Early Growth Response genes 2 and 3 are potent inhibitors of T-bet function for Interferon Gamma production in T cells

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Declaration

I hereby declare that the research presented in this thesis is my own work, except otherwise specified, and has not been submitted for any other degree. All illustrations have been adapted from the mentioned references and created and designed by myself in Microsoft Word and Power Point.

SIINFEKL Tetramer staining and CFSE labelling and flow cytometry for these

experiments was conducted by Professor Ping Wang. All work regarding maintaining the mice, administering virus, sacrificing mice and monitoring disease was done by Dr Su-ling Li at the animal facility, Brunel University London

Randeep Singh

Abstract

Early growth response (Egr) gene 2 and 3 are genes encoding transcription factors important for maintaining immune homeostasis. Here we define a fundamental role of Egr2 and 3 to control T cell proliferation and differentiation of effector T cells. Egr2 and Egr3 deficiency in T cells resulted in impaired T cell proliferation, but hyper-activation and excessive differentiation of T cells in response to viral infection, while, conversely, sustained Egr2 expression enhanced proliferation, but severely impaired effector differentiation in to T helper (Th) subsets, such as, Th1 and Th17 subtypes. T-bet is important for differentiation of effector T cells in response to pathogen and in particular it is a master regulator for modulating the T helper 1 lineage specific differentiation programme. Although T-bet has been extensively studied in T cells, the regulation of T-bet function is less well known. We have now discovered that Egr2 and 3 are potent inhibitors for T-bet function in CD4 and CD8 effector T cells. Together with Egr2 and 3, T-bet is induced in naïve T cells by antigen stimulation, but the expression was reciprocally regulated by IFNγ, which inhibited Egr2 and 3, but promoted T-bet expression. The expression of Egr2 and 3 in CD4 T cells under T_H2 and T_H17 condition was essential to suppress T_H1 differentiation *in vitro*. In response to viral infection, sustained Egr2 expression in T cells profoundly inhibited differentiation of effector cells, while Egr2 and 3 deficient T cells produced excessive levels of IFNγ. We found that both Egr2 and 3 can directly interact with the Tbox domain of T-bet, block its DNA binding and inhibit T-bet mediated production of IFNγ. Thus, Egr2 and 3 are antagonists for T-bet function in effector T cells and essential for the control of T cell differentiation and immune pathology.

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Finally I would like to thank my mum, Kamaljit Kaur, brother Jagjeet (a.k.a. Captain Steve Rogers) and sisters Simran and Jas for their love and support. I am grateful for their encouragement in my decision to do this Ph.D. and their invaluable contribution towards it. I would particularly like to thank my mum for her blessings and being a truly wonderful human being. My mum has been my inspiration in life and has always been there to support me through all life's challenges.

Thank you all for sharing this journey with me.

Dedication

This thesis is dedicated to my father the late Balvinder Singh Matharu, my mum Kamaljit Kaur Matharu and siblings, Simran, Jagjeet and Jasdeep and our lovely dog Ice. I hope this work can be an inspiration for everyone to pursue their dreams and believe anything is possible.

Happy the man, and happy he alone
He who can call today his own:
He who, secure within, can say
Tomorrow do thy worst, for I have lived today
Be fair or foul or rain or shine
The joys I have possessed, in spite of fate, are mine.
Not heaven itself upon the past has power,
But what has been, has been, and I have had my hour.

By John Dryden

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List of Abbreviations

Ag	Antigen
Amp	Ampicillin
APC	Antigen Presenting Cell
BAC	Bacterial Artificial Chromosome
CCR	Chemokine receptor
CD	Cluster of Differentiation
cDNA	complementary Deoxyribonucleic Acid
CFSE	Carboxyfluorescein Succinimidyl Ester
CIA	Collagen Induced Arthritis
CNS	Conserved Non-coding Sequence
Co-IP	Co-immunoprecipitation
CRAC	Ca^{2+} release activated Ca^{2+} channel
cTECs	cortical Thymic Epithelial Cells
CTL	Cytotoxic T Lymphocytes
DAG	Diacylglycerol
DC	Dendritic Cell
DN	Double Negative
DNA	Deoxyribonucleic Acid
DP	Double Positive
EAE	Experimental Autoimmune Encephalomyelitis
EGR	Early Growth Response
ELISA	Enzyme Linked Immunosorbent Assay
EMSA	Electrophoretic Mobility Shift Assay
ER	Endoplasmic Reticulum
FACS	Fluorescence Activated Cell Sorter
FBS	Foetal Bovine Serum
FCS	Foetal Calf Serum
EGFP	Enhanced Green Fluorescence Protein
HEK	Human Embryonic Kidney cells
HS	Hypersensitivity Site
HSC	Haematopoietic Stem Cell
IBD	Irritable Bowel Disease

IDDM	Insulin Dependent Diabetes Melitus
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRES	Internal Ribosome Entry Site
IRF	Interferon Regulatory Elements
ITAMs	Immunoreceptor tyrosine-based activation motif
K2-3	Egr2 and Egr3 knockout mice
KO	Knockout
MES	Mouse Embryonic Stem cells
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
MS	Multiple Sclerosis
mTECs	medullary Thymic Epithelial Cells
NK	Natural Killer cell
NKT	Natural Killer T cell
OVA-VV_{WR}	Ovalbumin – Vaccinia Virus Western Reserve
PAMP	Pathogen associated molecular patterns
PCR	Polymerase Chain Reaction
PFU	Plaque-forming Unit
PMA	Phorbol 12-myristate 13-acetate
PRR	Patter Recognition Receptors
RA	Rheumatoid Arthritis
Rag	Recombination activating gene
RNA	Ribonucleic Acid
RSS	Recombination Signal Sequences
RT	Reverse Transcriptase
RT-PCR	Real Time – Polymerase Chain Reaction
RV	Retrovirus
SDS-PAGE	Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis
SP	Single Positive
STAT	Signal Transducer and Activator of Transcription
T_{CM}	Central Memory

T_{EM}	Effector Memory
TCR	T Cell Receptor
Tg	Transgenic
TGF	Transforming Growth Factor
T_H	T helper
TLR	Toll Like Receptors
TNF	Tumour Necrosis Factor
TSP	Thymic Seeding Progenitors
TSS	Transcriptional Start Site
V(D)J	Variable (Diversity) Joining
WT	Wild-type
ZF	Zinc Finger

Chapter 1 - Introduction

1.1 The basic function of the immune system

The immune system is a complex system that provides protection from infection by viruses and other pathogenic microorganisms. Failure of the immune system to function effectively results in several acquired and congenital immune-deficiencies. The immune system consists of two major pathways of action, namely the innate and adaptive immune response. Innate immunity is rapid and identical qualitatively and quantitatively every time a pathogen is encountered. In contrast, the hallmarks of an adaptive response include the generation of long-lived antigen specific cells after initial exposure to antigen and a rapid response upon encountering the antigen a second time (Parkin and Cohen, 2001).

1.1.1 Innate Immunity

The innate immune response upon encountering an antigen utilises several families of pattern recognition receptors to initiate intracellular signalling events that lead to a rapid immune response, to eliminate invading pathogens as a first line of defence. The cellular components of the innate system include; macrophages, monocytes, dendritic cells and natural killer (NK) cells. Earlier understanding of the innate immune system indicated a non-specific recognition of pathogens, however, the discovery of Toll Like Receptors (TLRs) proved otherwise. These germ line encoded pathogen recognition receptors (PRRs) have evolved to detect pathogen associated molecular patterns (PAMPs). PAMPs take the form of nucleic acids, proteins, lipids and lipoproteins derived from a wide range of pathogens such as bacteria, viruses, fungi and parasites (Kawai and Akira, 2010). The complement system represents a major part of the innate immune system, and it is composed of a complex network of plasma and membrane proteins that have the ability to elicit cytotoxic and inflammatory immune responses to infectious organisms (Dunkelberger and Song, 2010).

1.1.2 Adaptive Immunity

Extensive research in the mechanisms of adaptive immunity has shown this system to be a highly complicated network of cellular and genetic events. The adaptive arm of an immune response is dominated by a group of cells known as lymphocytes and there are two types, namely, B and T cells. Adaptive immunity

is mediated by immunoglobulins (Ig) and T cell receptors (TCRs), which are generated through the recombination of variable (V), diversity (D) and joining (J) gene segments (Tonegawa, 1983). V(D)J rearrangement occurs in B and T cell precursors and the process is mediated by recombination-activating gene 1 (RAG1) and RAG2 which encode lymphoid specific proteins. These two proteins cooperate and target specific sequences known as recombination signal sequences (RSSs) and introduce double strand breaks. This process generates the diversity that manifests in Ig and TCR mediated immune response (Gellert, 2002). B cells express Igs as either membrane bound receptors or in soluble form where they become antibody secreting plasma cells. This antibody-mediated immunity is also known as humoral immunity (Mauri and Bosma, 2012). The TCR although undergoing the same gene V(D)J somatic re-combination process, remains membrane bound.

The molecular definition of the TCR established it as being composed of α and β chains forming a heterodimer linked together by disulphide bonds (Dembic, *et al*., 1986). Another small subset of T cells were also found to contain γ and δ chains (Brenner *et al*., 1986). Further investigation in its structure revealed a non-covalent association of the TCR heterodimer with other distinct polypeptides; CD3γ, CD3δ and CD3ε and a homodimer of ζ. The CD3 complex subunits contain immunoreceptor tyrosine-based activation motifs (ITAMs) in their cytoplasmic domains. These cytoplasmic tails are paramount in eliciting intracellular signalling cascades due to the tyrosine based motifs that are phosphorylated upon TCR-antigen binding. TCRs composed of α and β heterodimers are functionally reliant on polymorphic major histocompatibility complex (MHC) class I and II expressed by antigen presenting cells. In contrast TCRs composed of γ and δ chains function independently of MHC class I and II molecules (Wucherpfennip *et al*., 2010).

1.2 T Cell Immunology

1.2.1 T cell development in the Thymus

T cells form a critical part of the adaptive immune response to a plethora of pathogens in existence. Their contribution to the adaptive arm of the immune system takes the form of a combination of different functions, including, they are important for immunological memory, regulation of macrophage function, control autoimmunity, assist B cells in making antibodies and CD4 T cells influence CD8 mediated responses. These are just some major roles attributed to T cells (Zhu *et al*., 2010).

T cells arise from haematopoietic stem cells (HSCs) originating in the bone marrow and develop into mature CD4 and CD8 T cells in the thymus. The thymus is composed of four distinct highly specialised regions that provide the appropriate microenvironment for the different developmental stages involved in generating single positive (SP) CD4 and CD8 cells. These regions are known as the subcapsular zone, the cortex, the medulla and the corticomedullary junction. These regions have different cellular compositions. The subcapsular zone is composed of cortical thymic epithelial cells (cTECs). The cortex contains a combination of cTECs, macrophages and fibroblasts and the medulla contains dendritic cells (DCs) and medullary thymic epithelial cells (mTECs). The endothelial cells in the corticomedullary junction facilitate the entry and exit of thymocytes to and from the blood (Koch and Radtke, 2011).

1.2.2 $CD4^-$ $CD8^-$ Double Negative to $CD4^+$ or $CD8^+$ Single Positive T cells

Early progenitors entering the thymus are known as thymic seeding progenitors (TSPs) and once they encounter the thymic environment, the cells progress through several stages of development into mature T cells. Early progenitors do not express the CD4 or CD8 surface molecules and are therefore known as double negative (DN) cells. The DN cells progress through several stages and eventually express both CD4 and CD8 together, known as double positive (DP) cells. Finally these cells develop into CD4 and CD8 single positive T cells. The earliest DN stage referred to as DN1, further differentiate into different subsets from DN1-DN4 and can be identified by the lack of CD4 and CD8 surface

expression and differential expression of CD44, CD25 and CD117. Figure 1.1 illustrates the development of T cells and their respective surface markers.

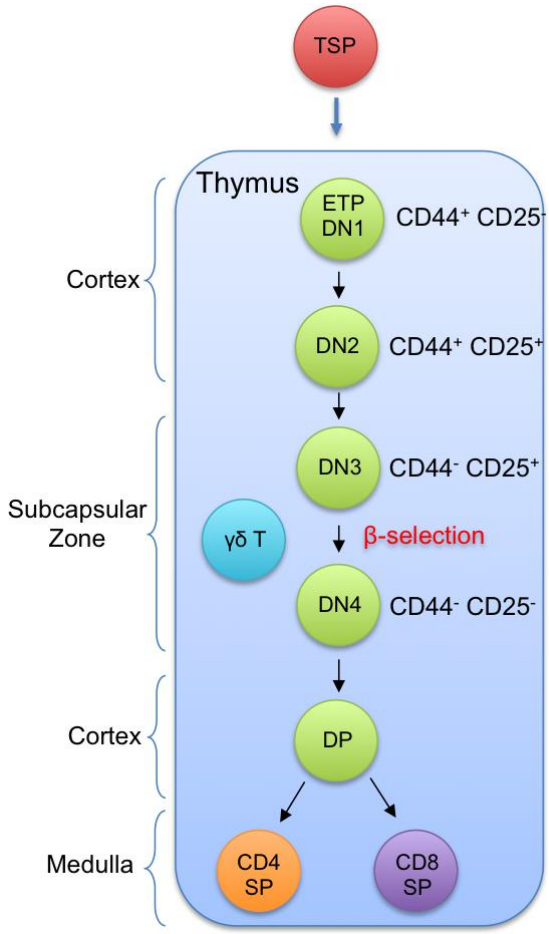

Figure 1.1 (left): T cell development in the thymus Overview of T cell development in the thymus. The diagram illustrates the maturation of T cells from its earliest thymic seeding progenitor (TSP) to mature single positive (SP) CD4 or CD8 T cells. Diagram adapted from Koch and Radtke 2011.

1.2.3 T cell negative and positive selection

During the developmental process there are critical checkpoints that are present to ensure there are not any developmental defects. The first critical checkpoint occurs at the DN1 stage and here an evolutionarily conserved signalling pathway, known as Notch signalling, inhibits alternative cell fates, such as myeloid and B cells. Once DN1 cells progress into the DN2 stage, further cell fates are inhibited and thus only have the potential to differentiate into NK cells. Further, *TCRγ, TCRδ* and *TCRβ* gene rearrangements are initiated at this stage of progression (Livak *et al*., 1999). DN2 cells are further distinguished by the level IL-7 receptor (IL-7R) expression, which are recognised as IL-7^{HIGH} and IL-7^{LOW} cells. Signalling through IL-7R leads to site-specific recruitment of histone

acetylases to the *TCRγ* locus. Therefore, IL-7R^{HIGH} cells are prone to differentiate into γδTCR T cells and IL-7R^{LOW} into αβTCR T cells (Kang *et al*., 2001). DN3 stage marks the specification of γδTCR and αβTCR -expressing T cells and β selection. Rearrangement at the γ, δ and β loci continue to generate a functional TCR and thus establishing γδ T cells fate with the aid of the transcription factor DNA binding protein inhibitor Id3 (Lauritsen *et al*., 2009). Signalling through a functional αβ pre -TCR is essential in a critical checkpoint known as β - selection. The pre-TCR should at this stage assume a correctly rearranged TCRβ chain, components of CD3 chain and a constant pre-TCR α chain (von Boehmer 2005).

Once the cells have reached the DN4 stage, they up-regulate CD4 and CD8 achieving a DP status and TCRα gene rearrangement is initiated to establish a functional αβTCR. Cell survival and differentiation into SP CD4 or CD8 is determined by TCR specificity and binding for major histocompatibility complex (MHC) ligands. Self-peptide MHC complexes presented to DP T cells undergo positive selection if they interact with intermediate voracity. Once the cells are selected, they commit to either CD4 or CD8 SP T cells and undergo negative selection. This process simply eliminates T cells with high affinity TCRs for self-antigens and ultimately prevents autoimmune pathology (Klien *et al*., 2009). The CD4 and CD8 SP T cells can now exit the thymus and into circulation.

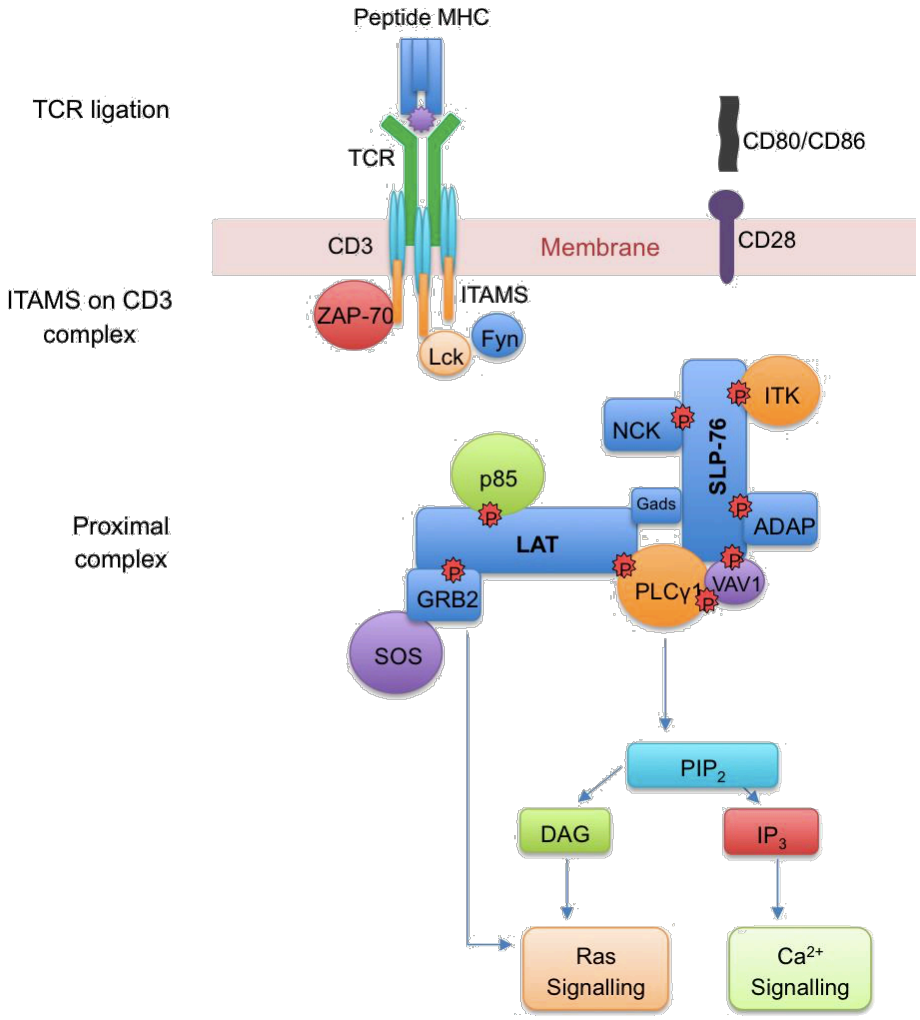

Figure 1.2: TCR Signalling Complex

Diagrammatic illustration of the TCR signalling complex. (Diagram adapted from Smith-Garvin *et al*., 2009)

1.2.4 TCR and antigen presentation

The TCR is a complex comprised of variable αβ or γδ chains associated with Cluster of Differentiation 3 (CD3) proteins. CD3 proteins are non-polymorphic in nature and exist as dimers of four polypeptides, (ε, γ, δ and ζ) identified as γε, ζζ and δε (Iwashima *et al*., 1994). The α and β chains together directly recognise peptide-MHC (pMHC) ligands. These chains are not transmembrane, therefore cannot transmit the signal to the internal components, unlike the CD3 molecule. CD3 dimers have transmembrane domains that are crucial for interaction with the internal machinery of the signalling cascade. Each of the cytoplasmic tails of the CD3 dimers contain immunoreceptor tyrosine-based

activation motifs (ITAMs), which are conserved sequences of peptides, that act as docking sites for other proteins inside the cell once the tyrosine residues on them are phosphorylated (Aivazian and Stern 2000). The CD4 and CD8 surface molecules assist the TCR in recognising pMHC class II and pMHC class I, respectively. The cytoplasmic segment of CD4 and CD8 is associated with the tyrosine kinase lymphocyte specific protein tyrosine kinase (Lck) (Figure 1.2).

