (Foster, 2025).

After creating and testing the *in vitro* assay system for Tfh cells, two commercially available SCD-1 inhibitors (CAY10566 and A939572) were used to target Tfh cell lipid metabolism. The results showed that SCD-1 inhibitors apparently decreased the frequency of Tfh cells (acute treatment; Figure 6.6) although this did not reflect a decreased cell count/frequency indicative of cell death (Figure 6.7). Upon further inspection, it was found that Tfh cells decreased the expression of CXCR5 but maintained all other factors associated with Tfh cell function and phenotype (Figures 6.8-6.10). This suggests that treatment of Tfh cells with SCD-1 inhibitors may lead to a decrease in the presentation/recycling of CXCR5 on the cell surface.

When CXCR5 protein is synthesised, co-translational translocation of CXCR5 to the ER is required for folding before surface presentation. Once inside the ER, the CXCR5 protein undergoes protein modification and folding, which is a highly regulated process and only allows correctly folded protein to be trafficked from the ER to the Golgi apparatus for post-translational modifications before being trafficked through endosomes to the plasma membrane (Comerford and Nibbs, 2005).

The rapid turnover of chemokine receptors through degradation and recycling, supported by ER stress, as reported by Son et al, 2020, suggests that SCD-1 inhibition drives dysfunctional ER responses and may hinder chemokine trafficking to the surface. Compared to other receptors, chemokine receptors are typically internalised more rapidly and have efficient recycling. In contrast, tyrosine kinase receptors (RTKs) and steroid receptors have less efficient internalisation and recycling. This is because chemokine receptor signalling must be short-lived so that immune cells remain responsive to changes in the external environment (Cosgrove et al., 2021, Neel et al., 2005). To determine if this is true, further experiments would need to be carried out, such as intracellular staining of CXCR5 to determine whether the chemokine receptor is still being produced but cannot be trafficked to the cell surface.

A decrease in CCR7 on the surface of Tfh cells treated with SCD-1 inhibitors, albeit not significantly, was also observed (Figure 6.10). If this finding was real, it would highlight a possible role of the ER in the ability of chemokine receptors to be presented on the surface of Tfh cells. Interestingly, ER stress has been linked to the upregulation of CXCL10 and CXCR3 on immune system cells during acute glaucoma; however, in most cases, it is documented that ER stress drives downregulation of chemokine receptors such as CCL23 on immune cells inside hepatic tumours (Karan, 2021) and CXCL1 on airway epithelial cells in Cystic Fibrosis (Tang et al., 2017). Furthermore, the finding that SCD-1 inhibition can decrease both CCR7 and CXCR5 is interesting, as normally, Tfh cells during differentiation downregulate CCR7 to upregulate the surface receptor CXCR5, allowing them to move to the B cell follicle (Ueno, 2018).

This could then, in turn, suggest the mechanism by which SCD-1 inhibition in Tfh cells increases ER stress response (Son et al., 2020), and then drives immunosuppression (shown previously in models of cancer and chronic inflammation that ER stress can be immunosuppressive) (Lee et al., 2014). Here, this suppression is achieved through the downregulation of CXCR5 and possibly CCR7, which prevents migration and aids in the early collapse of the GC response, as highlighted *in vivo* by Son et al. (2020) using the IAV X31 immunisation model. Further work would be needed to understand this fully, but it suggests a novel area of possible metabolic therapeutic targets, specifically by inducing ER stress through the manipulation of lipid metabolism. Similar ER stress-inducing therapies, such as Bortezomib, a proteasome inhibitor, which induces ER stress, have been shown to decrease the GC response in the murine model of transfused RBCs (Natarajan et al., 2017)

The use of SCD inhibitors has been explored as a treatment option for many diseases, such as cancer, non-alcoholic steatotic hepatitis and Alzheimer's disease. In cancer, inhibition of SCD-1 increases ER stress, and the cells undergo apoptosis as a result. This inhibition also drives increased recruitment of CD8⁺ T cells and DCs to the tumour microenvironment (Kato et al., 2022). These treatments are selectively targeted to cells that require SCD-1 and spare healthy cells, particularly in Human ovarian cancer cell lines and in Human blood mononuclear cells (Lee et al., 2024). They show promise in preclinical studies, with minor side effects in mice, such as increased eye discharge and hair loss, which have also been observed in humans (Kubota and Espenshade, 2022). However, long-term use may not be a viable option in the case of chronic inflammation, as SCD-1 inhibitors have been shown to drive skin, liver and arterial side effects (Brown and Rudel, 2010).

Skin dysfunction is a known side effect of SCD-1 loss, as the resulting epidermal lipid dysfunction leads to accelerated trans-epidermal water loss and can result in hair loss (Miyazaki et al., 2001). Interestingly, mice lacking SCD-1 had increased systemic inflammation and atherosclerosis, yet were protected against diet-induced obesity, hepatic steatosis and insulin resistance (Brown and Rudel, 2010). While SCD-1 inhibition protects against insulin resistance, its role in pancreatic β -cells is not supportive of its function in insulin production as SCD-1 inhibition has been shown to drive β -cell death, as they are extremely sensitive to saturated fatty acid lipotoxicity (Busch et al., 2005).

Conversely, our understanding of SCD-1 inhibitors has evolved over time, and balancing the side effects can be managed. Studies have shown that administering long-chain fatty acids, such as those found in fish oils, can help reduce the accumulation of saturated fatty acids and the inflammation that this can cause. This highlights that dietary intervention could be an effective treatment option if SCD-1 were used in the clinic to help mitigate adverse side effects (Brown et al., 2010)

Interestingly, recent finding in clinical studies have highlighted that using SCD1 inhibitors as a monotherapy is limited and that cancer cells have developed a mechanism for resistance, through fatty acid desaturase 2 (FADS2), which converts palmitic acid to sapienic acid: however this could be beneficial in some contexts, as this mechanism may allow other cells less responsive to lipotoxicity to process the lipid stress created by SCD-1 inhibition and further aid specificity in treatment by only target the cells that strongly depend on SCD-1 (Ikeda et al., 2024).

Ultimately, through careful development and crucially, understanding and mitigating the side effects driven by SFA lipotoxicity, this could open the door to SCD-1-targeted therapies against Tfh cells and may prevent their migratory capacity to the T/B border due to decreased CXCR5 expression. CXCR5 is crucial for the migration of the Tfh cells to the B cell follicle, as a chemokine ligand gradient for CXCL13 attracts them (Crotty, 2014)

A possible therapeutic avenue that could be explored would be to use CXCR5-mediated drug delivery systems. Similar strategies are currently being trialled in cancer treatments, using antibody-drug conjugate (ADC) against PD-1 (Fu et al., 2022). Through the utilisation of this anti-PD-1 ADU system, SCD-1 inhibitors could be delivered directly into cells expressing a large amount of the receptor on their surface. However, it may be better to aim for a receptor that is not expressed on skin epidermal or pancreatic β cells (PD-1 is

expressed on β cells) (Falcone and Foustari, 2020). A possible option would be CXCR5, which is not expressed on either of these cell types, allowing for a dual blocking mechanism against Tfh cells. Currently, therapies are being trialled using CXCR5-targeting ADC, and these are showing promising results in preclinical humanised models of mantle cell lymphoma (Schomber, 2023).

