

THE ROLES OF NR4A1 IN T CELL DEVELOPMENT AND ACTIVATION

BY

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Abstract

Nr4a receptors are activated upon T cell receptor (TCR) signalling and are thought to play redundant roles in T cell development and activation. Of the three Nr4a members, Nr4a1 expression is highly sensitive to TCR signals and is expressed during positive and negative selection in the thymus. The Nr4a1 receptor is also emerging as a therapeutic target within autoimmunity and cancer due to its negative regulatory functions. Understanding the function of Nr4a1, therefore, is of both fundamental and therapeutic interest. We utilised *Nr4a3*-Tocky mice as a tool for following NFAT-driven TCR signalling. *Nr4a3*-Tocky mice were crossed onto an *Nr4a1*-/- or *Nr4a1*+/- background. We compared T cell expression of Nr4a3-Timer and CD25 between *Nr4a3*-Tocky *Nr4a1*+/- to the *Nr4a3*-Tocky *Nr4a1*-/- mice. To determine the functional role of Nr4a1, ELISA's were used to measure interleukin-2 (IL-2) and interferon-γ (IFN-γ) produced by naïve CD4+ T cells activated in vitro and RNA-seq performed to analyse transcriptomic changes. We saw Increased evidence of TCR signalling in *ex vivo* analysis of *Nr4a1*-/- thymic T cells at all stages of development when compared to *Nr4a1*+/-. In contrast, no significant difference was observed within peripheral T cells from the spleen. We observed a significant increase in CD4SP CD25+ Treg cells within the *Nr4a1*-/- mouse, and Treg were enriched for apoptotic cells. Functional analysis revealed a redundant role for Nr4a1 in the control of IL-2 expression in activated CD4+ T cells. Our findings indicate that the absence of Nr4a1 results in more self-antigen driven signalling within the thymus and an expansion of autoreactive CD25+ Treg and Treg precursors. Nr4a1 appeared functionally redundant during peripheral T cell activation. We conclude that Nr4a1 may induce short-lived and T cell developmental stage-dependent effects that warrants further investigation.

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List of Contents:

Abstract……...2

Acknowledgements……..3

List of Contents……4

List of Figures……7

List of Abbreviations……9

1. Introduction……12

1.1 The Immune Response……12

1.1.1 Adaptive Immunity……..14

1.1.2 T Cells……...15

1.2 Thymic T Cell Selection……18

1.2.1 Development of Regulatory T Cells……20

1.3 The Nr4a Receptor Family……22

1.3.1 Nr4a Receptors and T Cell Regulation……23

1.3.2 Redundancy in the Nr4a Receptor Family……..25

1.3.3 *Nr4a3*-Timer of Cell Kinetics and Activity (Tocky) Reporter Mice……...29

1.4 Introduction Summary……30

2. Aims……...31

3. Materials and Methods……..32

3.1 Experimental Design……..32

3.2 Mice……..33

3.3 Tissue Preparation……34

3.4 Naïve CD4+ T Cell Purification……35

3.5 *In Vitro* Cultures……..……..35

3.6 Surface Staining……..……..36

3.7 Annexin V and Intracellular Staining……..…..37

3.8 Flow Cytometry……..…..38

3.8.1 Gating Strategies……38

3.9 ELISA……...41

3.10 RNA Isolation, Sequencing and Analysis…….……...…42

3.11 List of Reagents……45

3.12 Statistical Analysis and Graphical Presentation……..46

4. Results……..47

4.1 Loss of Nr4a1 Enhances TCR Signalling in Developing Thymic T Cells……..47

4.2 Splenic T Cells Display No Phenotypic Changes Due to Loss of Nr4a1……..50

4.3 Functional Redundancy of Nr4a1 Revealed by RNA-Seq……..52

4.4 Nr4a1-Deficient Naïve T Cells Show Unaltered Expression of Strong TCR Markers Following TCR Activation……56

4.5 CD3+CD28 Activation of Naïve CD4+ T Cells Show Higher Concentration of IL-2 When Compared to CD3-Only Activation, But Loss of Nr4a1 Causes No Effect on Cytokine Production……58

4.6 Absence of Nr4a1 Enhances Proliferation of T Cells……..……61

4.7 Annexin V Staining Reveals Higher Apoptotic Rate in T Cells Due to Loss of Nr4a1……..68

5. Discussion……75

5.1 Loss of Nr4a1 Causes a Temporary Expansion of Self-Reactive T Cells During Negative Selection in the Thymus……..75

5.2 Nr4a1 Exhibits Redundancy in Peripheral T Cells……77

5.3 Absence of Nr4a1 Escalates the Apoptosis of T Cells……79

5.4 Potential Effects of Global Nr4a1-Deletion on T Cell Selection and Function…..80

5.5 Discussion Summary……81

5.5 Future Directions……81

6. List of References……..83

List of Figures:

Figure 1: Signals Required for T Cell Activation (Showing CD4+ T Cell)……...16

Figure 2: Overview of T Cell Development……..18

Figure 3: Overview of the Nr4a1 Receptor in T Cell Regulation……..27

Figure 4: Schematic Showing Nr4a3 Blue and Nr4a3 Red Expression Categorisation on a Flow Cytometry Plot……29

Figure 5: Flow Chart of Experimental Design……31

Figure 6: Punnet Square Showing Predicted Genotypes from Cross Breeding of Mice……33

Figure 7: Table Showing Staining Panels for Experiments Respectively……...36

Figure 8: *Ex Vivo* Analysis Gating Strategy: Surface Staining……39

Figure 9: *In Vitro* Analysis Gating Strategy: Surface Staining……..40

Figure 10: *Ex Vivo* Analysis Gating Strategy: Annexin V and Intracellular Staining……...41

Figure 11: Table of Reagents or Resources Essential for This Project……..45

Figure 12: *Ex Vivo* Analysis of Double Positive, CD8+ and CD4+ T Cells from Whole Thymus……...48

Figure 13: *Ex Vivo* Analysis of Tregs from Whole Thymus……...49

Figure 14: *Ex Vivo* Analysis of CD8+ and CD4+ T Cells from Whole Spleen…….50

Figure 15: *Ex Vivo* Analysis of Tregs from Whole Spleen……51

Figure 16: Principal Component Analysis (PCA) Plot Showing Variation in Gene Expression……53

Figure 17: Heat Map Showing the Expression of Nr4a1 and Classical T Cell Activation Markers……55

Figure 18: *In Vitro* Analysis of Isolated Naïve CD4+ T Cells from Spleen Following 24-Hours of Activation……..57

Figure 19: Concentrations of Cytokines Following CD3-Only or CD3+CD28 Activation……60

Figure 20: Proliferation Analysis of Double Positive T Cells and CD8+ T Cells from Whole Thymus……..62

Figure 21: Analysis of CD25 and FoxP3 Expression in Thymic T Cells……..…...63

Figure 22: Proliferation Analysis of Tregs from Whole Thymus……..64

Figure 23: Analysis of CD25 and FoxP3 Expression in Splenic T Cells……..…65

Figure 24: Proliferation Analysis of CD8+ T Cells and Tregs from Whole Spleen…67

Figure 25: Apoptosis Analysis of Double Positive T Cells and CD8+ T Cells from Whole Thymus…….…….69

Figure 26: Apoptosis Analysis of Tregs from Whole Thymus…….……..71

Figure 27: Apoptosis Analysis of CD8+ T Cells and Tregs from Whole Spleen……73

List of Abbreviations:

α..……..Alpha

ANOVA……...Analysis of Variance

AP-1……Activator Protein 1

APC……Antigen Presenting Cell

ASK1…. ……..Apoptosis Signal-Regulating Kinase 1

BAC…… Bacterial Artificial Chromosome

Bcl-2…….B Cell Lymphoma-2

β……Beta

CAR…….Chimeric Antigen Receptor

CD……Cluster of Differentiation

CO_2……...Carbon Dioxide

CoREST……...Co-Repressor Element Silencing Transcription Factor

CTLA-4……...Cytotoxic T Lymphocyte Associated Protein-4

DAMP……Danger-Associated Molecular Pattern

DKO……..Double Knockout

DN……..Double Negative

DNA……..Deoxyribonucleic Acid

DP…….....Double Positive

ELISA……..Enzyme-Linked Immunosorbent Assay

FACS……Fluorescence-Activated Cell Sorting

FBS…….Foetal Bovine Serum

FoxP3...……..……Forkhead Box P3

FT……Fluorescent Timer

g……G Force

G…….Growth/Gap Phase

GITR……..Glucocorticoid-Induced TNFR-Related Protein

ICOS……....Inducible Costimulator

Ig……..Immunoglobulin

IL……Interleukin

LPS……...Lipopolysaccharide

MHC…….Major Histocompatibility Complex

mL……...Millilitre

MPEC……Memory Precursor Effector Cells

NFAT…….Nuclear Factor of Activated T Cells

ng…….….Nanogram

NK…….……Natural Killer Cell

nM…….Nanomolar

Nr4a……Nuclear Receptor Subfamily 4 Group A

OT-I……..…...MHC Class I-Restricted Ovalbumin-Specific CD8+ T Cells

OT-II…….MHC Class II-Restricted Ovalbumin-Specific CD4+ T Cells

PAMP……Pathogen Associated Molecular Pattern

PBS……..Phosphate Buffered Saline

PD-1……....Programmed Cell Death Protein 1

PRR…….Pattern Recognition Receptor

PS……. Phosphatidylserine

RBC……..Red Blood Cell

RNA……..Ribonucleic Acid

RPM……Revolutions Per Minute

RPMI……Roswell Park Memorial Institute

Runx3……..Runt-Related Transcription Factor 3

SEM……....Standard Error of the Mean

siRNA……..Small Interfering Ribonucleic Acid

SKO……....Single Knockout

SLEC……..……Short-Lived Effector Cells

SP……..Single Positive

STAT……..Single Transducer and Activator of Transcription

TCR……..T Cell Receptor

T_{FH}……..Follicular Helper T Cell

TGF-β……...Transforming Growth Factor Beta

Th……..T Helper Cell

TIM-3…..……..T Cell Immunoglobulin and Mucin Domain Containing-3

TKO……..Triple Knockout

Tocky……...Timer of Cell Kinetics and Activity

Treg……..Regulatory T Cell

wt……Absence of Fluorescent Timer

%.....Percentage

°C……Degrees Celsius

µL…….Microlitre

µM……Micromolar

+/-……..……Heterozygous

-/-……..…....Homozygous Knockout

1. Introduction

1.1 The Immune Response

The immune response is an intrinsic defence mechanism engineered by the immune system, which involves an intricate assortment of immune cells, barriers, and molecules that work together to protect the host. There are two branches of the immune system, innate and adaptive, that perform their functions synergistically to defend the body from potentially harmful diseases and substances it's exposed to daily (Marshall, Warrington, Watson and Kim, 2018). The innate immune system acts as the first line of defence for the body and includes physical barriers as well as tissue-resident macrophages, dendritic cells, neutrophils, natural killer (NK) cells and more (Amarante-Mendes et al., 2018). The physical barriers of the innate immune system are predominantly composed of endothelial and epithelial cells (Riera Romo, Pérez-Martínez and Castillo Ferrer, 2016). Mucosal surfaces such as oral epithelia, intestinal epithelia, and airway epithelia, all secrete mucus alongside antimicrobial substances to create an essential protective barrier to invading pathogens (Constant, Nice and Rauch, 2021). In the occasion that a pathogen does breach these physical barriers, innate immune cells are able to identify its presence through a class of receptors called pattern recognition receptors (PRRs) (Amarante-Mendes et al., 2018). Mainly expressed on macrophages, epithelia, neutrophils and dendritic cells of the innate immune system, PRRs recognise a diverse range of pathogen-associated molecular patterns (PAMPs), a typical example being lipopolysaccharide (LPS) which is present in the outer surface membrane of Gram-negative bacteria (Amarante-Mendes et al., 2018; Bertani and Ruiz, 2018).

Additionally, PRR's can recognise not only pathogenic molecules, but also endogenous molecules associated with cellular stress and injury called danger-associated molecular patterns (DAMPs). These DAMPs are often intracellular molecules that are released into the extracellular environment during cellular death, including heat shock proteins and DNA (Murao et al., 2021). On recognition of PAMPs or DAMPs, PRR's initiate downstream signalling pathways that can result in the formation of an inflammatory microenvironment or the elimination of dead or transformed cells (Cronkite and Strutt, 2018). For the generation of an inflammatory microenvironment, innate immune cells produce a collection of inflammatory cytokines and chemokines that rapidly recruit additional immune cells to the site of infection or damage. Innate immune cells use specific processes to cooperatively eradicate the foreign or damaged matter. For instance, neutrophils can perform phagocytosis while NK cells can release perforin and granzymes through degranulation (Cronkite and Strutt, 2018; Vivier et al., 2008). Activation of the complement system further aids the recruitment of immune cells and the release of pro-inflammatory cytokines to amplify the inflammatory cascade (Cronkite and Strutt, 2018; Schartz and Tenner, 2020).

The innate immune system is considered non-specific as it mounts an identical and acute response to any PAMPs or DAMPs detected in body. A more specialised response is engineered by the adaptive immune system, which is acquired over time and can develop long term immunological "memory" (Marshall, Warrington, Watson and Kim, 2018). Dendritic cells are the integral bridge between the innate and adaptive branches of the immune system and are crucial for the initial activation of the adaptive immune response (Patente et al., 2019).

1.1.1 Adaptive Immunity

The adaptive immune system is composed of T and B lymphocytes which are a type of immune cell that originate from the bone marrow. These T and B lymphocytes rely on antigen-presentation to initiate an appropriate response against a foreign substance. Antigens are molecules that bind T cell receptors (TCR) and antibodies, leading to the activation of the adaptive immune response. Antigens can be exogenous, “non-self”, which are from the external environment, or endogenous, “self”, which originate within the body. Ideally, “self” antigens are not flagged by immune cells as being dangerous and will be safe from attack, restricting the possibility of developing autoimmunity (Chaplin, 2010; Cronkite and Strutt, 2018; Geenen, 2021). In cases such as cancer, where cells originating from the body transform and become abnormal, antigens called tumour-specific antigens may be expressed, which although endogenous can be immunogenic (coined neoantigens) and marked as harmful to the body and an immune response will be initiated (Jiang et al., 2019).

While antibodies can bind to the native form of an antigen, T cells can only recognise antigens in an MHC restricted manner. This involves the presentation of antigens as small fragments called peptides, which are loaded onto major histocompatibility complexes (MHCs) of antigen-presenting cells (APCs) (Bosselut, 2019). As previously introduced in Chapter 1.1, dendritic cells play a key role in antigen presentation to T cells, however macrophages and B cells also have this ability (Cronkite and Strutt, 2018). The adaptive immune response will only attack the antigens it has been presented with, making this a highly specific response (Marshall, Warrington, Watson and Kim, 2018).

T cells mediate cellular immunity in the adaptive immune response while B cells are responsible for antibody immunity, also known as humoral immunity. Naïve B cells initially become activated by the binding of antigens to their surface immunoglobulin (Ig) receptors. Additional antigen-specific signals from T cells stimulate the proliferation and maturation of B cells, and their subsequent differentiation into plasma cells. Plasma cells are capable of producing antigen-specific antibodies that can circulate in the blood and neutralise invading pathogens (Doherty, Melo, Moreno-Olivera and Solomos, 2018). These processes by T and B cells can take days or even weeks to become fully effective, and after have the potential to create memory T and B cells that are antigen-experienced and can, if re-exposure occurs, produce a rapid and more potent attack (Chaplin, 2010; Marshall, Warrington, Watson and Kim, 2018).

1.1.2 T Cells

The prominent element of a T cell is its TCR, which in most cases is comprised of an alpha (α) and beta (β) chain. To optimise an immune response to unknown antigens invading or arising in the body, it is crucial that a diverse TCR repertoire is present. The greater the diversity of TCR generated, the higher the chance of recognition of an antigen, and the consequent activation of T cells which then mediate an attack (Cardinale, De Luca, Locatelli and Velardi, 2021).

The activation of a T cell is broadly defined into three signals, summarised in Figure 1. The first signal involves the presentation of antigens held on MHC molecules on the cell surface of APCs. Antigen-MHC class I complexes on nucleated cells can activate CD8+ T cells, while antigen-MHC class II complexes of APC's can activate

CD4+ T cells. The TCR binds to the antigen and initiates activation of the T cell. Signal two describes subsequent co-stimulation, where a surface molecule such as CD28 on the T cell will bind to either CD80 or CD86 on the surface of the APC (Sckisel et al., 2015). This co-stimulation during signal two stimulates proliferation of the specific T cell clone that is bound to the antigen, known as clonal expansion (Mayer, Zhang, Perelson and Wingreen, 2019). Signal three relies on the presence of cytokines to instruct this pool of T cell clones to differentiate into T cell subtypes which have distinct functions in the adaptive immune response (Kumar, Connors and Farber, 2018; Mayer, Zhang, Perelson and Wingreen, 2019).

Mature T cells are either CD4+, known as 'helper' T cells, or CD8+, known as 'cytotoxic' T cell. Specific subtypes of CD4+ and CD8+ T cells have distinct cytokine profiles which function to regulate either the activation or suppression of other innate and adaptive immune cells, as well as non-immune cells (Luckheeram, Zhou, Verma and Xia, 2012). At present, five subsets of CD4+ T cells have been described: T helper (Th)1, Th2, Th17, regulatory T cell (Tregs) and follicular helper T cells (T_{FH}). Th1, Th2 and Th17 cells each function against specific invading pathogens and have distinct cytokine profiles that are either pro- or anti-inflammatory (Geginat et al., 2014). For example, Th1 cells produce the highly pro-inflammatory cytokine IFN-γ, which stimulates macrophage activation and can promote the presentation of antigens on APCs (Tau and Rothman, 1999). Furthermore, Tregs are crucial for the negative regulation of the immune response and the prevention of damage to normal healthy tissue, while T_{FH} cells are responsible for the stimulation of B cell differentiation into plasma cells and the subsequent production of antibodies and memory B cells (Crotty, 2014).

For CD8+ T cells, there are two subtypes related to effector cells, those being type 1 CD8+ T cells, which produce IFN-γ, and type 2 CD8+ T cells which produce IL-4, IL-5 and IL-10 (Dobrzanski, Reome and Dutton, 2000). CD8+ T cells can further differentiate into either central or effector memory cells (Geginat, Lanzavecchia and Sallusto, 2003). The primary role of CD8+ T cells is to mediate clearance of pathogens by secreting cytokines, cytotoxic granules, and the induction of apoptosis through activation of the caspase cascade (Harari et al., 2009; Jaime-Sanchez et al., 2020).

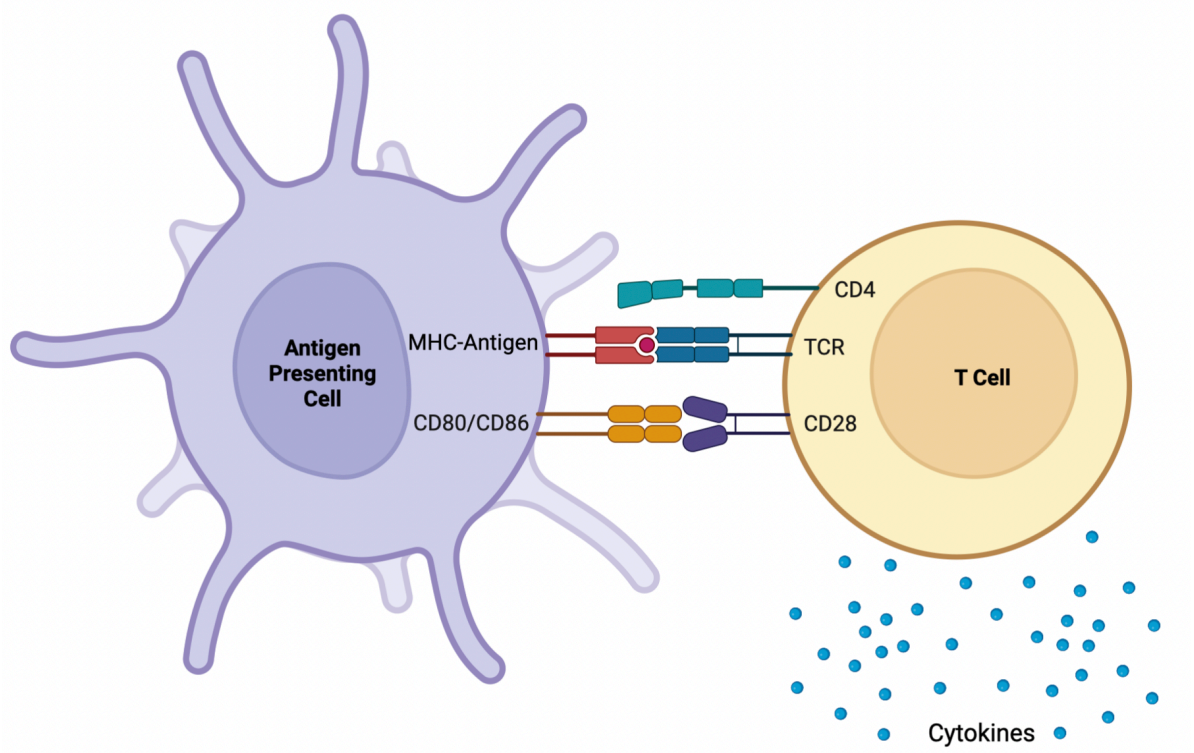


Figure 1. Signals Required for T Cell Activation (Showing CD4+ T Cell)
Signal 1 involves an antigen presenting cell (APC) presenting the MHC class I- or class II-antigen complex which is recognised by the TCR. Signal 2 co-stimulation involves the binding of the CD28 molecule on the T cell to CD80 or CD86 on the APC. Signal 3 requires the presence of cytokines to guide T cell differentiation into various subsets.
Figure made in BioRender.com.