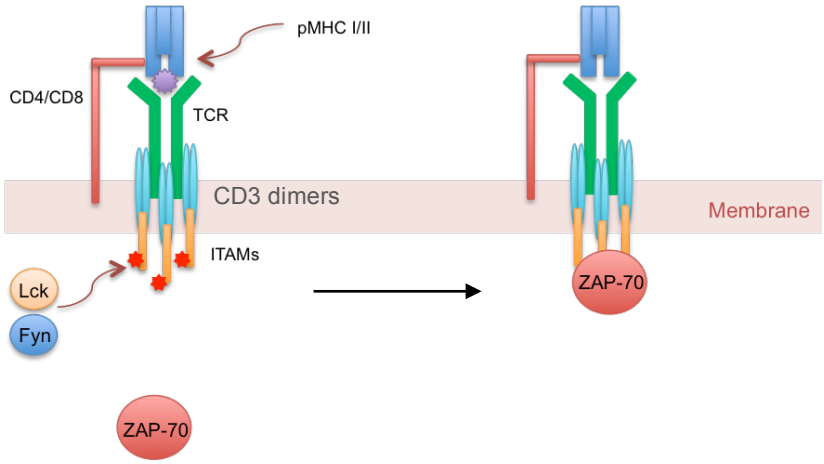

Figure 1.3: Tyrosine residues on the ITAMs phosphorylated by Lck and Fyn. This figure presents the earliest stage of TCR activation, where binding of αβ chains with pMHC promotes the phosphorylation of tyrosine residues on ITAMs, which leads to the recruitment of ZAP-70. (Diagram adapted from Smith-Garvin *et al*., 2009).

1.2.5 Initiation and progression of TCR signalling

Upon engagement of TCR with a high affinity pMHC, ITAMs on the CD3 complex are exposed to Lck and Fyn. Fyn is a proto-oncogene tyrosine protein kinase and both Fyn and Lck are members of the Src family of kinases, which phosphorylate the tyrosine residues on ITAMs. A portion of Lck is constitutively associated with the CD4 co-receptor (Wange and Samelson, 1996) therefore this brings Lck in close proximity to the CD3 ITAMs. The precise mechanism of TCR engagement and subsequent CD3 exposure for phosphorylation as of yet remains elusive. Nevertheless, this step is crucial as it leads to the recruitment of ZAP-70 (ζ-associated protein of 70 kDa) a PTK. The phosphorylation of the tyrosine residues on ITAMs acts as a docking site for ZAP-70 (Iwashima *et al*.,

1994). Following ITAM recruitment of ZAP-70, the Tyr-493 residue in the activation loop is phosphorylated by Lck. Zap-70 consequently autophosphorylates several tyrosine residues, which are important in the recruitment of effector and adapter molecules in order to form the initial TCR complex, referred to as the signalosome (Wange and Samelson, 1996). Fully activated ZAP-70 consequently has several targets for phosphorylation, which include, the adapter protein linker for the activation of T cells (LATs) and the Src homology 2 (SH2) domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76). The proximal complex is composed of several adapter proteins, which act as a scaffold and provide a framework around which other proteins are recruited in the correct spatiotemporal manner. LATs and SLP-76 are two such adapter proteins, which are recruited early in the TCR induced activation stage.

LAT interacts with four proteins, which include, growth factor receptor bound protein 2 (Grb2), Grb2 related adapter downstream of Shc (Gads), PLCγ1 (phospholipase C γ 1) and the p85 subunit of phosphoinositide 3 -kinase (PI3K). Phosphorylation of Gads consequently recruits SLP-76, which also binds Gads as a mutual binding partner. As mentioned, SLP-76 is yet another crucial adapter protein, which has two key regions required for interaction with other proteins. These regions are the proline rich region (PRR) and three tyrosines located at the N-terminal. The tyrosine residues once phosphorylated interact with VAV1, IL-2 induced tyrosine protein kinase (Itk) and the adapter protein Nck. The PRR is responsible for interacting with PLCγ1 and Gads, which are now mutual binding partners of LATs. SLP-76 also subsequently binds adhesion and degranulation-promoting adapter protein (ADAP). This proximal complex forms the basis of TCR activation, which follows the activation of PLCγ1 dependent pathways, Ca2+ signalling, DAG activation of the RasMAPK pathway, cytoskeleton arrangements and activation of integrin pathways (Liu *et al*., 1999).

Once LATs and SLP-76 has established the foundation of the proximal complex, Itk interacts with tyrosine 145 and the proline rich region (PRR) on SLP-76 and once it is localised here, it phosphorylates and thus activates

PLCγ1 (Bunnell *et al*., 2000). The PLCγ1 activated by Itk now sets in motion signalling cascades that induce the expression of transcription factors that play important roles in T cell activation. PLCγ1 does this initially by hydrolysing phosphatidylinositol 4,5-biophosphate (PIP_2), a membrane phospholipid, into two messenger molecules, Inositol triphosphate (IP_3) and Diacyglycerol (DAG), which are important second messengers (Sommers *et al*., 2004).

1.2.6 DAG mediated signalling pathway

DAG activates two signalling pathways that have a major role in the activation of key transcription factors essential for T cell activation and proliferation. DAG does this by activating Ras and Protein Kinase C theta (PKCθ). PKCθ is a member of the protein kinase C family of serine/threonine protein kinases. Ras, a guanine nucleotide binding protein, activates the well-studied MAPK/ERK signalling pathway. Primarily, Ras activates the serine-threonine protein kinase Raf-1, which is a Mitogen Activated Protein Kinase Kinase Kinase (MAPKKK). Following the classical three-tiered pathway, Raf-1 phosphorylates MAPKK, which in turn phosphorylates MAPK's extracellular signal-regulated kinase 1 (Erk1) and Erk2. The activity of Erk activates Elk1, which is a transcription factor that plays a key role in the activation of AP-1, a dimer of Jun/Fos and Elk1 is important in this case for regulating Fos expression (Li *et al*., 2001). Erk activity also activates another transcription factor, a member of the signal transducer and activator of transcription (STAT) family of transcription factors, STAT3. PKCθ mediates a second pathway, which is important in the activation of Nuclear Factor kappa-light-chain enhancer of activated B cells (NF-κB). NF-κB is associated with a molecule known as inhibitor of NF-κB (IκB) in the cytosol, which prevents it from entering the nucleus. Upon TCR activation IκB is phosphorylated and subsequently degraded (Bonizzi and Karin 2004). Consequently, this allows NF-κB to enter the nucleus and activate its target genes.

1.2.7 Ca^{2+} signalling pathway

IP_3, the second product of PIP_2 hydrolysis is involved in Ca^{2+} mediated signalling. IP_3 stimulates the Ca^{2+} channels on the endoplasmic reticulum (ER) leading to the release of Ca^{2+} stores into the cytoplasm. This further increases the influx of Ca^{2+} from its surroundings through the activation of Ca^{2+} release activated Ca^{2+} channels (CRAC). The transcription factor Nuclear Factor of Activated T cells (NFAT) resides in the cytosol but requires phosphorylation by calcineurin, which is activated by Ca^{2+} to translocate to the nucleus. NFAT in conjunction with AP-1 is essential for inducing Interleukin-2 (IL-2) expression in activated T cells (Rooney *et al*., 1995). IL-2 plays a vital role in several aspects of T cell immune response, which include; differentiation of CD4 T cells into effector sub-types, maintenance of regulatory T cells and contributes to CD8 mediated response by optimising cytotoxic T cell generation and differentiation into memory cells (Boyman and Sprent 2012).

An important factor in T cell activation through the TCR is the requirement of co-stimulation. Signalling through the TCR alone renders the cells to adopt a non-responsive state, known as tolerance. Cluster of Differentiation 28 (CD28) is a cell surface molecule expressed on T cells, which when bound to ligand in conjunction with TCR ligand binding, executes a robust T cell response. Co-stimulation with CD28 through a series of downstream events activates Atk (also known as Protein Kinase B). Akt has diverse roles once activated, which include prosurvival gene expression, inhibiting genes that promote cell cycle arrest and contributes to NF-κB and NFAT activation. Atk enhances the nuclear translocation of NF-κB and through activation of glycogen synthase kinase 3 (GSK-3), promotes the nuclear translocation of NFAT (Beals *et al*., 1997). Activation of T cells as has been discussed so far, involves a high degree of activity, which increases metabolic demand. Atk has also been reported to mediate glucose uptake and glycolysis (Frauwirth *et al*., 2002). In effect, co-stimulation is essential in T cell immune response because it is essential for cell survival, cellular metabolism, cytokine production and T cell proliferation.

TCR signalling is tightly regulated at several stages, from proximal components to distal ones, which is important to prevent autoimmune immunopathology. For instance Lck, responsible for activating ZAP-70, is phosphorylated on its inhibitory tyrosine residue by C-terminal Src kinase 3 (Csk), which keeps Lck in its inactive form (Vang *et al*., 2004). TCR ligand binding has also shown to distinguish between strong or weak ligand bindings. Stephanova and colleagues (2003) showed that weak ligand binding triggered a negative feedback loop, which lead to the recruitment of SHP-1 (SH2 domain containing protein tyrosine phosphatase). SHP-1 subsequently desensitised TCR signalling by inactivating Lck. In contrast, a positive feedback loop is initiated by SHP-1 inhibition by Erk upon strong ligand binding.

Two scaffold proteins known as downstream of kinase 1 (Dok1) and Dok2 have been shown to negatively regulate T cell signalling. In a study done in Dok1 and Dok2 deficient mice by Yasuda and colleagues (2007), they showed that these mice displayed elevated ZAP-70 activation, enhanced proliferation and cytokine production by T cells, and in contrast forced expression of these scaffold proteins displayed the opposite phenotype. Another study investigated the role of a member of HPK family of Serine/Threonine kinases HPK1 in negatively regulating T cell signalling. A knockout mouse model for HPK1 resulted in enhanced phosphorylation of SLP-76, PCγ1, Erk, increased production of cytokines and antigen specific antibodies. They also discovered that SLP-76's interaction with 14-3-3tau (a phosphorylated serine/threonine binding protein and negative regulator of TCR signalling) was markedly reduced in HPK1 deficient T cells (Shui *et al*., 2007). The only two members of a family of proteins known as the suppressor of T cell receptor signalling 1 (Sts-1) and Sts-2, displayed a role as negative regulators of TCR signalling. Mice deficient in Sts-1 and Sts-2 had increased ZAP-70 phosphorylation, increased cytokine production and the mice also showed an increased susceptibility to autoimmunity (Carpino *et al*., 2004).

1.3 CD8 T cell mediated immune response

The immune system does battle with infections but it also develops long-lived immunological protection, where the initial encounter with pathogen is

‘memorised’. Therefore, upon re-encounter with the same antigen a more robust and rapid response can be launched. Although both B and T cells are responsible for immunological memory, below only CD8 followed by CD4 T cells are discussed in detail for the purposes of this thesis.

CD8 T cell mediated response to acute viral and bacterial infection follows four distinct phases, which include, expansion, contraction, memory and recall. Naïve CD8 T cells once primed by antigen presenting cells (APCs) in the context of pMHC class I are activated and undergo differentiation and dramatic expansion into effector cells, and kill infected cells by releasing effector cytokines and other substances like perforins. Once the antigen has been cleared, the cells undergo a contraction phase where all except 5-10% of the initial antigen specific cells remain as memory cells, while others endure apoptosis. CTLs are maintained in the body in considerable numbers until they are re-encounter the same antigen, giving the immune system a distinct advantage (Zhang, 2011). Memory T cells can be localised in peripheral sites and can rapidly assume effector function status.

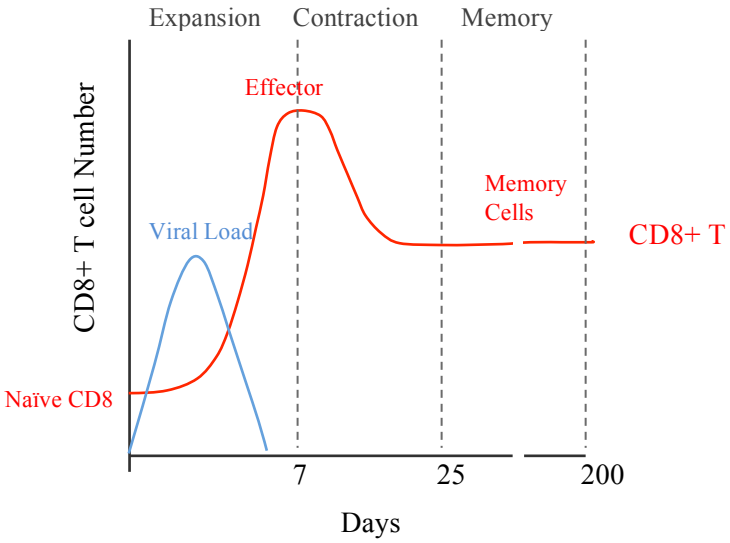

Figure 1.4: CD8 T cell response

This diagram illustrates the three phases of T cell immune response during acute infection, expansion, contraction and memory. Antigen specific CD8 T cells clonally expand and differentiate into CTLs. After antigen clearance, CTL reduce in number by apoptosis and this follows a pronounced number of cells, which serve as memory cells and can be maintained for a long time.

1.3.1 CD8 T cell activation, differentiation and expansion

The scale and quality of CTL mediated immune response can be affected by multiple factors, which include growth factors, inflammatory mediators co-stimulatory molecules and the availability of antigen. These factors contribute to both clonal expansion and differentiation upon antigen encounter of $CD8^+$ T cells. For instance, van Stipdonk and colleagues (2003) examined whether the duration of antigen stimulation during primary CTL activation can determine whether *in vivo* clonal expansion will be abortive or extensive. They found that antigen stimulation for 4 hours induced $CD8^+$ development and clonal expansion, but was not sustained. In contrast, 20 hour antigen stimulation produced a sustained $CD8^+$ T cell development and expansion with sustained expression of the IL-2 receptor alpha chain, CD25. Evidence suggests that the kinetics of a CD8 T cell response is independent of Ag quantity or the duration of infection. Once CD8 T cells are primed by Ag epitopes, they undergo

programming to differentiate into effector and memory cells in the first 1-2 days post-infection, without the need for further stimulation. For instance, the duration and quantity of Ag during *L. monocytogenes* infection was found not to determine the rate and duration of *in vivo* T cell proliferation (Mercado *et al*., 2000). Further, a study by Prlic and colleagues (2006) showed that the duration of Ag exposure directly correlated with clonal expansion but not the functionality of effector cells. Additionally, memory cells were able to induce a robust secondary response, which was also independent of the length of Ag exposure during primary response.

As described in the previous section, CD8 T cells are activated via Ag presentation by a professional antigen presenting cells in a MHC Class I context. This marks the first signal in the activation process. A secondary signal or more specifically, co-stimulation is necessary to determine the fate of the response and failure of this leads to functional tolerance. The most extensively studied pathway is the CD28/B7 interaction, where the CD28 receptor is expressed on T cells and the ligands B7.1 (CD80) and B7.2 (CD86) are expressed on dendritic cells (DCs). CD28 co-signalling promotes expansion and survival of T cells including production of IL-2. Aside from CD28, another molecule that appears to play an important role in directly co-stimulation of CD8 T cell response is 4-1BB (CD137). The 4-1BB is a tumour necrosis factor receptor (TNFR) family member and knockout studies of this receptor have demonstrated its importance in, optimal cellular response to influenza virus, IFNγ production and cell proliferation in response to mitogens (Kwon *et al* 2002).

Development of optimal, and in essence a complete CTL response, requires yet a third signal. The inflammatory cytokine IL-12 plays a crucial role in this process. During early encounter with Ag by phagocytes and DC, IL-12 is secreted into the surrounding environment, which interacts with the IL-12R expressed on activated T cells and NK cells. IL-12 is known to activate NK cells and induce the expression of IFNγ and thus favouring the differentiation of T

helper 1 (T_H1) cells. Several studies have indicated this notion of a third signal by IL-12 as an important contributing factor in CTL immune responses.

For instance, Curtsinger and colleagues (2003) showed that in the absence of IL-12, T cells fail to develop cytolytic effector function. CTLs produce granzyme B to counter Ag, but in the absence of IL-12 the production of granzyme B was impaired, strengthening the dependence of CD8 T cells on a third signal by IL-12 for a robust and complete immune response (Curtsinger *et al*., 2003).

1.3.2 CTLs contraction and memory formation

Once the pathogen has been cleared, CTLs arrive at the contraction phase, where pathogen specific effector CTLs are reduced in number by apoptosis. This process eliminates approximately 95 % of effector cells over the course of 10 days post infection. The mechanism/s that drive the formation of memory cells is still unclear, but several studies have recognized the importance of various surface markers and signalling cascades that influence this process. For instance, IL-7 signalling through the IL-7R expressed on T cells is crucial for the survival of naïve and memory T cells, but studies have found that IL-7 signalling during infection leads to increased Ag specific effector T cells but this does not correlate with increased number of memory cell formation (Sun *et al.,* 2006). Focus on cell intrinsic factors that are crucial for memory T cell formation have revealed some insightful participants. Like the Tbox transcription factor T-bet (T box expressed in T cells), which plays a prominent role in the differentiation of T_H1 cells and the production of IFNγ (Szabo *et al*., 2000). T-bet's role in cell-mediated immunity spans several key areas for it to acquire the title of 'master regulator' of gene encoding effector molecules. Eomesodermin (Eomes) is another Tbox transcription factor that is expressed in activated CD8 T cells and in resting and activated NK cells. Intlekofer and colleagues (2005) established a T-bet$^{-/-}$Eomes$^{-/-}$ mouse model and found that these two transcription factors are required for ample expression of CD122, required for cellular responsiveness for IL-15. IL-15 signalling is paramount for the survival of memory T cells (Intlekofer *et al.,* 2005). The IL-7Rα (CD127) has been proposed to mark effector cells destined to become memory cells. Bcl-2

interacting mediator of death (Bim) was found to be important in limiting T cell memory with $Bim^{-/-}$ mice having high functional memory T cells after viral infection with increased $CD127^{hi}$ cells (Wojciechowski *et al.,* 2006).