Here two commercial SCD-1 inhibitors demonstrate an effective range of 50-500 nm for CAY10566 and >500 nm for A939572 in decreasing CXCR5 on Tfh cells, highlighting the strong inhibitory effects of SCD on the Tfh phenotype. When comparing the effect of the SCD inhibitors in these assays to the IC₅₀ values reported (Liu et al., 2007, Xin et al., 2008) for CAY10566 (26nm-Human) and A939572 (37nm), the largest effects of SCD-1 inhibition were most pronounced between 5 nm and 50 nm for both compounds; however, they remained effective up to 500 nm, highlighting similar therapeutic potential.

The plausible next steps to assess the role of SCD-1 inhibitors in decreasing Tfh cell CXCR5 expression on the cell surface would be to test the functional consequences of this. A transwell migration assay using CXCL13 could be employed to understand if the migratory capacity of the cells (Shin et al., 2024) was diminished in response to SCD-1 inhibitors. Furthermore, understanding the role of lipid metabolism in chemokine receptor expression should be explored further to understand if any other metabolic pathways could facilitate similar outcomes to those observed with SCD-1 inhibitors. The enzymes involved in lipid desaturation pathways around SCD-1, such as SCD-2, and the FADS (1-3) should also be explored as they could offer similar therapeutic potential; however, caution should be taken as SCD-2 deletion in T cells can result in stronger type 1 interferon responses, which in the case of autoimmunity may not be favourable (Kanno et al., 2021).

This chapter highlights the potential use of SCD inhibitors to target Tfh cells, building on the pre-existing knowledge of the SCD-1 role in Tfh cells, as presented by Son et al. (2020). Here exposure to SCD inhibitors in *in vitro* Tfh cell cultures results in decreased CXCR5 expression, which may be due to lipid synthesis being required for the correct recycling of chemokine receptors, which rely on the rapid ability to internalise, recycle, and express new receptors in response to local environmental cues. This inability may be due to ER stress however, further research would be needed to confirm whether this is true in the human setting. Ultimately, this research also led to the development of a robust Tfh differentiation assay and key phenotyping and functional flow cytometric assays to

understand the effect of small molecule inhibitors on Tfh cells, both during differentiation and in acute dosing, to assess their impact on function.

Chapter 7: Main Discussion

7.1.1 Overview

Metabolism is a key determinant of immune cell function, and has emerged as a key area of interest over the past decade. This was catalysed by seminal work from pioneers such as Erika Pearce, who demonstrated that cellular metabolism extends beyond energy generation and can profoundly influence the fate of immune cells in many contexts (Pearce, 2010). Metabolic rewiring facilitates various effector functions, including T cell activation and differentiation, while maintaining the balance between pro-inflammatory and immune regulatory responses. Substantial inroads have been made in understanding and characterising effector and regulatory T cell immunometabolism; however, Tfh cells have often been overlooked, and much less is known about how these cells tailor their metabolic programs to support their function (Ma et al., 2024).

This knowledge gap is particularly evident in the context of type 1 vs type 2 immune responses, where cytokine environments, antigenic stimuli, and tissue-specific interactions could impose distinct metabolic profiles on the relevant Tfh cells. Previously, it has been shown that Tfh cells can adopt the flavour of the immune response they are responding to by expressing low levels of the TF which direct Th1, Th2, Th17, and Treg cell differentiation; however, the effect that the expression of these TF have on the metabolic signature of the Tfh cells is still relatively unknown (Kumar et al., 2024, Olatunde et al., 2021).

The major focus of this thesis was therefore to understand and define the metabolic profiles that underpin Tfh cell differentiation and function in distinct immune contexts. By comparing Tfh cells across type 1 and type 2 settings and contrasting them with their Th1 and Th2 counterparts, my work aimed to unravel the shared and distinct features of Tfh metabolism in different immune microenvironments. Particular attention was given to identifying key metabolic nodes, such as glycolysis, OXPHOS, FAS/FAO, and Lipid metabolism, as these had previously been described to be critical for Tfh cell biology (Crotty, 2019, Mayberry et al., 2022). However, I was particularly focused on the differences in expression of key metabolic genes/pathways in Tfh cell exposed to different inflammatory contexts to highlight areas of metabolic uniqueness in each Tfh subset.

Unravelling these metabolic distinctions holds considerable therapeutic promise, as it may lead to novel methods to target specific areas of the Tfh cell response (GC induction , cytokine production etc.). Abnormal Tfh cell responses have been implicated in a range of pathologies, including systemic lupus erythematosus, rheumatoid arthritis, Sjögren's syndrome, multiple sclerosis, and allergic diseases: aberrant activation of Tfh cells results in excessive GC formation and the production of high-affinity autoantibodies or IgE against self or allergens, which can drive tissue damage, fibrosis, and inflammation (Gensous et al., 2018, Crotty, 2014). To date, current immunotherapies and anti-inflammatory treatments for these diseases do not specifically target Tfh cells and focus predominantly on suppressing multiple different pathways involved in the immune response, rendering individuals susceptible to infection as a result (Crotty, 2019).

My hope is that by characterising Tfh cell metabolism in different immune contexts, this thesis lays the foundations for the development of context-dependent, Tfh-targeted interventions, particularly those that can selectively inhibit Tfh1 or Tfh2 cells without abrogating the entire Tfh compartment or conversely could be boosted to promote vaccine efficacy. This work may also allow us to differentiate Tfh cell populations based on transcriptional metabolic profiles that, with more research, may provide valuable insight as biomarkers for predicting infection or therapeutic outcome in patients

Overall, this work addresses a key gap in the immunometabolism field and provides foundational insights into how metabolic regulation integrates with cytokine signalling and transcriptional control to fine-tune the fate of Tfh cells. These findings have implications not only for our understanding of adaptive immunity but also for the rational design of next-generation therapeutics for autoimmune and allergic diseases

7.1.2 Tfh cells have a distinct Immunometabolism compared to Th2 cells

In Chapter 3, following the increase in mTOR activation (Fig.3.6/3.7) observed between naïve and Tfh cells, I also found that Tfh cells have a distinct metabolic phenotype compared to Th2 cells. Th2 cell displayed higher neutral lipid accumulation and the expression of nutrient transporters compared to Tfh cells. This is interesting as comparing unactivated naive T cells and Tfh, you would expect metabolic changes such as mTOR activation , however what is striking is the fact that when you compare two activated T cells (Tfh and Th2) you also see distinct metabolic signatures.

mTOR activation is critical in the regulation of lipid synthesis and storage, as well as increasing nutrient transport into T cells (Waickman and Powell, 2012). Neutral lipid accumulation, primarily in the form of lipid droplets, has been previously identified in CD4⁺ T cells, and DGAT1 is involved in their formation (Zhang et al., 2021). It has been suggested that neutral lipids can influence T cell function through serving as energy reserves, buffering lipid toxicity whilst also influence cell signalling and differentiation (Cai et al., 2021); however, their role in Tfh and Th2 cells has never fully been explored.

One key difference between Tfh and Th2 cells is the lower expression of PPAR- γ by Tfh cells. This could be one of the key drivers in the increased lipid metabolic profiles exhibited by Th2 cells, as it has been shown that PPAR- γ expression was associated with increased fatty acid uptake and lipolysis (Angela et al., 2016). Another plausible reason for the decreased reliance on lipid storage in Tfh cells compared to Th2 cells may be the differences in cell proliferation (as demonstrated in Chapter 3 (Fig. 3.11), where I showed that Tfh cells exhibit lower Ki-67 expression than Th2 cells. When T cells proliferate, they require new membranes, which are derived from lipids, especially cholesterol, which is interspersed within the phospholipid bilayer and makes up a significant proportion (~40%) of membrane lipid. It functions primarily as a bidirectional regulator, key in membrane fluidity (Pohnl et al., 2023, Bietz et al., 2017), so it could be suggested that the increased proliferative potential of Th2 cells is why we see increased neutral lipid storage, allowing the cells to divide rapidly when they reach the site of infection. This requirement of lipids for proliferation was shown to be critical recently, as patients with chronic lymphocytic leukaemia have impaired T cell proliferation, which has been linked to disrupted lipid metabolism, particularly those pathways involved in cholesterol metabolism (Jacobs et al., 2025).