1.2 Thymic T Cell Selection

T cells develop from progenitors called thymocytes, which originate from hematopoietic stem cells in the bone marrow (Kumar, Connors and Farber, 2018). The thymocytes migrate to the cortex of the thymus, where they begin their maturation process, shown in Figure 2. At this initial stage, thymocytes are classed as being double-negative (DN) due to their lack of expression of both CD4 and CD8 receptors. This DN population undergo random TCR loci rearrangements, resulting in a diverse repertoire of thymocytes that each express a unique TCRαβ complex. The thymocytes that successfully assemble a functional TCR will proceed to express both CD4 and CD8 receptors, known as CD4+CD8+ double-positive (DP) thymocytes (Montel-Hagen and Crooks, 2019). Selection processes then ensue to ensure only the thymocytes with ideal interactions with peptides survive.

First, is positive selection where DP thymocytes are presented with MHC class I and II molecules. Those that have a TCR with a high affinity for the MHC molecules are given intracellular signals that allow them to escape cell death, while the DP thymocytes with a low affinity TCR will die because of neglect. The surviving thymocytes go on to downregulate one of their CD4 and CD8 receptors, becoming either CD4+CD8– or CD4–CD8+ single positive (SP) thymocytes (Klein, Kyewski, Allen and Hogquist, 2014; Kondo, Ohigashi and Takahama, 2018).

Second, is negative selection, where the positively selected SP thymocytes enter the medulla of the thymus and are exposed to self-antigens embedded in MHC Class I and II molecules. The SP thymocytes that have a high affinity to self-peptides are eliminated by induction of apoptosis, a type a programmed cell death often regarded

as ‘cell suicide’. In contrast, the SP thymocytes that have a low affinity towards the self-peptides survive negative selection and are now fully mature naïve CD4+ or CD8+ T cells that can be released into the circulation (Kondo, Ohigashi and Takahama, 2018). The selection processes of the thymus generate central tolerance, which significantly narrows the opportunity for self-reactive T cells to escape into the periphery and provoke an autoimmune attack.

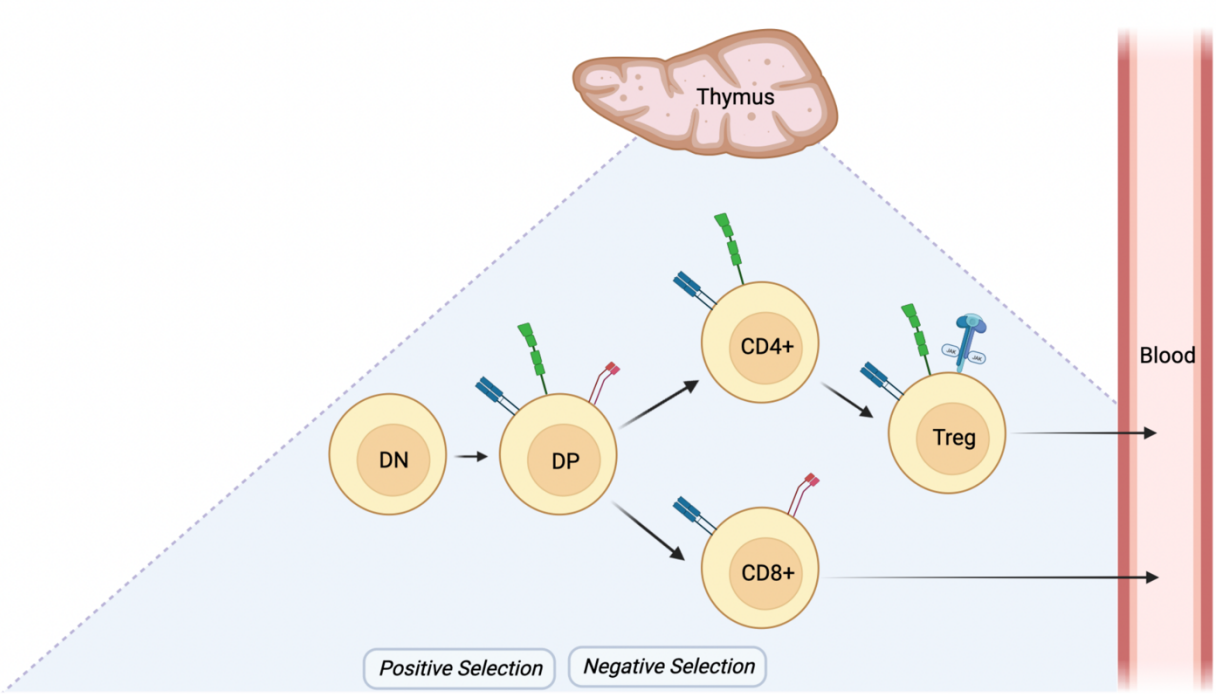


Figure 2. Overview of T Cell Development

T cell progenitors develop in the thymus as thymocytes. First, they begin as double negative (DN), expressing neither a CD4 nor a CD8 receptor. After underdoing random TCR loci rearrangements, DN thymocytes will express a TCRαβ complex. If their TCRαβ complex is functional, they will be positively selected and transform into double positive (DP) thymocytes by upregulating both a CD4 and a CD8 receptor. DP thymocytes with an appropriate tolerance to self-peptide survive negative selection and downregulate either their CD8 or CD4 receptor, becoming single positive (SP). CD4+ T cells can further differentiate into regulatory T cells (Tregs). SP thymocytes and Tregs can mature and leave the thymus into the periphery through the circulation.

Figure made in BioRender.com.

1.2.1 Development of Regulatory T Cells

Although central tolerance provides a meticulous mechanism for the prevention of self-reactive T cell escape, it is not a flawless system. Even in healthy individuals, a small portion of self-reactive T cells can survive negative selection in the thymus and enter the periphery. To counterbalance this, specialised CD4+ Tregs are established primarily in the thymus which infiltrate the periphery and implement peripheral tolerance (Rocamora-Reverte, Melzer, Würzner and Weinberger, 2021).

During their development in the thymus, a small fraction of CD4+ T cells receiving strong TCR stimulation will upregulate CD25, a high affinity receptor that binds interleukin-2 (IL-2), producing CD4+CD25+FoxP3- Treg precursors (Owen, Sjaastad and Farrar, 2019; Wang, Cui and Qian, 2018). Subsequent signalling of IL-2 results in phosphorylation of STAT proteins and the expression of the forkhead box P3 (FoxP3) protein (Zorn et al., 2006). FoxP3 is a master regulatory transcription factor that distinguishes Tregs from other CD4+ T cell populations (Zhang and Zhou, 2019). Moreover, CD4+ T cells can also differentiate into Tregs outside of the thymus. Similarly to thymic CD4+ T cells, strong TCR activation can evoke expression of CD25 in peripheral CD4+ T cells. However, unlike thymic CD4+CD25+FoxP3- Treg precursors, it has been well described that signalling by the cytokine transforming growth factor-beta (TGF-β) is essential for the upregulation of FoxP3 in peripherally differentiated Tregs (Caramalho, Nunes-Cabaço, Foxall and Sousa, 2015; Luckheeram, Zhou, Verma and Xia, 2012), although the stability of this FoxP3 expression is not yet explicit (Freudenberg et al., 2018). Naturally arising Tregs, whether differentiated in the thymus or the periphery, all have this characteristic

CD4+CD25+Foxp3+ phenotype (Rocamora-Reverte, Melzer, Würzner and Weinberger, 2021).

Recent reports have suggested an alternative route of Treg differentiation, whereby mature Tregs develop from CD4+CD25-FoxP3+ T cells (Marshall, Sinclair, Tung and Seddon, 2014; Tai et al., 2013). In populations of CD4+ T cells that are CD25-FoxP3+, FoxP3 promotes pro-apoptotic signatures to emerge, such as Puma, while pro-survival factors, such as Bcl-2, are supressed, resulting in FoxP3-induced cellular death. However, it has now been described that T cells of this phenotype compete for IL-2 cytokine signalling, when available, and if successful are able to mature into CD4+CD25+FoxP3+ Tregs (Schuster et al., 2019; Tai et al., 2013).

It is well defined that Tregs have a fundamental role in mediating the suppression of immune cells, including other T cells, as to maintain peripheral self-tolerance and prevent the development of autoimmune disease. Since their initial discovery, a variety of additional functions observed have been for Tregs. Examples proposed include initiating tolerance against antigens from food, suppression of allergy, initiating maternal tolerance to the foetus, and even the maintenance and protection of commensal bacteria. Whether all Tregs have these abilities or just distinct subsets is not well-defined (Corthay, 2009).

Immune homeostasis by Tregs is primarily controlled by use of cytotoxic T-lymphocyte-associated protein-4 (CTLA-4), which is an inhibitory molecule that can downregulate immune responses. CTLA-4 shares ligands with the activation molecule CD28, and acts by regulating the CD28 pathway in immune cells (Walker,

2013), as has been previously described in section 1.1.2. Anti-inflammatory cytokines such as IL-10 and TGF-β are secreted by Tregs, and along with CD28 regulation, work to supress the proliferation, activation, and cytokine production of immune cells. Most prominently, Tregs regulate CD8+ T cells. CD8+ T cells, often regarded as 'cytotoxic' T cells, function to kill cells expressing the target 'foreign' antigen, by release of the apoptosis-inducing proteins perforin and granzyme (Marshall, Warrington, Watson and Kim, 2018). Additionally, Tregs regulate other populations of CD4+ T cells, and have also been observed to regulate dendritic cells, macrophages, neutrophils, B cells (Okeke and Uzonna, 2019) and even non-immune cells such as the intestinal epithelial cells (Cosovanu and Neumann, 2020).

Overall, Tregs have the ability to regulate a variety of cell types and their actions during an immune response, and therefore are imperative for the maintenance of self-tolerance, making Treg development and function an appealing area of research that continues to uncover novel information.

1.3 The Nr4a Receptor Family

Nr4a receptors are a family of orphan nuclear receptors encoded by immediate early response genes, meaning they respond rapidly to a large variety of stimuli. Nr4a receptors act as transcription factors, with their activity being ligand independent and regulated instead through their expression levels. The Nr4a family comprises of three members: Nr4a1 (Nur77), Nr4a2 (Nurr1) and Nr4a3 (Nor-1) (Odagiu et al., 2021). The *NR4A1* gene that encodes the Nr4a1 protein was the first to be discovered by the biochemist Thomas Hazel in 1988 who was investigating several immediate early genes within mouse fibroblasts during cell cycle transition from the

G0 phase to the G1 phase (Hazel, Nathans and Lau, 1988). Thereafter, Nr4a2 and Nr4a3 were identified. Nr4a receptors are associated with many essential functions throughout body, including proliferation, apoptosis, fatty acid metabolism (Odagiu et al., 2021), and even in long-term memory formation in the hippocampus of the brain (McNulty et al., 2012).

1.3.1 Nr4a Receptors and T Cell Regulation

Since their discovery, the Nr4a receptors have been extensively researched, however, only more recently have their functions in immune cells been explored. All three members of the Nr4a receptor family have been linked to the regulation of T cell function. Previous reports indicate Nr4a1 to have the most influence in T cells, although Nr4a2 and Nr4a3 have been found to have their own respective roles. For example, Nr4a2 has been previously shown to be highly expressed in T cells that secrete IL-17, most notably Th17 cells. A study utilising a mouse model where transfection of siRNA was used to block Nr4a2 expression found that loss of Nr4a2 caused the prevention of Th17 differentiation. It was revealed that Nr4a2 controls autocrine signalling of IL-21, and the initiation of IL-17 transcription, which are both essential processes for the maturation of Th17 cells (Raveney, Oki and Yamamura, 2013). Furthermore, it's been previously highlighted that Nr4a2 has a contributing role in the expression of FoxP3 in CD4+ T cells. It was demonstrated that loss of Nr4a2 can lead to the production of Tregs that are prone to lose their FoxP3 expression. This was found to be caused by the ability of Nr4a2 to directly bind to the regulatory regions of the *FoxP3* gene and co-ordinate histone modifications required for FoxP3 expression (Sekiya et al., 2011). A distinct role for Nr4a3 has been described, relating to the differentiation of CD8+ T cells into distinguished subsets during acute infection. It was shown that Nr4a3 can influence the chromatin

accessibility of specific genes that dictate the early transcriptional programme of CD8+ T cell differentiation preferentially into short-lived effector cells (SLEC) as opposed to memory precursor effector cells (MPEC) (Odagiu et al., 2020).

It has been displayed in several reports that Nr4a1 may have a role in cellular apoptosis (He et al., 2020; Odagiu et al., 2021; Safe and Karki, 2021). One report demonstrated that Nr4a1 can directly control the transcription of the *Runx3* gene in CD8+ T cells, a mediator of cell proliferation and apoptosis. Nr4a1 was revealed to initiate CoREST, an inhibitor of *Runx3*, which consequently supressed the proliferation of and induced apoptosis in both thymic and peripheral CD8+ T cells (Vogiatzi, De Falco, Claudio and Giordano, 2006). Another report has described an alternate Nr4a1-associated pathway of cellular apoptosis. It was suggested that in response to TCR stimulation by stress signals, ASK1, a regulator of apoptosis, initiates a signalling cascade that causes downstream upregulation of Nr4a1 and eventual apoptosis of the cell (Huo, Xu and Lam, 2020).

Conversely, additional reports have challenged the association of Nr4a1 in apoptosis pathways. It has been suggested that Nr4a1 does not directly contribute to apoptosis pathways of cells, but instead plays a role in the regulation of cellular metabolism. It was described that Nr4a1 can influence the metabolic profile of T cells to preferentially utilise mitochondrial respiration, rather than the more advantageous use of aerobic glycolysis, consequently limiting the effector function of the CD4+ T cells. It was further found that Nr4a1 can restrict the progression of CD4+ T cells through the cell cycle, resulting in an indirect increase in cellular apoptosis (Liebmann et al., 2018).

Not only does Nr4a1 contribute to natural T cell development and function, but also emerges in the dysfunctional 'exhaustion' state of CD8+ T cells. This 'exhausted' phenotype is defined by a loss of effector functions, and the sustained expression of inhibitory receptors such as PD-1 in response to chronic infection or cancer (Wherry, 2011). A recent study explored Nr4a receptors within CAR T cells that were limited in their therapeutic potential due to their 'exhausted' phenotype. It was observed that Nr4a1 expression had a strong positive correlation with the expression of the inhibitory receptors PD-1 and TIM-3 (Chen et al., 2019). Another study looking a Nr4a1 within T cell dysfunction discovered that overexpression of Nr4a1 can supress the differentiation of effector T cells. It was described that Nr4a1 preferentially binds and inhibits AP-1, a transcription factor associated with effector-gene expression. Using Nr4a1-deficient mice, it was found that loss of Nr4a1 promoted effector T cell functions against cancer and chronic infection (Liu et al., 2019).

1.3.2 Redundancy in the Nr4a Receptor Family

Previous studies have explored the use of mice with either single knockout (SKO), double knockout (DKO) or triple knockout (TKO) Nr4a genotypes in order to investigate similarities and distinctions between the Nr4a family members.

The compound deletion of all Nr4a receptors in TKO mice have been reported to cause fatal abnormalities. The absence of Nr4a receptors caused the mice to develop with thymic deformities, a complete loss of all thymic and peripheral Tregs, an enhanced Th2-driven inflammatory state, and overall systemic multiorgan autoimmunity that resulted in their early death (Kasler et al., 2012; Odagiu et al., 2021; Sekiya et al., 2013).

Interestingly, in *Nr4a1-/- Nr4a3-/-* DKO mice, but not *Nr4a2-/- Nr4a3-/-* DKO mice, a comparable phenotype to the Nr4a TKO has been observed, whereby negative selection in the thymus fails, the production of FoxP3+ Tregs is lost, and the consequent survival of self-reactive T cells results in fatal autoimmunity of the mice (Sekiya et al., 2015). However, this principal effect was not present in *Nr4a1-/-* or *Nr4a3-/-* SKO mice studies (Hiwa et al., 2021). The only variances found occurred in the *Nr4a1-/-* SKO mice, where there was an extremely subtle alteration in the production of PD-1 and CD69 during negative selection in the thymus (Fassett et al., 2012; Rajpal, 2003). In contrast, the dual overexpression Nr4a1 and Nr4a3 has been shown to induce thymocyte apoptosis during selection events. Again, these findings were not replicated in mice overexpressing individual Nr4a1 or Nr4a3 receptors (Odagiu et al., 2021). It has been suggested that both Nr4a1 and Nr4a3 are upregulated in negative selection, but only Nr4a1 contributes to the regulation of positive selection, although this remains uncertain (Baldwin and Hogquist, 2007; Odagiu et al., 2021). It was demonstrated in one *Nr4a1-/-* SKO mouse model using OT-II mice, that there was an increase in thymocytes positively selected in the thymus (Fassett et al., 2012). Although, this effect on positive selection was not replicated in another Nr4a1-/- SKO mouse model, which used OT-I mice (Hu, Suen, Henao Caviedes and Baldwin, 2017).

These findings imply that the individual members of the Nr4a receptor family may possess their own characteristic functions, however it is evident that redundancy is present for the roles of the Nr4a receptors, particularly for Nr4a1 and Nr4a3, whose roles in T cell function appear to bear considerable overlap. Despite the emerging

research surrounding Nr4a2 and Nr4a3 receptors, knowledge of their individual significance for T cell function is limited. The Nr4a1 receptor has been much more extensively researched, and the suggested roles of Nr4a1 appear to be associated with processes that can significantly impact T cell development and function to a higher degree than Nr4a2 and Nr4a3. Therefore, the focus of this project solely explores the roles of the Nr4a1 receptor within T cells during their development and activation.

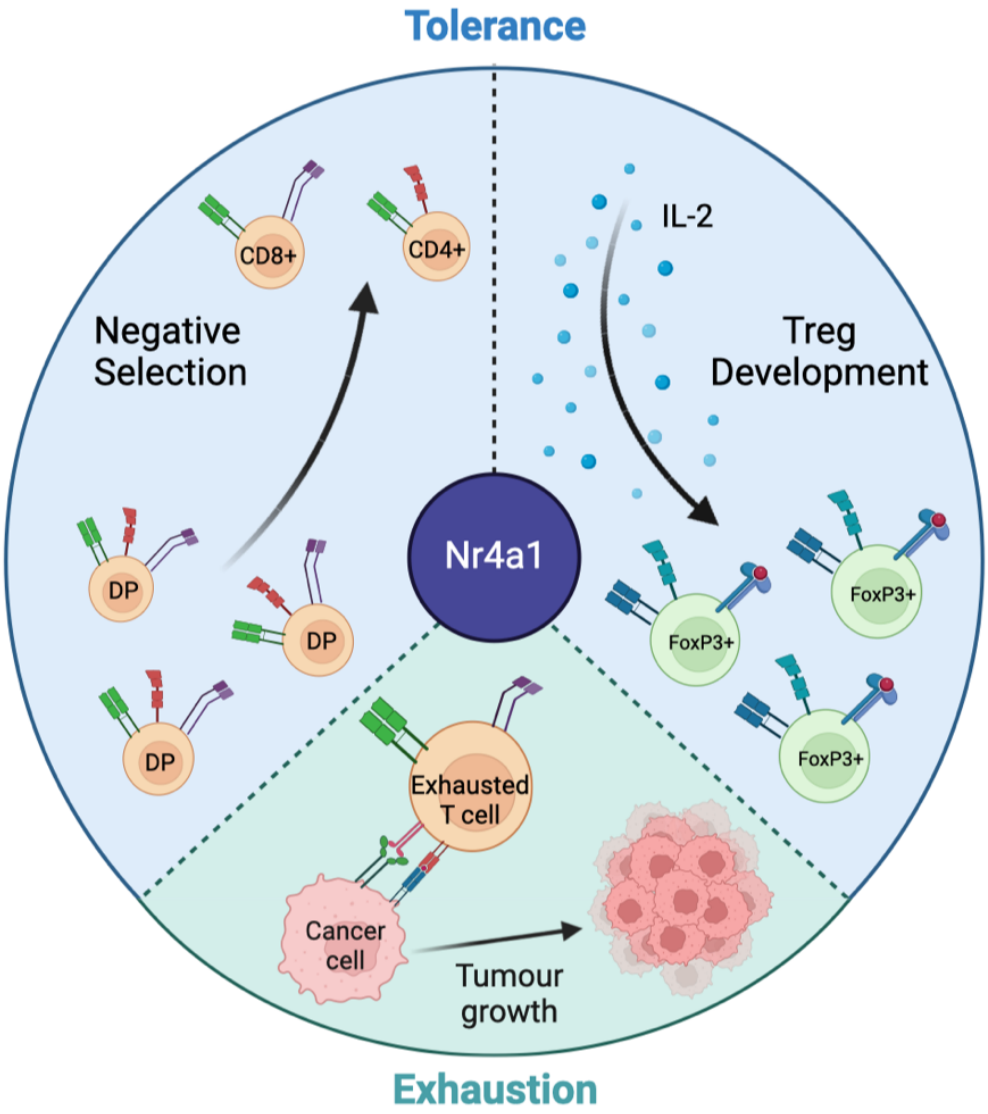


Figure 3. Overview of the Nr4a1 Receptor in T Cell Regulation

Nr4a1 has been identified in important processes in T cells, including successful negative selection during T cell development in the thymus, the differentiation of regulatory T cells by FoxP3 upregulation and the failure of T cell effector function in dysfunctional state of 'exhaustion'.

Figure adapted from: Lith, Os, Seijkens and Vries, 2020.

Figure made in BioRender.com.

1.3.3 *Nr4a3*-Timer of Cell Kinetics and Activity (Tocky) Reporter Mice

The NFAT family of transcription factors have been long recognised in T cells as key regulators of activation. It was confirmed by the work of Bending et al. (2018) that following TCR stimulation, calcium signalling initiates the calcineurin-NFAT1 pathway, directly inducing downstream *Nr4a3* expression with its peak expression occurring within 2 hours of TCR stimulation. Using this knowledge, they developed a novel transgenic reporter mouse model that has an artificial bacterial chromosome (BAC) which, upon *Nr4a3* transcription, produces a Fluorescent Timer (FT) fast mCherry protein (Subach et al., 2009). This FT protein initially occurs in an unstable form that emits a blue fluorescence, with a half-life of 4 hours, and over time matures into a stable form that emits a red fluorescence. The shift of emission of the protein is spontaneous and irreversible and allows analysis of the temporal and dynamic aspects of T cell signalling by flow cytometry. For its unique abilities, this system is named Timer of cell kinetics and activity (Tocky) (Bending et al., 2018).