As well as cell autonomous and intrinsic mechanisms for CD8 memory formation, CD4 T cells help also has an important role in this process. Using $CD4^{+/+}$ and $CD4^{-/-}$ mice Shedlock and Shen (2003) discovered that memory CD8 cells generated in $CD4^{+/+}$ mice when transferred to $CD4^{-/-}$ mice responded normally when challenged with the same antigen a second time. In contrast, memory T cells generated in $CD4^{-/-}$ mounted a defective recall response when transferred into $CD4^{+/+}$ mice. This suggests that CD4 T cells are essential in priming CD8 T cells in the initial response to antigen for them to mount a strong secondary response. Sun and colleagues (2004) later found memory CD8 T cells gradually decreased in number in the absence of CD4 T cells, suggesting the significance of CD4 T cells also in maintenance of memory T cells after acute infection.

1.3.3 Central Memory (T_{CM}) and Effector Memory (T_{EM})

Memory T cells are characterised by two types, most prominent by their phenotype and functionality, known as central memory (T_{CM}) and effector memory (T_{EM}). The proposed subsets are based on the role of L-selectin (CD62L) and CC-chemokine receptor 7 (CCR7), which serve as homing molecules. T_{CM} cells display a $CD62L^{hi}$ and $CCR7^{+}$ phenotype whereas T_{EM} cells display the opposite, $CD62L^{lo}$ and $CCR7^{-}$. T_{CM}, $CD62L^{hi}$ $CCR7^{+}$ primarily reside in lymphoid organs whereas T_{EM} $CD62L^{lo}$ $CCR7^{-}$ are found in other sites around the body, such as liver and lungs (Unsoeld and Pircher, 2005). CD62L interacts with peripheral-node addressin (PNAd), which is expressed on high endothelial venules. Once CD62L attach to PNAd, memory cells exhibit a rolling action, which ultimately localises them in lymphoid organs, such as lymph nodes and spleen (Arbonés *et al*., 1994). CCR7 is a chemokine receptor, which initiates arrest of lymphocytes in the lymphoid organs and responds to the chemokines CCL19 and CCL21 (Förster *et al*., 2008). T_{CM} allows these cells to survey non-lymphoid tissues and mucosal sites, where infections are generally

initiated. Consequently, effector cells have a decreased potential to migrate into lymphoid organs due to reduced expression of CD62L and CCR7, and in contrast, their potential to migrate to inflammatory tissues is enhanced due to the expression of CCR5 and CCR2. Memory T cells are further maintained for a long time due to homeostatic proliferation, which takes place at a slow and constant rate. The homeostatic proliferation is coupled with equal rate of cell death, which ensures that the memory cells remain at a steady level. Cytokine signalling has been implicated to play and important role in this process, such as IL-15 and IL-7 (Kaech *et al*., 2002).

There are interesting questions being asked to elucidate how memory T cells determine which organ they will reside in as tissue specific T_{EM}. Interestingly, the DCs used to prime CTL can determine the tissue homing properties of the effector memory cells to the site where the antigen was first encountered. Mora and colleagues (2003) found that DC cells from Peyer's patches for instance, used to stimulate CD8 T cells induced high levels of alpha4beta7 (α4β7) and CCR9 receptor for the gut-associated chemokine receptor TECK/CCL25 and collectively these form intestinal homing molecules. These findings indicate effector memory T cells can be induced to express homing molecules, which allow cells access to sites that will most likely contain the antigen that induced effector memory cell formation at the first instance.

1.4 CD4 T cells

CD4 T cells recognize antigens presented by MHC II molecules on antigen presenting cells. Unlike CD8 T cells, CD4 T cells do not directly affect target cells, but play essential roles in regulating different types of responding immune cells for their function in adaptive immune responses (Zhu *et al*., 2010). Therefore, they are termed as helper cells. CD4 T cell differentiation into distinct effector populations, established during the last 25 years provides a fundamental link between the innate and adaptive arms of the immune system. The differentiation process is governed by distinct cytokines and transcription factors. These limited set of transcription factors are induced early during the differentiation process, driving clonal expansion of individual lineages with their

unique cytokine profiles. The following section provides a detailed view of the key processes and molecules involved in this process.

1.4.1 CD4 T helper differentiation

$CD4^+$ T cells have the capacity upon antigen encounter, to subdivide into T helper 1 (T_H1), T_H2, T_H17, follicular T_H (T_{fh}) and regulatory T_H cells (T_{Reg}) (Figure 1.5). These are the subsets most commonly reported and have varied functions to deal with the invading pathogens (Yamane and Paul, 2013). Upon TCR stimulation by antigen presented in a MHC Class II context, the helper T cells undergo programmed cell differentiation. Hence naïve T cells differentiate into functionally distinct subclasses with a distinct cytokine/s expression profile. CD4 cells form an indispensable role in adaptive immunity; they assist B cells in producing antibodies, mediate immunological memory, regulate macrophage function, have the capacity to deal with a wide range of pathogens and regulate the degree of effectiveness of response (Table 1.1). Their spectacular functional influence in adaptive immunity is made possible by their ability to differentiate into T helper cells of varied function known as effector cells and/or memory cells.

In a landmark paper published in 1986 by Mosmann and Coffman, they discovered two subsets of CD4 T cells, which were distinguished by their cytokine profiles. These were known as T_H1 and T_H2 cells. These two subsets were defined by characteristic cytokine expression profiles and were functionally different. Along with signature cytokines, the T cell differentiation programme is driven by key transcription factors specific to each lineage, known as master regulators. As well as master regulators, STAT proteins are critical in the signalling process and different members of the STAT family are involved in relaying specific lineage differentiation signals. STAT proteins, in collaboration with master regulators induce the expression of other important transcription factors and cytokines.

1.4.2 T-bet and T helper 1 cells (T_H1)

Differentiation of CD4 T cells into IFNγ producing T_H1 cells is crucial for defence against intracellular pathogens. When challenging CD4 T cells with a T_H1 inducing stimulus, they rapidly induce IFNγ production and differentiation (Lighvani *et al.,* 2001). IFNγ production is essential for the differentiation process since its expression also induces T-bet. T-bet is a T box transcription factor expressed in T cells, that has been identified as the master regulator for this lineage. For T_H1, T-bet has a central role in T_H1 cell development (Szabo *et al*., 2002). Although there are transcription factors termed the master regulators of individual T helper lineages, other transcription factors induced by TCR or cytokine signalling, or may be constitutively expressed, all perform critical roles that assist in the development of specific lineages.

T-bet induces the expression of genes necessary for T_H1 differentiation and function. In contrast, T-bet also supresses genes essential for the development of other lineages, such as T_H2. This was demonstrated by over expressing T-bet in T_H2 cells, which effectively resulted in the cells becoming competent IFNγ producing cells and consequently losing the ability to produce IL-4 (Szabo *et al*., 2000). Hsieh and colleagues (1993) demonstrated *in vitro* that the cytokine IL-12 was important for the development of T_H1 cells in response to heat killed Listeria monocytogenes, through macrophage production of IL-12. It was later found that T-bet induces the expression of IL-12 receptor beta (IL-12Rβ) and thus sensitizing the cell to IL-12 signalling. IL-12 serves to act as a growth signal, which induces survival and cell division and prolongs IFNγ expression (Mullen *et al*., 2001). T-bet$^{-/-}$ cells are unable to differentiate into T_H1 cells and are skewed toward a T_H2 phenotype. In an interesting study by Usui and colleagues (2006), they showed that a prominent role for T-bet is to regulate GATA3 activity, a master regulator for T_H2 lineage differentiation, since T-bet$^{-/-}$ cells were unable to exhibit normal T_H1 differentiation, provided GATA3 levels are regulated at early stages of activation with anti-IL-4. T-bet is essential for IFNγ production not only by CD4 T_H1 cells but also CD8 cells, but more prominent in CD8 cells is another T box transcription factor known as eomesodermin (Eomes). Double knockouts of T-bet and Eomes renders these

CD8 cells unable to produce sufficient levels of IFNγ, but not in single knockouts. Developing CD8 T cells express Eomes, which regulates IFNγ production and their cytolytic effector function. But Eomes also has a functional significance in CD4 T cells, where it contributes to T_H1 development (Suto *et al*., 2006). T-bet and Eomes are both responsible for the expression of CD122 in CD8 T cells, which is a receptor for IL-15 responsiveness, required for the maintenance of memory T cells (Intlekofer *et al*., 2005).

STAT proteins in conjunction with other transcription factors induce the expression of master regulators and cytokines. STAT1 is activated by IFNγ, which can then induce the expression of T-bet for T_H1 differentiation. Lighvani and colleagues (2001) showed that IFNγ induction of T-bet was specific and STAT1 dependent. In essence IFNγ regulates the expression of a transcription factor that promotes its own production, in an autocrine loop. The IFNγ mediated induction of T-bet is not limited to the lymphoid lineage but also myeloid cells. Another STAT signalling pathway utilised by T_H1 cells is STAT4. STAT4 is activated by IL-12, and it is important in T_H1 mediated response to *Taxoplasma gondii*. Signalling through STAT4 is important for the induction of IFNγ in the absence of T-bet. Yang and colleagues (2001) showed that IL-12 in fact works synergistically with IL-18 to induce IFNγ, which is TCR independent. This is an interesting find as it provides some vital information illustrating how after initial encounter with antigen, TCR signalling does not need to be sustained in order for T_H1 cells to produce IFNγ.

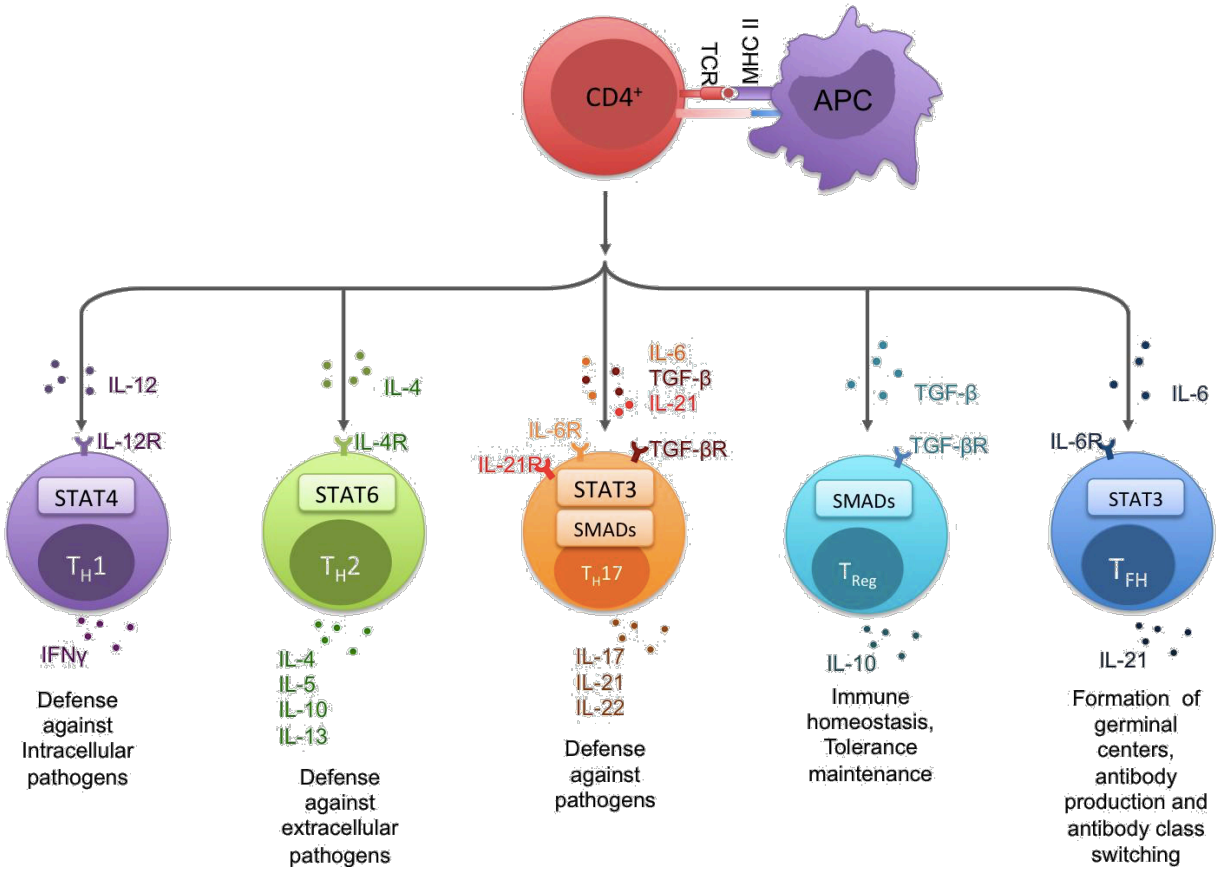

Figure 1.5: CD4$^+$ T cell polarization

The figure shows the differentiation of CD4$^+$ T helper cells upon antigen activation and the individual functions attributed to each lineage, mainly dictated by the cytokine environment. The diagram illustrates potent cytokine inducers, via the STAT or SMAD proteins with signature cytokines unique to each lineage (Diagram was adapted from Kaplan *et al*., 2015).

1.4.3 GATA3 and T helper 2 cells (T_H2)

T_H2 cells have IL-4, IL-5 and IL-13 as their signature cytokines and also produce IL-2 but at rather moderate levels to T_H1 (Swain *et al*., 1990). The master regulator for the T_H2 differentiation programme is a transcription factor that belongs to the GATA family of transcription factors, known as GATA3. Following this discovery by Mossman and Coffman in 1986, it consequently led to finding several other T helper subsets with characteristic cytokine expression profiles, unique master regulators and exclusive functional properties. T_H2 cells are responsible for strong antibody production, eosinophil activation and to

provide a phagocyte-independent response. T_H2 has GATA3 as its master regulator with IL-4 as the characteristic cytokine and also include IL-5 and IL-13. Expression of GATA3 is up-regulated during T_H2 differentiation and down-regulated during T_H1 (Zhang *et al*., 1997). Zhu and colleagues (2004) generated a conditional GATA3 knock out mouse cell line and they found that IL-4 dependent and independent T_H2 differentiation was abolished including the expression of IL-5 and IL-13 but not IL-4 production.

As with T_H1 lineage differentiation, STAT play an integral part in T_H2 differentiation. The member of STAT family most prominent for this lineage is STAT6. IL-4 mediated expansion and differentiation of T_H2 lineage is dependent upon STAT6 signalling. The significance of STAT6 was clearly illustrated by Kurata and colleagues (2001), where they showed that expression of STAT6 in developing T_H1 cells induced IL-4 and GATA3 and supressed IFNγ and the IL-12Rbeta2 chain expression. STAT5 signalling is also required for T_H2 differentiation. Although STAT6 is critical for IL-4 mediated T_H2 development (Kurata *et al*., 1999), STAT5 is also an important contributing factor to this lineage. STAT5 binding to the IL-4 receptor α (IL - 4Rα) gene elements is primarily induced by IL-2 signalling upon T_H2 differentiation. STAT5a and STAT5b both have numerous targets of genes in T_H2 lineage cells and play and integral role in this process (Liao *et al*., 2008).

1.4.4 RORγt and T helper 17 cells (T_H17)

Subsequent discoveries involved the characterisation of several other T helper subsets, which include T_H17, T regulatory helper cells (T_{Reg}) and T follicular helper cells (T_{fh}). T_H17 cells have RAR-related orphan receptor gamma (RORγt), a member of nuclear receptor family of transcription factors, as their master regulator, essential for their differentiation upon TCR stimulation. Like T_H1 and T_H2, T_H17 are tailored to provide adaptive immunity against specific pathogens, such as extracellular bacteria. These cells secrete IL-17A as their signature cytokine upon differentiation and produce other cytokines, such as; IL-6, IL-21 and IL23. STAT3 signalling is crucial for this T helper subset, since in the absence of STAT3, CD4 T cells are unable to produce IL-17A (Mathur *et al*.,

2007). STAT3 was discovered to bind directly to the IL-17A locus and hence defective IL-17A production in STAT3 negative T cells. STAT3 signalling also functions to induce RORγt and IL-23R expression (Chen *et al*., 2006). RORγt and STAT3 both collaborate to achieve IL-17A expression. All these signalling events occur downstream of IL-6, which induces IL-21 expression, IL-21 promotes the production of more IL-21 and IL-23 which induce RORγt expression and promote T_H17 differentiation and IL-17A production. Studies conducted *in vitro* only require IL-6 and TGFβ for T_H17 differentiation (Zhou *et al*., 2007). TGFβ is a cytokine that upregulates the expression of IL-23R and thus allowing for IL-23 responsiveness (Mangan *et al*., 2006).

A key theme in CD4 T helper cell differentiation is the interplay of certain transcription factors between each lineage. For instance, in developing specific T helper cell, other subclasses are supressed since their expertise is not required for that particular response. For example, GATA3 is over expressed during T_H2 development, which down regulates the expression of STAT4 required for IL-12 signalling in T_H1 cells (Usui *et al*., 2003). STAT5 contributes by inhibiting T bet expression and thus promoting T_H2 development. Similarly, T-bet inhibits GATA3 during T_H1 differentiation (Usui *et al*., 2006).

1.4.5 Bcl6 and T follicular helper cells (T_{fh})

There is yet another subset of CD4 T cells important in the generation of immunological memory, known as Follicular T cells (T_{fh}). In response to T cell dependent antigens, they help B cells produce antibodies and are also important in the induction and control of immunoglobulin class switching and somatic hypermutation. What makes T_{fh} distinguishable from other T helper cells includes; the expression of the chemokine receptor CXCR5, their location and migratory patterns to B cell follicles and as mentioned, their ability to assist B cells in antibody production. Assistance in B cell antibody production involves the stimulation of B cells via IL-21R signalling, where the IL-21 is produced by T_{fh} to induce the differentiation of B cells into antibody producing cells (King *et al*., 2008). The B cell lymphoma 6 (Bcl6), a transcription factor, has been discovered as their master regulator. These cells are also important in the

formation and maintenance of germinal centres, home to B cell differentiation into plasma and memory cells (Dent *et al*., 1997).

A controversial topic in the existence of T_{fh} cells is whether they are a distinct lineage parallel to T_H1, T_H2 and T_H17, or exist as a phenotypic state of each of the other subsets as determined by environmental cues, which include how the cells are primed. In a study by King and Mohrs (2009), they found that following helminth infection CD4 T cells displayed phenotypic properties of both T_{fh} and T_H2 cells. IL-4 is crucial for T_H2 differentiation, but they discovered that IL-4 producing T cells were found in B cell follicles. These IL-4 producing cells also expressed high levels of CXCR5, IL-21, programmed cell death protein 1 (PD-1) and Bcl6, characteristic of T_{fh} cells. Deletion of IL-4 in this scenario resulted in defective B cell expansion and maturation. They effectively established that T_{fh} cells in B cells follicles were responsible for the production of IL-4 *in vivo*. It seems apparent that T_{fh} cells interacting with B cells in the germinal centre produce either IL-4 or IFNγ, depending on how they have been primed.