Using a commercially available stain for neutral lipids, I observed that Th2 cells store significantly more lipid in this form than Tfh cells. The reason for this remains unknown; however, findings from my transcriptomic analysis suggest that Th2 cells (Figure 4.10), compared to Tfh cells, have upregulated pathways associated with sterol and lipid metabolism (Figure 7.1). These pathways are fundamental in lipid synthesis, as sterols like cholesterol and lipids like triglycerides are stored as neutral lipid droplets in cells, particularly in a nutrient-poor environment (Howie et al., 2017b). Further work would be required to understand the functional consequences of these pathways; however, their upregulation and subsequent increase in neutral lipid accumulation underscore the need for effective lipid management by Th2 cells.

A possible reason for why Th2 cells store neutral lipid may also be to act as reservoirs for cholesterol, required for synthesis of steroid hormone precursors. Th2 cells have also been shown to produce pregnenolone through CYP11A1, a key rate-limiting enzyme in steroid synthesis pathways; this enzyme catalyses the conversion of cholesterol to pregnenolone (Mahata et al., 2014). Our results showed that CYP11A1 was expressed at the highest level in Th2 cells compared to Tfh and naive T cells in both uninfected and *H. polygyrus* infected mice. This was also accompanied by an increase in the expression of the lipid transporter gene SLC27A6, which is crucial for the uptake of large fatty acids. This suggests that the increased upregulation of sterol and lipid metabolism may be linked to Th2 cells production of pregnenolone. Interestingly, Tfh cells also express CYP11A1; however, it remains unknown if they also produce pregnenolone like Th2 cells and whether this could have any therapeutic implications (Mahata et al., 2014).

Pregnenolone has been shown to have effects on both innate and adaptive immune responses; particularly it can promote degradation of proteins such as TLR 2 and TLR4 in macrophages and microglial cells, which drive suppression of inflammation through preventing TNF- α and IL-6 release driving resolution towards immune homeostasis (Murugan et al., 2019). It has also been reported that pregnenolone can suppress Th cell proliferation (Mahata et al., 2014). This could prove to be an invaluable understanding as through manipulation of Th2 cell metabolism to increase the synthesis of lymphosteroids like pregnenolone, we could drive in part resolution of aberrant Th2 cell responses, for instance in allergy (Libert and Dejager, 2014). Also understanding if Tfh cells synthesise and secrete this steroid precursor may give us a new insight into how Tfh cells control the GC response as this hormone has been shown to alter class switching in B cells (Mahata et al., 2014).

The role of lipid synthesis, which is upregulated during early Th cell activation, is better understood than our understanding of lipid storage in T cells. Lipid synthesis is upregulated to facilitate cellular proliferation, and this is commonly driven by mTORC1, which activates the SREBP family of TFs to promote lipid metabolism through upregulation of nutrient transporters and enzymes responsible for lipid synthesis (Waickman and Powell, 2012). To understand whether increased FAS was possibly facilitating increased neutral lipid accumulation in Th2 cells compared to Tfh cells, I explored the expression of a key enzyme in this pathway, ACC1, which is responsible for the conversion of acetyl-CoA to Malonyl-CoA, a key substrate in FAS. I demonstrated in Chapter 3 (Fig. 3.12) that similar protein levels of this enzyme are present in both Tfh and

Th2 cells using METFLOW, however, a limitation to this work was not being able to measure the flux through this pathway, so this conclusion could not be stated until this is proven.

In Chapter 3 (Figure 3.8), I also observed that Tfh cells had decreased expression of CD71 and CD98 on their surface compared to Th2 cells (Figure 7.1). CD98 is mainly involved in the uptake of large amino acids, particularly Leucine and arginine, which are critical for activating mTOR signalling (Drummond and Rasmussen, 2008), which is required by Th2 cells for differentiation (Yang et al., 2013). CD71 is essential in cellular iron uptake through transferrin and is also critical in Th cell differentiation and effector function (Voss et al., 2023). Thus, while Tfh cells exhibited increased expression of these key transporters compared to naïve T cells, their differential expression suggests a role for distinct metabolic signatures between Tfh and Th2 cells.

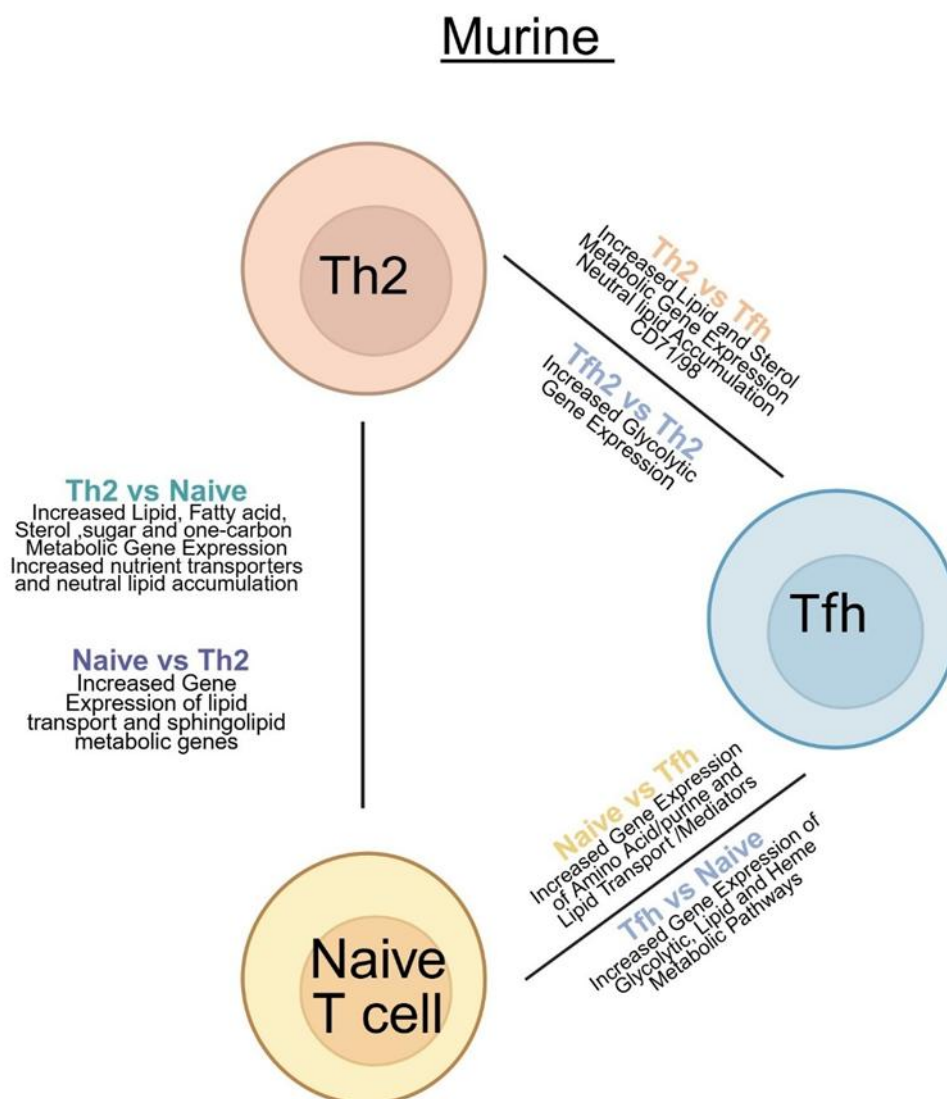


Figure 7.1. Summary of Different Metabolic Pathways employed by Tfh, Th2 and Naïve T cells in the mLN.