Figure 4 demonstrates the visual representation of the emission of the FT protein on a flow cytometry plot. Signals from activated TCRs can be categorised into new, persistent, or arrested.

The *Nr4a3*-Tocky mouse model has proven to be a reliable readout of TCR signalling, with no influence caused by external factors such as co-stimulation with CD28 and the presence of exogenous IL-2 (Bending et al., 2018). This novel system offers an exceptional method of studying temporal and dynamic TCR signalling, especially within the context of complex diseases derived from autoimmunity or cancer.

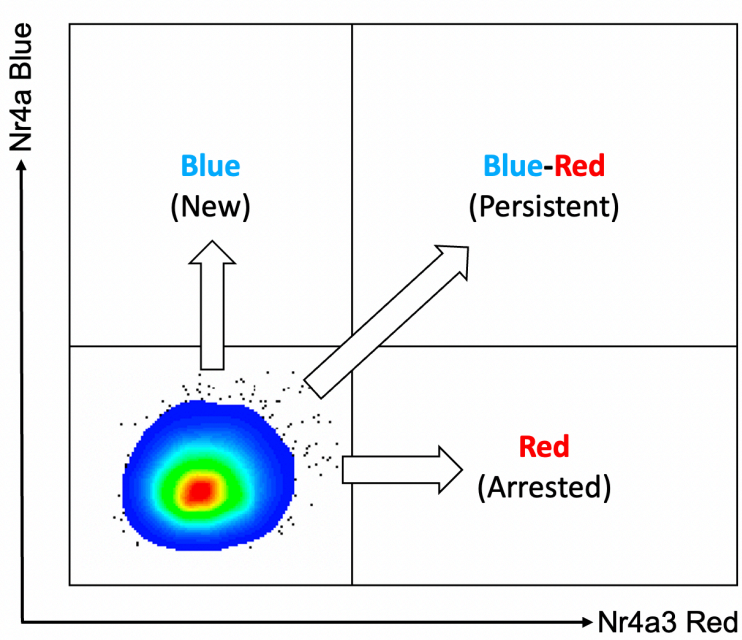


Figure 4. Schematic Showing Nr4a3 Blue and Nr4a3 Red Expression Categorisation on a Flow Cytometry Plot

Following TCR stimulation, initial expression of the Timer Blue protein will appear in the top left quadrant of the plot. Overtime, the TCR signal will become arrested, and the Timer Blue protein will mature into the Timer Red protein, which will appear in the bottom right quadrant of the plot. TCR signals that remain persistent and are in steady state emerge in the top right quadrant of the plot. No TCR stimulation will be shown in the bottom left quadrant of the plot, as shown in this figure.

1.4 Introduction Summary

The explicit controversy surrounding the roles of Nr4a1 in T cells from the current literature remains unresolved. This indicates an undeniable discrepancy in the present information available and highlights the importance of the continuation of Nr4a receptor research. The potential roles of Nr4a1 links to crucial processes in T cell development and signalling that are required for the maintenance of immune homeostasis in the body, with further potential for optimising T cell therapies in response to devastating autoimmune diseases and cancers. Further investigation to reveal a distinct role or mere redundancy of Nr4a1 is essential.

2. Aims

From what can be drawn of the existing literature, we hypothesise that Nr4a1 is functionally redundant, particularly within peripheral T cells. To test our hypothesis, the novel *Nr4a3*-Tocky reporter mice will be used to explore the roles of Nr4a1 in both developing, central T cells of the thymus, and mature, peripheral T cells of the spleen. For *ex vivo* analysis of the Nr4a1 receptor, surface stains including key markers of TCR activation will be used and analysed by flow cytometry. For functional analysis of the Nr4a1 receptor, *in vitro* cultures of purified of CD4+ T cells will be performed at different time points for analysis of RNA by RNA-Sequencing (RNA-Seq), cytokine production by ELISAs, as well as surface staining of additional TCR activation markers by flow cytometry. To gain an insight into the of cellular proliferation, apoptosis and FoxP3 expression of the thymic and splenic T cells, annexin V and intracellular staining will be performed and analysed by flow cytometry. These methods will allow the following aims to be explored:

Aim 1) To determine how Nr4a1 influences TCR signaling during T cell development

Aim 2) To reveal the functional roles of Nr4a1 during T cell activation

3. Materials and Methods

3.1 Experimental Design

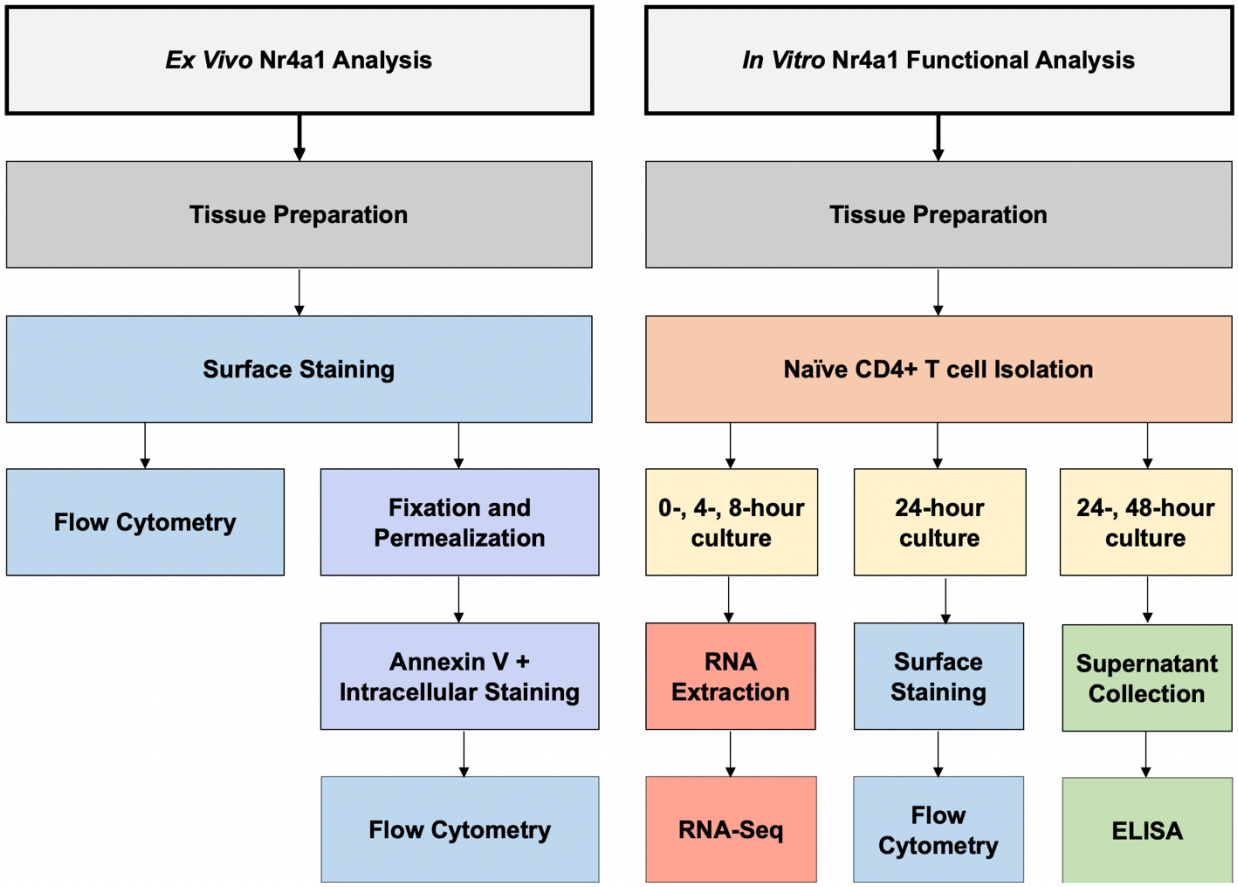


Figure 5. Flow Chart of Experimental Design

For ex vivo analysis of Nr4a1, thymic and splenic tissue from *Nr4a3*-Tocky mice were prepared, stained for surface markers and were either immediately run on the Fortessa, or were first stained for Annexin V and intracellular markers before flow cytometry analysis. For in vitro analysis of Nr4a1, splenic and lymph node tissue from *Nr4a3*-Tocky mice were prepared and then naïve CD4+ T cells were isolated and activated on 96-well plates. After 0-, 4- and 8-hours of activation, RNA was extracted, and RNA-Sequencing was performed. After 24-hours of activation, surface staining and flow cytometry analysis was performed. After 24- and 48-hours of activation, supernatant was collected and ELISA's for IL-2 and IFN-γ were completed.

3.2 Mice

The animal experiments performed in this project were all in compliance with the Home Office project licence P18A92E0A, held by David Bending. Animal procedures were carried out in the Biomedical Services Unit at the University of Birmingham, all in accordance with the local Animal Welfare and Ethical Review Body. Animals were housed in a specific pathogen-free environment under appropriate husbandry conditions in accordance with the NC3Rs. Schedule 1 killing of the animals was performed by David Bending or other lab members who hold a personal licence.

For this project, *Nr4a3*-Tocky mice (Bending *et al*., 2018), founder line 323 (obtained from Dr. Masahiro Ono, Imperial College London under MTA), were crossed onto a *Nr4a1-/-* background, producing *Nr4a3*-Tocky mice that were either heterozygous (*Nr4a1+/-)* or homozygous knockout (*Nr4a1-/-*) for the *Nr4a1* gene. Figure 6 shows a Punnett Square of this mice breeding, with “FT” denoting the presence of the fluorescent timer protein (FT) and “wt” denoting the absence of the fluorescent timer protein.

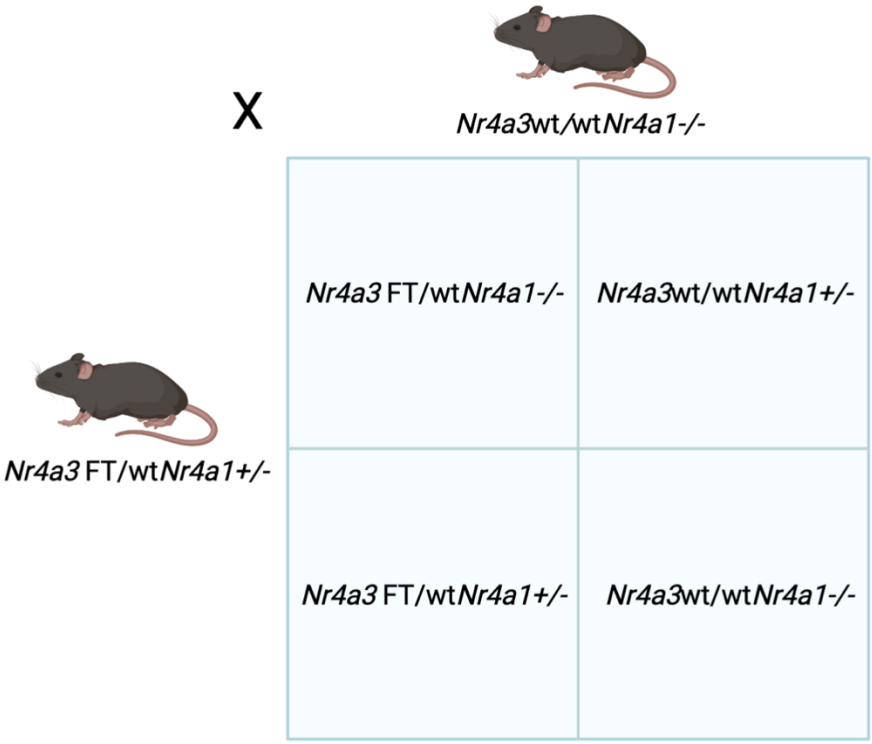


Figure 6. Punnett Square Showing Predicted Genotypes from Cross Breeding of Mice
Cross breeding of *Nr4a3* FT/wt*Nr4a1+/-* mice with *Nr4a3*wt/wt*Nr4a1-/-* is predicted to produce mice with frequency of genotypes as follows: 25% *Nr4a3* FT/wt*Nr4a1+/-*, 25% *Nr4a3* FT/wt*Nr4a1-/-*, 25% *Nr4a3*wt/wt*Nr4a1+/-*, and 25% *Nr4a3*wt/wt*Nr4a1-/-*.
Figure made in BioRender.com.

3.3 Tissue Preparation

Whole thymus, spleen or lymph nodes obtained from *Nr4a3*-Tocky mice were mechanically disrupted by being pushed through 70-µm cell strainers (Greiner), producing single cell suspensions in complete media (RPMI 1640 with 2nM L-Glutamine and 10% foetal bovine serum (FBS)) (Gibco), unless otherwise described. For the spleens, subsequent red blood cell (RBC) lysis was completed (eBioscience) as reported by the manufacturer's instructions. Both thymic and splenic cells were counted using a 1:1 dilution of Trypan Blue Solution (Gibco) and a haemocytometer according to manufacturer's instructions. Cells were prepared at appropriate cell densities as described throughout. Where cells were to be cultured, this was

performed using standard aseptic technique in a Class II tissue culture hood. Centrifugation of cells was completed using 1400-1500 RPM at 4°C for 5 minutes with an acceleration and deceleration speed of $9g_n$, unless otherwise stated.

3.4 Naïve CD4+ T Cell Purification

Cell suspensions of whole spleen combined with lymph nodes were prepared as described in Chapter 3.3, although complete media had the addition of Pen-Strep (RMPI 1640 with 2nM L-Glutamine, 10% FBS and 1% Pen-Strep) (Gibco), and all steps were performed using standard aseptic technique.

CD4+ T cell populations were negatively selected through magnetic isolation using the MojoSort Mouse CD4 Naïve T Cell Isolation kit (Biolegend) as per the manufacturer's instructions.

3.5 *In Vitro* Cultures

96-well flat-bottom plates (Corning) were coated with PBS containing functional grade anti-CD3 mouse monoclonal antibody (eBioscience) on its own or in combination with functional grade anti-CD28 mouse monoclonal antibody (eBioscience), as reported by the manufacturer's instructions. Coated plates were placed in an incubator for 2 hours or in the fridge overnight. Next, naïve CD4+ T cells were purified as described in Chapter 3.4. Cells were counted using a 1:1 dilution of Trypan Blue Solution (Gibco) and a haemocytometer according to manufacturer's instructions. Cells were resuspended at a cell density of $1x10^6$ per mL, and 250,000 cells were transferred to 1.5mL microtubes (Eppendorf) ready for RNA isolation (0-hour timepoint). Cells were then plated at 250,000 cells per well on the coated 96-

well flat-bottom plates (Corning). A tissue culture (TC) grade β-mercaptoethanol preparation (RPMI 1640 with 10% FBS and 4% β-mercaptoethanol) was added to each well of cells. A brief centrifuge at 100g for 1 minute was completed to gather cells to the bottom of the wells in the plate, and uniformly begin their time points. Cells were placed in the incubator for time points 4-, 8-, 10-, 24- and 48-hours and at the indicated time points analysed for flow cytometry, RNA expression and cytokine production by ELISA. The incubator used was set to optimal conditions for cell cultures with a temperature of 37 °C, 95% humidity and 5% CO_2. All steps for *in vitro* cultures were performed using standard aseptic technique.

3.6 Surface Staining

Whole spleen and thymus were prepared as described in Chapter 3.3 and plated at a cell density of $1x10^6$ cells per well in 96-well round-bottom plates (Corning). Cells were stained with eFluor 780 fixable viability dye (eBioscience) alongside cell surface antibodies (Figure 7), in FACS buffer (Ca/Mg+ free PBS with 10% FBS) (Gibco), as reported by the manufacturer's instructions.

Purified naïve CD4+ T cells were cultured as described in section Chapter 3.4 and plated at a cell density of $2.5x10^5$ cells per well in 96-well round-bottom plates (Corning). Cells were stained with eFluor 780-flurescent fixable viability dye (eBioscience) alongside cell surface antibodies (Figure 7), in FACS buffer, as reported by the manufacturer's instructions.

For both whole spleen and thymus, and purified naïve CD4+ T cells, once staining was complete, cells were washed once and resuspended using FACS buffer, then transferred into 5mL polystyrene tubes (BD Biosciences) before flow cytometry analysis.

Whole Spleen and Thymus Surface Staining Panel		Purified Naïve CD4+ T cell Surface Staining Panel		Fixed Whole Spleen and Thymus Intracellular Staining Panel	
Antibody/Protein/Dye	**Fluorophore**	**Antibody/Protein/Dye**	**Fluorophore**	**Antibody/Protein/Dye**	**Fluorophore**
Viability Dye	eFluor780	Viability Dye	eFluor780	Viability Dye	eFluor780
CD4	BUV737	CD4	BUV395	CD4	BUV737
CD8	BUV395	OX40	APC	CD8	BUV395
TCRb	AF700	ICOS	AF700	TCRb	AF700
CD25	APC	CD25	PerCP-Cy5.5	CD25	PE-Cy7
CD69	FITC	CD69	FITC	Annexin V	FITC
CD44	PE-Cy7	GITR	PE-Cy7	Ki-67	PE
Fluorescent Timer Blue	BUV421	Fluorescent Timer Blue	BUV421	FoxP3	APC
Fluorescent Timer Red	PE-Texas Red	Fluorescent Timer Red	PE-Texas Red		

Figure 7. Table Showing Staining Panels for Experiments Respectively
The staining panel shown in this figure for whole spleen and thymus, and purified naïve CD4+ T cell flow cytometry analysis used a mixture of antibodies, proteins and dyes for staining surface markers of TCR stimulation. The staining panel shown in this figure for fixed whole spleen and thymus flow cytometry analysis used a mixture of antibodies, proteins and dyes for both surface and intracellular staining of TCR stimulation, proliferation and apoptosis.

3.7 Annexin V and Intracellular Staining

Surface staining was completed as described in Chapter 3.6, using the antibodies shown in Figure 7. Firstly, cells were washed with Annexin V Binding Buffer (eBioscience), then stained for Annexin V FITC (BioLegend) and washed once more in the Binding Buffer as per the manufacturer's instructions. Secondly, cells were fixed using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) as reported by the manufacturer's instructions. Lastly, cells were stained for Ki-67 PE

(BioLegend) and Foxp3 APC (ThermoFisher) in accordance with the manufacturer's instructions.

3.8 Flow Cytometry Analysis

Samples along with single stains were analysed using the BD LSRFortessa X-20 Cell Analyser. The initial blue form of the Nr4a3-Timer protein was excited off the 405 nm laser and detected in the blue (450/40 nm) channel, and the mature red form of the Nr4a3-Timer protein was excited off the 561 laser and detected in the mCherry (610/20 nm) channel. FlowJo 10.8.0 was used for the compensation of overlapping emissions, with use of the single stains, and was used for gating on cells of interest.

3.8.1 Gating Strategies

Flow cytometry data was collected as stated in Chapter 3.8. For *ex vivo* analysis of whole spleen and thymus using surface stains, gates were drawn around cell populations of interest. First, lymphocytes were gated, then single cells, live cells, CD4+/CD8+ and DP T cells for the thymus, CD4+/CD8+ T cells for the spleen, and finally TCRβ for each T cell population. Within the TCRβ gate, TCR activation markers and Nr4a3 timer expression were analysed (Figure 8).

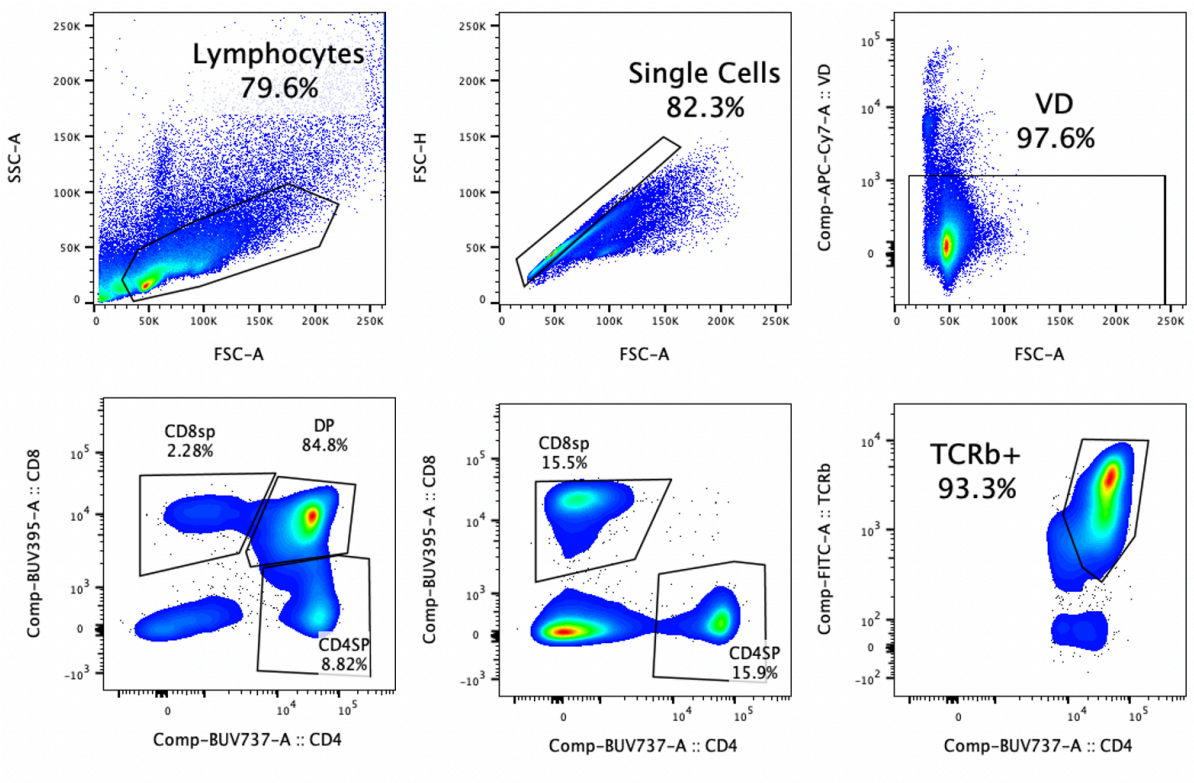


Figure 8. *Ex Vivo* Analysis Gating Strategy: Surface Staining

Data was analysed using FlowJo software. Compensation was completed using single stains and unstained samples. Lymphocytes were first gated with the axis set to FSC-A and SSC-A, single cells were next gated on with the axis set to FSC-A and FSC-H. Viability dye was then used to gate on live cells. Next, CD4 and CD8 staining was used to gate on these populations in the spleen and thymus as well as DP T cells in the thymus. Finally, TCRβ was gated on to only look at the T cells that were functionally active.