1.4.6 Foxp3 T regulatory cells (T_{Reg})

Another subclass of T cells known as T regulatory cells (T_{Reg}) appear to be primarily involved in controlling the function of other subsets. The transcription factor forkhead box P3 (Foxp3) has been known to be a master regulator for this lineage. T_{Reg} cells are primarily involved in the maintenance of immunological self-tolerance by actively supressing self-reactive lymphocytes (Vignali *et al*., 2008).

T helper lineage	Hallmark cytokines	Hallmark Transcription Factors	Cytokine inducers	Cytokine signalling pathways	Function and Host defence	Pathology
T_H1	IFNγ IL-2	T-bet	IFNγ IL-12	STAT1 STAT4	- Macrophage activation - IgG production - Defence against Intracellular Pathogens	Inflammatory disorders, Autoimmunity
T_H2	IL-4 IL-5 IL-13 IL-25 IL-10	GATA3	IL-4 IL-2	STAT5	- IgE production - Activation of Mast cell and Eosinophil - Defence against Extracellular parasites	Allergy and Asthma
T_H17	IL-21 IL-17A IL-17F IL-22	RORγt	TGF-β IL-6 IL-21 IL-23	STAT3	- Defence against extracellular bacteria, Fungi	Inflammatory disorders, Autoimmunity
T_{Reg}	TGF-β IL-35 IL-10	Foxp3	TGF-β IL-2	STAT5	- Immune tolerance - Lymphocyte homeostasis - Regulation of immune responses	Loss of immune tolerance, Autoimmunity
T_{fh}	IL-21	Bcl6	IL-6 IL-21	STAT3	- B Cell maturation	Autoimmunity

Table 1.1 Summary of T helper cells*

*Adapted from Zhu *et al.,* 2010

1.5 Cytokines

Cytokines form an integral part of the immune system with diverse roles in immunoregulation, immune cell development and modulating immune effector functions. Aberrant expressions of particular cytokines have considerable implications to the host with regards to the development of autoimmunity and autoimmune disease (O'Shea *et al.,* 2002). Cytokines such as IL-2, which have historically been recognised as a factor that promotes proliferation of T cells and IFNγ as an inflammatory cytokine has dramatically changed over recent years. These cytokines have demonstrated to have a broad range of modulatory effects on immune cells. In contrast, immunosuppressive cytokines, such as IL-10 and TNF-β primarily produced by T_{Reg} cells have inhibitory effects in an autoimmune environment (Sanjabi *et al*., 2009) (Table 1.2). This staggering effect of cytokines on immune cells invites a challenging prospect for researchers to investigate the intricate mechanisms that govern their expression and function.

As mentioned, IL-2 has a key role in promoting lymphoid proliferation, but IL-2 and IL-2 receptor (IL-2R) knockout mouse models have highlighted a far more complex picture. Under homeostatic conditions, IL-2 is mainly produced by CD4 T helper cells in secondary lymphoid organs. Other cells that can produce IL-2 are, CD8 T cells, Natural Killer T cells (NKT) and Natural Killer (NK) cells (Boyman and Sprent,. 2012). The expression of IL-2 is further induced in CD4 and CD8 T cells after activation, although CD8 cells to a lesser extent than CD4 cells (Malek, 2008). The impact of IL-2 on CD8 T cells is vital during immune responses. IL-2 contributes to primary expansion, contraction and memory generation and secondary expansion. *Il2*$^{-/-}$ mice displayed a threefold lower primary expansion in response to viral infection compared to WT mice (Boyman *et al*., 2010). The differentiation of CD8 cells into short lived or long-lived memory cells are affected by the strength and duration of IL-2 signals, during an immune response. CD25 is the third chain of the trimeric IL-2R and although it does appear to play a role in signal transduction directly, it increases receptor affinity for IL-2. Kalia and colleagues (2010) found that the affinity of IL-2 on cells with respect to CD25 expression was important for development of long-

lived memory cells. They found that during acute viral infection, a subset of virus-specific T cells sustain CD25 expression longer than the rest. These $CD25^{hi}CD8^{+}$ cells upregulated CD127 and CD62L to give rise to functional long-lived memory cells. $CD25^{hi}$ cells also appear to be terminally differentiated with a more pronounced effector phenotype.

IL-2 is also essential for the survival and homeostasis of naturally occurring T_{Reg} cells. Yu and colleagues (2009) showed that IL-2RBeta-deficient mice developed severe autoimmunity. Expression of IL-2Beta chains in these mice resulted in the production of a normal proportion of natural T_{Reg} cells that suppressed the development of severe autoimmunity. Foxp3 as described earlier, is a master regulator of the T_{Reg} lineage. In naturally occurring T cells, IL-2 signalling was found to be important in maintaining high expression levels of Foxp3 (Fontenot *et al*., 2005). IL-2 also plays and important role in maintaining homeostasis of CD4 T helper subsets, including T_H1, T_H2 and T_H17 along with controlling CD8 memory cell homeostasis (Letourneau *et al.,* 2009, Sharma *et al*., 2007). Due to its diverse role in the immune system under homeostatic and infection conditions, IL-2 is regulated by several mechanisms. One mechanism involves the silencing of the *Il2* gene by the transcription factor B lymphocyte-induced maturation protein 1 (Blimp1). Professor Calame's group who discovered this transcription factor developed a Blimp1 knockout mouse model, which resulted in the mice developing aberrant T cell homeostasis and fatal colitis. They discovered that Blimp1 was essential for directly repressing *Il2* expression. Upon induction of IL-2 in activated T cells, IL-2 induces *Prdm1* expression (gene encoding Blimp1), which forms an auto-regulatory loop for its regulation (Martins *et al*., 2008).

Transforming growth factor beta (TGFβ) is yet another pleotropic cytokine with functions in a range of cells of the immune system (Li and Flavell, 2008). Its cellular origin extends to all cells of the leukocyte lineage, including; lymphocytes, macrophages and dendritic cells (Letterio and Roberts, 1998). TGFβ's function is primarily attributed to the maintenance of tolerance by regulating lymphocyte proliferation, differentiation and survival (Li *et al*., 2006).

Gorelik and Flavell (2000) demonstrated that abolition of TGFβ signalling in T cells lead to spontaneous T cell differentiation and autoimmune disease. As discussed earlier, other cytokines such as IFNγ, IL-4, IL-17A, IL-21 and TNFα play important roles in defining lineage commitments of both CD4 and CD8 T cells. Table 1.2 shows some common cytokines and their functions in the immune system.

Cytokine	Source	Cellular targets and biological effects	Signalling pathways and disease	Reference
IFNγ	– $CD4^+$ T_H1 – $CD8^+$ CTL – NK cells	– Up-regulation of MHC class I and class II on APCs such as B cell, DC cells, Macrophages – Influences T_H1 development – Induction of antiviral enzymes such as PKR (serine/threonine kinase) – Promote cell cycle and growth – Inhibition of IL-17 production – Stimulation of T_{Reg} cell function.	STAT-1 mediated signalling. Aberrant expression involves development of autoimmune disease	Schroder *et al*., 2004 Kelchtermans *et al*., 2008.
IL-2	– $CD4^+$ T helper cells – $CD8^+$ cells – NKT – NK cells	– $CD4^+$ T cell differentiation after antigen stimulation – Expansion and differentiation of $CD8^+$ T cells. – Inhibition of T follicular helper cell differentiation. – Development, expansion and function of $CD4^+CD25^+T_{reg}$ cells.	STAT-5 mediated signalling. Absence of IL-2 signalling leads to enhanced susceptibility to autoimmune disease.	Ballesteros-Tato *et al*., 2012 Boyman and Sprent, 2012 Nelson, 2004
IL-4	– Activated T cells – Mast cells – Basophils – Eosinophils	– $CD4^+$ T_H2 cell differentiation upon antigen stimulation. – Prevent T_H2 cells from apoptosis. – Regulate proliferation, differentiation and apoptosis of myeloid, mast, dendritic, endothelial, muscular and neuronal cells.	STAT-6 mediated signalling Allergic and autoimmune disease	Luzina *et al*., 2012 Zubiaga *et al*., 1992.
IL-17A	– $CD4^+T_H17$ – γδ T cells – αβ iT_H17 cells	– Important for the clearance of extracellular bacteria – Promotes production of pro-inflammatory cytokines and chemokines	ACT-1 dependent signalling pathway Dysregulated IL-17A expression linked to autoimmune diseases such as; MS, RA and IBD*	Jin and Dong, 2013 Gaffen, 2009
TNF	– Activated T cells	– Inducer of apoptotic cell death – Production of pro-inflammatory cytokines and	Activation of NF-κB and AP-1	Sedger and McDermott, 2014.

	– Macrophages – Activated NK cells	chemokines	Deregulation of TNF is associated with inflammatory disorders such as; arthritis and IBD*	Brenner *et al*., 2015
TGF-β	– Lymphocytes – Macrophages – Dendritic cells	– Inducer of apoptotic cell death – Proliferation, differentiation and survival of lymphocyte	Signalling through SMAD proteins TGF-β is important for T_{Reg} cells to mediate immunosuppression and maintaining peripheral tolerance	Taylor, 2009.

Table 1.2: Some common cytokines and functions in the immune system

*MS Multiple Sclerosis, RA Rheumatoid Arthritis, IBD Irritable Bowel Disease

1.5.1 Interferon Gamma (IFNγ): A type II interferon

The IFNs are classified into type I and type II according to receptor specificity and sequence homology. The IFN type I are comprised of IFNα, IFNβ, IFNω and IFNτ, which are structurally related and bind to a common heterodimeric receptor. Many different cell types secrete type I IFNs, and haematopoietic cells are the major producers of IFNα and IFNω upon viral infection. IFNγ is the sole member of type II family and what differentiates it from type I is that it binds to a different receptor, which is structurally unrelated and encoded by a separate locus (Bach *et al*., *1997*). Many cells of the immune system produce IFNγ but the major producers of this cytokine are, CD4 T_H1, CD8 CTLs, NK, NKT and professional antigen presenting cells (APC) such as, dendritic cells and macrophages (Frucht *et al*., 2001, Szabo *et al*., 2002).

IFNγ is a very interesting cytokine that has diverse effects on the cells of the immune system, which it implements through transcriptional regulation of immunologically relevant genes (Schroder *et al*., 2004). IFNγ was discovered almost 50 years ago as a substance capable of inhibiting the cytophathic activity of Sindbis virus (Wheelock, 1965). This cytokine has since been extensively studied, increasing our understanding of its significance in immunology, and posing some challenging questions due to its complex nature of function. Although initially discovered as an agent with antiviral activity, it is involved in many immunological scenarios, including; influence on cell proliferation and cell death, stimulation of antigen presentation through MHC class I and II and stimulation of bactericidal activity of phagocytes (Boehm *et al*., 1997). IFNγ is essential for immune function, which was apparent when mice with homologous disruption of the IFNγ gene, infected with Leishmania major were unable to clear the infection (Wang *et al* 1994). As well as agents in viral and bacterial infection, IFNγ alone has proved to have anti-cancer properties, which has been demonstrated both by use of tumour cells altered to express IFNγ to promote IFNγ dependent T cell mediated anti-tumour response (Tannenbaum and Hamilton 2000).

1.5.2 Interferon Gamma (IFNγ) and its receptor

Interferon was initially found to be induced in human leukocytes by phytohemagglutinin, a compound derived from red kidney bean (Wheelock, 1965). Although IFNγ was then identified as a compound with anti-viral activity, research conducted until now has established it as a cytokine that modulates all phases of immune and inflammatory responses. The IFNγ receptor consists of two IFNR1 chains for ligand binding and two IFNR2 chains for signal transduction. IFNR1 is expressed in excess but IFNR2 is regulated under strict conditions and its expression depends upon the cellular state, i.e. activated or differentiating. This is especially important in T_H1 and T_H2 development and how the lack of IFNR2 expression counters the growth inhibitory effects of IFNγ. T_H1 cells express low levels of IFNR2 therefore it blocks the inhibitor effects of IFNγ and thus this population continues to proliferate. On the contrary, T_H2 cells when exposed to IFNγ expressing high levels of IFNR2 are subjected to apoptosis. Bach and colleagues (1995) showed that IFNγ, through a feedback loop extinguishes the IFNR2 receptor expression required for signal transduction in T_H1 as a negative feedback control and in T_H2 cells, a mechanism for cellular desensitization to this cytokine. Mice deficient in $IFN\gamma^{-/-}$ and $IFNR1^{-/-}$ showed no significant defects in immune system development, although natural resistance to bacterial and viral infections were compromised (Huang *et al*., 1993).

1.5.3 IFNγ Signalling in T cells

IFNγ primarily signals through the JAK-STAT pathway, which subsequently involves the activation of members of the Janus family of kinases (Jak 1-3 and Tyk2) and STATs (STAT1-6), ultimately controlling the expression of target genes. The pathway is initiated upon ligand binding, which initially induces Jak2 auto-phosphorylation and activation, which in turn phosphorylates Jak1. The activated Jak1 phosphorylates tyrosine residues on the IFNR1 chain creating a docking site for STAT1, which is involved in a further phosphorylation event by Jak2. The activated STAT1 travels to the nucleus and binds to key gene elements regulated by IFNγ, which includes a wave of transcription factors, such as Interferon Regulatory Factors (IRF) (Igarashi *et al*., 1994). STAT1

therefore forms an essential part of IFNγ signalling and studies have shown that STAT1$^{-/-}$ mice are unable to launch a potent response against viral and microbial infections. For instance, Durbin and colleagues (1996) disrupted the STAT1 gene in mouse embryonic stem cells (MES) and found that these mice were unresponsive to IFNγ, but were responsive to other cytokines. This showed that STAT1 functions in an exclusively IFNγ dependent fashion, but does not completely hinder immune system development. These mice were extremely susceptible to viral disease. Negative regulation of IFNγ signalling is an important aspect to prevent organism from the destructive force of this cytokine when over expressed. One recorded mechanism by which this occurs is by the degradation of ligand:receptor complex upon internalisation, thereby down-regulating IFNR1 expression and IFNγ responsiveness (Celada and Schreiber, 1987). Another mechanism for negatively regulating IFNγ signalling, involves Suppressor of cytokine signalling (SOCS1 and SOCS3). Song and Shuai (1998) showed how SOCS1 and SOCS3 inhibited the tyrosine phosphorylation and nuclear translocation of STAT1 in response to IFNγ signalling. In addition SOCS1 exhibited a much stronger inhibitory effect than did SOCS3.

1.5.4 Regulation of IFNγ in T cells

The fate of CD4 T cells when differentiating into effector cells is largely controlled by cytokine cues released by cells of the immune system, which guide the differentiation process towards a distinct T cell lineage (Balasubramani *et al*., 2010). As described earlier, the cytokine IFNγ plays an important role in commitment of CD4 T helper cells to the T_H1 lineage with T-bet as its master regulator. The *Ifng* locus is tightly regulated and it has been studied extensively to ultimately establish key transcription factors and cis elements in the *Ifng* locus, that are important in providing new insights into the epigenetic control and transcription of *Ifng* in T cells. Epigenetic processes that allow cells to adapt a particular gene expression profile regulate T lineage commitment, but at the same time retain a degree of plasticity to be able to change according to environmental cues (Wilson *et al*., 2009). Transcription factors, T-bet, STAT1 and STAT4 play important roles directing naïve CD4 T

cells into competent IFNγ producing T_H1 cells. Differentiation in to T_H1 cells takes the form of two phases; Schulz and colleagues (2009) analyse expression kinetics of T-bet, IFNγ and IL-12Rβ2 during T_H1 differentiation and found IFNγ signalling induced T-bet expression initially and upon TCR signalling termination, IL-12 signalling maintained T-bet expression and thus promoting IFNγ expression. Interestingly, they found the second phase of T_H1 differentiation upregulated the expression of the transcription factors Runx3 and Hlx, which further accommodate IFNγ expression. To date, multiple transcription factors have been identified to regulate T_H1 differentiation with binding sites for these transcription factors on the *Ifng* locus, but much research is needed to decipher how they contribute to T_H1 differentiation and IFNγ expression.

Soutto and colleagues (2002) analysed distal regulatory regions surrounding the IFNγ gene and found that a human *IFNg* BAC transgene that contained approximately 90 kb of flanking 5' and 3' sequence was necessary for T_H1 specific expression of IFNγ. These regulatory DNA elements conserved across species have been termed conserved non-coding sequences (CNS) and thus far several have been identified that are critical for the regulation of the *Ifng* locus (Figure 1.6). The CNS regions correspond with DNase 1 Hypersensitivity sites (HS), including a considerable number in naïve CD4 T cells, which increase upon T_H1 differentiation and diminish upon T_H2 differentiation (Balasubramani *et al*., 2010). Although there are multiple sites that are DNase I hypersensitive, CNS-22 displayed a lineage unrestricted DNase I hypersensitivity and in addition both CNS-22 and CNS-34 appeared to be in an active state with elevated histone methylation (H3K4) status in naïve CD4 T cells. In fact, Hatton and colleagues (2006) found that deletion of CNS-22 in the context of an *Ifng* reporter transgene ablated *Ifng* expression T cells. Other regions including CNS-70 displayed lineage unspecific DNase I hypersensitivity indicating an important role for these CNS in early structural organisation of the *Ifng* locus.

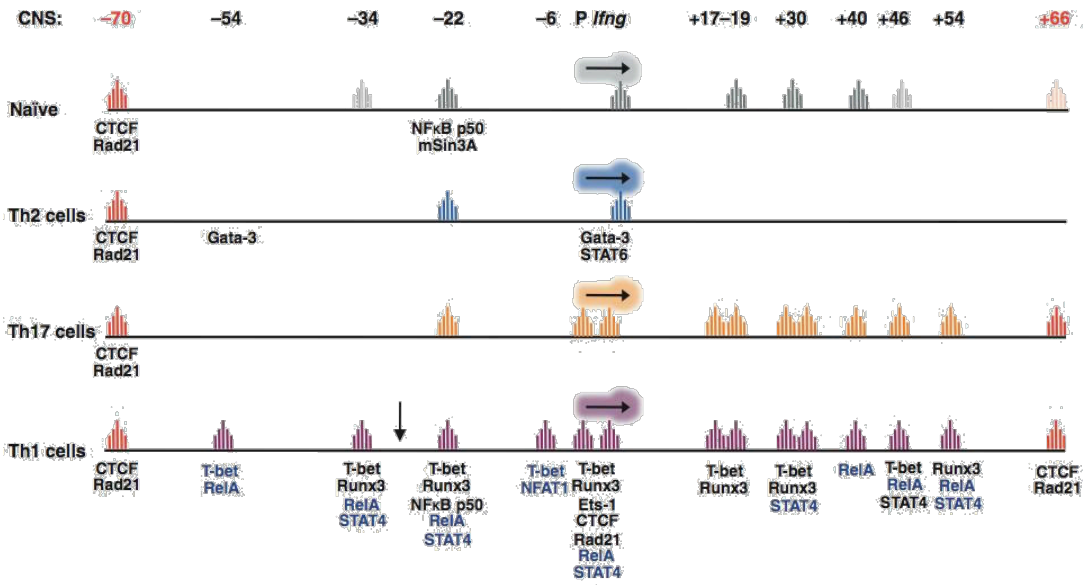

Figure 1.6 DNase I Hypersensitivity regions on *Ifng* locus in CD4$^+$ T cells
Coloured peaks indicate DNase I HS sites and downward arrow for T_H1 represents a HS site not in a conserved region. Binding sites for various transcription factors are indicated across the *Ifng* locus involved in enhancing or inhibiting transcriptional activity.