This figure illustrates the induction/repression of various metabolic pathways used by Tfh, Th2 in *H. polygyrus* infection, and naïve T cells in uninfected mice in the small intestine draining mLN. The figure was created in BioRender.

Interestingly, in Chapter 4, we observed that Th2 cells exhibited higher MYC expression compared to Tfh cells. Although MYC has many targets, it has been demonstrated that MYC can induce CD71 expression; therefore, this may be the reason why Th2 cells exhibit higher CD71 expression (O'Donnell et al., 2006). Th2 cells have been shown to have larger iron pools than Th1 cells (Thorson et al., 1991). It could be suggested that limiting iron availability may be important in preserving Tfh cell identity, as Bcl-6 represses MYC in conditions of limited iron availability in the GC, thereby preventing excess metabolic activity in Tfh cells (Oestreich et al., 2014). However, in patients with SLE, labile iron accumulation has been shown to drive Tfh differentiation, highlighting the complex interplay and control of iron by Tfh cells (Scindia et al., 2022, Nguyen et al., 2017).

Interestingly, whilst Th cells require iron for function and differentiation (Obeagu, 2025), systemic iron availability in the body can have profound effects on immune polarisation; it has been suggested that iron deficiency can drive Th2 skewing of the immune response and can lead to increased proliferation of Th2 lymphocytes (Roth-Walter, 2022). It should be noted that iron deficiency may actually be hindering the activation of other Th cell subsets, while Th2 cells, which have larger chelated iron pools, are protected (Oestreich et al., 2012). This increased pool of iron may serve as a survival advantage of Th2 cells, highlighting that Th1/Th2 balance may be due to iron availability rather than uptake. Together, these data and my own suggest that Th2 cells favour the build-up of nutrients such as iron and neutral lipids in reservoirs, but it is still unknown why.

A possible mechanism for this could be that Th2 cells, unlike Tfh cells, create nutrient pools to survive during their migration and when they reach the tissue, which Tfh cells are not required to do. Th2 cells essentially create a “packed lunch” that they utilise in conditions where nutrients are limited. This idea links both my work and the work previously defined in our lab by (Heieis, 2019), as when Th2 cells differentiate in the LN, they increase neutral lipid accumulation and have increased nutrient receptor expression, which builds internal stores of nutrients. They also show increased expression of pathways associated with glycolysis and FAS compared to naïve T cells from uninfected mice, essentially making the “packed lunch”. However, Heieis, (2019) showed that when Th2 cells reach the tissue, there is a metabolic rewiring through ST2/PD-1, which changes the metabolism of Th2 cells to favour FAO and OXPHOS (Heieis, 2019). This could, in part, mean that Th2 cells are breaking down their internal neutral lipid stores to fuel ATP generation; in part, using my analogy, they are now eating their “packed lunch”.

7.1.3 Tfh cells have Increased Glycolytic Gene Expression compared to Th1/Th2 cells in the Reactive LN

In chapters 4 and 5, I witnessed increased glycolytic gene expression in Tfh cells compared to Th1 and Th2 cells, which together suggest that Tfh cells are more glycolytic than conventional Th cells during *H. polygyrus* and IAV infections. This might at first appear counterintuitive, given the prevailing literature (Lee, 2023) and one study that suggests that Th1 cells are more glycolytic than Tfh in IAV infection (Ray et al., 2015) however, this increased glycolytic gene expression in Tfh cells over T effector cells was apparent in both inflammatory settings.

Tfh cells are less proliferative and often described as being more metabolically quiescent than other effector T cells (Stark et al., 2019). However, previous research carried out by Dr Graham Heieis in our lab suggested that Tfh cells increase glucose uptake compared with naïve and Th2 cells during *N.brasiliensis* infection in the LN, coupled with my findings in chapter 4 (Fig. 4.4/4.9) and chapter 5 (Fig. 5.4/5/7) suggest that Tfh cell may have increased glycolytic gene expression over both effector and naïve T cells in the mLN irrespective of infection setting. A possible explanation for higher glycolytic gene expression in Tfh cells over effector T cells may stem from the role of MYC in the early differentiation of Tfh cells, which drives high glycolytic gene expression in Tfh cells (Ray et al., 2015) however, further research would have to be undertaken to understand how this relates to MYC expression in other T effector cells (Figure 7.2).

Further to this, Tfh cells, unlike Th1/Th2, reside in nutrient-deprived hypoxic areas of the LN. The stresses induced by this microenvironmental conditioning result in the usage of multiple different metabolic pathways are used to support energetic and biosynthetic demands. Hypoxia has been shown to stabilise HIF-1 α in Tfh cells (Dong et al., 2019), which we demonstrated was significantly upregulated in expression compared in Tfh cells compared to Th2 cells (Chapter 3; Fig. 3.1). HIF-1 α can upregulate glycolytic enzymes and glucose transport, which may be the resulting factor in the increased gene expression of glycolytic genes in Tfh compared to naïve and CD4⁺ Th effector T cell populations (Mayberry et al., 2022, Huang et al., 2022, Zhu et al., 2019b).

Overall, I have shown that Tfh cells have higher glycolytic gene expression compared to Th1 and Th2 cells, whilst Th2 cells favour the increase in expression of nutrient transporter on their cell surface as well as increased neutral lipid accumulation linked to increased

gene expression of cholesterol/sterol metabolic pathways. This highlights 2 key findings, Tfh cells are metabolically active and also that they are metabolically divergent when compared to other T effector cells during infection. This highlights that these major metabolic pathways might be sensible therapeutic targets. Next, we looked for differences between Tfh cell immunometabolism directly, to understand if Tfh cells from different immune contexts show similar or distinctive metabolic signatures.

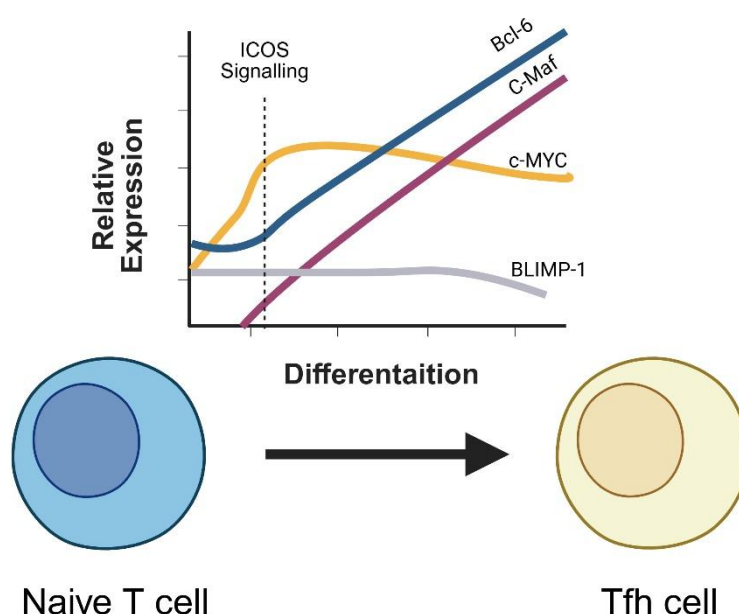


Figure 7.2. TF Induction in Tfh Cells During Differentiation.