For *in vitro* analysis of isolated naïve CD4+ T cells, gates were drawn around lymphocytes, then single cells, live cells, CD4+ T cells, and finally negative Nr4a3 timer was gated on. The positive Nr4a3 timer surrounding the negative Nr4a3 timer gate was used to analyse the expression of markers associated with strong TCR stimulation (Figure 9).

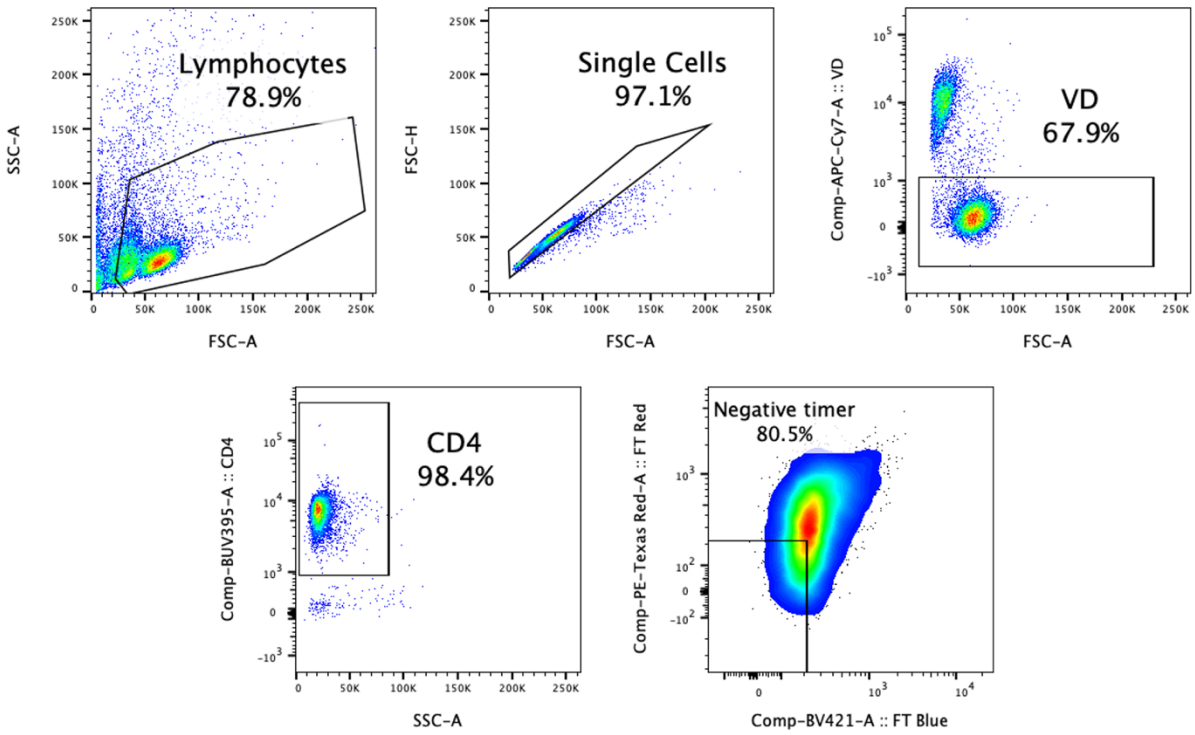


Figure 9. *In Vitro* Analysis Gating Strategy: Surface Staining

Data was analysed using FlowJo software. Compensation was completed using single stains and unstained samples. Lymphocytes were first gated with the axis set to FSC-A and SSC-A, single cells were next gated on with the axis set to FSC-A and FSC-H. Viability dye was then used to gate on live cells. Next, CD4 staining was used to gate on this population alone. Finally, a gate for the negative expression of Nr4a3 timer was used, with the “events inside” box unticked, so that only Nr4a3 positive cells would be analysed.

For *ex vivo* analysis of whole spleen and thymus using annexin V and intracellular staining of FoxP3 and Ki-67, gates were first drawn around lymphocytes, then single cells, CD4+/CD8+ and DP T cells for the thymus, CD4+/CD8+ T cells for the spleen, and TCRβ within each population of T cell. Within the DP T cell gate, TCRβ - and TCRβ + gates were drawn. Within each TCRβ + population of T cells, apoptosis, proliferation and Treg markers were analysed (Figure 10).

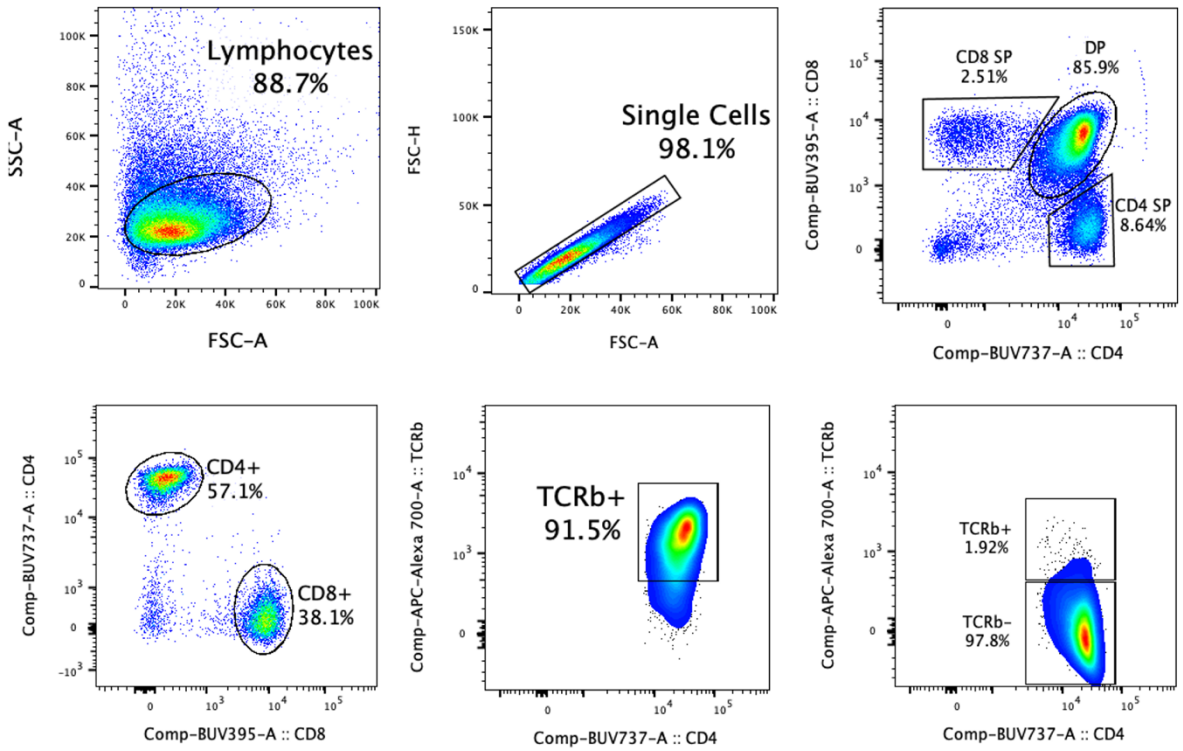


Figure 10. *Ex Vivo* Analysis Gating Strategy: Annexin V and Intracellular Staining
Data was analysed using FlowJo software. Compensation was completed using single stains and unstained samples. Lymphocytes were first gated with the axis set to FSC-A and SSC-A, single cells were next gated on with the axis set to FSC-A and FSC-H. Next, CD4 and CD8 staining was used to gate on these populations in the spleen and thymus as well as DP T cells in the thymus. TCRβ was gated on to only look at the T cells that were functionally active. Finally, withing the DP T cell gate, TCRβ + and TCRβ - was gated to analyse these specific populations.

3.9 ELISA

In vitro cultures were completed as described in Chapter 3.5. At time points 24- and 48-hours, plates were centrifuged at 1400-1500 RPM at 4 °C for 3 minutes with an acceleration and deceleration of $9g_n$. 50uL of supernatant was transferred to 1.5mL microtubes (Eppendorf) and placed in the freezer at -20 °C and thawed at room temperature when ELISA's were ready to be completed. All stages were completed using standard aseptic technique.

Absorbance of the cytokines IL-2 and IFN-y from the supernatant were obtained by solid phase sandwich ELISA through use of R&D Systems DuoSet ELISA kit as per the manufacturer's instructions.

3.10 RNA Isolation, Sequencing and Analysis

Naïve CD4+ T cells were cultured as described in Chapter 3.5. After time points 0-, 4-, 8- and 10- hours, RNA was extracted and isolated from cells using the RNeasy Mini Kit, in accordance with the manufacturer's instructions.

For the preparation of libraries, the Lexogen's QuantSeq 3'mRNA kit was used in accordance with the manufacturer's instructions. First strand cDNA synthesis was initiated using oligodT priming which contained the Illumina-specific Read 2 linker sequence, causing degradation of RNA. Random priming and a DNA polymerase was next used to carry out second strand synthesis. Magnetic beads allowed the purification of DNA. Following this, libraries were amplified, and the cluster generation sequences along with sample indexes were added. NextSeq 500 was used for the sequencing of libraries. Using the the BlueBee QuantSeq FWD pipeline, FASTQ files were uploaded to the BlueBee cloud for further analysis (Lexogen). Bbduk v35.92 from the bbmap suite allowed the trimming and exclusion of low-quality tails, poly(A)read-through and adaptor contamination. The alignment of reads to the mouse GRCm38 (mm10) genome was completed using STAR v2.5.2a aligner. HTSeq-count v0.6.0 was used to generate read counts for mRNA species and subsequent mapping statistics. Raw read counts in the .txt format were further analysed using DESeq2 (Love et al. 2014 Genome Biol) in R. Read counts were normalized and transformed using the regularised log (rlog) transformation. Principal

component analysis was performed within the DESeq2 package and heatmap analysis was performed on the rlog transformed data using the R package gplots.

The following code was used for RNA-seq analysis:

1. Blue bee software used to align files and generate raw HTseq count files mapped to the ensemble gene id

```
cts=as.matrix(read.table("raw.txt"))#count matrix prepared for DESeq2
coldata=read.csv("coldata.csv", row.names=1)#provides sample information
library(DESeq2)
dds=DESeqDataSetFromMatrix(countData=cts,colData=coldata,design=~condition)
keep=rowSums(counts(dds))>=10#removes low count genes
dds=dds[keep,]
dds=DESeq(dds)
```

2. rlog function normalisation of library and plotting of PCA

```
rld=rlog(dds,blind=F)
write.csv(assay(rld),file="norm.csv") #rlog of counts file
pdf("PCAall.pdf")
plotPCA(rld, intgroup="condition", ntop=1000) #performs PCA analysis
dev.off()
```

3. The following code was used to retrieve gene names and the generation of a heatmap in R

```
hm=read.csv("norm.csv")
colnames(hm)[1]="ensembl_gene_id"
e=hm[,1]
mart=useMart(biomart="ensembl",dataset="mmusculus_gene_ensembl")
x=getBM(mart=mart,values=e,filter=c("ensembl_gene_id"),attributes=c("ensembl_ge
 ne_id","external_gene_name"))#retrieves gene names
m2=m[!duplicated(m[,20]),] #removes duplicated rows
rownames(m2)=m2[,20]
m2=m2[,c(-1,-20)]
genes=m2[c("Nr4a1","Nr4a2","Nr4a3","Il2ra","Il2","Ifng","Tnfrsf4","Icos","Cd69"),]
 library(gplots)
pdf("hm.pdf")
heatmap.2(genes,scale="row",col=bluered(100), Colv=F, Rowv=F,
 density.info="none",trace="none") #generates z-score normalised heatmap
dev.off()
```

3.11 List of Reagents

Reagent or Resource	Source	Identifier
Antibodies		
Anti-CD4 BUV737 Rat Anti-Mouse Monoclonal GK1.5	BD Sciences	Catalogue Number: 612761 RRID: AB_2870092
Anti-CD8α BUV395 Rat Anti-Mouse Monoclonal 53-6.7	BD Sciences	Catalogue Number: 563786 RRID: AB_2732919
Anti-TCRβ AF700 Armenian Hamster Anti-Mouse Monoclonal H57-597	BioLegend	Catalogue Number: 109224 RRID: AB_1027648
Anti-CD25 APC Rat Anti-Mouse Monoclonal PC61	BioLegend	Catalogue Number: 102012 RRID: AB_312861
Anti-CD69 FITC Armenian Hamster Anti-Mouse Monoclonal H1.2F3	BioLegend	Catalogue Number: 104506 RRID: AB_313109
Anti-CD44 PE-Cy7 Rat Anti-Mouse Monoclonal IMA	BioLegend	Catalogue Number: 103030 RRID: AB_830787
Anti-CD4 BUV395 Rat Anti-Mouse Monoclonal GK1.5	BD Sciences	Catalogue Number: 563790 RRID: AB_2738426
Anti-OX40 APC Rat Anti-Mouse Monoclonal OX-86	BioLegend	Catalogue Number: 119413 RRID: AB_2561723
Anti-ICOS AF700 Armenian Hamster Monoclonal C398.4A	BioLegend	Catalogue Number: 313528 RRID: AB_2566126
Anti-CD25 PerCP-Cy5.5 Rat Anti-Mouse Monoclonal PC61	BioLegend	Catalogue Number: 102030 RRID: AB_893288
Anti-GITR PE-Cy7 Rat Anti-Mouse Monoclonal DTA-1	BioLegend	Catalogue Number: 126317 RRID: AB_2563385
Anti-CD25 PE-Cy7 Rat Anti-Mouse Monoclonal PC61	BioLegend	Catalogue Number: 102015 RRID: AB_312864
Anti-Ki-67 PE Rat Anti-Mouse Monoclonal 16A8	BioLegend	Catalogue Number: 652404 RRID: AB_2561525
Anti-FoxP3 APC Rat Anti-Mouse Monoclonal FJK-16s	ThermoFisher	Catalogue Number: 17-5773-82 RRID: AB_469457
Media and Supplements		
Gibco RPMI 1640 Medium with L-Glutamine	ThermoFisher	Catalogue Number: 21875034
Gibco PBS, pH 7.4	ThermoFisher	Catalogue Number: 10010023
Gibco Heat Inactivated FBS, EU-Approved, South American Origin	ThermoFisher	Catalogue Number: 11550356
Penicillin-Streptomycin (10,000 U/mL)	ThermoFisher	Catalogue Number: 15140122
UltraPure 0.5 EDTA, pH 8.0	ThermoFisher	Catalogue Number: 15575020
Gibco 2-Mercaptoethanol	ThermoFisher	Catalogue Number: 21985023
Commercial Assays		
MojoSort Mouse CD4 Naïve T Cell Isolation Kit	BioLegend	Catalogue Number: 480040
Mouse IL-2 DuoSet ELISA	R&D Systems	Catalogue Number: DY402
Mouse IFN-gamma DuoSet ELISA	R&D Systems	Catalogue Number: DY485
RNeasy Mini Kit	Qiagen	Catalogue Number: 74104
Invitrogen Superscript IV Reverse Transcriptase	ThermoFisher	Catalogue Number: 18090050
Invitrogen Random Hexamers	ThermoFisher	Catalogue Number: N8080127
eBioscience Fixable Viability Dye eFluor780	ThermoFisher	Catalogue Number: 65-0865-14
FITC Annexin V	BioLegend	Catalogue Number: 640905
Annexin V Binding Buffer	BioLegend	Catalogue Number: 422201
eBioscience Foxp3/Transcription Factor Staining Buffer Set	ThermoFisher	Catalogue Number: 00-5523-00
QuantSeq 3' mRNA-Seq Library Prep Kit FWD for Illumina, 24 Preps	Lexogen	Catalogue Number: 015.24

Figure 11. Table of Reagents or Resources Essential for This Project

The table in this figure lists the reagents or resources used for this project along with the supplier they were sourced from, and includes information of their individual identifiers e.g. catalogue number.

3.12 Statistical Analysis and Graphical Presentation

Statistical analysis was performed, and graphs were made using GraphPad Prism 8 software. Unpaired t-tests were used to compare two means, and two-way ANOVA with Tukey's multiple comparisons test was used to compare two independent variables. Variance is reported as mean ± SEM. P values, as shown graphically, represent significance as follows: *p=<0.05, **p=<0.01, ***p=<0.001, ****p=<0.0001. Standard curves for the interpolation of ELISA data were produced using a hyperbole curve, where X is concentration, and each interpolated value reported with its 95% confidence interval.

4. Results

4.1 Loss of Nr4a1 Enhances TCR Signalling in Developing Thymic T Cells

Flow cytometry analysis comparing the *Nr4a3*-Tocky *Nr4a1+/-* genotype to *Nr4a3*-Tocky *Nr4a1-/-* of thymic T cells revealed unaltered expression of Nr4a3 Timer in the double negative populations (Figure 12 A-C). Although, both the CD4SP and CD8SP T cell populations had significantly elevated TCR signalling in the Nr4a1-deficient mice. The overall percentage of Nr4a3 Timer expression and the median fluorescence intensity (MFI) of Nr4a3 Timer red expression was considerably increased in the CD4SP and CD8SP populations with loss of Nr4a1 (Figure 12 D-I).

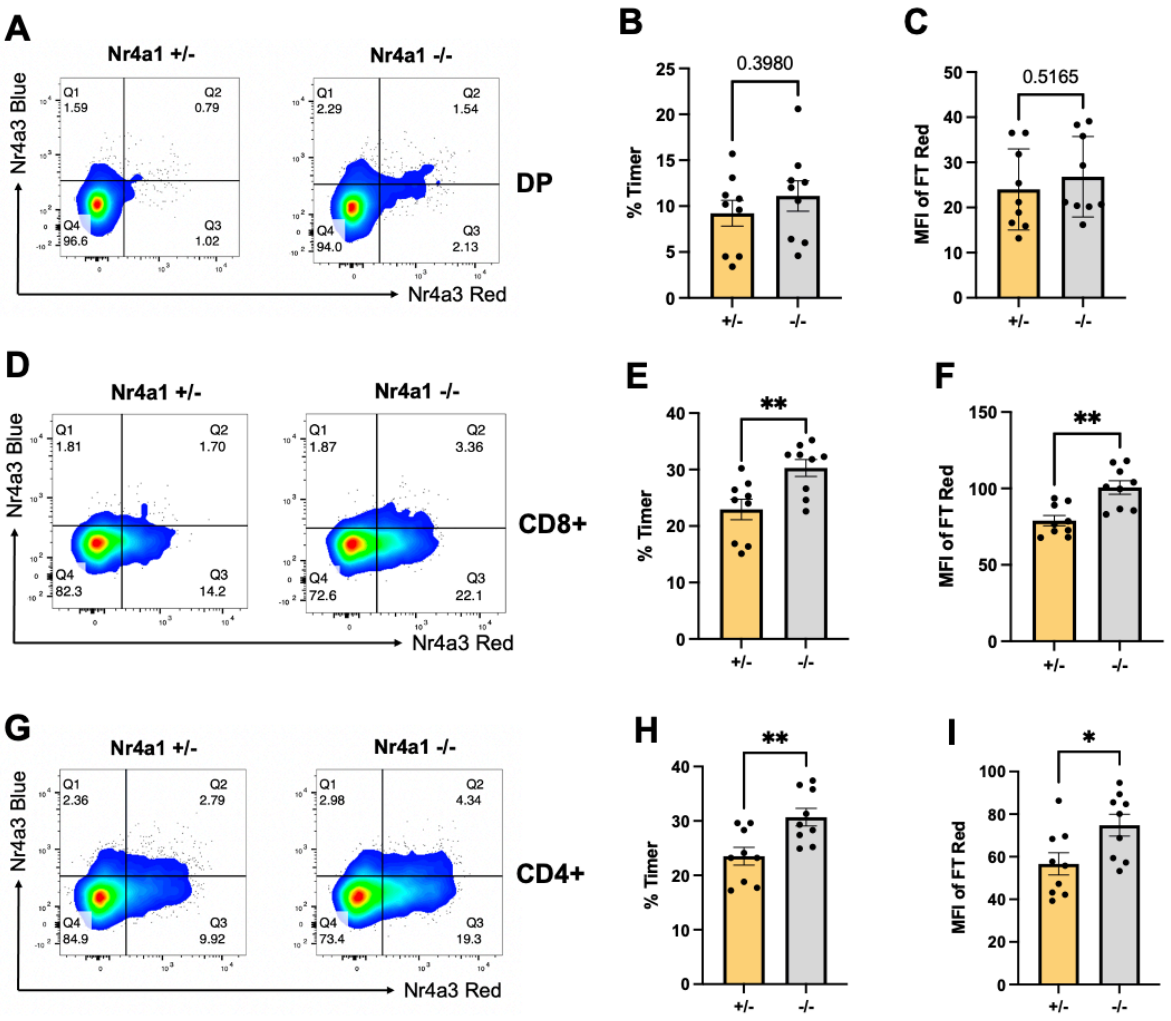


Figure 12. *Ex Vivo* Analysis of Double Positive, CD8+ and CD4+ T Cells from Whole Thymus

A) Flow cytometry plot of double positive (DP) thymocyte Timer protein expression. B) Bar chart showing % Timer protein expression in DP thymocytes. C) Bar chart showing medium fluorescence intensity (MFI) of fluorescent timer (FT) red in DP thymocytes. D) Flow cytometry plot of CD8+ T cell Timer protein expression. E) Bar chart showing % Timer protein expression in CD8+ T cells. F) Bar chart showing MFI of FT red in CD8+ T cells. G) Flow cytometry plot of CD4+ T cell Timer protein expression. H) Bar chart showing % Timer protein expression in CD4+ T cells. I) Bar chart showing MFI of FT red in CD4+ T cells. Error bars represent mean ± SEM. Overall n=9, combined from 3 experiments of n=3. Statistical analysis by unpaired t-tests. Significance represented is as follows: *p=<0.05, **p=<0.01, ***p=<0.001, ****p=<0.0001. +/- denotes the presence of Nr4a1, -/- denotes the absence of Nr4a1.