Transcription factors recruited to the *Ifng* locus (figure 1.6), the binding regions of which span several CNS sites, such as T-bet, Runx3 and STAT4 have dynamic roles in regulating the transcriptional state of the *Ifng* locus. Factors such as long range histone acetylation that drives T_H1 differentiation and IFNγ expression depends heavily on the presence of T-bet or STAT4 (Chang and Aune, 2005). As described earlier, STAT4 dependent signalling by IL-12 is essential to maintain T-bet expression in the absence of TCR stimulation. In addition to these lineage specific transcription factors, other transcription factors that are commonly associated with multiple cell lineages are important in the induction of gene expression also. For instance RelA of the NF-kB family of transcription factors, have multiple binding sites on the *Ifng* locus including CNS regions flanking 5' and 3' of *Ifng* gene, have an important role in T_H1 polarisation (Balasubramani *et al.*, 2010).

1.5.5 IFNγ in disease

Although IFNγ is an important immune modulator, aberrant expression or absence of IFNγ signalling can have considerably dire consequences. IFNγ transgenic mice have shed light on the role of IFNγ in various autoimmune diseases, such as, lupus-like syndrome, insulin dependent diabetes mellitus (IDDM), rheumatoid arthritis (RA) and multiple sclerosis (MS) (Baccala *et al*., 2005). For instance, mice with IFNγ over-expression in the epidermis develop a lupus-like syndrome by 3 months of age, characterised by production of IgG anti-ds DNA, anti-histone and anti-nucleosome autoantibodies. A third of the females developed immune mediated glomerulonephritis (Seery, 2000). Sarvetnick and colleagues (1990) demonstrated that the progressive destruction of pancreatic islets in diabetes is mediated by lymphocytes and that the pancreatic expression of IFNγ can result in the loss of tolerance to normal islets. Pollard and colleagues (2012) found that targeting IFNγ pathways could potentially have therapeutic implications for systemic autoimmunity. They found that in mercury-induced systemic autoimmunity (mHgIA) absence of IFNγR1 resulted in reduction of disease, while reduction in genes promoting IFNγ had little or no effect. A study by Richards and colleagues (2001) examined the role of IFNγ in lupus nephritis induced by treatment of mice with a hydrocarbon oil pristane. IFNγ$^{-/-}$mice failed to develop the disease when treated with pristane indicating an important role in the induction of nephritis (Richards *et al*., 2001).

Although IFNγ is predominantly regarded as a pro-inflammatory cytokine identified in T_H1 dominated autoimmune disease, it also has a protective role discovered in autoimmune animal models. Billiau and colleagues (1988) induced acute experimental allergic encephalomyelitis (EAE) in C57BL/6J and SJL/J mice where both mice developed the disease with the SJL/J mice showing a highly aggressive form of the disease. C57BL/6J mice were less susceptible displaying low morbidity rates compared to SJL/J mice. Interestingly, SJL/J mice with high susceptibility to induced EAE when treated with IFNγ resulted in reduced morbidity and mortality, suggesting an essential role for IFNγ in limiting the disease. Collagen induce arthritis (CIA) in mice is one of several animal models used as a model to study RA (Williams, 2004).

Mice with a deficiency in the IFNγR lead to the occurrence of severe CIA with accelerated onset compared with their wild-type counterparts (Manoury-Schwarz *et al*., 1997). In an interesting study by Wang and colleagues (2006) they found that IFNγ was essential for the conversion of $CD4^+CD25^-$ T cells into $CD4^+$ T_{Reg} cells. They found that treatment of $CD4^+CD25^-$ with IFNγ *in vitro* upregulated Foxp3 and subsequent conversion of these cells into $CD4^+$ T_{Reg} cells. These cells were observed to have regulatory properties as evidenced by suppression of EAE by adoptive transfer. Collectively these results illustrate the significance of IFNγ in modulating the immune response and how regulation of its expression is critical to prevent immune pathology.

1.6 Early growth response (Egr) gene family

1.6.1 Egr zinc finger domain

The early growth response gene family of transcription factors is composed of four members, Egr1 (also called NGFI-A and Zif268), Egr2 (also called Krox20), Egr3 (also called PILOT) and Egr4 (also called NGFI-C). Chavrier and colleagues (1988) first discovered Egr2 to be induced in fibroblasts during the G_0/G_1 phase *in vitro* (Chavrier *et al*., 1998). The zinc finger domain is a DNA binding domain, which contains 3 C2H2 type zinc finger motifs. A zinc ion is individually coordinated by four ligands. The metal is associated with two histdinies and two cytosines and stabilises the structure through these interactions (Luisi 1992). The crystal structure of one of the member of Egr family, Egr1 was determined (Pavletich and Pabo, 1991). The structure of the zinc finger domain consists of two anti-parallel beta strands and alpha helix (Figure 1.7). The Egr2 protein binding sequence was identified as a 9 bp sequence and each motif binds to 3 bp of DNA.

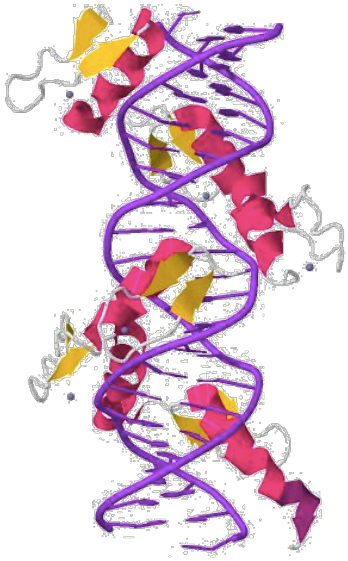

Figure 1.7: Egr1 zinc finger domain. α-helices shown in red and β-sheets in yellow. Zinc ion represented by tiny grey circles, which is bound to two cysteine and two histidine residues. Protein Data Bank (PDB) ID 1P47.

1.6.2 Egr DNA binding

The high affinity DNA binding sequence for Egr1 was established by Christy and Nathans (1989) with the aid of DNase I footprinting, methylation interference and by use of synthetic oligonucleotides. Induced rapidly in particular regions of the brain, they sought to establish the binding site to explore the function further by identifying possible target genes regulated by Egr1. They found that Egr1 binds to GC rich sequences with the consensus binding sequence being GCGGGGGCG. This sequence is the established consensus sequence for Egr1, Egr2 and Egr3 as it was determined that these members of Egr family could activate transcription from this sequence (Swirnoff and Milbrandt, 1995). In experiments involving protein-DNA interactions in Electrophoretic Mobility Shift Assay (EMSA) for this project, the Egr2 binding probe was designed based in this sequence.

1.6.3 Egr proteins function outside the immune system

Egr proteins were initially discovered to have important functions outside the immune system. For instance, Egr proteins have been found to play a critical role in hindbrain development and myelination of the peripheral nervous system (O'Donovan *et al* 1999 and Topilko *et al*., 1994). Topilko and colleagues found that Egr2 was activated in Schwann cells before the onset of myelination and in the absence of Egr2, differentiation of Schwann cells at the early stages was

affected, and an overall prevention of myelination in the nervous system. Schneider-Maunoury and colleagues (1993) found that Egr2's expression was restricted to rhombomeres (r) 3 and 5 and inactivation of Egr2 resulted in a marked reduction or elimination of r3 and r5. Mutation in the Egr2 gene has also been associated with Charcot-Marie-Tooth (CMT) disease characterised as a hereditary motor and sensory neuropathy (Chung *et al* 2005).

In addition, mutations in the zinc finger DNA binding domain are associated with CMT, Dejerine-Sottas syndrome and congenital hypomyelination neuropathy (Warner *et al.,* 1999). Egr1 has been shown to play an important role in angiogenesis, which is the formation of new blood vessels from pre-existing vasculature (Khachigian 2004). Tourtellotte and Milbrandt (1998) generated Egr3 deficient mice and these mice developed gait ataxia, increased frequency of perinatal mortality, scoliosis, resting tremors and ptosis. They found that Egr3 deficient mice lacked muscle spindles and was highly expressed in muscle spindles. Collectively, these results demonstrated that type I myotubes are dependent on Egr3-mediated transcription for spindle development.

1.6.4 Egr proteins in T cells

Egr1 and Egr3 were induced during thymocyte differentiation in response to pre-TCR signalling, suggesting a potentially important role in thymocyte differentiation during pre-TCR stages. Carter and colleagues (2007) found Egr1 and Egr3 deficient mice develop severe thymic atrophy and impaired thymocyte differentiation. Their findings suggest Egr1 and Egr3 cooperate to promote thymocyte development. Activation of pre-TCR complex serves as an important checkpoint for immature thymocytes ($CD4^-CD8^-$) to progress beyond the beta selection checkpoint. Carleton and colleagues (2002) demonstrated that Egr1, Egr2 and Egr3 are important molecular effectors of pre-TCR signalling. Defect in Egr function resulted in impaired development of $CD4^-CD8^-$ DN thymocytes into $CD4^+CD8^+$ DP cells. Although expression of Egr1, Egr2 and Egr3 are important for thymocyte development, its apparent they play selective roles in this process, since there are differences in genes modulated by enforced expression of individual members. For instance, Xi and Kresh (2004) showed

Egr3 deficient mice had a reduced numbers of thymocytes compared to WT and this is due to poor proliferation during DN to DP transition (Xi and Kresh., 2004). Egr3 transgenic mice with constitutively increased Egr3 expression when bred onto a Rag1(-/-) background, displayed increase thymocyte proliferation. These results demonstrate Egr3 as an important regulator of proliferation of developing thymocytes in response to pre-TCR signals. Interleukin-2 (IL-2) drives proliferation of activated T lymphocytes during an immune response. IL-2 is recognised to modulate activated T cells to undergo cell cycle progression (Smith 1988). Egr1 was demonstrated to be an important activator of the IL-2 gene with a binding site present upstream of the binding site for NFAT on the IL-2 promoter region (Decker *et al*., 1998). Decker and colleagues also showed that IL-2 transcription induced by a combination of Egr1 and NFAT was increased by 200-fold.

Lazarevic and colleagues (2009) demonstrated an important role for Egr2 in Natural Killer T cell (NKT) survival and development. NKT cells have a grand role to play in the immune system, including, cell-mediated immunity against viruses, bacteria and tumours and regulation of self-tolerance (Berzins and Ritchie 2014). In mice deficient in calcineurin-NFAT signalling, Lazarevic and colleagues (2009) demonstrated, NFAT signalling was essential for NKT cells development. In addition, mice deficient in Egr2 had impaired NKT cells development through the positive selection stage and thus NKT cells matured inefficiently. Although Egr1 and 3 deficient mice have normal NKT cell development, they were shown to compensate for the loss Egr2. The ability of the Egr family members to compensate for each other is a recurring theme. Egr2 has also been shown to be important in the development of both B and T cells. Li and colleagues (2011) analysed the expression of Egr1, 2 and 3 at different stages of B and T cells development. They discovered that Egr2 was essential for progression of $CD4^+CD8^+$ DP cells to mature into $CD4^+$ or $CD8^+$ SP cells in the thymus and immature B cells into mature B cells in the bone marrow.

1.6.5 Egr proteins in autoimmunity

Egr2 have been found to be important in inducing clonal anergy in CD4 T cells (Harris *et al*., 2004). Harris and colleagues (2004) found that Egr2 is required for full induction of clonal anergy in CD4 T cells. They found that Egr2 gene expression persisted in non-proliferating anergized A.E7 T cells, whereas Egr1, a related protein, displayed very little or no expression in this state. This was further illustrated by prevention of anergy induction in A.E7 T cells with silenced Egr2 gene by interfering RNA. E3 ligases are involved in promoting anergy induction (Heissmeyer *et al*., 2004). Safford and colleagues (2005) found that overexpression of Egr2 and 3 was associated with an increase in E3 ubiquitin ligase Cbl-b and inhibition of T cell activation. Our group (Zhu *et* al., 2008) found with the aid of a conditional CD2-Egr2 knockout mouse model, that Egr2 played an important role in controlling the tolerance of self-reactive T cells and the development of a late onset of lupus like autoimmune disease. They found that Egr2 was induced in $CD4^{+}CD44^{high}$ T cells and in the absence of Egr2, $CD4^{+}CD44^{high}$ T cells were hyper-proliferative and hyper-reactive. The accumulation of $CD44^{high}CD4^{+}$ leads to the development of late-onset lupus like autoimmune disease characterised by the accumulation of IL-17A and IFNγ. They also found that the expression of cyclin-dependent kinase inhibitor p21cip1was impaired in CD2-$Egr2^{-/-}$ T cells and Egr2 directly interacted with the promoter of p21cip1 *in vivo*. In conclusion, these findings revealed a potent role for Egr2 in controlling the self-tolerance of T cells and preventing autoimmunity by controlling cytokine production and proliferation of T cells.

Although CD2-$Egr2^{-/-}$ mice developed a late onset of systemic autoimmune disease, due to a functional overlap between Egr2 and Egr3, our group subsequently developed CD2-$Egr2/3^{-/-}$ double knockout mice and found that these mice displayed an alarming early onset of systemic autoimmune disease, characterised by lymphocytic infiltration in multiple organs, high levels of self-antibodies and glomerulonephritis (Li *et al*., 2012). These mice had splenomegaly and super enlarged lymph nodes with increased numbers of highly activated B and T cells. Although Safford and colleagues (2005) had previously shown Egr2 and 3 as negative regulators of T cell activation,

unusually Egr2 and 3 deficient T cells displayed impaired proliferation in response to antigen stimulation *in vitro* and impaired IL-2 production. In addition, production of pro-inflammatory cytokines such as, IFNγ, IL-17, IL-21, GM-CSF and IL-4 was enhanced. Our group went on to establish hyper-activated STAT1 and STAT3 and reduced SOCS1 and SOCS3 expression. Egr2 was found to directly induce the expression of SOCS1 and SOCS3, which defined the hyperactive states of STAT1 and STAT3, respectively. These results brought to prominence the importance of Egr2 and 3 in prevention of autoimmune disease and limiting immunopathology during T cells responses (Li *et al*., 2012).

1.7 Egr2 and Egr3 conditional knockout mouse model

1.7.1 Development of CD2-Egr2/3$^{-/-}$ and CD2-Egr2 Tg mouse models

To investigate the function of Egr2 and 3 in T cell responses, conditional knockout for Egr2 and 3 in B and T cells were developed by Professor Wang's group. In addition, they also developed a conditional transgenic mouse with enhanced Egr2 CD2^{+} lymphocytes. The proceeding paragraphs describe the process of how these mice were generated.

The CD2-Egr2/Egr3$^{-/-}$ mice were generated on a C57BL/6 background and this process is described here briefly. The transcription factors Egr2 and Egr3 are required for hindbrain development and myelination of the peripheral nervous system (Taillebourg *et al*., 2002). Germ line deletion of Egr2 and Egr3 causes severe developmental defects and the mice die at a very young age. Therefore, a conditional knockout model was generated, where the Egr2 and Egr3 genes were deleted in B and T lymphocytes. This was achieved by utilizing the Cre-loxP system. This system is derived from P1 bacteriophage, where the Cre-recombinase will excise any region of DNA placed between two loxP sites. A loxP site is a 34 bp nucleotide sequence that can be targeted around a gene of interest. The expression of Cre-recombinase can be categorically induced in B and T lymphocytes by cloning a CD2 promoter upstream of Cre. CD2 is exclusively expressed in B and T cells, therefore Cre-recombinase under the control of CD2 promoter, will excise the target DNA of interest in B and T cells

only. Egr2 is expressed during development in immature and myelinating Schwann cells and has been observed in neuronal populations of the cortex (Herdegen *et al*., 1993). Postnatal lethality of $Egr2^{-/-}$ mice prevented the analysis of late functions and so conditional knockouts were generated. $Egr2^{LoxP/LoxP}$ mice were obtained from P. Charnay's group (National de la Santé et de la Recherche Médicale, Paris, France), where the LoxP site flanked 5' immediately preceding exon 2, which encodes that largest part of the protein and the zinc finger DNA binding domain (figure 1.8). The other loxP site is located on the 5' of exon 2 and 3' of the neomycin cassette (Taillebourg *et al*., 2002) (figure 1.8). These mice, referred to as $Egr2^{Flox/Neo}$ mice were interbred with human-CD2-Cre (hCD2-Cre), which induces Cre mediated recombination in CD2 specific B and T cells as represented in figure 1.8.

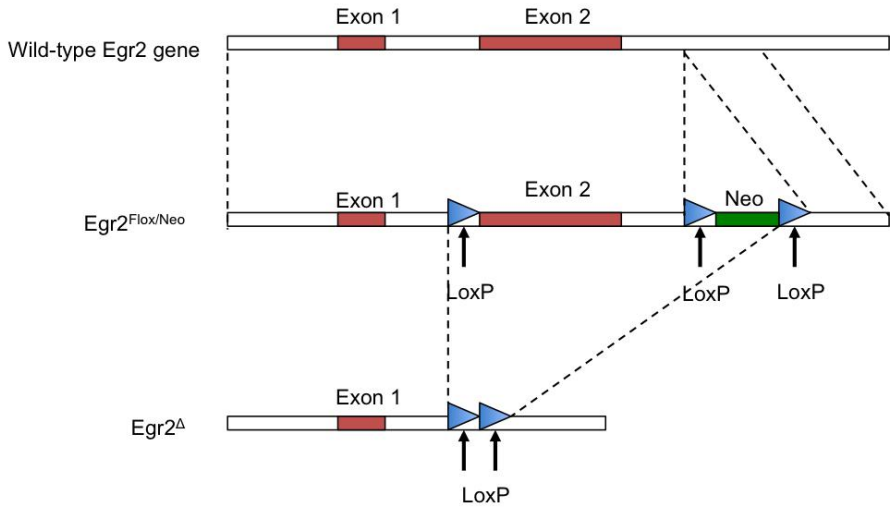

Figure 1.8: Diagrammatic representation of generation of single Egr2 knockout

The wild-type Egr2 gene contains 2 coding exons, as indicated. Exon 2 is responsible for encoding a large part of the protein and the zinc finger DNA binding domain. Therefore, Exon 2 is deleted by homologous recombination by Cre-LoxP system, but not exon 1. LoxP sites are represented by blue triangles (This figure was adapted from Taillebourg and colleagues 2002).