This figure illustrates the induction/repression of various TFs crucial for the development of Tfh cells. The figure was created in BioRender.

7.1.4 Tfh2 have an Increased Lipid Gene Expression Signature compared to Tfh1

In Chapter 5, I explored the differences observed between Tfh cells isolated from *H. polygyrus* infection and those from IAV infection. Even though the two pathogens (*H. polygyrus* and IAV) infect two different tissues, intestine and the lung, I am confident that the location alone does not impose metabolic differences, as we compared naïve T cells from each location and observed no significant upregulation of any pathways involved in

metabolism (Figure 5.11). This would suggest that no metabolic programming of naïve T cells occurs in the reactive lymph node of either infection prior to activation.

Interestingly, I found that Tfh cells from *H. polygyrus* had an increased gene signature associated with lipid metabolism compared to Tfh cells from IAV infection. This finding raises many questions and highlights an area of future interest, as a myriad of different factors may drive this difference in metabolic signature, including distinct tissue environments (GC microenvironments), cytokine milieus etc. that could alter the metabolic demands of Tfh cells under these conditions, forcing Tfh cells from type 2 immune responses to rely heavily on lipid metabolism (Olatunde et al., 2021)

A possible reason for the increased lipid signature may be the nature of the infection itself, as Type 2 responses often occur at mucosal sites where lipid availability is higher due to epithelial turnover and dietary intake (Jewell et al., 2021). Also mucosal sites are covered in mucus, which is abundant in lipids (Paone and Cani, 2020), this may mean that lipids are used as a fuel source as they are plentiful in this environment. However, whether these factors affect the LNs draining mucosal sites is unknown. It is also possible that this decreased reliance of Tfh1 cells on lipid metabolism compared to Tfh2 cells could be attributed to increased IL-12/IFN- γ signalling in the type 1 context (Shi et al., 2025), which may drive glycolytic reprogramming in Tfh cells and hence reduce the dominance of lipid metabolism. However, this metabolic reconditioning is not observed to be significantly different between the two Tfh subsets in *H. polygyrus* and IAV infection

Another plausible explanation may be prolonged B-cell interactions. In the type 2 response, to support IgE and IgG1 class switching, Tfh cells are required to carry out prolonged B cell interactions in the GC. These sustained interactions may be better supported by increased lipid synthesis or utilisation, which may be due to limited glucose availability as the GC proceeds. Tfh cells' metabolic plasticity may enable the alternative upregulation of metabolic pathways to meet their energetic demands. In contrast, Tfh cells in Th1 contexts require short-lived interactions aimed at rapid antibody production (IgG2a/c) (Xiong et al., 2012). This may necessitate rapid ATP generation via glycolysis; however, this increased metabolic demand does not coincide with further upregulation of glycolytic gene expression, likely because Tfh cells already exhibit high baseline expression of key glycolytic genes, as demonstrated by their enhanced glycolytic signature compared to Th2 cells (Figure 4.9).

Overall, Tfh cells in both contexts appear to be active in lipid metabolism and glycolysis. However though, when comparing Tfh from Th1 and Th2 contexts, Tfh cells in the Th2 environment show a specific increased dependence for expression of lipid metabolism genes. This highlights to us that Tfh cells in a type 2 context may be more susceptible to therapeutic manipulation of their lipid metabolism compared to those in a Th1 inflammatory context. Given the importance of lipid metabolism in Tfh cells, I decided to test the therapeutic potential of manipulating these pathways directly in *in vitro-derived* human Tfh cells, to understand this altering Tfh cell metabolism can lead to phenotypic changes of these cells

7.1.5 Role of Stearoyl CoA Desaturase-1 in Human *in vitro* Derived Tfh Cells

Importantly, to bridge the gap between basic and translational research in murine and human Tfh cells, I decided to focus on the role of lipid metabolism in the differentiation and function of human *in vitro* differentiated Tfh cells in Chapter 6. I focused on the role of SCD-1, a key component of the fatty acid synthesis pathway responsible for catalysing the conversion of saturated fatty acids into monounsaturated fatty acids. This creates oleic and palmitoleic acid from Stearate and Palmitate, and is crucial in controlling cellular lipid abundance, cell signalling and metabolic states within cells (Sen et al., 2023).

Our data suggested that inhibition of SCD-1 in human Tfh cells *in vitro* decreased Tfh cells' ability to express CXCR5 (Figure 7.3) (and possibly CCR7) on their surface (Figure 6.8). Previous studies have reported that SCD-1 inhibition induces murine Tfh cell ferroptosis via endoplasmic reticulum (ER) stress *in vivo* (Son et al., 2020) However, this research failed to address the impact on human Tfh cells. Interestingly, I did not observe a significant increase in cell death in our *in vitro* assays, in contrast to the murine data from (Son et al., 2020). Our results only showed a decrease in CXCR5 expression and highlight possible differences in the role of SCD-1 between murine and human Tfh cells.

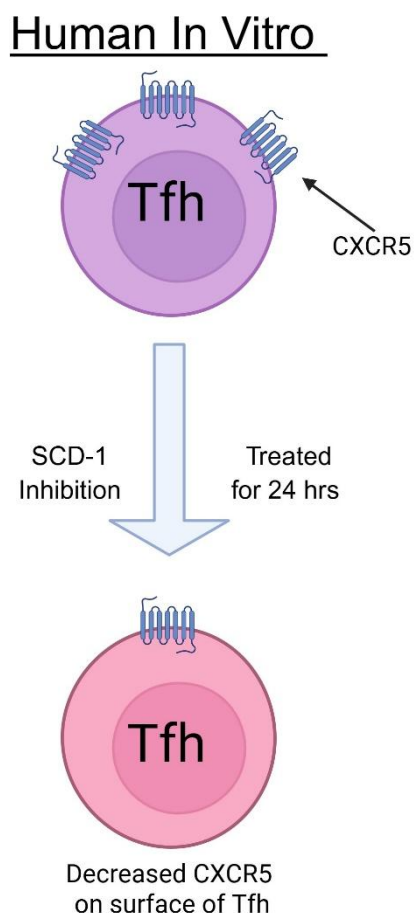


Figure 7.3. Stearoyl CoA Desaturase 1 Inhibition leads to decreased CXCR5 expression on the surface of Human *in vitro*-derived Tfh cells.

This figure illustrates the reduced expression of CXCR5 on human *in vitro*-derived Tfh cells when treated with SCD-1 inhibitors (CAY10566 and A939572) for 24 hours, as shown in Chapter 6. The figure was created in BioRender.

Notably, our data suggest that inhibition of SCD-1 may serve as a potential therapeutic strategy by preventing Tfh cells from expressing CXCR5 on their surface (Figure 6.8). Previously, it has been shown that altering lipid metabolism can affect CXCR5 expression in murine Tfh cells, as shown by (Fu et al., 2021), providing compelling evidence that targeting lipid metabolism in Tfh cells could warrant further investigation to understand if there is a therapeutic rationale to develop treatments against these metabolic pathways in Tfh cell. Through decreasing CXCR5, which is a key chemokine receptor mediating Tfh cell migration into B cell follicles, its downregulation could impair Tfh cell positioning and, consequently, the GC reaction preventing autoantibody generation in diseases such as systemic lupus erythematosus (Crotty, 2019).