Furthermore, T cells expressing both CD4 and CD25 receptors showed the most sizable difference in TCR signalling between the *Nr4a1* genotypes. Nr4a1-deficiency caused a dramatic increase in the overall expression of Nr4a3 Timer, and the most notable increase in MFI of Nr4a3 Timer red expression (Figure 13 A-C). When looking at the percentage of CD25, the CD4+CD25+ population of T cells had an overall significantly elevated expression of CD25 with loss of Nr4a1 (Figure 13 D-E).

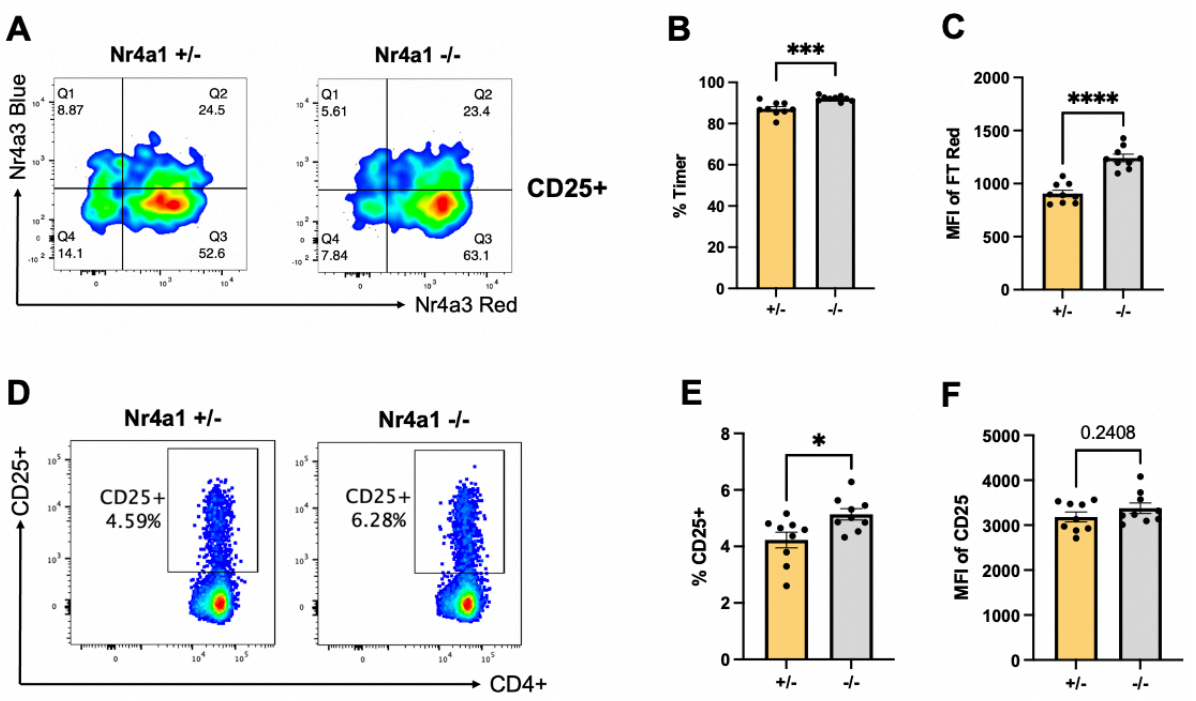


Figure 13. *Ex Vivo* Analysis of Tregs from Whole Thymus

A) Flow cytometry plot of CD4+CD25+ T cell Timer protein expression. B) Bar chart showing % Timer protein expression of CD4+CD25+ T cells. C) Bar chart showing MFI of FT red in CD4+CD25+ T cells. D) Flow cytometry plot showing CD25 expression of CD4+ T cells. E) Bar chart showing % CD25 expression in CD4+CD25+ T cells. Error bars represent mean ± SEM. Overall n=9, combined from 3 experiments of n=3. Statistical analysis by unpaired t-tests. Significance represented is as follows: *p=<0.05, **p=<0.01, ***p=<0.001, ****p=<0.0001. +/- denotes the presence of Nr4a1, -/- denotes the absence of Nr4a1.

4.2 Splenic T Cells Display No Phenotypic Changes Due to Loss of Nr4a1

Flow cytometry analysis comparing the *Nr4a3*-Tocky *Nr4a1+/-* genotype to *Nr4a3*-Tocky *Nr4a1-/-* of splenic T cells demonstrated no significant change in the TCR signalling of CD4SP or CDSP populations. Overall, Nr4a3 Timer expression and MFI of Nr4a3 Timer red expression saw no changes with loss of Nr4a1 (Figure 14 A-F).

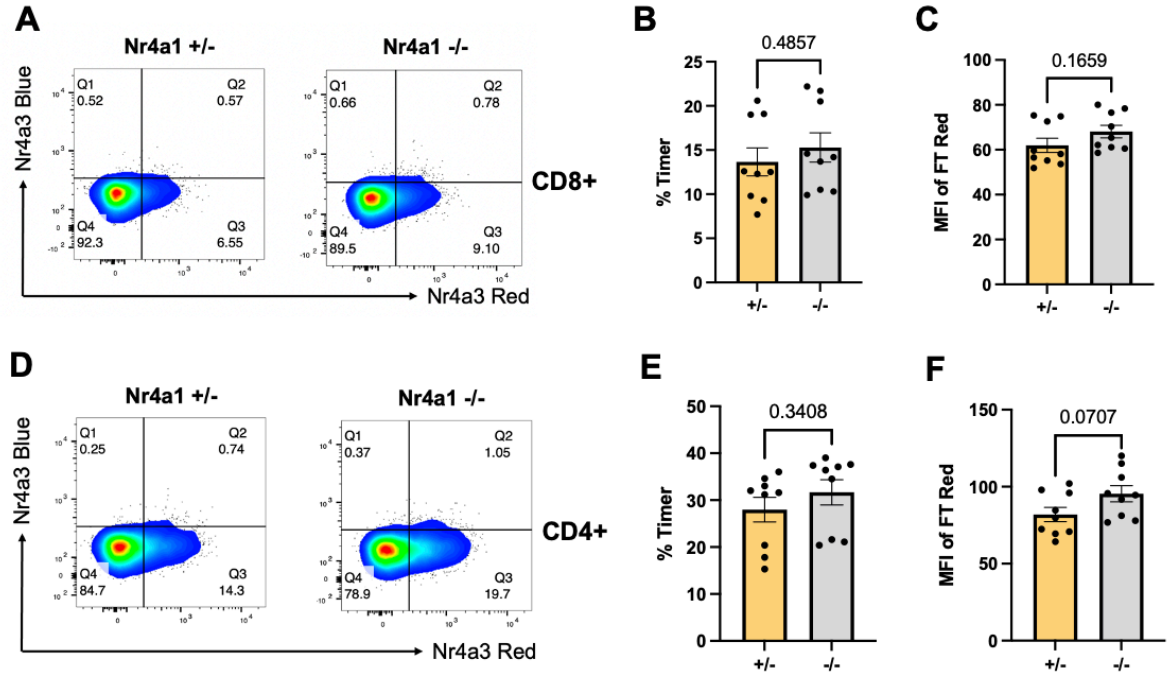


Figure 14. *Ex Vivo* Analysis of CD8+ and CD4+ T Cells from Whole Spleen
A) Flow cytometry plot of CD8+ T cell Timer protein expression. B) Bar chart showing % Timer protein expression in CD8+ T cells. C) Bar chart showing median fluorescence intensity (MFI) of fluorescent timer (FT) red in CD8+ T cells. D) Flow cytometry plot of CD4+ T cell Timer protein expression. E) Bar chart showing % Timer protein expression in CD8+ T cells. F) Bar chart showing MFI of FT red in CD8+ T cells. Error bars represent mean ± SEM. Overall n=9, combined from 3 experiments of n=3. Statistical analysis by unpaired t-tests. +/- denotes the presence of Nr4a1, -/- denotes the absence of Nr4a1.

Analysis of the CD4+CD25+ population displayed unaltered TCR signalling in the Nr4a1-deficient mice. Both the percentage of overall Nr4a3 Timer expression and MFI of Nr4a3 Timer red remained unaffected by loss of Nr4a1 (Figure 15 A-C). Moreover, the percentage of CD25 receptor expression also remained unaffected between the *Nr4a1* genotypes (Figure 15 D-E).

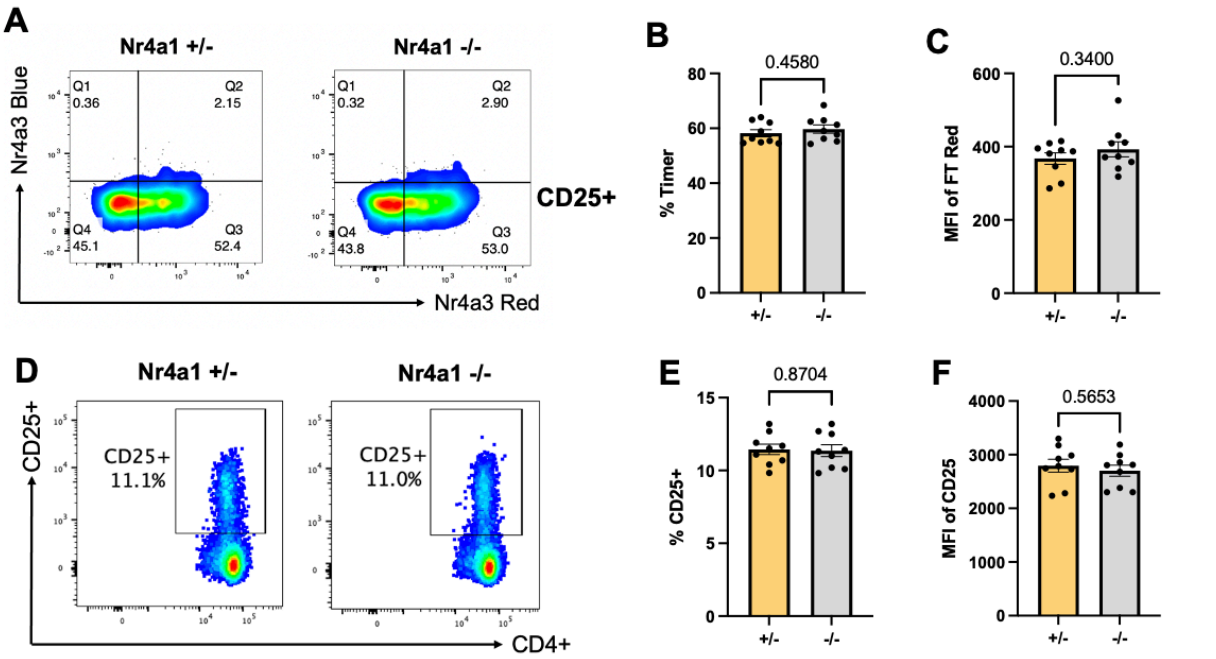


Figure 15. *Ex Vivo* Analysis of Tregs from Whole Spleen

A) Flow cytometry plot of CD4+CD25+ T cell Timer protein expression. B) Bar chart showing % Timer protein expression of CD4+CD25+ T cells. C) Bar chart showing MFI of FT red in CD4+CD25+ T cells. D) Flow cytometry plot showing CD25 expression of CD4+ T cells. E) Bar chart showing % CD25 expression in CD4+CD25+ T cells. Error bars represent mean ± SEM. Overall n=9, combined from 3 experiments of n=3. Statistical analysis by unpaired t-tests. +/- denotes the presence of Nr4a1, -/- denotes the absence of Nr4a1.

4.3 Functional Redundancy of Nr4a1 Revealed by RNA-Seq

Given the differences between thymic and splenic T cell populations, we wished to gain an unbiased insight into the potential non-redundant roles of Nr4a1 during T cell activation. Given the phenotype in CD4SP and CD4+ Treg precursors, we focused on analysing the role of Nr4a1 during naïve CD4+ T cell activation. Naïve CD4+ T cells of the spleen from *Nr4a3*-Tocky *Nr4a1+/-* and *Nr4a3*-Tocky *Nr4a1-/-* mice were isolated and activated for 0, 4 or 8 hours with anti-CD3 and anti-CD28 before RNA extraction and library preparation for RNA-sequencing (RNA-seq). RNA-seq analysis using R revealed one differentially expressed gene between the *Nr4a3*-Tocky *Nr4a1-/-* and *Nr4a3*-Tocky *Nr4a1-/-* genotypes, this being the *Nr4a1* gene for the time points 4- and 8-hours only.

Principle component analysis showed no separation in the overall transcriptomic make up between the *Nr4a1* genotypes, however, did illustrate the variation in gene expression of the T cells at the 0-, 4- and 8-hour time points post-TCR stimulation (Figure 16).

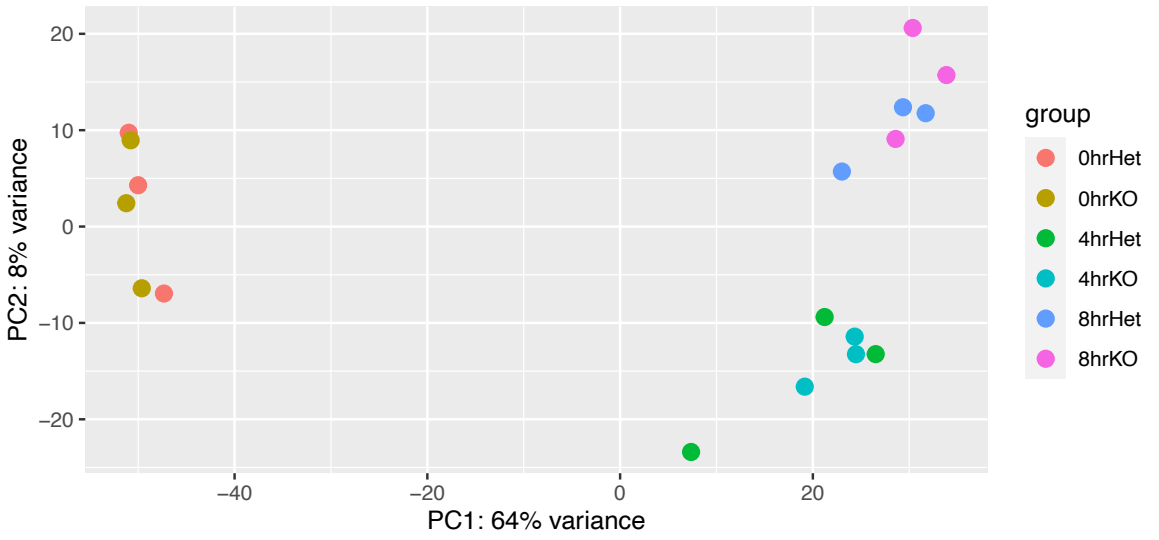


Figure 16. Principle Component Analysis (PCA) Plot Showing Variation in Gene Expression Whole spleens of *Nr4a3*-Tocky *Nr4a1+/-* and *Nr4a3*-Tocky *Nr4a1-/-* mice were processed, naïve CD4+ T cells were isolated and activated. After 0-, 4- and 8- hours of activation, RNA was extracted from cells. The plot in this figure shows the divergence and correlation of gene expression between *Nr4a3*-Tocky *Nr4a1+/-* and *Nr4a3*-Tocky *Nr4a1-/-* mice at their respective time-points post-stimulation. Each sample has been assigned a colour for this graphical representation; the colour key can be found on the right of the PCA plot. The colour key can be found to the right of the PCA plot, where "Het" represents the *Nr4a3*-Tocky *Nr4a1 +/-* (heterozygous) mice, and "KO" represents the *Nr4a3*-Tocky *Nr4a1-/-* (knockout) mice. Overall, n=3, from one experiment.

Generation of a heatmap, as shown in Figure 17, allowed interpretation of the upregulation and downregulation of specific genes between the *Nr4a1* genotypes at the 0-, 4- and 8- hour time points post-activation. The genes focused on for this experiment were *Nr4a1*, *Nr4a2*, *Nr4a3*, *IL2RA*, *IL-2*, *IFN-g*, *TNFRSF4* (*OX40*), *ICOS* and *CD69*, as these are well-described markers of T cell activation. At 0-hours, the T cell activation markers remained low, illustrating the lack of TCR stimulation. At 4-hours post-TCR stimulation, the expression of all markers considerably rose, with no identifiable differences between the presence and absence of Nr4a1. The only exception is the expression of Nr4a1 in the mice that were Nr4a1-deficient, where there was less Nr4a1 expression compared to the *Nr4a1+/-* genotype. At the 8-hour

time point, there was downregulation in the *ICOS*, *CD69*, *Nr4a2* and *Nr4a3* genes in comparison to the 4-hour timepoint, whereas the remaining markers showed no change in expression. Again, no differences were seen between the *Nr4a1* genotypes for any of the T cell activation marker, except for the expression of Nr4a1 which was absent in the Nr4a1-deficient mice. Overall, this data is a clear presentation of the upregulation of genes following TCR stimulation, their peak expression timepoints, and the demonstration of functional redundancy during early T cell activation (Figure 17).

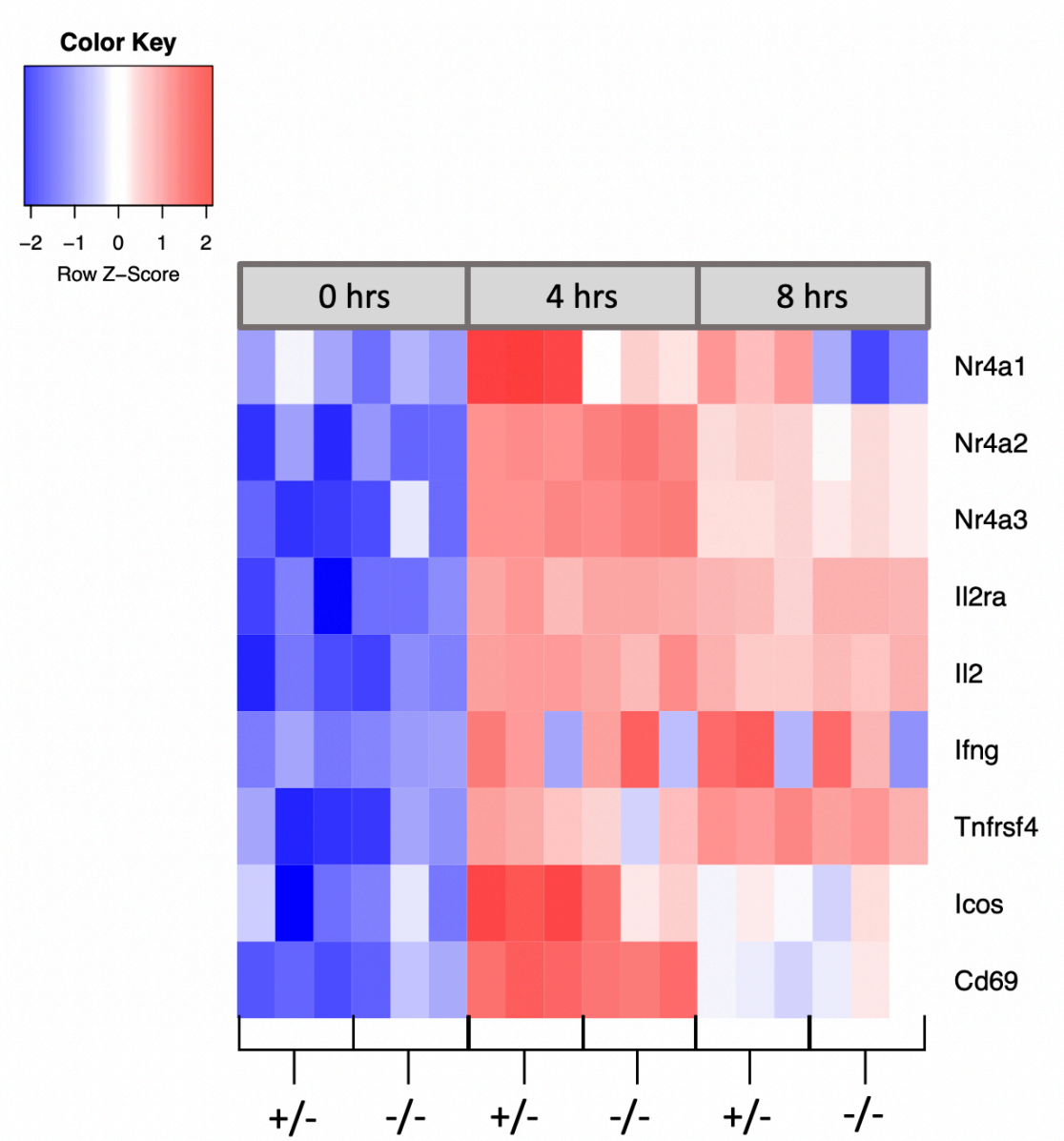


Figure 17. Heatmap Showing the Expression of Nr4a1 and Classical T Cell Activation Markers

Whole spleens of *Nr4a3*-Tocky *Nr4a1+/-* and *Nr4a3*-Tocky *Nr4a1-/-* mice were processed, and naïve CD4+ T cells were isolated and activated *in vitro*. After 0-, 4- and 8- hours of activation, RNA was extracted from cells. The map in this figure shows upregulation and downregulation of gene expression between *Nr4a3*-Tocky *Nr4a1+/-* and *Nr4a3*-Tocky *Nr4a1-/-* mice at their respective time-points post-stimulation. Brighter red colouring represents higher gene expression, while brighter blue colouring represents lower gene expression; the colour key is at the top right of the heat map. Overall, n=3, from one experiment. +/- denotes the presence of Nr4a1, -/- denotes the absence of Nr4a1.

4.4 Nr4a1-Deficient Naïve CD4+ T Cells Show Unchanged Expression of Strong TCR Markers Following TCR Activation

To determine whether the results discussed in Chapter 4.1 and 4.2 were an accurate representation of Nr4a1-deficiency, or whether a potential indirect effect was being imposed by other cell types present in the microenvironment, T cells were purified prior to analysis of Nr4a1. Isolated naïve CD4+ T cells from a combination of spleens and lymph nodes were analysed by flow cytometry following 24-hours of TCR activation, comparing the *Nr4a3*-Tocky *Nr4a1+/-* genotype to *Nr4a3*-Tocky *Nr4a1-/-*.