1.7.2 Egr3 knockout mice

Egr3 was deleted systemically and Egr3-deficient mice were obtained from Tourtellotte group (Washington University, School of Medicine, St Louis, USA) and briefly they were generated as follows. Egr3-deficient mice were generated by targeted mutagenesis in embryonic stem cells. A DNA fragment containing the whole *Egr3* gene was subcloned into a pBluescript vector, and a 1.5kb fragment containing the neomycin resistance selection cassette pMC1NeopA was used to disrupt the gene coding sequence by deleting a 0.9 kb fragment encoding the zinc finger domain and the remaining carboxyl terminus of the protein (Tourtellotte *et al* 1998) generating Egr3 knockout mice ($Egr3^{-/-}$) (Figure 1.9).

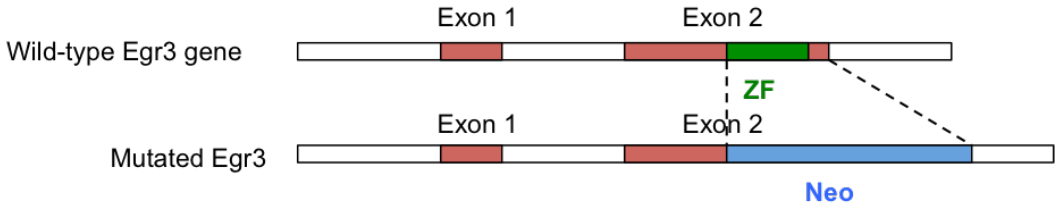

Figure 1.9: $Egr3^{-/-}$ knockout mice

To generate Egr3 deficient mice, inserting a neomycin resistant cassette disrupted exon 2 of the Egr3 gene. Exon 2 is responsible for housing the coding information for the zinc finger (ZF) DNA binding domain. The neomycin cassette therefore disrupts the ZF domain at the remainder of the carboxyl terminus. This figure was generated in Microsoft Power Point and is a diagrammatic representation of the disruption of the Egr3 gene by the neomycin cassette.

T and B cell conditional Egr2 and Egr3 knockout mice were generated by interbreeding hCD2-$Egr2^{-/-}$ with $Egr3^{-/-}$ on C57BL/6 background identified as hCD2-$Egr2^{-/-}$$Egr3^{-/-}$. hCD2-$Egr2^{-/-}$$Egr3^{-/-}$ mice were backcrossed with C57BL/6 mice more than 30 times to minimize the homeostatic disorder that develops in hCD2-$Egr2^{-/-}$$Egr3^{-/-}$ mice. These mice will be referred to as CD2-$Egr2/3^{-/-}$ from here on.

1.7.3 hCD2-Egr2 transgenic mice

To generate hCD2-Egr2 transgenic mice the Egr2 gene was cloned in the pBABE-Egr2 EGFP plasmid. The hCD2 was cloned into this vector with the aid of appropriate restriction sites. The hCD2-Egr2 construct was injected into the pro-nuclei of fertilized oocytes on C57BL/6 background mice. All mice used in this study were of 7-8 weeks of age unless otherwise stated.

1.8 Aims of study

The aim of the project is to establish whether Egr2 and Egr3 control T cell activation and differentiation *in vivo* in response to viral infection. Also, to investigate the mechanism of how Egr2 and 3 regulate IFNγ expression in activated T cells.

Specific objectives of the study are:

- To investigate T cell activation and proliferation during viral infection in mice by examining cell surface marker expressions such as, CD44, CD62L, CD69, and Ki67.
- Study T cell differentiation *in vivo* in mice response to viral infection and T helper differentiation *in vitro* with respect to effector cytokine production.
- Generate IFNγ luciferase reporter gene with IFNγ promoter and enhancer regions to study IFNγ expression *in vitro*.
- Study the expression of T-bet in Egr2 and 3 deficient cells and generate T-bet expression vectors to study protein interactions.
- Investigate a potential feedback regulation of Egr2 and Egr3 expression by effector cytokines, such as IFNγ and IL-12.

Chapter 2: Methods and Materials

2.1 Mice

2.1.1 Maintenance of mice

All wild-type (WT) mice are C57BL/6, which were used as controls in all experiments. The Egr2 and 3 double knockout (K2-3 or CD2-Egr2/3$^{-/-}$) and Transgenic (Tg or CD2-Egr2 Tg) mouse models were generated on C57BL/6 background. All mice were analysed at 7-8 weeks of age and both sexes were included without randomization or 'blinding'. Mice were maintained in the Biological Services Unit, Brunel University and according to the established institutional guidelines under the authority of a UK Home Office project license.

2.1.2 Virus and Infection

All work regarding maintaining the mice, administering virus, sacrificing mice and monitoring disease was done by Dr Su-ling Li at the animal facility, Brunel University London. All mice were infected with Western Reserve (WR) strain of ovalbumin encoding vaccinia virus (OVA-VV_{WR}). Mice were infected intranasally with 2 x 10^5 PFU of vaccinia virus in 10 µl of physiological saline. The mice were weighed and observed for illness daily. OVA-VV_{WR} stocks were grown using TK143 cells in T175 flasks infected at a multiplicity of infection of 0.5 and were harvested at 72 hours. Virus was isolated by rapid freeze thawing the cell pellet three times in 5 ml DMEM containing 10 % fetal calf serum (FCS). Cell debris was removed by centrifugation. Clarified supernatant was frozen at -80°C as virus stock. OVA-VV_{WR} stocks were titrated using TK143 cells.

2.2 Cell Culture

2.2.1 Isolation of $CD4^+$ and $CD8^+$ T cells

Secondary lymphoid organs including the spleen and lymph nodes were extracted from sacrificed mice under sterile conditions. A cell suspension was prepared by homogenization of these organs with 1 X phosphate buffered saline (PBS) solution. The cells were pelleted by centrifugation at 1200 rpm and lysed with 0.8 % ammonium chloride solution by incubation at 37 °C for 5 minutes. This step lyses the erythrocytes but maintains the viability of lymphocytes, provided the lyses step is stopped at 5 minutes. The cells were

centrifuged at 1200 rpm for 5 minutes at which stage all other cells apart from red blood cells pellet, which was re-suspended in 1 x PBS.

To isolate $CD4^+$ or $CD8^+$ counterparts, cells were isolated by Magnetic Activated Cell Sorting (MACS) positive selection kit (Miltenyi Biotec) according to the manufacturer's instruction. The cells were incubated with anti-CD4 or anti-CD8 antibodies attached to magnetic beads (Miltenyi Biotec) at 4 °C for 20 minutes in beads buffer (PBS containing 0.5 % Foetal Bovine Serum, FBS) The anti-CD4 or anti-CD8 antibodies attached to magnetic beads bind to their respective cell types during this incubation period. After centrifugation, the pellet was re-suspended and washed in 1 x PBS solution to remove any unbound antibodies, and the cell suspension run through a magnetic column. The anti-CD4 and anti-CD8 antibodies attached to $CD4^+$ or $CD8^+$ cells bind to the magnetic column as the cells pass through the column. After washing the column a few times with beads buffer, CD4 or CD8 cells were eluted with Roswell Park Memorial Institute (RPMI) 1640 medium.

2.2.2 Isolating Naïve $CD4^+$ $CD62L^+$ $CD44^{low}$ T cells

To isolate naïve CD4 T cells, once red blood cells have been lysed by ammonium chloride and removed by centrifugation as described in 2.2.1, the remaining cells are directly labelled with fluorescent antibodies for naïve T cell markers and sorted by FACS. Naïve CD4 T cells are identified by the expression of CD62L and low expression of CD44. To exclude activated cells and T Regulatory cells (T_{Reg}), cells negative for CD25 expression were gated on. Therefore, fluorescently labelled antibodies for these markers were used to stain the cells and gate on $CD4^+$, $CD62L^+$ and $CD44^{low}$. Dr Gary Warnes at Queen Mary University, Blizard Institute sorted Naïve CD4 T cells on the FACS Aria sorter (BD Immunocytometry systems).

2.2.3 Cell Culture and Stimulation and cytokine production in T cells

$CD4^+$ and $CD8^+$ T cells were maintained in RPMI 1640 medium, which was supplemented with 10% Foetal Bovine Serum (FBS), 300 mg/L L-glutamine, 50 µM β -mercaptoethanol (2-ME) and 50 µg/ml gentamicin (all from Invitrogen).

Cells were cultured at 37 °C in 5 % CO_2. To stimulate the cells two different approaches were used; one involved using plate bound anti-CD3 and at 5 µg/ml and anti-CD28 at 2 µg/ml for approximately 16-18 hours unless otherwise specified. In the second approach the cells were stimulated with a combination of soluble phorbol 12-myristate 13-acetate (PMA) and Inomycin at 200 ng/ml for both, which stimulates the cells by implementing the Ca^{2+} signalling pathway (Chatila *et al*., 1989). To analyse cytokine expression by flow cytometry, after stimulation with anti-CD3 and anti-CD28 for 16-18 hours, cells were further stimulated further with PMA and Inomycin for 3 hours in the presence of Golgi Stop (BD Biooscіences) according to the manufacturer's instruction. Human Embryonic Kidney 293 (HEK293) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with Penicillin and Streptomycin (P/S). These cells did not require any stimulation.

2.2.4 Cytokine production by antigen specific T cells

Purified CD8 cells (5×10^4) were incubated with OVA-VV_{WR} infected LB27.4 cells at 1:1 or 1:5 ratios in 96-well plates in triplicate for 48 hours, in the presence of Golgi Stop (BD Biosciences) according to the manufacturer's instruction. LB27.4 cells are B cell hybridoma cells that express both MHC class I and II proteins of the H-2 Kb haplotype. These cells were maintained in DMEM supplemented with 10% FCS, 2 mM L-glutamine, and antibiotics. Cells were routinely tested for mycoplasma contamination. Cells were infected with OVA-VV_{WR} at a multiplicity of infection of ~ 5 rounds and harvested between 8 and 12 hours after infection. Cells were then re-suspended in complete RPMI 1640 and used to stimulate CD8 cells.

2.2.5 T Helper Differentiation

Naïve $CD4^+$ $CD62L^+$ $CD44^{Low}$ cells from WT C57BL/6 mice were stimulated by anti-CD3 (5µg/ml) and anti-CD28 (2 µg/ml) in the presence of T helper differentiation inducing agents. Each lineage required a unique combination of cytokines and antibodies, described in table 2.1. The cells were analysed for Egr2 and T-bet expression by flow cytometry. To examine cells for cytokine expression, such as IFNγ, IL-4 and IL-17A, the cells were further stimulated for

3 hours with PMA/INO in the presence of Golgi Stop (BD Biosciences) according to the manufacturer's instruction. Analysis of transcription factors and cytokines were achieved by staining with fluorescently labelled antibodies and detected by flow cytometry.

T helper cell	Compound	Concentration	Company
T helper 0 (T_H0)	Interleukin-2 (IL-2)	20 ng/ml	R&D systems 402-ML-020
T helper 1 (T_H1)	IL-2	20 ng/ml	R&D systems 402-ML-020
	IL-12	20 ng/ml10	R&D system 419-ML-010
	anti-IL-4	10 µg/ml	Biolegend 504102
T helper 2 (T_H2)	IL-2	20 ng/ml	R&D systems 402-ML-020
	IL-4	100 ng/ml	Biolegend 574302
	anti-IL-12	10 µg/ml	Biolegend 505303
	anti-IFNγ	10 µg/ml	Biolegend 505702
T helper 17 (T_H17)	TGFβ	2ng/ml	R&D system 240-B-002
	IL-6	100ng/ml	Biolegend 575702
	anti-IL-4	10µg/ml	Biolegend 504102
	anti-IFNγ	10µg/ml	Biolegend 505702

Table 2.1 *In vitro* T helper differentiation conditions

2.2.6 Developing a mouse-T-bet expressing HEK293 cell line

Human Embryonic Kidney (HEK) 293 cells (American Type Culture Collection, ATCC) were utilized to generate a stable cells line expressing mouse-Tbet. Calcium phosphate method was used to transfect the cells (see section 2.2.7). HEK293 cells were transfected by Myc-tagged T-bet in pDNA3.1 (-) vector, which also houses the GFP protein sequence. After transfection, the cells were cultured for 16-18 hours at 37 °C and 5% CO_2. GFP positive cells were sorted by Dr Gary Warnes at the Blizard Institute, Queen Mary University on the FACS Aria sorter (BD Immunocytometry systems). GFP positive cells were cultured for a further 6-7 days followed by another sorting event of GFP positive cells. In total, GFP positive cells were sorted four times until a stable expression of T-bet was achieved without the need for any stimulation. Cells were subsequently stained for T-bet with anti-Tbet PE.cy7 to determine the percentage of T-bet positive cells.

2.2.7 Calcium Phosphate transfection of HEK293

To introduce DNA into HEK-293 cells (ATCC) the calcium phosphate method was used, which was developed by Graham and van der Eb (1973). In principle, this method works by the formation of calcium phosphate-DNA precipitate, and the calcium phosphate facilitates DNA binding to the cell surface. Precipitated DNA on the cell surface then enters the cell by endocytosis. This is a very simple method of transfecting cells with good transfection efficiency. Briefly, in 24 well plates, 50 x 10^3 HEK293 cells were seeded per well, in 500 µl of DMEM medium supplemented with penicillin and streptomycin (P/S). The cells were incubated at 37 °C 5 % CO_2 for 16-18 hours. The following the day the medium in the wells was replaced by fresh medium 2-3 hours prior to transfection. In a 50 µl reaction volume, the DNA was mixed with 1 x TE, 2.5 M $CaCl_2$ and H_2O to make up to 50 µl. To this 50 µl of 2 x HBS (Hepes Buffered Saline) was added, while continuously agitating the tube. The now 100 µl of reaction volume was pipetted into the HEK293 cells per well drop wise. The cells were then incubated for a further 16-18 hours at 37 °C 5 % CO_2.

HEK293 cells were transfected with Flag tagged mouse Egr2 or Flag tagged Egr2 zf, Flag tagged Egr3, Myc tagged T-bet or Myc tagged Tbox either singly or in various combinations by calcium precipitation. The expression of each tagged protein was confirmed by immunoblotting with antibodies against Myc for T-bet and Tbox, or Flag for Egr2, Egr2 zf and Egr3.

2.2.8 Transforming competent cells

When competent cells, such as DH10α *Escherichia coli* bacteria are subjected to temperatures of 42 °C, their cellular membranes are compromised and foreign material, such as DNA can seep into the cells. As the cells divide, the foreign DNA is copied, maintained in each progeny and thus produced in large quantities. Cells that house the transfected vector can be selected based the presence of an antibiotic resistance gene, for instance ampicillin. Cells transformed with vector are grown on agar supplemented with ampicillin and only cells positive for the ampicillin resistant gene are able to grow. Positive

clones are picked under sterile conditions and are grown further, analysed by restriction digestion and sequencing to ensure the sequence is correct.

To transform bacterial competent cells, briefly, to 25 µl of competent cells, the ligation product was added followed by heat shock at 42 °C for 30 seconds. The cells were then immediately transferred onto ice for 5 minutes. To this 100 µl of SOC medium (New England Biolabs) was added and spread on agar plates containing 100 µg/ml of ampicillin. The following day, 8 clones were picked, grown further in LB medium supplemented with 100 µg/ml of ampicillin. Transformed vector was isolated from these clones by QIAquick spin mini-prep kit (Qiagen) according to the manufacturer's instructions. In order to test whether the cloning was successful, the extracted vector was digested restriction enzymes (New England Biolabs) according to the manufactures. Detailed information regarding restriction sites are described for individual vectors in section 2.6, Generating Constructs.

2.3 Protein studies

2.3.1 Nuclear Protein Extraction

$CD4^+$ T cells were isolated from WT and CD2-Egr2/3$^{-/-}$ mice by positive selection with the MACS system (Miltenyi Biotec), see section 2.2.1. The cells were stimulated *in vitro* with plate bound anti-CD3 (5 µg/ml) and anti-CD28 (2µg/ml) for 16-18 hours to induce expression of proteins of interest. HEK293 transfected cells with singly or combinations of vectors such as, Flag tagged mouse Egr2, Flag tagged Egr2 zf, Flag tagged Egr3, Myc tagged T-bet or Myc tagged Tbox, were subject to this method of nuclear protein extraction also.

To extract nuclear protein, cells were first washed with 1 X PBS and the pellet was re-suspended in cytoplasmic lysis buffer (10mM HEPES pH7.9, 10mM KCl (Potassium Chloride), 0.1mM EDTA (Ethylenediaminetetraacetic acid), 0.2mM EGTA (ethylene glycol tetraacetic acid), 1mM DTT (Dithiothreitol), 100mM PMSF (phenylmethanesulfonyl fluoride)). PMSF is essential to add from this stage forward, as a serine protease inhibitor, which prevents protein degradation. The cells were left on ice for 15 minutes during which they swell.

To this 20% NP-40 (4-Nonylphenyl-polyethylene glycol) was added, a detergent that solubilises membrane and cytosolic proteins for isolation. Proteins can retain their native state in the presence of this buffer and protein-protein interactions can be preserved. The cells were vortexed for 30-60 seconds after adding 20 % NP-40. After centrifugation, the supernatant now containing cytosolic protein was removed and the protein was stored at – 80 °C if required for any future experiments. To continue extracting nuclear protein, the pellet was re-suspended in nuclear protein lysis buffer (NPI) and PMSF and agitated for 1 hour at 4 °C. Finally the cell suspension was centrifuged at 13000 rpm for 10 minutes and the supernatant transferred to a clean tube, which contains nuclear protein.

2.3.2 Protein Quantification – Bradford Assay

The Bradford reagent was used to determine protein concentration. In principle, the Bradford reagent contains a dye named Brilliant Blue G, which when mixed with the protein lysate forms a complex with the proteins in the solution. The protein dye complex causes a shift in the absorbance from 495 nm to 595 nm and the amount of absorption is proportional to the protein present. Absorbance was measured at 595 nm in a KC4 luminometre (Bio-Tek Instrument Inc). A standard curve was prepared using a serial dilution from 0 mg/ml to 10 mg/ml of purified Bovine Serum Albumin (BSA) 100 X (New England Biolabs). A standard curve was plotted with absorbance value against BSA concentration and the protein concentrations of unknown samples were determined from this standard curve. Protein samples of unknown concentration were diluted by 1:250 in H_2O, to which an equal volume of Bradford reagent was added. The standard curve was also plotted with a 1:250 dilution in H_2O. After a brief incubation at room temperature for 5 minutes, absorbance values were determined at 595 nm and the data analysed.