7.1.6 Future Directions of Research

Overall, this thesis aimed to answer 3 unknown questions about Tfh cells' metabolism in both mice and in humans. I set out to define the immunometabolism of murine Tfh cells compared to effector T cells during *H. polygyrus* and IAV infection, whereby I highlighted several key findings such as Tfh cells having higher glycolytic gene expression compared to Th1 and Th2 cells, and also that Th2 cells favour increased nutrient transporter expression and neutral lipid accumulation compared to Tfh cells during *H. polygyrus* infection. Further to this, I also explored whether the immunometabolism of Tfh cells is similar/distinct depending on the inflammatory context (type 1 or type 2 inflammation). I found Tfh cells from type 2 inflammation have higher dependency on lipid metabolism gene expression than Tfh cells from type 1 context. I then explored whether manipulation of lipid metabolism in human Tfh *in vitro* had therapeutic potential and found that inhibition of SCD-1 resulted in decreased CXCR5 expression on the surface of human *in vitro-derived* Tfh cells. Overall, I feel like my thesis adequately addressed the aims presented; however, in doing so, it has also opened new avenues of unanswered questions and possible next steps.

One key question is understanding the primary driving force behind high glycolytic gene expression in Tfh cells over other CD4⁺ T effector cells in the LN, which may lead to potential ways to alter their differentiation and function in specific contexts. A possible approach to exploring this would be to understand the role of MYC in the early differentiation of Tfh cells (Ray et al., 2015), to determine if this drives the high glycolytic gene expression in Tfh, and if modulation of MYC could decrease the magnitude of glycolysis employed (Figure 7.2).

Further to this, understanding the chromatin accessibility of glycolysis genes during the induction of MYC and subsequently Bcl-6 in Tfh cells would reveal whether high glycolytic gene expression is driven directly by MYC during early differentiation and whether it is maintained after the induction of Bcl-6, a glycolytic repressor (Oestreich et al., 2014). This approach would enable me to determine whether a high glycolytic signature is a prerequisite for Tfh cells during differentiation and whether modulating early differentiation can have a significant impact on the induction of Tfh cells. Research suggests that Bcl-6 should decrease glycolytic gene expression (Oestreich et al., 2014); however, coupled with environmental factors such as hypoxia, which can induce HIF-1 α , (Dong et al., 2019) and enhance glycolysis, understanding how all of these pathways

intertwine to create this expression profile would give invaluable insight into the dynamics of Tfh cell metabolism.

Likewise, understanding the differential lipid gene expression observed between Tfh cells in type 1 and type 2 contexts should be explored further. For example, blocking upregulated lipid genes through CRISPR-CAS 9 technology (used by (Huang et al., 2022)) or therapeutic modulation with small-molecule inhibitors (employed by (Son et al., 2020)), may yield interesting results as these experiments would determine which pathways are essential for each aspect of Tfh biology, and could identify specific pathways or control points that would allow therapeutic intervention. This may allow for the tailored depletion of Tfh cells in the type 2 context, while not affecting Tfh cells arising in type 1-mediated inflammation. Further work on this topic, such as proteomics of Tfh1 vs Tfh2, or after specific gene knock-out through CRISPR-CAS9, would be necessary to understand whether the gene expression changes are translated to the protein level and result in differential activation, to understand the role that each of these genes plays in Tfh cell biology.

Specifically, it would be interesting to build on the findings I made regarding the role of SCD-1 in human *in vitro*-derived Tfh cells by exploring further how inhibition of SCD-1 decreases the expression of CXCR5 (possibly CCR7) on the surface of these cells. In particular, by determining whether SCD-1 promotes chemokine receptor expression or whether it is the inhibition that prevents the trafficking of CXCR5 to the surface of Tfh cells would allow for a deeper understanding of how lipid metabolism can affect chemokine receptor biology.

Understanding if ER stress, as described by Son et al. (2020) in murine Tfh cells resulting from SCD-1 inhibition, is conserved across species in human *in vitro*-derived Tfh cells, and whether this is the driver behind the decreased CXCR5 on the surface, warrants further investigation and if this is the case, does decreasing CXCR5 affect the migratory capacity of Tfh cells? To address this, we should utilise the transwell migration assay to investigate how inhibition of SCD-1 affects the migratory capacity of Tfh cells in response to CXCR5's ligand, CXCL13. This approach could thereby determine whether SCD1 inhibition could be potentially therapeutically utilised to prevent Tfh and B cell cross-talk and migration, critical for the induction of the GC response (Crotty, 2019).

Whilst these are all critical questions referring to advancements of the work proposed in this thesis, these findings should act as a catalyst for understanding Tfh cell metabolism across different inflammatory contexts. The next steps should be to compare Tfh cell metabolism during autoimmunity, chronic vs acute infection, and new vs established disease to truly understand the plasticity in Tfh cell immunometabolism to drive novel therapeutic discovery. Further to this, understand how the metabolism of Tfh cell in mice relates to humans is also fundamental as failing to appreciate this may lead to hindered translation of therapeutic avenues into drug discovery. For example, our finding of inhibition of SCD-1 in human Tfh cells ability to decrease CXCR5 on the surface of Tfh cells, whilst novel and exciting must lead to a functional decrease in migratory capacity to warrant further investigation, as if the Tfh cells can still migrate towards CXCL13 in a similar fashion, this would not affect the ability of Tfh cells to drive GC formation *in vivo*, emphasising that a decrease in receptor expression may not translate to an alteration in its function.

7.1.7 Summary of Key Findings

Overall, this thesis highlights several key findings that will advance our understanding of the immunometabolism of Tfh cells in both type 1 and type 2 immune responses. I have identified potential metabolic differences among naïve, effector (Th1/Th2), and Tfh cells. Additionally, I found that Tfh cells exhibit decreased neutral lipid accumulation and protein expression of amino acid and iron transporters compared to Th2 cells. My findings also highlight differences in metabolic gene expression between Tfh and effector T cells, such as increased glycolytic gene expression in Tfh cells compared to Th2 cells. Furthermore, I also demonstrated that Tfh cells derived from type 2 immune responses exhibit an increased lipid signature compared to those derived from type 1 immune responses.

I was also able to translate findings from our murine *in vivo* results into the human *in vitro*-derived Tfh cells assay, yielding promising therapeutic results. Specifically, I found that SCD-1 inhibition decreases the expression of CXCR5 on the surface of human Tfh cells. Overall, while being a predominantly descriptive investigation into Tfh cell biology, the data presented will advance our understanding of immunometabolism of Tfh cells and provide a solid foundation for the continued investigation of metabolism as a therapeutic strategy for manipulating Tfh cells and consequent GC formation, antibody production. Through this, my hope is that maybe this research will yield new strategies for treating

diseases like autoimmunity and allergy more specifically than conventional therapies, which fail to do so, limiting the side effects experienced by the individual whilst also driving resolution of the aberrant immune responses to favour the patient's needs.