The following TCR activation marker were used: CD25, CD69, ICOS, OX40 and GITR. CD25 and CD69 are both well-established markers of TCR stimulation, with CD25 also being an indicator of the differentiation of CD4+ T cells into Tregs. Previous research in the lab has identified an association of the *OX40*, *ICOS* and *GITR* genes with strong TCR signals (Elliot et al., 2021). We have therefore used these strong TCR markers in this project to investigate whether Nr4a1 could regulate the strength of TCR signals.

T cells activated for 24-hours exhibited high levels persistent TCR signalling, although no significant differences were found between the *Nr4a1* genotypes (Figure 18 A-C). The MFI of CD25 and CD69 were both at high levels, and the expression of CD25 and CD69 was unaltered in the Nr4a1-deficient mice (Figure 18 D-F). The markers associated with strong TCR signals, ICOS, OX40 and GITR, all displayed high levels of signalling, although no consequence was observed in response to Nr4a1-deficiency (Figure 18 G-L).

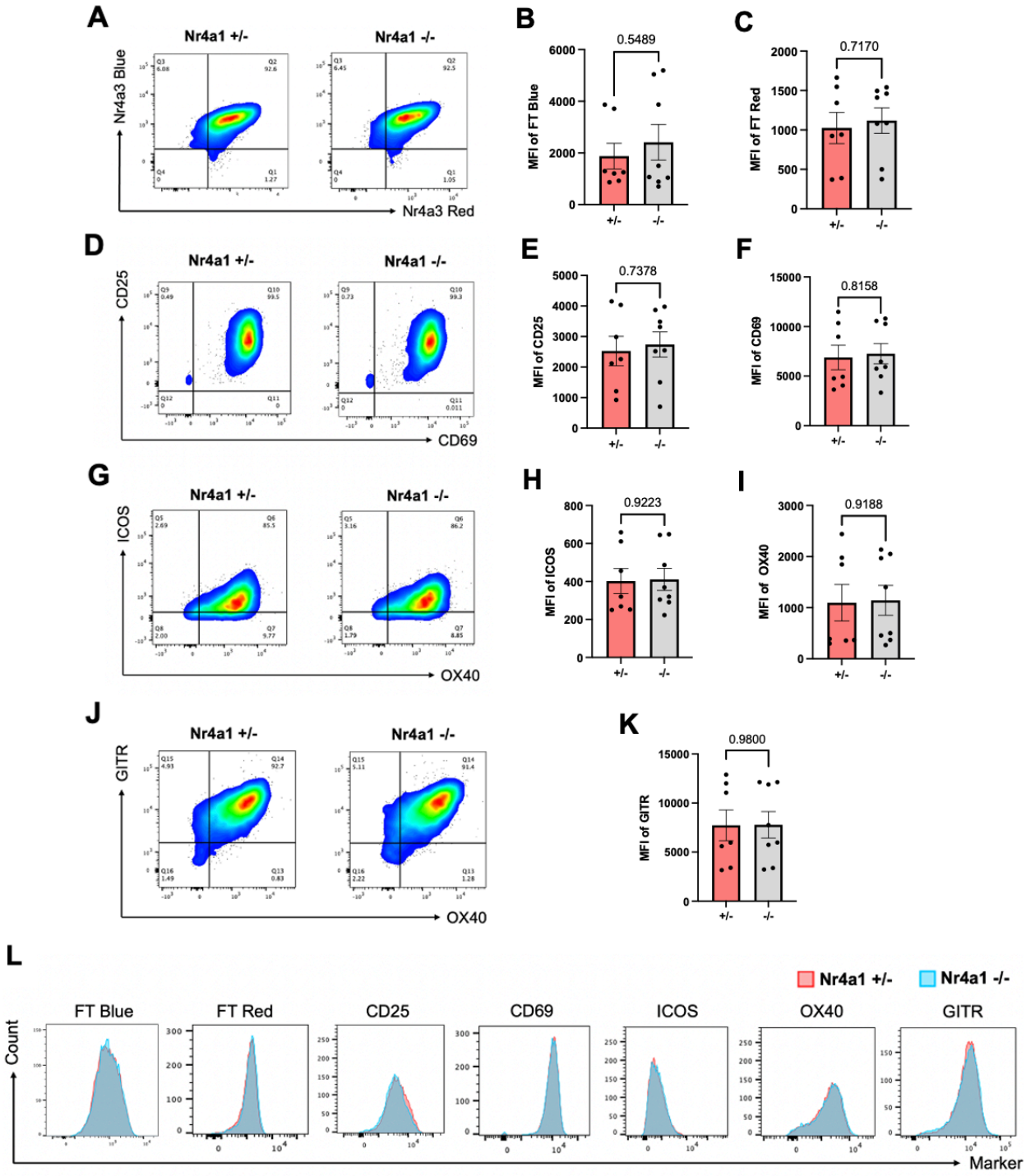

A
Nr4a1 +/-
Nr4a1 -/-
Nr4a3 Blue
Nr4a3 Red
B
0.5489
MFI of FT Blue
+/-
-/-
C
0.7170
MFI of FT Red
D
CD25
CD69
E
0.7378
MFI of CD25
F
0.8158
MFI of CD69
G
ICOS
OX40
H
0.9223
MFI of ICOS
I
0.9188
MFI of OX40
J
GITR
K
0.9800
MFI of GITR
L
Nr4a1 +/-
Nr4a1 -/-
FT Blue
FT Red
CD25
CD69
ICOS
OX40
GITR
Count
Marker

Figure 18. *In Vitro* Analysis of Isolated Naïve CD4+ T Cells from Spleen Following 24-Hours of Activation

A) Flow cytometry plot of Timer protein expression. B) Bar chart showing median fluorescence intensity (MFI) of fluorescence timer (FT) blue expression. C) Bar chart showing MFI of FT red expression. D) Flow cytometry plot of CD25 and CD69 expression. E) Bar chart showing MFI of CD25 expression. F) Bar chart showing MFI of CD69 expression. G) Flow cytometry plot of ICOS and OX40 expression. H) Bar chart showing MFI of ICOS expression. I) Bar chart showing MFI of OX40 expression. J) Flow cytometry plot of GITR and OX40 expression. K) Bar chart showing MFI of GITR expression. L) Histograms showing comparison of marker expression between *Nr4a3*-Tocky *Nr4a1-/-* and *Nr4a3*-Tocky *Nr4a1-/-* mice. Error bars represent mean ± SEM. Overall n=7, combined from 3 experiments of the following: n=2, n=2 and n=3. Statistical analysis by unpaired t-tests. +/- denotes the presence of Nr4a1, -/- denotes the absence of Nr4a1.

4.5 CD3+CD28 Activation of Naïve CD4+ T Cells Show Higher Concentration of IL-2 When Compared to CD3-Only Activation, But Loss of Nr4a1 Causes No Effect on IFN-g or IL-2 Concentration

Previous activation had incorporated signal 1 and signal 2. We investigated whether the effects of Nr4a1 may be more apparent under conditions of only anti-CD3 stimulation. Isolated naïve CD4+ T cells from a combination of spleen and lymph nodes were activated for 24- and 48-hours with either anti-CD3 functional-grade antibody on its own, or a combination of anti-CD3 and anti-CD28 functional grade antibodies. Supernatant was collected at the end of the respective time points and analysed by ELISA for IL-2 and IFN-γ cytokines. Both *Nr4a3*-Tocky *Nr4a1+/-* and *Nr4a3*-Tocky *Nr4a1-/-* mice were used to analyse the effect of Nr4a1-defiency in this experiment.

Following 24-hours of CD3-only activation, the naïve CD4+ T cells produced minor levels of IL-2, although this increased sharply in cells activated by a combination of CD3 and CD28. Neither condition displayed an effect in response to Nr4a1-deficiency (Figure 19 A). 48-hour activation of the T cells showed uniformity with the

results of the 24-hour activation period, with an increase again occurring in IL-2 production in CD3 and CD28 activation compared to CD3-alone, and no differences found between the *Nr4a1* genotypes (Figure 19 B).

After 24-hours of CD3-only activation, levels of IFN-γ were extremely low, with a slight elevation with seen in IFN-γ in response to CD3 and CD28 activation (Figure 19 C). After 48-hours of CD3-only activation, the levels of IFN-γ were higher in comparison to 24-hours of activation, but not to a significant degree (Figure 19 D). The production of IFN-γ in combined CD3 and CD28 activation remained constant following both 24-hour and 48-hour activation of the T cells. Overall, no significant effects were found in IFN-γ production between the two activation conditions or between the *Nr4a1* genotypes following both 24- and 48-hours of activation.

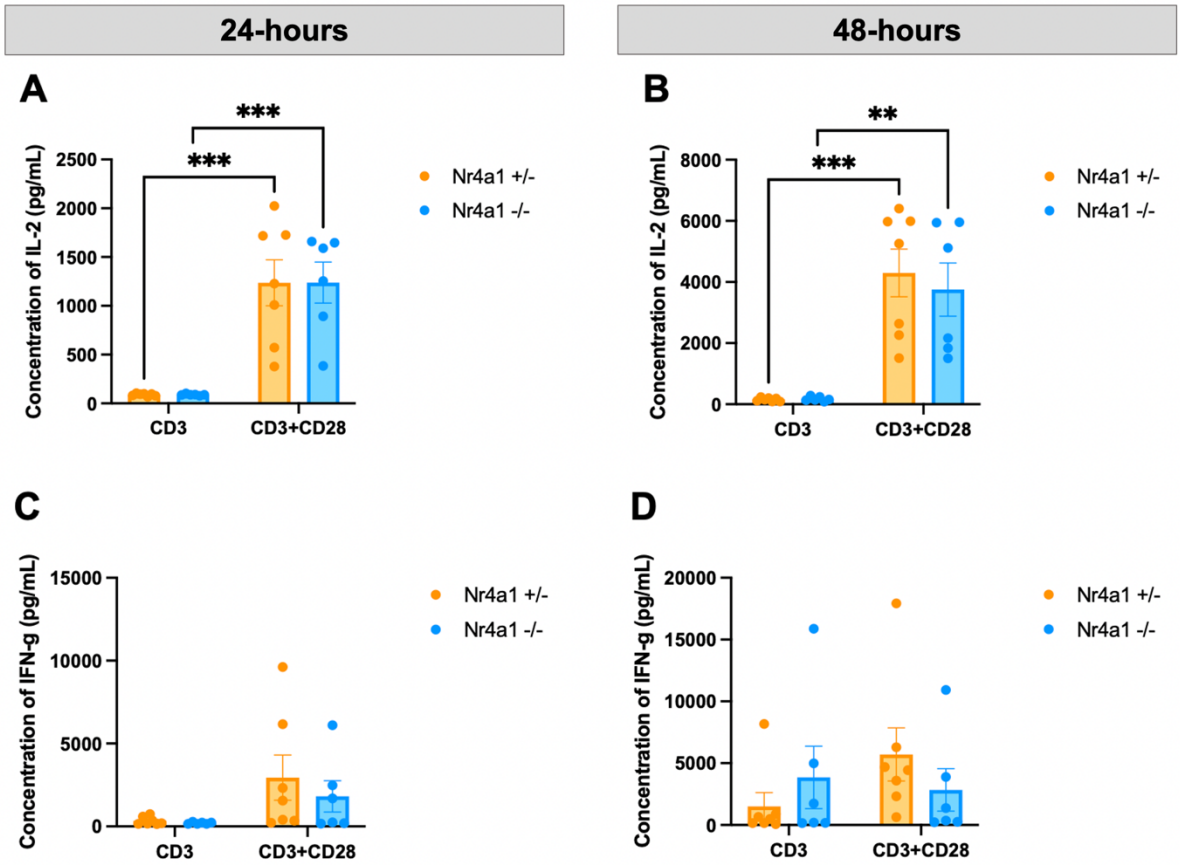


Figure 19. Concentrations of Cytokines Following CD3-only or CD3+CD28 Activation

Whole spleens of *Nr4a3*-Tocky *Nr4a1+/-* and *Nr4a3*-Tocky *Nr4a1-/-* mice were processed, naïve CD4+ T cells were isolated and activated with either anti-CD3 on its own or in combination with anti-CD28. After 24- and 48- hours of activation, supernatant was collected and ELISAs were run. A) Bar chart showing concentration of interleukin-2 (pg/mL) after 24-hours of activation. B) Bar chart showing concentration of interleukin-2 (pg/mL) after 48-hours of activation. C) Bar chart showing concentration of interferon-gamma (pg/mL) after 24-hours of activation. D) Bar chart showing concentration of interferon-gamma (pg/mL) after 48-hours of activation. Error bars represent mean ± SEM. Overall n=6 or n=7, combined from 3 experiments of the following: n=3, n=2 and n=2 (for Nr4a1+/-) and n=1 (for Nr4a1-/-). Statistical analysis by two-way ANOVA with Tukey's multiple comparison test. Significance represented is as follows: *p=<0.05, **p=<0.01, ***p=<0.001, ****p=<0.0001. +/- denotes the presence of Nr4a1, -/- denotes the absence of Nr4a1.

4.6 Proliferation of T Cells is Boosted in the Absence of Nr4a1

Given the redundancy of Nr4a1 in the activation of peripheral T cells, we returned to examine whether Nr4a1 could exert T cell developmental stage-specific effects. Given that thymic T cell subsets were enriched for Nr4a3+ cells, but this was not seen in the periphery, we wanted to investigate proliferation and apoptosis rates. In order to explore the potential for Nr4a1 to promote proliferation of cells, staining for Ki-67 was completed. The Ki-67 protein is only expressed within cells during the active G1, S, G2 and mitosis phases of the cell cycle, although is not present during the resting G0 phase of the cell cycle, making it a specific and powerful marker of cellular growth and proliferation (Scholzen and Gerdes, 2000).

Looking at the thymic DP T cells that are yet to form a TCRαβ complex, shown as DP TCRβ - in Figure 20 A-B, a slight increase in proliferation was observed with loss of Nr4a1. The same effect was seen in DP T cells that possess a TCRαβ complex, shown as DP TCRβ + in Figure 20 C-D. For thymic CD8+ T cells, there was again a slight rise in the Ki-67 present in the Nr4a1-deficient mice in comparison to the heterozygous *Nr4a1* genotype (Figure 20 E-F). However, none of these results reached significance.

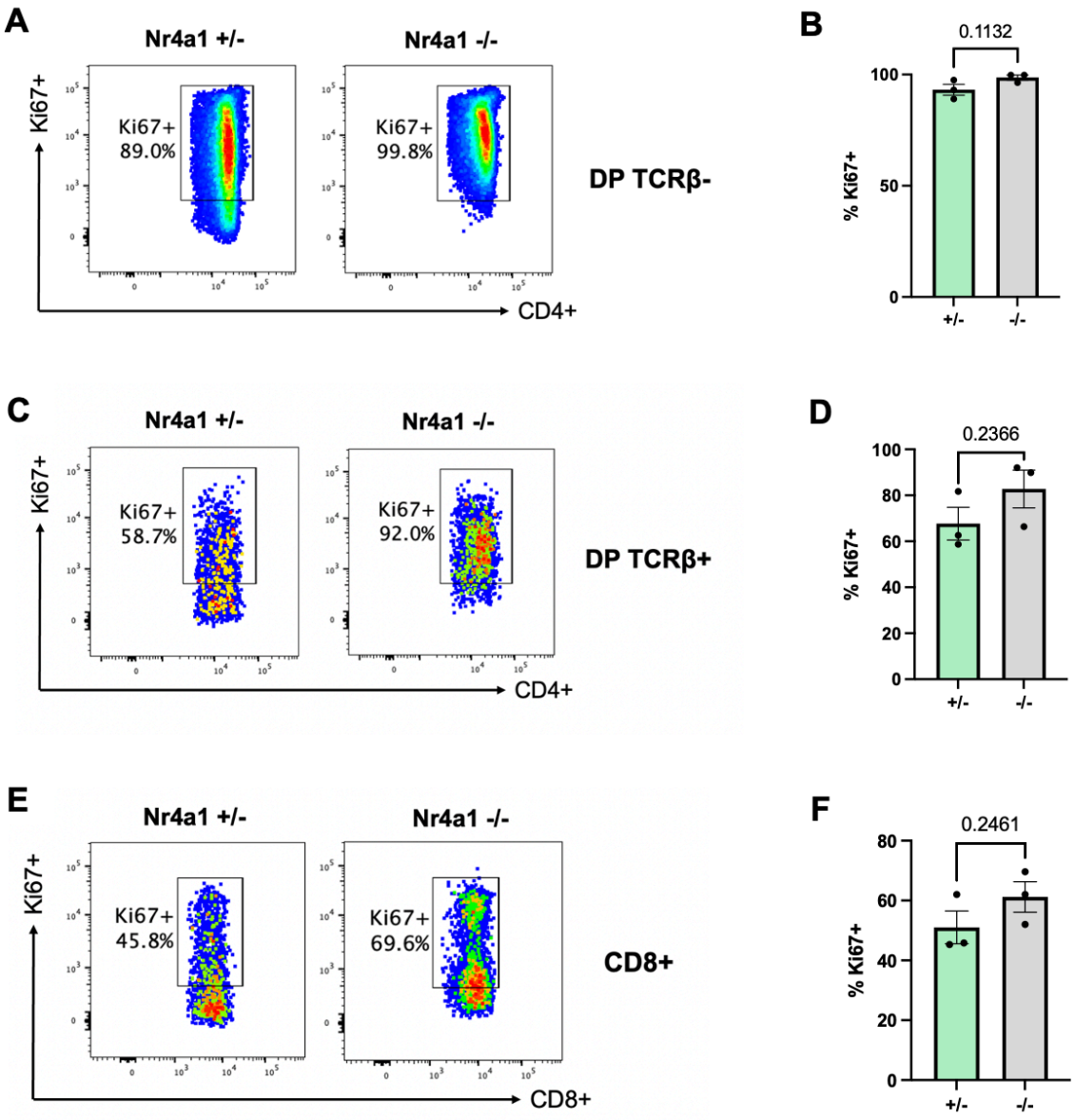


Figure 20. Proliferation Analysis of Double Positive and CD8+ T Cells from Whole Thymus

A) Flow cytometry plot of DP TCRβ - T cell Ki-67 expression. B) Bar chart showing the number of DP TCRβ- T cells expressing Ki-67. C) Flow cytometry plot of DP TCRβ + T cell Ki-67 expression. D) Bar chart showing the number of DP TCRβ + T cells expressing Ki-67. E) Flow cytometry plot of CD8+ T cell Ki-67 expression. F) Bar chart showing the number of CD8+ T cells expressing Ki-67. Error bars represent mean ± SEM. Overall n=3, from one experiment. Statistical analysis by unpaired t-tests. Significance represented is as follows: *p=<0.05, **p=<0.01, ***p=<0.001, ****p=<0.0001. +/- denotes the presence of Nr4a1, -/- denotes the absence of Nr4a1.

Further CD25 and FoxP3 staining was completed for analysis of specific Treg populations within the thymus. Overall, within thymic CD4+ T cells, percentage CD25 expression was again significantly increased in the Nr4a1-deficient mice, supporting the results shown in Figure 13. Further analysis of thymic CD4+ T cells revealed no changes in percentage FoxP3 expression due to loss of Nr4a1 (Figure 21).

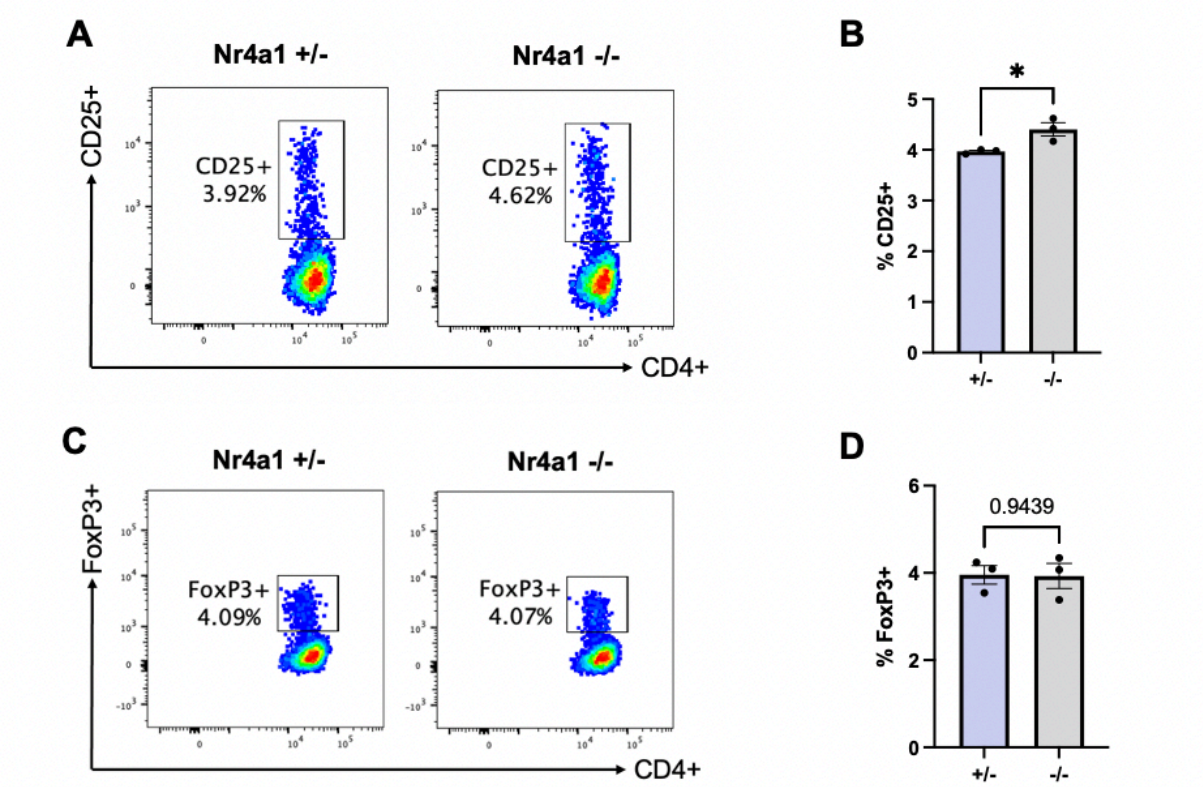


Figure 21. Analysis of CD25 and FoxP3 Expression in Thymic T Cells
A) Flow cytometry plot of DP CD4+ T cell CD25 expression. B) Bar chart showing % CD25 expression of CD4+ T Cells. C) Flow cytometry plot of DP CD4+ T cell FoxP3 expression. D) Bar chart showing % FoxP3 expression of CD4+ T Cells. Error bars represent mean ± SEM. Overall n=3, from one experiment. Statistical analysis by unpaired t-tests. Significance represented is as follows: *p=<0.05, **p=<0.01, ***p=<0.001, ****p=<0.0001. +/- denotes the presence of Nr4a1, -/- denotes the absence of Nr4a1.