2.3.3 Co-Immunoprecipitation

Co-immunoprecipitation (Co-IP) experiments were carried out to determine physiologically relevant protein-protein interactions. The method relies on antibodies (monoclonal or polyclonal) with high affinity for target proteins. This

antibody-protein complex is precipitated and leads to the indirect capture of proteins that are bound to the target protein in question. This protein complex is resolved on a polyacrylaminde denaturing gel (see SDS-PAGE and Western Blotting, 2.3.4), which will consequently give information about the protein-binding partners. Nuclear cell lysates were prepared as described in 2.3.1, from both primary CD4 T cells and transfected HEK293 cells where indicated.

Prior to the first step before capturing the target protein with specific antibodies, the crude cell lysate is cleared of immunoglobulins that can bind non-specifically to protein A or G (protein G used in this project). The cells were agitated for 1 hour at 4 °C with protein G beads (New England Biolabs). To remove protein G beads after 1 hour of agitation, the lysate was centrifuged and the supernatant with the pre-cleared cell lysate was transferred into a clean eppendorf tube. A total of 5 µg of antibody against either T-bet (eBioscience - cat 12-5825-80, clone 4B10), Egr2 (eBioscience cat 17-6691-82, clone erongr2), anti-Myc for Tbet or Tbox and anti-Flag for Egr2 Egr2, Egr2 zf and Egr3 (anti-Myc and Flag antibodies from Cell Signaling Technology) was used to capture the protein in question by incubation on a circular rotor for 2 hours at 4 °C. To this approximately 25 µl of magnetic protein G beads were added (New England Biolabs) and incubated for a further hour on a circular rotor at 4 °C. The magnetic beads should now be in complex with the antibody plus the antibody specific protein. The beads were washed in a wash buffer (10mM HEPES pH7.9, 10mM KCl 0.1mM EDTA, 0.2mM EGTA, 1mM DTT, 100mM PMSF) 3 times and finally suspended in sample loading buffer (see SDS-PAGE for buffer details). At this stage the magnetic protein G beads were separated from its constituent target captures by heating at 95 °C for 5 minutes. A magnetic field was applied to draw the beads away leaving a protein lysate in sample loading buffer, which was loaded into the wells of a denaturing polyacrylamide gel for separation, blotting and development.

2.3.4 SDS-PAGE and Western Blotting

Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) allows the separation of proteins based on their size. Although the proteins are separated based on their size, their migration through the electric field is dependent upon both its size and charge. The proteins migrate through a denaturing gel from negative to positive. The charge of the protein is not proportional to its size, unlike DNA molecules. The proteins are treated with the detergent SDS, which consequently coats the proteins with negatively charged suphate groups. The hydrophobic dodecyl chains of SDS interact with the hydrophobic amino acids in the proteins. The amount of hydrophobic residues is usually proportional to the size of the protein, therefore the amount of associated SDS and consequently the degree of negative charge is also proportional to the size. When the proteins are run on a polyacrylamide gel in an electric field, proteins treated with SDS will migrate depending on their size and are separated by electrophoresis. A 10 % separating gel (Deionized H_2O 7.95 ml, TRIS 1.5M (pH 8.8) 5 ml, 10 % SDS 200 µl, Protogel 30 % 6.65 ml, 10 % APS – 200 µl, TEMED 12.5 µl) and a 5 % stacking gel (Deionized H_2O 3.725 ml, TRIS 0.5 M (pH 6.8) 625 µl, 10 % SDS 50 µl, Protogel 650 µl, 10 % APS 25 µl, TEMED 5 µl) were made for this process.

First the protein concentration was determined with the aid of the Bradford Assay (see Protein Quantification section 2.3.2) to ensure equal loading. To load the protein into the wells, the samples were mixed with sample loading buffer (187.5 mM Tris-HCl (pH 6.8), 6 % w/v SDS, 30 % Glycerol, 150 mM DTT, 0.03 % w/v Bromophenol Blue, 2 % β-mercaptoethanol) and heated at 95 °C for 5 minutes. After heating, the proteins were immediately transferred onto ice to cool. 15-20 µl of protein sample was loaded into each well and an electric potential of 100-150 V applied for approximately 35-40 minutes. The gel was carefully removed ensuring it does not break or dehydrate, followed by transfer onto a nitrocellulose membrane (Amersham). All component of the transfer cassette, for instance, sponge, filter papers were soaked in transfer buffer (NuPAGE) before assembling. The nitrocellulose membrane was soaked in

methanol for 30-60 seconds followed by soaking in distilled water for 2-3 minutes and then finally transferred into 1 X transfer buffer from NuPAGE.

All components for transfer as indicated above were placed in a transfer cassette in this wet transfer system. An electric potential of 30 V was applied for 1.5-2 hours at 4 °C. The negatively charged proteins migrate towards the positive, however when the proteins reach the nitrocellulose membrane, they are arrested and thus preventing further migration. Proteins are aligned in the same manner as they were on the gel. The nitrocellulose membrane was removed after transfer and unspecific binding sites were blocked by 5% skimmed milk or 5% BSA prepared in TBS-T (Tris Buffered Saline 0.1% Tween-20) at room temperature for 30 minutes before being incubated with primary and secondary antibodies. The primary antibodies were diluted according to the manufacturer's instruction in TBS-T and the incubated with membrane over night at 4 °C with continuous agitation. The following day unbound antibody was washed from membrane with several rounds of TBS-T, and further incubated with Horseradish Peroxidase conjugated secondary antibodies diluted according to the manufacturer's instruction in TBS-T for 1 hour at room temperature with continuous agitation. Information of primary and secondary antibodies used is provided in table 2.2.

Primary Antibodies		Secondary Antibodies	
Antibody	Dilution	Antibody	Dilution
Egr2 Mouse (eBioscience) cat 17-6691-82, clone erongr2	1:1000	Mouse monoclonal Anti-Rat Kappa Light Chain HRP (abcam, ab99692)	1:100000
T-bet Rabbit polyclonal (Santa-Cruz H-210)	1:1000	Anti-Rabbit HRP (Cell Signaling Technologies)	1:100000
Mouse **Myc**-Tag mAB (Cell Signaling Technologies, 71010)	1:1000	Anti-mouse HRP (Cell Signaling Technologies, 7076)	1:10000
Mouse **Flag**-Tag mAB (Sigma Life Science FI804)	1:1000	Anti-Mouse HRP (Rockland 18-8817-30)	1:1000

Table 2.2 Antibodies for Immunoblotting

2.3.5 Protein detection with Chemiluminescence

Proteins on the nitrocellulose membrane were detected by the ECL (Enhanced Chemiluminescence) plus kit (GE Health Care UK Ltd for both), according to the manufacturer's instruction. The kit contained two solutions Solution A; Luminol Enhancer Solution (RPN2232V1) and Solution B; Peroxidase Solution (RPN2232V2) (GE Health Care UK Ltd for both). These were mixed at a 1:1 ratio prior to use. Briefly, the nitrocellulose membrane was placed on a cling film and the mixture of chemiluminescence solution A and B poured on to it, making sure all areas are covered generously. This was left for 5 minutes at room temperature in the dark. The membrane was rid of all excesses chemiluminescence solution and completely covered by the cling film; this was then developed onto Hyperfilm ECL (Amersham).

2.3.6 Electrophoretic Mobility Shift Assay (EMSA)

EMSA is a technique that allows the detection of protein-DNA complexes. The observation is based on the principle that the electrophoretic mobility of a

protein-nucleic acid complex is typically less than that of free nucleic acid. EMSA probes labelled with Cyanine5 (Cy5) for T-bet was adopted from Szabo and colleagues (2001), incorporating a T-bet consensus binding sequence. The binding consensus for Egr2 was adapted from (Chavrier *et al* 1990). Sense and anti-sense oligonucleotides were annealed by firstly mixing together, followed by heating for 5 minutes at 95 °C and allowed to gradually cool at room temperature. Heating at 95 °C denatures any secondary structures that may be present in the oligonucleotides so that they can bind to the complementary sequence upon cooling.

The consensus probe for Egr2 (5'-TGTAGGGGCGGGGGCGGGGTTA-3') or T-bet (5'- GACAGCTCACACTGGTGTGGAGCAGGG-3') were labelled with Cyanine5 (Cy5) on both sense and anti-sense strands by Sigma Aldrich. Labelled probes for Egr2 and T-bet were individually incubated with a protein mixture in the presence of other components necessary to promote protein-DNA binding. Nuclear extracts were from CD4 T cells stimulated with anti-CD3 (5 µg/ml) and anti-CD28 (2 µg/ml) for 16 hours and HEK293 cells transfected with a combination of vectors, including Myc tagged T-bet and Tbox and Flag tagged Egr2, Egr2 zf and Egr3. The combination of vectors are specified for individual experiments.

A typical reaction mixture contained, EMSA buffer (50mM KCl, 20mM HEPES pH7.9, 1mM EDTA, 1mM DTT), salmon-sperm DNA (ssDNA), 50mM $MgCl_2$, Nuclear protein extract (10 µg or protein), specific antibodies such as anti-Tbet (sc-21003X, Santa Cruz Biotechnology) or anti-Egr2 (eBioscience) and non-specific antibodies for negative controls, such as anti-IgG for supershift reactions. dH_2O was used to make up the desired reaction volume, which for most experiments was 20 µl, unless otherwise stated. Once all reaction components were mixed, the reaction was incubated at room temperature for 20 minutes in the dark. Samples with antibodies for supershift reactions were further incubated at room temperature for 10 minutes to allow antibody binding to its target protein complex. The samples were electrophoresed on 5 % polyacrylamide gels (30% Acrylamide 1.5 ml,

10 x TBE (Amresco) 600 µl, 10 % Ammonium persulphate (APS) 200 µl, TEMED (N,N,N',N'tetramethylethylene diamine) 40 µl, H_2O 9.7 ml) in 0.5 x Tris/Borate/EDTA (TBE). The gels were run at 100 V for 1.5 hours at 4 °C. The gels were scanned using a Typhoon 9400 imager (Amersham Biosciences).

Probe sequence – sense and anti-sense	**Labelled or Un-labelled**	**Transcription Factor specific for**
5'-TGTAGGGGCGGGGGCGGGGTTA-3' 3'-ACATCCCCGCCCCCGCCCCAAT-5'	Cyanine5	Egr2
5'-GACAGCTCACACTGGTGTGGAGCAGGG-3'* 3'-CTGTCGAGTGTGACCACACCTCGTCCC-5'	Cyanine5	T-bet

*The red highlighted bases represent T-bet binding consensus sequence from Szabo *et al* 2001. These sequences are derived from the IFNγ promoter.

Table 2.3 EMSA Probes

2.3.7 Enzyme-Linked Immunosorbent Assay (ELISA)

To measure IFNγ concentration in the cell culture supernatant of CD4 cells during phases of TCR stimulation for 5 hours with anti-CD3 (5ug/ml) and anti-CD28 (2ug/ml), and rest for 16 hours, a mouse ELISA kit (BD Biosciences OptEIA™ Cat 555138) was used. The procedure was performed according to the manufacturer's instructions. Briefly, 96 well plates were coated with coating buffer (0.1M Calcium Carbonate solution) with capture antibody 250x overnight at 4 °C. The coating buffer was washed 5x with wash buffer (0.05 % Tween-20 in 1x PBS), after which the plate was blocked with fresh blocking buffer (10 % FBS in 1x PBS) at room temperature for 1 hour. The plate was washed 5 times with wash buffer, followed by addition of 100 µl of each sample per well, including a IFNγ standard serial dilution (2000 pg/ml, 1000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.3 pg/ml and 0) to generate a standard curve, according to the manufacturer's instruction. The plate was incubated at

room temperature for 2 hours and subsequently washed 5 times. Detection antibody plus AvidinHRP (100 µl) was added to each well and incubated at room temperature for 1 hour. After incubation, this plate was aspirated and washed 10 times, immediately followed by the addition of substrate solution, which contains tetramethylbenzidine (TMB), and a further incubation for 30 minutes in the dark at room temperature. The absorbance was read at 450 nm and the data was analysed.

2.4 Polymerase Chain Reaction

2.4.1 RNA Extraction

Total RNA was extracted using TRI-reagent (Sigma 93289). This method is adopted from the Phenol Chloroform extraction method. TRI-reagent is a monophasic solution of phenol, guanidine isothiocyanate and a combination of other chemicals, which facilitate the isolation of nucleic acids from other cellular components. In principle, the acidic conditions result in the separation of the RNA from the DNA and proteins following phenol chloroform extraction. Proteins such as RNAse are denatured by guanidine isothiocyanate, which is a strong denaturing agent. The RNA was extracted according to the manufacturer's protocol.

RNA was isolated from CD4 T cells, approximately 3-5x10^6 cells were collected and frozen at -80 °C for RNA extraction. Briefly, CD4 T cells were pelleted and then lysed in 500 µl of TRI reagent (Sigma 93289), vortexed for 30-60 seconds and incubated at room temperature for 10-15 minutes. To this 350 µl of chloroform (Sigma C2432) was added and immediately vortexed for 30 seconds followed by centrifugation at 13000 rpm at 4 °C for 20 minutes. Following centrifugation, there were three clearly separate layers, a red organic phase and a clear aqueous phase separated by a white interphase. DNA and proteins reside in the organic phase and the RNA in the aqueous phase, which was carefully pipetted into a clean tube. To this 300 µl of isopropanol, 2 µl of glycogen and 3 µl of sodium acetate (Sigma S-7899) were added. Nucleic acids are not soluble in alcohol and so the RNA precipitates out of the solution.

Glycogen also co-precipitates with the RNA, which aid in the visualization of the pellet. Before centrifugation the cells were incubated at -20 °C for 1 hour. After centrifugation, the RNA pellet, which should be fairly visible, was washed in 80% ethanol and re-suspended in RNAse-free water for use in further experiments.

2.4.2 First strand cDNA synthesis

The next stage was to reverse transcribe RNA into cDNA with the aid of the enzyme reverse transcriptase. Reverse transcriptase uses the RNA as a template and synthesizes its complementary cDNA. RNA was converted to first strand cDNA with SuperScript^{TM} III (Thermo Fisher Scientific). This system provides a one step cDNA synthesis and PCR amplification in the same tube. The reverse transcriptase (RT) is a version of Murine Moloney Leukaemia Virus (M-MLV), which can be used to synthesis cDNA from total RNA. First strand cDNA synthesis was performed on 1 µg of RNA, the concentration of which was determined by measuring absorbance at 260 nm. RNA was incubated with oligo-dT nucleotides that act as primers (random primers) for RT. Volumes of solution were chosen to give 1 µg of RNA per reaction, 40 ng of oligo-dTs and H_2O supplemented with RNAse inhibitor. These were then heated at 70 °C for 5 minutes to denature any secondary structures present in the RNA and facilitating the binding of oligo-dT primers. The samples were then placed on ice. For PCR, reagents were finally added to give a final concentration of 10 mM of dNTPs, 25 mM of $MgCl_2$, 1 unit/µl of RNAse inhibitor and 1 µl of SuperScript^{TM} III RT (200 Units/µl) and 1 X RT buffer. The reaction mixture was heated to 25 °C for 5 minutes and then 42°C for 1 hour. The reaction was inactivated by heating at 70 °C for 15 minutes. The samples were stored at -20 °C.

2.4.3 Real-Time PCR

An antibody for Egr3 has not yet been produced; therefore to measure Egr3 expression, RT-PCR was used and western blot where necessary. As described in section 2.4.1 and 2.4.2, RNA was extracted from CD4 T cells and cDNA synthesized by reverse strand synthesis. The concentration of cDNA was

determined by measuring absorbance at 260 nm. RT-PCR is a highly sensitive technique, which is based on the same principles as PCR with the addition of allowing quantitative comparison of gene expression in different samples. Quantification of a target gene expression is based on the relative expression of a reference gene, referred to as a housekeeping gene. Housekeeping genes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β -actin are known to be stably and constitutively expressed at high levels in tissues and cells. Quantitation of newly synthesized PCR products in real-time is made possible by SYBR Green fluorescent dye, which directly binds to double stranded DNA. Following each round of PCR product synthesis SYBR Green binds to newly formed double stranded DNA and quantified by measurement of fluorescence.

Each RT-PCR reaction was set-up with SuperFAST SYBR green (A&B) 1 X as per manufacturer's instruction with 1 µl of cDNA and a final concentration of 2 µM sense and anti-sense primers for Egr3. RT-PCR was performed on Rotor-Gene systems (Corbett Robotics) using the program; 95 °C 5 minutes and 35 cycles with 95 °C 30 seconds, 62 °C 30 seconds and 72 °C 30 seconds.

At 95 °C the double stranded DNA is denatured. During each elongation step, fluorescent data is collected. Upon completion of the cycles, a melting curve analysis if performed, during which fluorescent intensity is monitored as the temperature is gradually increased from 50°C – 90 °C. As the melting temperature (Tm) reaches an optimum for each DNA molecule present in the reaction mixture, 50 % of the molecule is denatured which is detected as a decrease in fluorescence. The calculated Tm for each product then allows the detection of non-specific products such as primer dimers, since SYBR green will bind to any double stranded DNA molecule. The PCR products were routinely run on agarose gel to confirm that there was only one specific band of the correct size.

Rotor Gene Software was used for RT-PCR data analysis. Every reaction displays an exponential increase when the number of copies of DNA doubles during a cycle, called the exponential phase. This phase is followed by a plateau phase where increase after each cycle is comparatively small, which is normally when one of the reagents becomes limiting. A threshold value is chosen in the exponential phase and the number of cycles (Ct) it takes the fluorescence to reach this threshold value for each sample. The Ct values were normalized with GAPDH or β-actin, which corrects small variances in mRNA amount. The relative expression of Egr3 with reference to GAPDH or β-actin was calculated by the following equation: Relative expression = 2^ ($Ct_{(GAPDH)}$)-$Ct_{(Egr3)}$) x 10,000. All samples were run in triplicates.

2.5 Flow Cytometry

With the aid of flow cytometry, properties of individual cells can be investigated such as expression of cellular markers, transcription factors and cytokine expression at the single cell level. Antibodies that have high affinity for the protein of interest conjugated with fluorescent markers enables their detection. The cells are first stained for cell surface markers after which they are fixed with formaldehyde to stabilize the cell membrane and permeabilize with the detergent saponin to allow entry of antibodies into the cell, to stain intracellular proteins such as cytokines or nuclear proteins such as transcription factors.