Chapter 8: Appendix

23.1.1 *H. polygyrus* transcriptomics (RStudio code)

All code presented below is representative of the code used to analyse and create the figures of the data presented in Chapter 4

The following packages (Library) were installed in order to analyse the data presented;

- `library("ggplot2")`
- `library("RColorBrewer")`
- `library(gplots)`

The following code was used to load tables into RStudio ;

- `em = read.table("D:\\PhD RNA seq. Tfh cells H.polyflu new\\SL2_2\\SL2_2\\all_genes\\de_workflows\\CD44_I_LOW_vs_CD44_N_LOW\\data\\de_annotated.csv", sep = "\t", row.names=2, header = TRUE)`
- `ss = read.table("D:\\PhD RNA seq. Tfh cells H.polyflu new\\processed\\processed\\ss_2.csv", sep = "\t", row.names=1, header = TRUE)`

To make a single gene boxplot, the following representative code was used;

- `# wrangle data`
- `gene_data = data.frame(t(em[["PHD2"],11:29]))`
- `gene_data$group = ss$SAMPLE_GROUP`
- `names(gene_data) = c("Expression", "Group")`
- `# make plot`
- `ggp = ggplot(gene_data, aes(x = Group, y= Expression, fill = Group)) +
geom_boxplot()+scale_x_discrete(limits =
c("CD44_N_LOW","CD44_I_LOW","TFH_I","TH2_I"))`
- `print(ggplot(gene_data, aes(x = Group, y= Expression, fill = Group)) +
geom_boxplot()+scale_x_discrete(limits =
c("CD44_N_LOW","CD44_I_LOW","TFH_I","TH2_I")))`
-

To make a heatmap from multiple selected genes, the following representative code was used;

- `em_symbols_glycolysis
=data.frame(em[c("HK1","HK2","HK3","LDHA","LDHAL6A","LDHAL6B","LDHB","LDHC","PDHA1","PDHA2","PDHB","PFKL","PFKM","PFKP","PGAM1","PGAM2","PGAM4","PGK1","PGK2","PGM1","PGM3","PKLR","TPI1"
,"ALDH7A1","ALDH9A1","ALDOA","ALDOB","ALDOC","BPGM","DLAT","DLD","ENO1","ENO2","ENO3","FBP1","FBP2","G6PC","G6PC2","GALM","GAPDH","GAPDHS","GCK","GPI","ACSS1","ACSS2","ACYP1","ACYP2","ADH1A","ADH1B","ADH1C","ADH4","ADH5","ADH6`

- ```
"ADH7","ADHFE1","AKR1A1","ALDH1A3","ALDH1B1","ALDH2","ALDH3A1","ALDH3A2",
,"ALDH3B1","ALDH3B2"),30:33])
```
- `em_symbols_glycolysis = na.omit(em_symbols_glycolysis)`
  - `row.names(em_symbols_glycolysis)`
  - `em_symbols_glycolysis_matrix <- as.matrix(em_symbols_glycolysis)`
  - `heatmap(em_symbols_glycolysis_matrix)`
  - `print(heatmap)`

To make a complex heatmap from multiple selected genes loaded from a table in Excel, the following representative code was used;

- `Lipid = read.table("D:\\PhD RNA seq. Tfh cells H.polyflu new\\Gene List for Analysis\\Lipid Genes Table.csv", sep = "\t", header = TRUE)`
- `em_symbols_Lipid = data.frame(em[(Lipid$Name),30:33])`
- `em_symbols_Lipid = na.omit(em_symbols_Lipid)`
- `colnames(em_symbols_Lipid) = c("CD44 Low_N", "CD44 Low_I", "Tfh_I", "Th1_I")`
- `row.names(em_symbols_Lipid)`
- `em_symbols_Lipid_matrix <- as.matrix(em_symbols_Lipid)`
- `heatmap.2(em_symbols_Lipid_matrix)`
- `heatmap.2(em_symbols_Lipid_matrix, main = "Lipid Gene Expression", margins = c(5,6), xlab = "Cell type", ylab = "Genes", key=TRUE, key.xlab = "Expression", cexCol = 1.4, line=4, scale = "row", offsetCol = 1, trace="none", adjCol = c(0.5,0.2), srtCol = 0, density.info = "none", Colv = "row", col=brewer.pal(5, "Blues"))`

To make a bar chart of multiple selected genes, the following representative code was used;

- `candidate_genes = c("ADD GENES OF INTEREST")`
- `# create the table`
- `gene_data = em[candidate_genes,11:29]`
- `gene_data = data.frame(t(gene_data))`
- `#z-score`
- `library(dplyr)`
- `gene_data_Z = gene_data %>%`
- `mutate_at(c(1:7), funs(c(scale(.))))` – Change to accomodate how many genes
- `gene_data_Z$groups = factor(ss$SAMPLE_GROUP,`  
`levels=c("CD44_N_LOW", "CD44_I_LOW", "TFH_I", "TH2_I"))`
- `# melt`
- `gene_data.m = melt(gene_data_Z, id.vars = "groups")`
- `ggp = ggplot(gene_data.m, aes(x=variable, y=value, fill = groups)) +`
- `geom_boxplot()+`
- `geom_jitter(position=position_dodge(0.8))+`
- `ggtitle("Amino Acid Metabolism") +`
- `xlab("Gene") + ylab("z-score")+`
- `theme(axis.text.x = element_text(angle = 45, hjust = 1)) +`
- `scale_fill_manual(values = c("#88CCEE", "#CC6677", "#DDCC77", "#117733"))`
- `print(ggp)`



To make a Heatmap of the Log Fold Change of multiple selected genes, the following representative code was used;

- `em = read.table("D:\\PhD RNA seq. Tfh cells H.polyflu new\\SL2_2\\SL2_2\\all_genes\\de_workflows\\CD44_I_LOW_vs_CD44_N_LOW\\data\\de_annotated.csv", sep = "\t", row.names=2, header = TRUE)`
- `ss = read.table("D:\\PhD RNA seq. Tfh cells H.polyflu new\\processed\\processed\\ss_2.csv", sep = "\t", row.names=1, header = TRUE)`
- `UPLIPID = read.table("D:\\PhD RNA seq. Tfh cells H.polyflu new\\Gene List for Analysis\\HP CD44 LOW TFH LIPID DEGRADATION Upregulated.csv", sep = "\t", header = TRUE)`
- `em_symbols_UPLIPID = data.frame(em[(UPLIPID$Name),c(11:14,20:24)])`
- `colnames(em_symbols_UPLIPID) = c("CD44 Low1_N", "CD44 Low2_N", "CD44 Low3_N", "CD44 Low4_N", "Tfh1_I", "Tfh2_I", "Tfh3_I", "Tfh4_I", "Tfh5_I")`
- `em_symbols_UPLIPID1 <- log(em_symbols_UPLIPID)`
  
- `em_symbols_UPLIPID1$CD44LOWFC1 <- em_symbols_UPLIPID$`CD44 Low1_N` - em_symbols_UPLIPID$`CD44 Low1_N``
- `em_symbols_UPLIPID1$TfhFC1 <- em_symbols_UPLIPID$Tfh1_I/em_symbols_UPLIPID$`CD44 Low1_N``
- `em_symbols_UPLIPID1$TfhFC2 <- em_symbols_UPLIPID$Tfh2_I/em_symbols_UPLIPID$`CD44 Low2_N``
- `em_symbols_UPLIPID1$TfhFC3 <- em_symbols_UPLIPID$Tfh3_I/em_symbols_UPLIPID$`CD44 Low3_N``
- `em_symbols_UPLIPID1$TfhFC4 <- em_symbols_UPLIPID$Tfh4_I/em_symbols_UPLIPID$`CD44 Low4_N``
- `em_symbols_UPLIPID1$TfhFC5 <- em_symbols_UPLIPID$Tfh5_I/em_symbols_UPLIPID$`CD44 Low4_N``
  
- `row.names(em_symbols_UPLIPID1)`
- `Matrix_UPLIPIDFC = as.matrix(as.data.frame(em_symbols_UPLIPID1[c("CD44LOWFC1", "TfhFC1", "TfhFC2", "TfhFC3", "TfhFC4", "TfhFC5")]))`
- `colnames(Matrix_UPLIPIDFC) = c("CD44 Low", "Tfh1", "Tfh2", "Tfh3", "Tfh4", "Tfh5")`
- `heatmap.2(Matrix_UPLIPIDFC,`
- `main = "Lipid Degradation Fold Change",`
- `margins = c(5,7), xlab = "Cell type",`
- `ylab = "Genes",`
- `key=TRUE,`
- `key.xlab = "Expression",`
- `cexCol = 1.3 ,`
- `cexRow = 0.8,`
- `line=3 ,`
- `offsetCol = 1,`
- `trace="none",`
- `adjCol = c(0.5,0.2), srtCol = 0,`
- `density.info = "none",`
- `Colv= "row",`
- `col=brewer.pal(5,"Blues"))`