Focusing on the Treg populations of CD4+ T cells in the thymus, CD4+CD25+FoxP3-, CD4+25-FoxP+ and CD4+CD25+FoxP3+ Tregs were shown to have a minor increase in Ki-67 expression due to loss of Nr4a1, although this difference was not statistically significant (Figure 22).

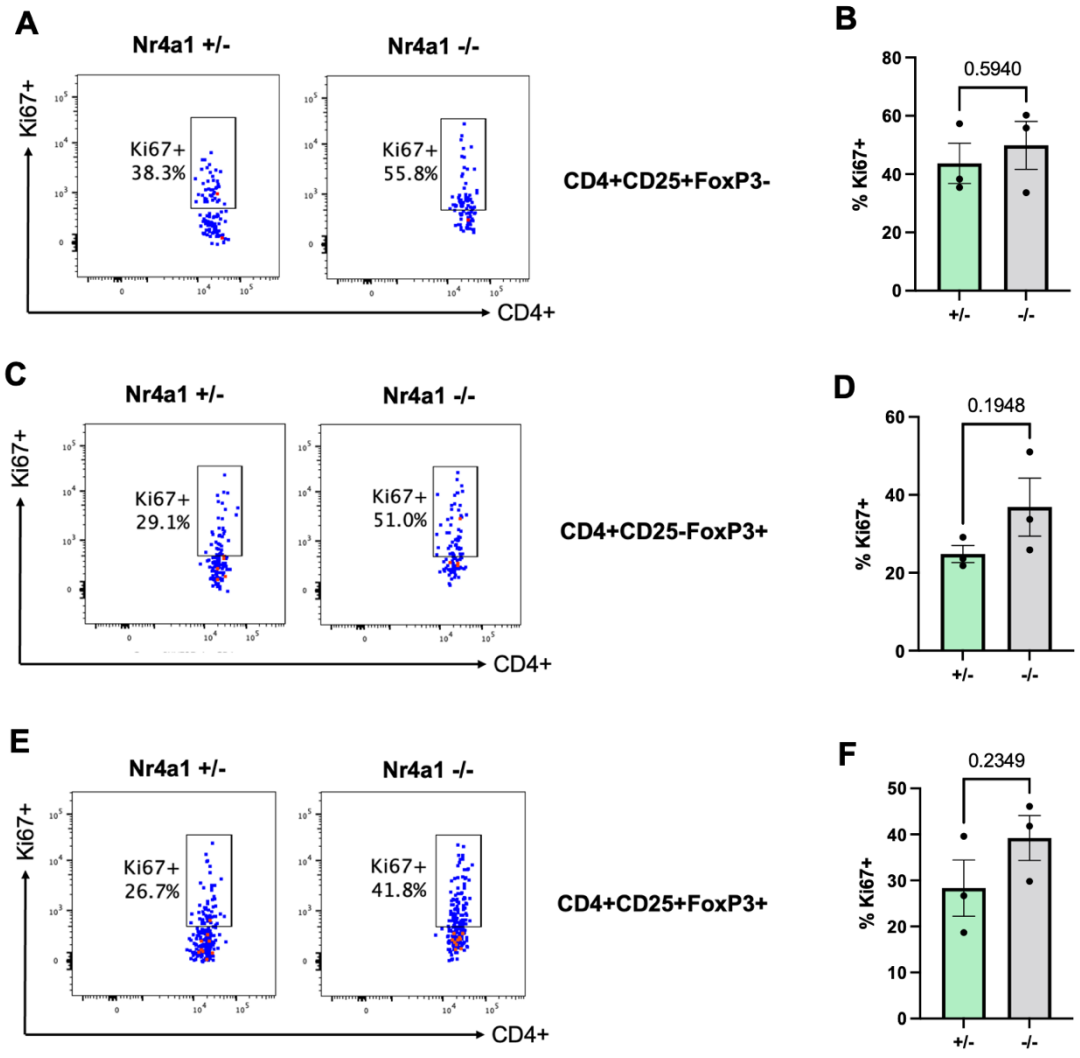


Figure 22. Proliferation Analysis of Tregs from Whole Thymus

A) Flow cytometry plot of CD4+CD25+FoxP3- T cell Ki-67 expression. B) Bar chart showing the number of CD4+CD25+FoxP3- T cells expressing Ki-67. C) Flow cytometry plot of CD4+CD25-FoxP3+ T cell Ki-67 expression. D) Bar chart showing the number of CD4+CD25-FoxP+ T cells expressing Ki-67. E) Flow cytometry plot of CD4+CD25+FoxP3+ cell Ki-67 expression. F) Bar chart showing the number of CD4+CD25+FoxP3+ T cells expressing Ki-67. Error bars represent mean ± SEM. Overall n=3, from one experiment. Statistical analysis by unpaired t-tests. Significance represented is as follows: *p=<0.05, **p=<0.01, ***p=<0.001, ****p=<0.0001. +/- denotes the presence of Nr4a1, -/- denotes the absence of Nr4a1.

We again performed CD25 and FoxP3 staining in order to establish any effects of Nr4a1-deficiency within peripheral T cells of the spleen. Although not significant, both the percentage CD25 and percentage FoxP3 expression in splenic CD4+ T cells dropped with the loss of Nr4a1 (Figure 23).

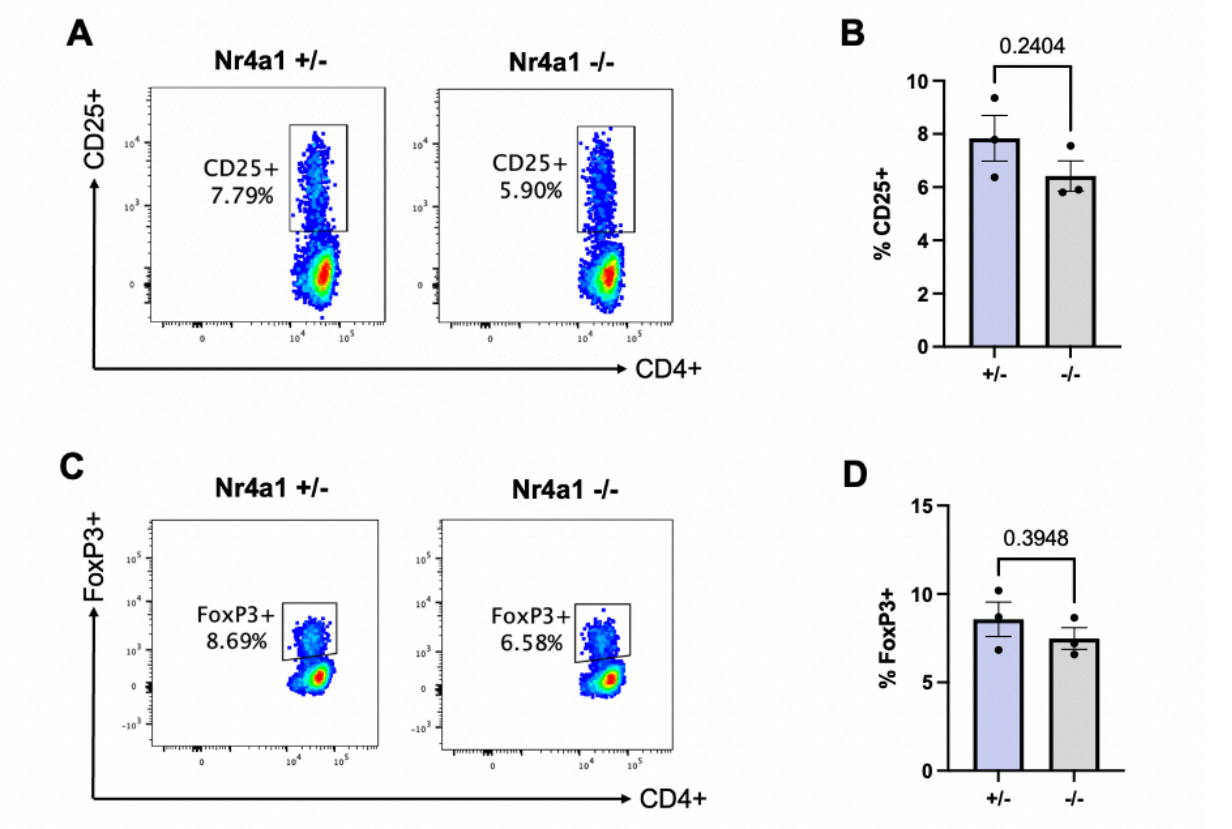


Figure 23. Analysis of CD25 and FoxP3 Expression in Splenic T Cells
A) Flow cytometry plot of DP CD4+ T cell CD25 expression. B) Bar chart showing % CD25 expression of CD4+ T Cells. C) Flow cytometry plot of DP CD4+ T cell FoxP3 expression. D) Bar chart showing % FoxP3 expression of CD4+ T Cells. Error bars represent mean ± SEM. Overall n=3, from one experiment. Statistical analysis by unpaired t-tests. Significance represented is as follows: *p=<0.05, **p=<0.01, ***p=<0.001, ****p=<0.0001. +/- denotes the presence of Nr4a1, -/- denotes the absence of Nr4a1.

In the spleen, although slightly trending upwards with loss of Nr4a1, none of the T cell populations had a significant change in their Ki-67 expression. This includes splenic CD8+, CD4+CD25+FoxP3-, CD4+CD25-FoxP3+ and CD4+CD25+FoxP3+ T cells (Figure 24).

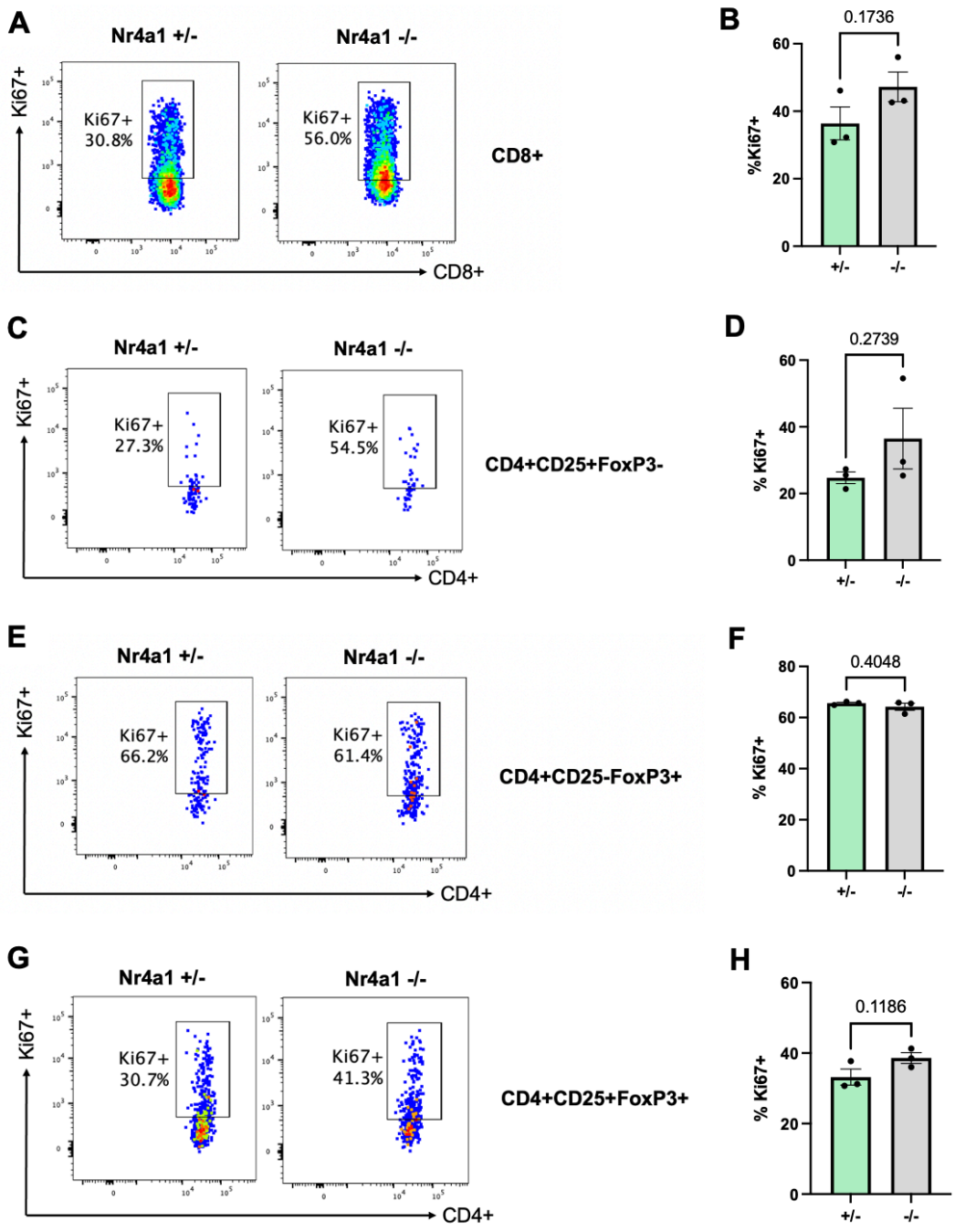


Figure 24. *Ex Vivo* Analysis of CD8+ T cells and Tregs from Whole Spleen

A) Flow cytometry plot of CD8+ T cell Ki-67 expression. B) Bar chart showing the number of CD8+ T cells expressing Ki-67. C) Flow cytometry plot of CD4+CD25+FoxP3- T cell Ki-67 expression. D) Bar chart showing the number of CD4+CD25+FoxP- T cells expressing Ki-67. E) Flow cytometry plot of CD4+CD25-FoxP3+ cell Ki-67 expression. F) Bar chart showing the number of CD4+CD25-FoxP3+ T cells expressing Ki-67. G) Flow cytometry plot of CD4+CD25+FoxP3+ cell Ki-67 expression. H) Bar chart showing the number of CD4+CD25+FoxP3+ T cells expressing Ki-67. Error bars represent mean ± SEM. Overall n=3, from one experiment. Statistical analysis by unpaired t-tests. Significance represented is as follows: *p=<0.05, **p=<0.01, ***p=<0.001, ****p=<0.0001. +/- denotes the presence of Nr4a1, -/- denotes the absence of Nr4a1.

4.7 Annexin V Staining Reveals Higher Apoptotic Rate in Thymic and Peripheral CD4+, CD8+ and FoxP3+ T Cells

To examine the potential for Nr4a1 to initiate apoptosis in T cells, annexin V and viability dye were used. Viability dye is a great detector of the presence of dead cells. As it is an amine-reactive dye, viability dye can enter the disrupted cell membrane of dead cells and irreversibly react with amines in the cytoplasm of the cell (Perfetto et al., 2010). Annexin V staining allows for the identification of cells that are in the process of apoptosis. In the early stages of apoptosis, there is a conformational change to the plasma membrane of a cell, resulting in the exposure of phosphatidylserine (PS), which annexin V can bind specifically (van Engeland et al., 1998). The combination of viability dye and annexin V staining allows for the detection of cells which are still alive, are confirmed dead, or are beginning apoptosis.

For the DP TCRβ -, DP TCRβ + and CD8+ populations of T cells from the thymus, a trending increase in annexin V staining and therefore apoptosis was observed, although not reaching statistical significance (Figure 25).

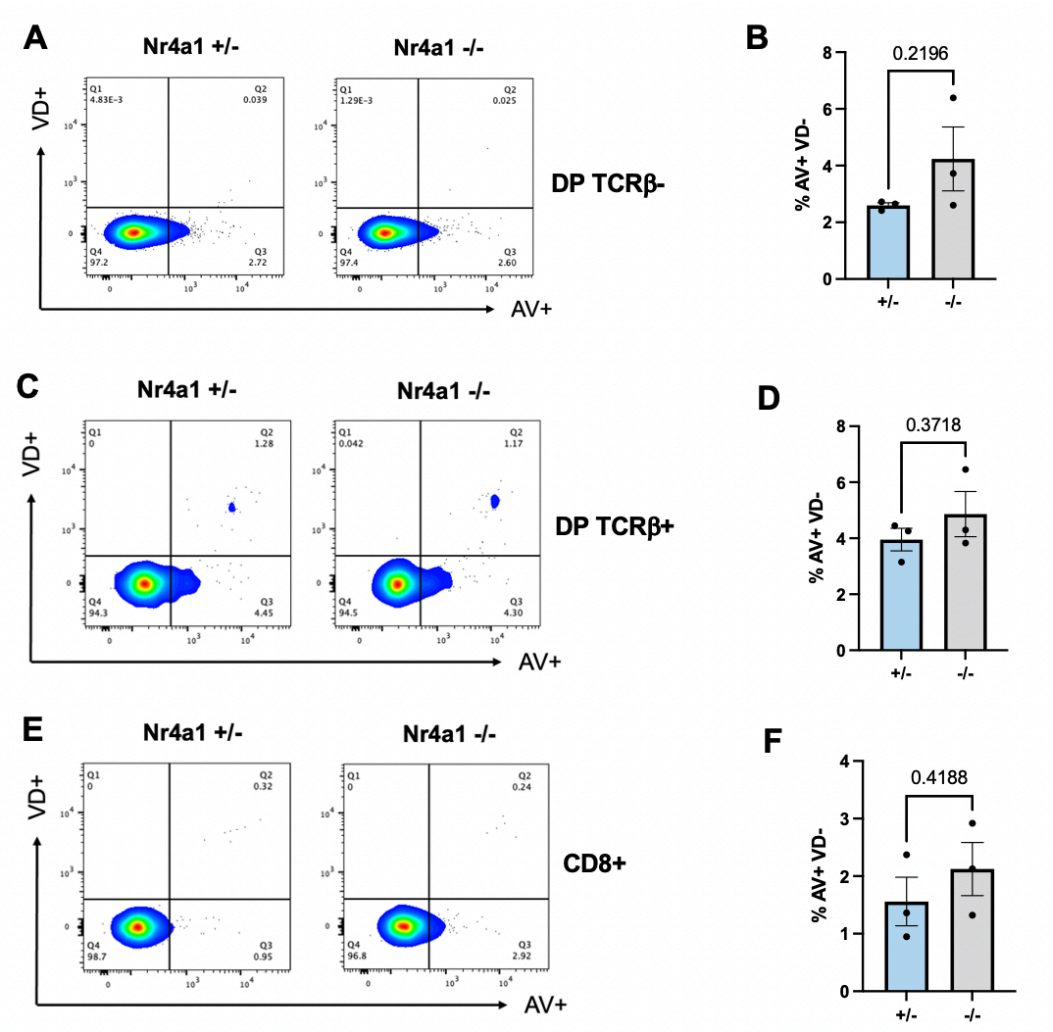


Figure 25. Apoptosis Analysis of Double Positive T Cells and CD8+ T cells from Whole Thymus

All bar charts represent the cell numbers within the lower right quarter of the quadrant drawn on each flow cytometry plot (AV+ and VD-). A) Flow cytometry plot of DP TCRβ - T cell staining by viability dye and Annexin V. B) Bar chart showing the number of DP TCRβ - T cells stained for Annexin V, but not viability dye. C) Flow cytometry plot of DP TCRβ + T cell staining by viability dye and Annexin V. D) Bar chart showing the number of DP TCRβ + T cells stained for Annexin V, but not viability dye. E) Flow cytometry plot of CD8+ T cell staining by viability dye and Annexin V. F) Bar chart showing the number of CD8+ T cells stained for Annexin V, but not viability dye. Error bars represent mean ± SEM. Overall n=3, from one experiment. Statistical analysis by unpaired t-tests. Significance represented is as follows: *p=<0.05, **p=<0.01, ***p=<0.001, ****p=<0.0001. VD+ on x axis represents staining by viability dye, and AV+ on y axis represents Annexin V staining. +/- denotes the presence of Nr4a1, -/- denotes the absence of Nr4a1.

Annexin V staining of thymic Tregs increased in the CD4+CD25+FoxP3- and CD4+CD25-FoxP3+ populations in the absence of Nr4a1 (26 A-D). Additionally, the CD4+CD25+FoxP3+ Tregs displayed a considerable rise in annexin V staining and therefore apoptosis in the Nr4a1-deficient mice, with this being a statistically significant effect (26 E-F).