### 23.1.1 IAV transcriptomics (RStudio code)

To analyse and make figures for the IAV dataset, similar code was used as above however it was manipulated to analyse the IAV samples rather than the *H.polygyrus* dataset

All code presented below is representative of the code used to analyse and create the figures of the data presented in chapters 5

The following packages (Library) were installed in order to analyse the data presented;

- `library("ggplot2")`
- `library("RColorBrewer")`
- `library(gplots)`

The following code was used to load tables into RStudio ;

- `em = read.table("D:\\PhD RNA seq. Tfh cells H.polyflu new\\SL2_1\\SL2_1\\all_genes\\de_workflows\\CD44_I_LOW_vs_CD44_N_LOW\\data\\de_annotated.csv", sep = "\\t", row.names=2, header = TRUE)`
- `ss = read.table("D:\\PhD RNA seq. Tfh cells H.polyflu new\\processed\\processed\\ss_1.csv", sep = "\\t", row.names=1, header = TRUE)`

To make a single gene boxplot, the following representative code was used ;

- `gene_data = data.frame(t(em["ACAT",11:25]))`
- `gene_data$group = ss$SAMPLE_GROUP`
- `names(gene_data) = c("Expression", "Group")`
- `# make plot`
- `ggp = ggplot(gene_data, aes(x = Group, y= Expression, fill = Group)) + geom_boxplot()`
- `print(ggplot(gene_data, aes(x = Group, y= Expression, fill = Group)) + geom_boxplot())`

To make a complex heatmap from multiple selected genes loaded from a table in Excel, the following representative code was used;

- `candidate_genes = c("PDCD1","CXCR5","BCL6", "MAF")` – Add genes of interest
- `# create the table`
- `gene_data = em[candidate_genes,11:25]`
- `gene_data = data.frame(t(gene_data))`
- `#z-score`
- `library(dplyr)`
- `gene_data_Z = gene_data %>%`
- `mutate_at(c(1:4), funs(c(scale(.))))`
- `gene_data_Z$groups = factor(ss$SAMPLE_GROUP,`  
`levels=c("CD44_N_LOW","CD44_I_LOW","TFH_I", "TH1_I"))`
- `# melt`
- `gene_data.m = melt(gene_data_Z,id.vars = "groups")`
- `ggp = ggplot(gene_data.m, aes(x=variable, y=value, fill = groups)) +`
- `geom_boxplot()+`
- `geom_jitter(position=position_dodge(0.8))+`
- `ggtitle("Tfh Cell Key Differentiation Genes ") +`
- `xlab("Gene") + ylab("z-score")+`
- `theme(axis.text.x = element_text(angle = 45, hjust = 1))+`
- `scale_fill_manual(values = c("#88CCEE", "#CC6677", "#DDCC77", "#117733"))`
- `print(ggp)`

To make a Heatmap of Log Fold Change of multiple selected genes, the following representative code was used

Note; (SL2\_1 – is IAV samples even though it says *H.polygyrus*, which were SL2\_2)

- `# load tables`
- `em = read.table("D:\\PhD RNA seq. Tfh cells H.polyflu new\\SL2_1\\SL2_1\\all_genes\\de_workflows\\CD44_I_LOW_vs_CD44_N_LOW\\data\\de_annotated.csv", sep = "\t", row.names=2, header = TRUE)`
- `ss = read.table("D:\\PhD RNA seq. Tfh cells H.polyflu new\\processed\\processed\\ss_1.csv", sep = "\t", row.names=1, header = TRUE)`
- `#UPLIPID = read.table( "D:\\PhD RNA seq. Tfh cells H.polyflu new\\Gene List for Analysis\\Flu Fatty acid metabolism upregulated naive vs tfh.csv", sep = "\t", header = TRUE)`
- `#UPLIPID = read.table( "D:\\PhD RNA seq. Tfh cells H.polyflu new\\Gene List for Analysis\\Flu Lipid Transport upregulated naive vs tfh.csv", sep = "\t", header = TRUE)`
- `UPLIPID = read.table( "D:\\PhD RNA seq. Tfh cells H.polyflu new\\Gene List for Analysis\\Flu Lipid Transport upregulated naive vs tfh.csv", sep = "\t", header = TRUE)`
- `em_symbols_UPLIPID =data.frame(em[(UPLIPID$Name),c(11:14,19:22)])`

- `colnames(em_symbols_UPLIPID)= c("CD44 Low1_N","CD44 Low2_N","CD44 Low3_N","CD44 Low4_N","Tfh1_I","Tfh2_I","Tfh3_I","Tfh4_I")`
- `em_symbols_UPLIPID1<- log(em_symbols_UPLIPID)`
- `em_symbols_UPLIPID1$CD44LOWFC1 <- em_symbols_UPLIPID$`CD44 Low1_N` - em_symbols_UPLIPID$`CD44 Low1_N``
- `em_symbols_UPLIPID1$TfhFC1 <- em_symbols_UPLIPID$Tfh1_I/em_symbols_UPLIPID$`CD44 Low1_N``
- `em_symbols_UPLIPID1$TfhFC2 <- em_symbols_UPLIPID$Tfh2_I/em_symbols_UPLIPID$`CD44 Low2_N``
- `em_symbols_UPLIPID1$TfhFC3 <- em_symbols_UPLIPID$Tfh3_I/em_symbols_UPLIPID$`CD44 Low3_N``
- `em_symbols_UPLIPID1$TfhFC4 <- em_symbols_UPLIPID$Tfh4_I/em_symbols_UPLIPID$`CD44 Low4_N``
- `row.names(em_symbols_UPLIPID1)`
- `Matrix_UPLIPIDFC = as.matrix(as.data.frame(em_symbols_UPLIPID1[c("CD44LOWFC1","TfhFC1","TfhFC2","TfhFC3","TfhFC4")]))`
- `colnames(Matrix_UPLIPIDFC) = c("CD44 Low","Tfh1","Tfh2","Tfh3","Tfh4")`
- `heatmap.2(Matrix_UPLIPIDFC,`
- `#main = "Fatty Acid Metabolism Fold Change",`
- `#main = "Lipid Transport Fold Change",`
- `main = "Lipid Metabolism Fold Change",`
- `margins = c(5,7), xlab = "Cell type",`
- `ylab = "Genes",`
- `key=TRUE,`
- `key.xlab = "Expression",`
- `cexCol = 1.3 ,`
- `cexRow = 0.8,`
- `line=3 ,`
- `offsetCol = 1,`
- `trace="none",`
- `adjCol = c(0.5,0.2),srtCol = 0,`
- `density.info = "none",`
- `Colv= "row",`
- `col=brewer.pal(5,"Blues"))`

- References), xlab = "Cell type",
- ylab = "Genes",
- key=TRUE,
- key.xlab = "Expression",
- cexCol = 1.3 ,
- cexRow = 0.8,
- line=3 ,
- offsetCol = 1,
- trace="none",
- adjCol = c(0.5,0.2), srtCol = 0,
- density.info = "none",
- Colv = "row",
- col=brewer.pal(5,"Blues"))

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