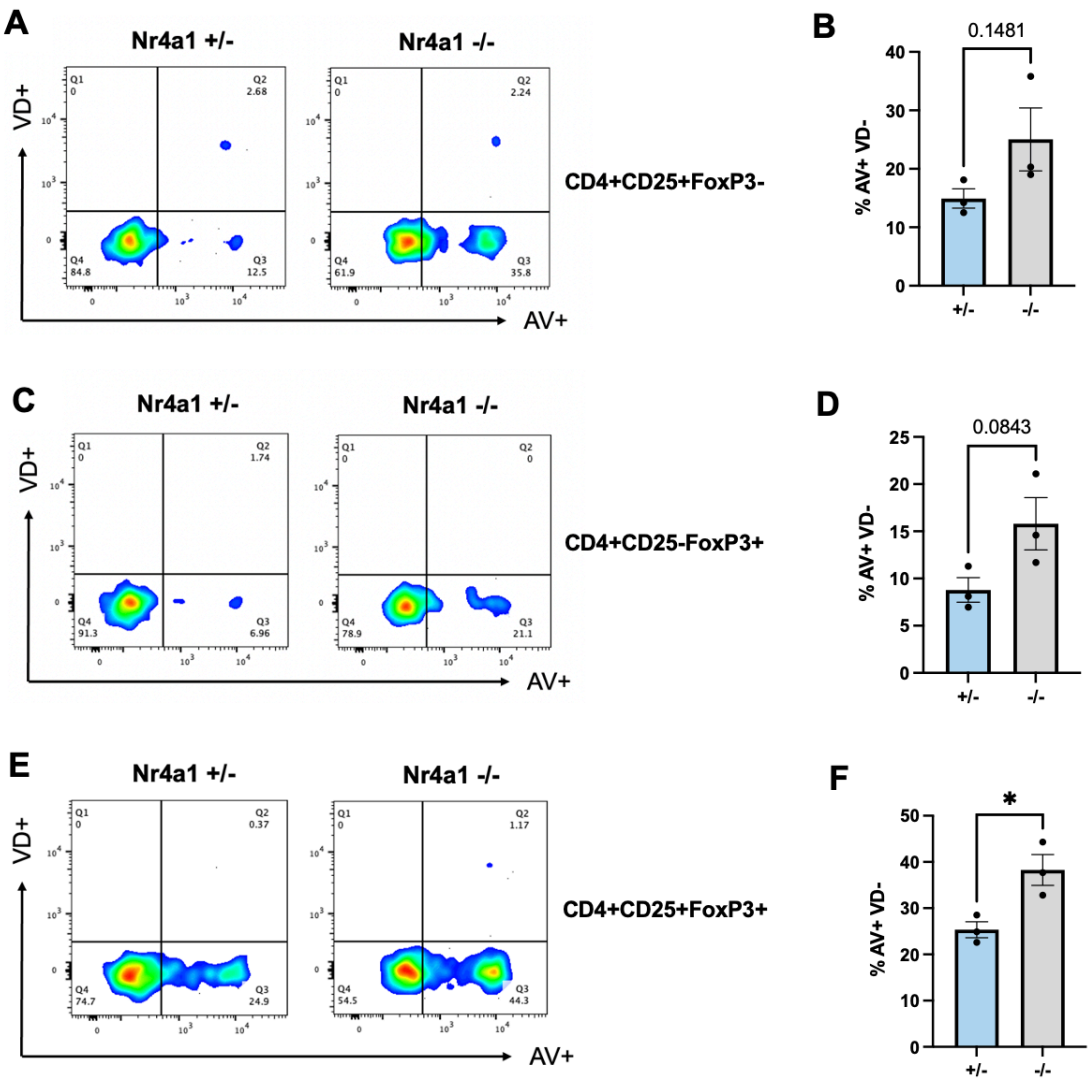


Figure 26. Apoptosis Analysis of Tregs from Whole Thymus

All bar charts represent the cell numbers within the lower right quarter of the quadrant drawn on each flow cytometry plot (AV+ and VD-). A) Flow cytometry plot of CD4+CD25+FoxP3- T cell staining by viability dye and Annexin V. B) Bar chart showing the number of CD4+CD25+FoxP3- T cells stained for Annexin V, but not viability dye. C) Flow cytometry plot of CD4+CD25-FoxP3+ T cell staining by viability dye and Annexin V. D) Bar chart showing the number of CD4+CD25-FoxP3+ T cells stained for Annexin V, but not viability dye. E) Flow cytometry plot of CD4+CD25+FoxP3+ T cell staining by viability dye and Annexin V. F) Bar chart showing the number of CD4+CD25+FoxP3+ T cells stained for Annexin V, but not viability dye. Error bars represent mean ± SEM. Overall n=3, from one experiment. Statistical analysis by unpaired t-tests. Significance represented is as follows: *p=<0.05, **p=<0.01, ***p=<0.001, ****p=<0.0001. VD+ on x axis represents staining by viability dye, and AV+ on y axis represents Annexin V staining. +/- denotes the presence of Nr4a1, -/- denotes the absence of Nr4a1.

Moreover, in response to the loss of Nr4a1, splenic CD8+ T cells revealed a slight decrease of annexin V staining, whereas the splenic Treg populations CD4+CD25+FoxP3- and CD4+CD25-FoxP3+ displayed a slight increase in annexin V staining (Figure 27 A-F). Finally, the CD4+CD25+FoxP3+ Tregs revealed no changes in annexin V between the *Nr4a1* genotypes (Figure 27 G-H).

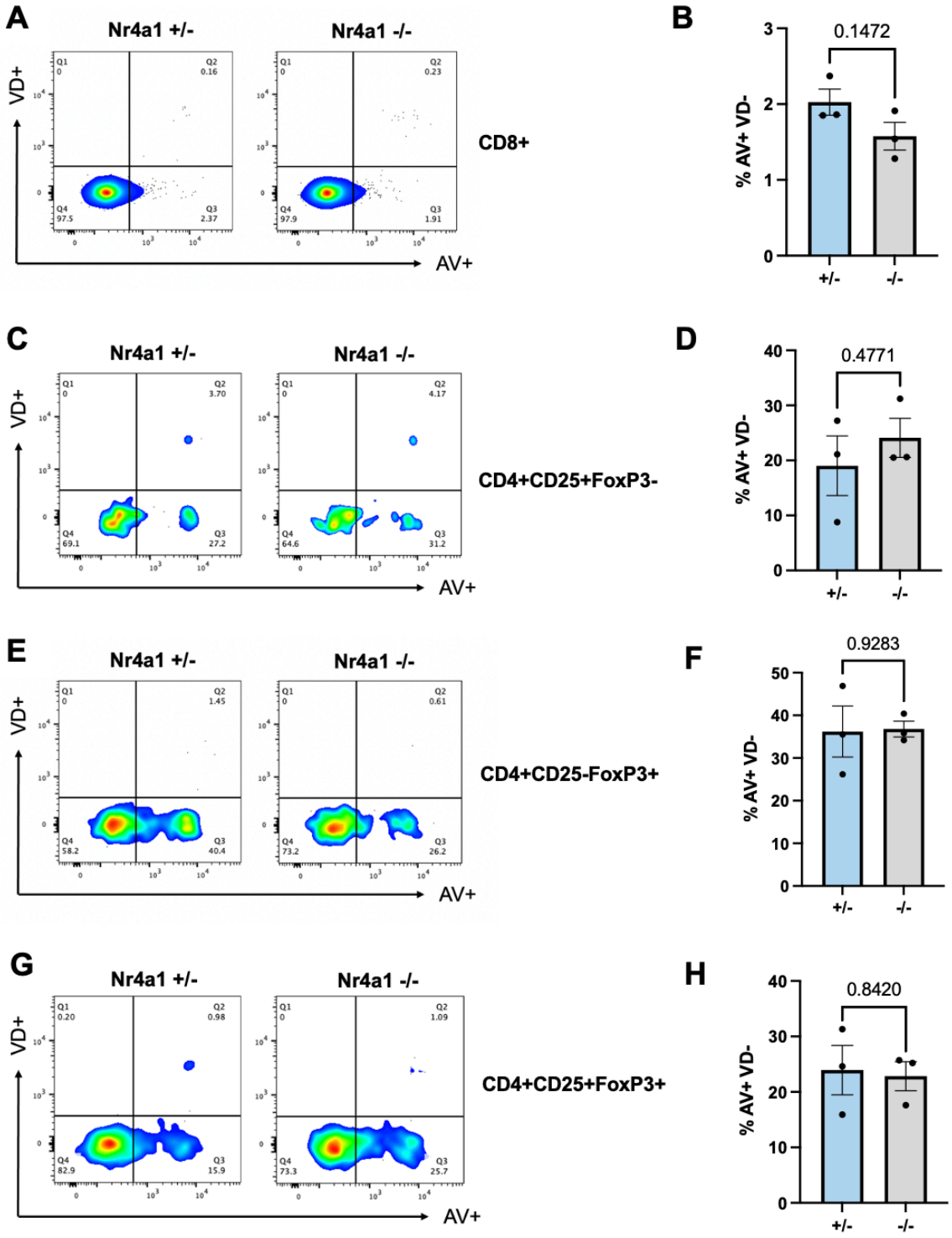

A
Nr4a1 +/-
Nr4a1 -/-
VD+
AV+
Q1 0
Q2 0.16
Q4 97.5
Q3 2.37
Q1 0
Q2 0.23
Q4 97.9
Q3 1.91
CD8+
B
0.1472
% AV+ VD-
3
2
1
0
+/-
-/-
C
Nr4a1 +/-
Nr4a1 -/-
VD+
AV+
Q1 0
Q2 3.70
Q4 69.1
Q3 27.2
Q1 0
Q2 4.17
Q4 64.6
Q3 31.2
CD4+CD25+FoxP3-
D
0.4771
% AV+ VD-
40
30
20
10
0
+/-
-/-
E
Nr4a1 +/-
Nr4a1 -/-
VD+
AV+
Q1 0
Q2 1.45
Q4 58.2
Q3 40.4
Q1 0
Q2 0.61
Q4 73.2
Q3 26.2
CD4+CD25-FoxP3+
F
0.9283
% AV+ VD-
50
40
30
20
10
0
+/-
-/-
G
Nr4a1 +/-
Nr4a1 -/-
VD+
AV+
Q1 0.20
Q2 0.98
Q4 82.9
Q3 15.9
Q1 0
Q2 1.09
Q4 73.3
Q3 25.7
CD4+CD25+FoxP3+
H
0.8420
% AV+ VD-
40
30
20
10
0
+/-
-/-

Figure 27. Apoptosis Analysis of CD8+ T Cells and Tregs from Whole Spleen

All bar charts represent the cell numbers within the lower right quarter of the quadrant drawn on each flow cytometry plot (AV+ and VD-). A) Flow cytometry plot of CD8+ T cell staining by viability dye and Annexin V. B) Bar chart showing the number of CD8+ T cells stained for Annexin V, but not viability dye. C) Flow cytometry plot of CD4+CD25+FoxP3- T cell staining by viability dye and Annexin V. D) Bar chart showing the number of CD4+CD25+FoxP3- T cells stained for Annexin V, but not viability dye. E) Flow cytometry plot of CD4+CD25-FoxP3+ T cell staining by viability dye and Annexin V. F) Bar chart showing the number of CD4+CD25-FoxP3+ T cells stained for Annexin V, but not viability dye. G) Flow cytometry plot of CD4+CD25+FoxP3+ T cell staining by viability dye and Annexin V. H) Bar chart showing the number of CD4+CD25+FoxP3+ T cells stained for Annexin V, but not viability dye. Error bars represent mean ± SEM. Overall n=3, from one experiment. Statistical analysis by unpaired t-tests. Significance represented is as follows: *p=<0.05, **p=<0.01, ***p=<0.001, ****p=<0.0001. VD+ on x axis represents staining by viability dye, and AV+ on y axis represents Annexin V staining. +/- denotes the presence of Nr4a1, -/- denotes the absence of Nr4a1.

5.Discussion

5.1 Loss of Nr4a1 Causes Expansion of Self-Reactive T Cells During Negative Selection in the Thymus

The initial findings of this project give insight into the potential redundancy of Nr4a1 in positive selection of developing T cells in the thymus. No changes in TCR signalling were observed in the DP populations with loss of Nr4a1, implying the redundancy of Nr4a1 for this process in T cell development. Although, the latter findings of this project using Ki-67 staining revealed a subtle increase in the proliferation of both DP TCRβ- and DP TCRβ+ T cells with loss of Nr4a1, suggesting a negative regulatory role for Nr4a1 during positive selection. Despite this, the results of this project regarding proliferation are very preliminary, and any subtle effects observable would need further investigation. Therefore, a potential role for Nr4a1 in positive selection remains unclear.

However, it does appear that Nr4a1 may have a more pronounced regulatory role during negative selection of developing T cells in the thymus. It was seen that in response to Nr4a1-deficiency, TCR stimulation was considerably increased in both CD4+ and CD8+ T cells derived from the thymus. This indicates there may be an increase in the numbers of CD4+ and CD8+ T cells appearing in the thymus. Alternatively, this could be due to an increase in the frequency and strength of their TCR signalling, or it may potentially be a result of both. It could be suggested that this is a result of defective negative selection, whereby more DP thymocytes are able to survive and mature into CD4+ or CD8+ T cells. With the purpose of negative selection being the clearance of DP thymocytes whose TCR have a threateningly

strong affinity for self-peptide, it is also conceivable that these surplus surviving CD4+ and CD8+ T cells are receiving more frequent and stronger TCR signals due to their heightened reactivity.

These findings are consistent with previous reports, whereby Nr4a1 is upregulated during both positive and negative selection in the thymus. Despite the expression of Nr4a1 during positive selection in the thymus, it has been previously shown that no impact on positive selection of thymocytes is caused by Nr4a1-defiency alone (Xue et al., 1997). Furthermore, it is well described that Nr4a1 is highly expressed in T cells during negative selection in the thymus (Hiwa et al., 2021; Mittelstadt, Taves and Ashwell, 2019; Odagiu et al., 2021). Although some previous studies have indicated no impairment of clonal deletion in response to the loss of Nr4a1 alone (Hiwa et al., 2021; Hu and Baldwin, 2015), another study found contradicting results with clear defects observed in clonal deletion when using Nr4a1-deficient mice (Fassett et al., 2012).

Within the thymus, the most notable change observed between the *Nr4a1* genotypes was in the upregulation of CD25. The CD25 receptor is a widely used marker of naturally occurring Tregs (Chen and Oppenheim, 2011). CD4+ T cells receiving strong TCR signals can differentiate into CD4+CD25+ Tregs. Marking the CD25 receptor can also be a sign of recent strong TCR activation, allowing CD25 to give valuable insight into the population of CD4+ T cells that are established Tregs, or are in the process of differentiating into Tregs. In this project, the significant rise in CD25 expression found in the Nr4a1-deficient mice suggests a boost in the numbers of CD4+ T cells differentiating into Tregs. The preliminary proliferation analysis of Tregs

supports this by indicating an increased proliferation of Tregs in the thymus with the absence of Nr4a1.

It appears that without Nr4a1, there is promotion of Treg differentiation from CD4+ T cells in the thymus. This could either be occurring directly due to the loss of Nr4a1 or could be a response to the abundance of self-reactive T cells surviving negative selection, as a prospective attempt to restore immune homeostasis.

5.2 Nr4a1 Exhibits Redundancy in Peripheral T Cells

The observations seen in TCR signalling of peripheral T cells highlight a seemingly redundant role of Nr4a1 in mature T cells. TCR signalling remained unaltered in the *ex vivo* flow cytometry analysis of splenic CD4+ and CD8+ T cells in response to loss of Nr4a1, surprisingly including the expression of the Treg marker CD25. It could be suggested that any effect Nr4a1 may have within mature T cells is normalised due to other contributing factors in the periphery of the thymus. This prompted the following experiments of this project in which naïve CD4+ T cells were isolated from spleens combined with lymph nodes and activated *in vitro*. Following 24-hours of activation, although TCR signalling was globally high, no defect was observed due to loss of Nr4a1.

The comparison of CD3-only and a combination of CD3 and CD28 for the activation of T cells gives an insight into how powerful co-stimulation is for TCR stimulation. CD3-only activation mimics thymic TCR stimulation (Levelt, Ehrfeld and Eichmann, 1993), whereas the combination of CD3+CD28 represents how T cells are activated in the periphery (Poltorak et al., 2020). In this project, CD3-only or CD3+CD28

activation of naïve CD4+ T cells was performed for 24- and 48-hour time periods. The T cells given both CD3 and CD28 produced a significantly higher amount of IL-2 at both time points. However, no significant difference in IL-2 was found due to Nr4a1-deficiency. In addition, following 24- and 48-hours of activation, the naïve CD4+ T cells exhibited no defects in their production of IFN-γ due to loss of Nr4a1. This further emphasises the consistency of the effector functions of peripheral T cells regardless of the presence of the Nr4a1 receptor.

With no distinct phenotype of Nr4a1 being found in peripheral T cells, it can be presumed that there is redundancy for Nr4a1 outside of the thymus. Further analysis supported this idea, whereby RNA was extracted from peripheral CD4+ T cells activated for 0-, 4- and 8-hours. RNA-seq revealed no differentially expressed genes between the *Nr4a1* genotypes, other than the explicit difference of the *Nr4a1* gene. However, the RNA extracted from sampled was at the lower end (10ng) giving a shallower read depth of the transcriptome.

Prior to this project, it has been described that Nr4a1 is upregulated in both CD8+ and CD4+ peripheral T cells (Odagiu et al., 2021). Expression of Nr4a1 was observed in CD8+ T cells, causing suppression in their effector function and cytokine production, and eventual adoption of the ‘exhausted’ dysfunctional state (Liu et al., 2019). Moreover, Nr4a1 has also been found to increase the frequency of CD8+ T cells in the periphery (Nowyhed et al., 2015). In CD4+ peripheral T cells, it has been demonstrated that loss of Nr4a1 increases proliferation and cytokine production, including IFN-γ (Liebmann et al., 2018). These previous reports suggest a non-redundant role for Nr4a1 in mature CD8+ and CD4+ T cells in the periphery,

however this notion contradicts the results of this project thus far. The differences could be explained by the timings and nature of the stimulus. In this system we used anti-CD3 and anti-CD28 that provide very strong stimulus and may obscure non-redundant effects of Nr4a1 that may exert during weak TCR signalling events such as those during thymic selection.

5.3 Absence of Nr4a1 Escalates the Apoptosis of T Cells

With previous reports illustrating the direct role Nr4a1 can play in the apoptosis of T cells, as discussed in Chapter 1.3.1, it was surprising to see little effect, and in some cases even an increase, in the apoptosis of T cells in the thymus and the periphery of Nr4a1-deficient mice. In particular, CD4+CD25+FoxP3+ Tregs from the thymus and the periphery exhibited a significant rise in apoptosis in response to loss of Nr4a1.

From this, it could be proposed that Nr4a1 may not have such a strong correlation with the induction of apoptosis, and that the other members of the Nr4a receptor family may also have apoptosis-inducing abilities and can compromise for the loss of Nr4a1, or perhaps death by apoptosis is occurring through another pathway altogether. It is important to mention how the production of IL-2 is essential for the survival of Tregs by maintaining their FoxP3 expression. A previous report explored the maintenance of the Treg niche size in the thymus (Weist et al., 2015). It was illustrated that IL-2 plays a major role in the preservation of the thymic Treg niche size, as mature Tregs will supress the development of new Tregs through competition of IL-2. This theory supports the findings of this project where no changes in IL-2 were found, regardless of the *Nr4a1* genotype. Knowing this, it is

possible that the cause of the increased apoptotic rate of thymic Tregs is due to the limited IL-2 available, causing loss of FoxP3 expression and induced apoptosis.

5.4 Potential Effects of Global Nr4a1-Deletion on T Cell Selection and Function

A crucial aspect of this project is the use of the *Nr4a3*-Tocky mice crossed onto an *Nr4a1-/-* background. Although invaluable for insight into TCR signalling for this project, the *Nr4a3*-Tocky/*Nr4a1-/-* mice have *Nr4a1* knocked out globally, meaning all cell types are Nr4a1-deficient. As previously described in Chapter 1.3, Nr4a1 is found widely throughout the body, and has been linked to essential processes in a multitude of different immune and non-immune cell types. A global Nr4a1 knockout will inevitably cause other immune cells as well as non-immune cells to function abnormally. For example, it is possible that the increased TCR signalling observed in the thymus is due to the promotion of antigen-presentation by APCs, as a result of their loss of Nr4a1. A recent report explored the effects of Nr4a1-deficiency on dendritic cell function. It was found that the loss of Nr4a1 in dendritic cells enhances inflammatory responses, causing increased antigen-presentation to T cells, and consequently escalating T cell proliferation (Tel-Karthaus et al., 2018). Additionally, another report looking into the effect of Nr4a1-deficiency on macrophages revealed that with loss of Nr4a1, thymic resident macrophages fail to develop normally, and lose their ability to clear apoptotic T cells during the selection events of the thymus. This ultimately was suggested to cause impairment of positive and negative selection of developing thymic T cells (Tacke, Nowyhed, Wu and Hendrick, 2013).

5.5 Discussion Summary

With Nr4a1 contributing to such a diverse range of cellular functions in the body, it is challenging to predict and pinpoint the cause of any effects appearing due to the absence of Nr4a1. The data gathered in this project suggest that with loss of Nr4a1, there is an expansion of T cells, potentially of specific clones, within the thymus. These T cells appear to only temporarily survive and promptly die by apoptosis, possibly due to the aberrant function of other cell types in response to global Nr4a1-deletion, or because of limited essential resources for their survival. Overall, it appears Nr4a1 may exert short-lived and T cell developmental stage-dependent effects within thymic T cells, but remains functionally redundant in peripheral T cells.

5.5 Future Directions

Future investigations to expand our knowledge of Nr4a1 in T cell activation and development would enable better understanding of the findings of this project. It remains unclear what the distinct roles of Nr4a1 may have within thymic T cells. Exploring the signalling of the TCR in thymic T cells at more detailed stages of their development, for instance during their TCRαβ complex formation, would give great insight into how Nr4a1 may affect these processes. Using *in vivo* anti-CD3 TCR stimulation, as opposed to *in vitro* anti-CD3 and anti-CD28 stimulation, may offer an exacerbated response of the thymic T cells and help tease out any minor effects occurring due to the loss of Nr4a1. Further analysis of thymic T cell IL-2 production would also be of interest to explore the theory behind the maintenance of a thymic Treg niche size.

Moreover, the initial experiments of this project were subject to some batch effects when combining data of several experiments. This was likely a consequence of a slight shift in the ages of the mice. Although the variability in ages in the mice were only by a few weeks, due to the rapid maturation rate of mice this can have a substantial impact on both the morphology of the thymus and the development and function of T cells. Future experiments exploring T cell ontogeny may help to pin-point time-dependant functions of Nr4a1.

Finally, it would be of great benefit to explore the use of Cre/l*ox* recombination to flox the *Nr4a1* gene, creating a mouse model with a T cell-specific Nr4a1-deficiency. This would allow analysis of T cell developmental and functional effects which are driven directly by the loss of Nr4a1 within T cells themselves, limiting the impact of other cell types in the analysis of Nr4a1 within T cells.

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