Fluorescence	Antibody specific for	Catalogue Number and Clone	Company
Fluorescein isothiocyanate (FITC)	CD4	cat 11-0041-81 clone: GK1.5	eBioscience
	CD8	cat 11-0452-81 clone 53-6.7	
	IFNγ	cat 11-7311-81 clone XMG1.2	
	TCRβ	cat 11-5961-81 clone H57-597	
Phycoerythrin (PE)	CD4	cat 12-0041-81 clone GK1.5	
	CD8	cat 12-0081-81 clone 53- 6.7	
	CD25	cat 12-0251-81 clone PC61.5	
	CD62L	cat 12-0621-81 clone MEL-14	
	CD69	cat 12-0691-81 clone H1.2F3	
	IL-2	cat 12-7021-81 clone JES6-5H4	
	IL-4	cat 12-7041-81 clone 11B11	
	IL17A	cat 12-7177-81 clone 17B7	
	Ki67	cat 12-5698-82 clone SolA15	
	Egr2	cat 12-6691-80 clone erongr2	
	Granzyme B	cat 12-8898-80 clone NGZB	
Allophycocyanin (APC)	CD44	cat 17- 0441-81 clone IM7	
	Egr2	cat 17-6691-82 clone erongr2	
	TNFα	cat17-7321-81 clone MP6- XT22	
PE-cy7	CD44	cat 25-0441-81, clone IM7	
	T-bet	cat 12-5825-80 clone 4B10	
Un-labeled	CD28	cat 557393 clone 37.51	BD Biosciences
	Myc	cat 2276, clone 9B11	Cell Signaling Technology
	Flag	cat 8146, clone M2	
FITC	MHC Tetramers H2Kb peptides	MHC molecules bearing OVA-SIINFEKL	National Institutes of Health Tetramer Core Facility, Emory University, Atlanta GA

Table 2.4: Antibodies Information

2.5.1 Nuclear and cytokine staining with fluorescent antibodies for flow cytometry.

Cells to be stained were pelleted and re-suspended in 1 X PBS to wash, followed by centrifugation. To stain cells for cell surface markers, 2 µl of antibody was added and the cells were incubated at room temperature for 15-20 minutes. After which, the cells were washed and re-suspended in 1 X PBS for analysis by flow cytometry. For intracellular or nuclear staining, the cell pellet was re-suspended in 300 µl of 1 X Fixation/Permiabilisation (Fix/Perm) buffer (eBioscience) and incubated at room temperature for 15 minutes. The Fix/Perm solution was prepared according to the manufacturers standard. Following centrifugation, the cell pellet was re-suspended in 1 X Permeabilisation buffer (eBioscience) and incubated at room temperature for a further 10 minutes. To the tube now containing the cells in 1 x permeabilisation buffer, 1 x Permeabilisation/Wash (P/W) (BD Biosciences) buffer was added. After this wash step, the cell pellet was re-suspended in relevant antibodies diluted in P/W buffer and incubated in the dark for 20 minutes at room temperature. Finally, the cells were washed to remove un-bound antibodies and re-suspended in 1 X PBS for immediate analysis by flow cytometry. T cells from OVA-VV infected mice after incubation with OVA-VV_{WR} infected LB27.4 cells *in vitro* were stained with SIINFEKL H2-Kb tetramer, prior to staining for surface molecules. The cells were subsequently stained for surface antigens and intracellular proteins as indicated. Professor Ping Wang did SIINFEKL Tetramer staining for detecting antigen specific T cells.

2.5.2 Proliferation

Naïve CD4 cells were labelled with CFSE (Invitrogen). CFSE is a protein dye and this amino reactive dye forms stable covalent bonds with cell proteins. Cell division is measured as successive halving of the fluorescent intensity of CFSE by flow cytometry The cells were stimulated for 72 hours with 5µg/ml anti-CD3 and 2µg/ml anti-CD28 before analysis by flow cytometry. These experiments were done by Professor Ping Wang.

2.6 Generating constructs

2.6.1 Cloning Interferon Gamma (IFNγ) promoter (-468) and CNS-22 in pGL2-b luciferase construct (IFNγ-pGL2-b)

Designing IFNγ-pGL2-b construct on UGENE

The IFNγ gene DNA sequence was obtained from Ensembl (Mouse IFNγ gene ENSEMBL ID: ENSMUST00000068592), which contains 4 exons. The -468 promoter region is located immediately upstream of the transcriptional start site (TSS). The Conserved non-coding sequence (CNS) located -22 kb upstream of the promoter was obtained from Hatton *et al* (2006). The -468 IFNγ promoter and CNS-22 fragments were first cloned into pGL2-b on UGENE, which is a free open-source bioinformatics software and can be used to create and edit nucleic acid and protein sequences. The vector chosen for this study is the pGL2-basic, which carries the coding region for firefly luciferase (*Photinus pyralis*). This provides a sensitive and rapid quantification of reporter activity. Vector sequences, such as pGL2-b can be downloaded into the software and DNA sequences of interest can be cloned into it in the desired orientation. This software was also used to identify potential restriction sites for cloning and to design primer sequences accordingly.

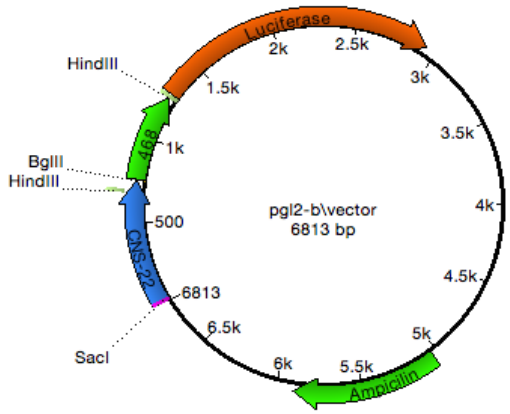

Figure 2.1: Circular view of CNS-22 and -468 IFNγ enhancer and promoter regions in pGL2-b: indicating, HindIII, BgIII and SacI restriction sites used for cloning. HindIII has 2 site, the second site is located in the CNS-22 region

Designing primers for -468 and CNS-22

The primers for the IFNγ promoter -468 and CNS-22 were designed based on their respective sequence on PerlPrimer. The sense and anti-sense primer for -468 was generated with the addition of restriction sites for Bg1II and HindIII, respectively, which will aid in the cloning process. Primers for CNS-22 were

designed to house the restriction sites SacI and BglII. In addition to this, a start codon was incorporated into the primer to aid transcription (box 2.1). These primers were used in PCR reactions to amplify -468 and CNS-22 from mouse genomic DNA.

Box 2.1: Primer sequences for the IFNγ promoter -468 and CNS-22 with restriction sites as indicated.

-468 (IFNγ promoter)

BglII
Sense 5'-TTTTCAGATCTAAAAGTTTGAAAAGGCTTCCCCC-3'
HindIII
Anti-sense 5'-ATGCCAAGCTTTGTCTTCTCTAGGTCAGCCG-3

CNS-22

SacI Start
Sense - 5'-TACCGAGCTCCATGCAAGTATTAACATGC-3'

BglII
Anti-sense - 5'- CTTTTAGATCTGAAAATTAGACACCTAATTTGC-3

PCR amplification for -468 and CNS-22 fragments

PCR reactions were setup as, 10x Pfx amplification buffer, 10mM dNTP mixture, 50 mM $MgSO_4$, Primers 10 µM, mouse cDNA template, and platinum pfx DNA polymerase (Invitrogen) plus dH_2O to make up the final reaction volume of 50 µl. The three step cycling PCR experiment was set-up as follows; 94 °C 5 minutes and 35 cycles of 94 °C 15 seconds, 53 °C 30 seconds, 68 °C 120 seconds and finally 68 °C 10 minutes. These PCR reaction conditions were for amplifying both -468 and CNS-22. The PCR products were resolved on a polyacrylamide gel by electrophoresis. Specific bands for -468 and CNS-22 were extracted from the gel with QIAquick gel extraction kit according to the manufacturer's instruction.

Cloning -468 into pGL2-b

After amplification of IFNγ -468, the PCR product for -468 and the pGL2-b vector were digested with BglII and HindIII (New England Biolabs) according to

the manufacture's instructions. The pGL2-b vector and the digested PCR product were mixed together with T4 ligase (New England Biolabs) as per manufactures instructions and the ligation reaction was allowed to progress at 16 °C for one hour. Prior to the ligation step, the digested product was treated with shrimp alkaline phosphatase (New England Biolabs) at 37 °C for 15 minutes. This step removes phosphorylated ends of DNA and prevents self re-ligation of linearized DNA. The ligation reaction should result in the - 468 IFNγ promoter cloned upstream of firefly luciferase in pGL2-b (figure 2.2).

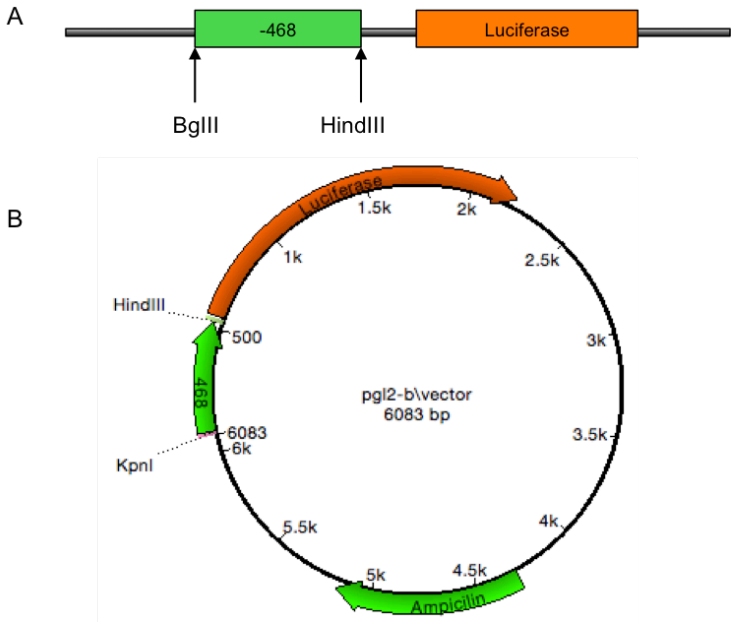

Figure 2.2: Circular view of pGL2-b-468IFNγ

A: Diagrammatic representation of -468 fragment upstream of CNS-22 and the restriction sites BglII and HindIII. **B:** Circular image of pGL2-b-468 construct from UGENE showing KpnI and HindIII restriction sites, which were at a later stage used to digest and confirm successful ligation of -468 in pgl2-b luciferase vector.

Cloning CNS-22 into pGL2-b-468IFNγ

The pGL2-b vector now containing the -468 fragment was used as a vector to clone the CNS-22 fragment. The sense primers for CNS-22 were designed to house the SacI restriction site and the anti-sense primer was designed to have the BglII restriction site. The CNS-22 fragment and pGL2-b-468 vector were digested with SacI and BglII and treated with shrimp alkaline phosphatase before ligation into pGL2-b-468. The ligation product was transformed into competent cells as described in section 2.2.8, and positive clones picked for plasmid extraction. Positive clones were grown further in LB medium supplemented with 100 µg/ml of ampicillin.

Plasmid extraction was performed with QIAquick spin mini-prep kit (Qiagen) according to the manufacture's instruction. To determine whether the plasmid now contains the CNS-22 fragment, it was digested with HindIII (New England Biolabs), of which there are 2 restriction sites in the vector (figure 2.3). The digestion should result in two fragments, a 577 bp and a 6236 bp fragment. The positive clones were subsequently sent for sequencing (Source Bioscience) to ensure no mutations have occurred prior to the plasmids being used for further experiments. From here on pGL2-b vector containing the IFNγ -468 promoter and CNS-22 will be known as IFNγ-pGL2-b.

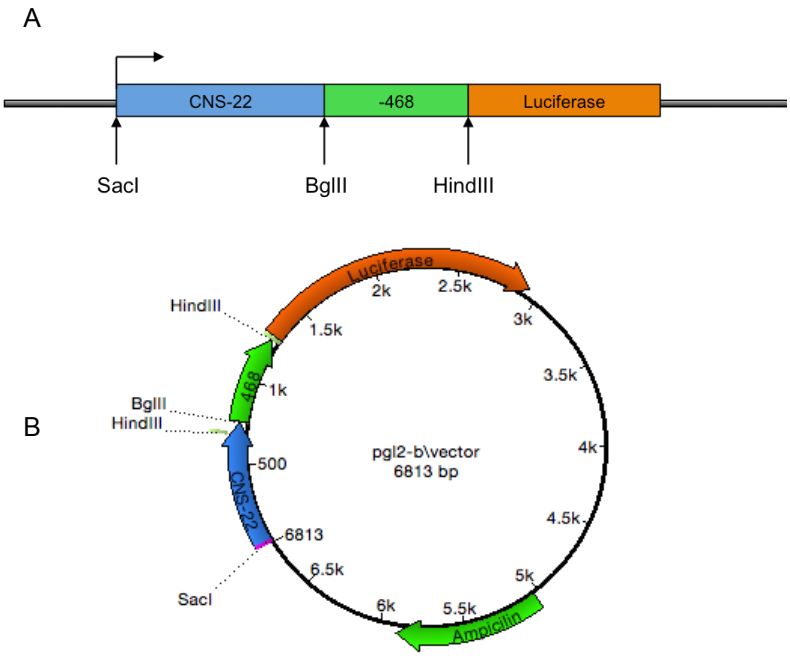

Figure 2.3: Diagrammatic presentation of CNS-22 IFNg promoter fragment cloned into a pgl2-b luciferase vector.

A: This diagram illustrated how with the aid of restriction sites SacI, BgIII and HindIII the IFNγ promoter and CNS-22 were cloned into pgl2-b. The diagram also illustrates the orientation of the two fragments in juxtaposition to each other and the luciferase gene. **B:** A circular view of pGL2-b with IFNγ -468 and CNS-22 with HindIII restriction sites indicated.

2.6.2 Cloning Tbox in pcDNA3.1 and Tbet in pcDNA3.1

T-bet-pcDNA3.1 and Tbox-pcDNA3.1 Vector design on UGENE

The vectors were first designed on UGENE. Tbet and Tbox consensus coding sequence (CCDS) were derived from NCBI CCDS database, CCDS ID: 25315.1. Tbox is a 615 bp fragment and T-bet is considerably bigger at 1644 bp. The vector pcDNA3.1 sequence was used as a template into which the Tbet and Tbox sequences were cloned on UGENE. Gene expression is driven by a cytomegalovirus (CMV) enhancer-promoter, resulting in high-level expression of gene of interest. T-bet and Tbox were separately cloned into pcDNA3.1 in the correct orientation upstream of IRES-EGFP. IRES is an Internal Ribosome Entry Site, which allows translation to begin in the middle of mRNA and thereby enhancing the translation efficiency. EGFP is an Enhanced Green Fluorescent

Protein, which emits green fluorescence expressed. This is useful in identifying positive clones after transfection of suitable cell lines. Figure 2.4 shows the vector design on UGENE.

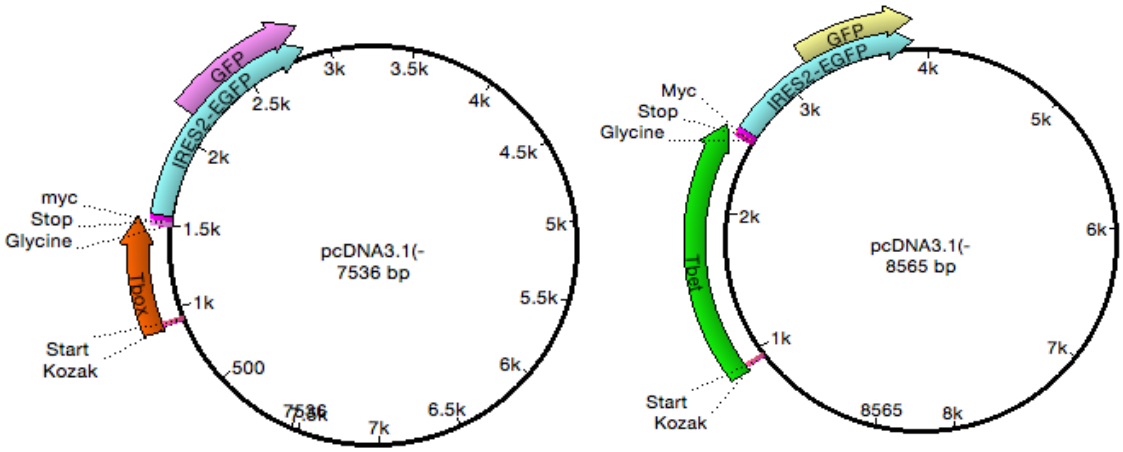

Figure 2.4 pcDNA3.1-Tbox and pcDNA3.1-Tbet in UGENE

Circular pcDNA3.1 vectors from UGENE illustrate the Tbet (A) and Tbox (B) fragments. Key sequences such as, Kozak, Myc, Start and Stop are also indicated. The T-bet and Tbox fragments are cloned upstream of IRES-EGFP.

Designing Tbet and Tbox primers

Constructs were developed to generate Tbet and Tbox proteins for protein interaction studies. Primers for T-bet and Tbox were designed using PerlPrimer and the Tbet-RV construct was used as a template to amplify Tbet and Tbox sequences in PCR reactions. The Tbet-RV construct was a generous gift from Professor Kenneth Murphy. Both primers for T-bet and Tbox were designed in a similar fashion, with respect to restriction sites and Myc tagged sequences as described below.

To design the primers, gene sequences for T-bet and the Tbox region of T-bet were obtained from Ensembl (Gene ID: Tbx21 ENSMUSG00000001444) the consensus coding sequence (CCDS) was derived from NCBI CCDS database, CCDS ID: 25315.1. This sequence was used as a basis for designing primers for T-bet and Tbox region within it. The sense primer was designed with the XhoI restriction site at the 5’ (5’-CTCGAG-3’), immediately followed by a Kozak

sequence (5'-GCCACC-3') and a start codon (5'-ATG-3'). The Kozak sequence flanking the AUG initiation codon influences its recognition by eukaryotic ribosomes (Kozak 1987), which will enhance the translation of this sequence in the transfected cells. The antisense primer contained an EcoRI restriction site (GAATTC), immediately followed by Myc sequence (CAGATCCTCTTCTGAGATGAGTTTTTGTTC), and a stop codon. The Myc sequence was derived from Mouse.

The sense primer for T-bet is 38 nucleotides in length with 68 % GC content and a melting temperature of 79.1 °C, which was calculated by using the Wallace rule. The anti-sense primer for T-bet is much longer at 66 nucleotides as it needs to accommodate the Myc tag, with a melting temperature of 70.5 °C and 39 % GC content. The sense primer for Tbox composed of 36 nucleotides with a GC content of 61% and a melting temperature of 73.6 °C. The anti-sense primer considerably longer with 61 nucleotides and has a melting temperature of 74.3 °C with 49 % GC content (see box 2.2 and 2.3).

Box 2.2: Sense and anti-sense primers for Tbet, highlighting restriction sites and Myc.

Myc tagged **T-bet** primers

Sense:

5'-CTAGACTCGAGGCCACCATGGGCATCGTGGAGCCGGGC-3'

XhoI Kozak Stop

Anti-sense:

EcoRI Stop Myc

5'TGGTGGGAATTCTCACAGATCCTCTTCTGAGATGAGTTTTTGTTCGCCGTTG GGAAAATAATTATA-3'

Box 2.3: Sense and anti-sense primers for Tbox, highlighting restriction sites and Myc

Myc tagged **T-box** primers

Sense:

XhoI Kozak Stop

5'- CTAGACTCGAGGCCACCATGAAGCTGAGAGTCGCGC-3'

Anti-sense:

EcoRI Stop Myc

5'GGTGGAATTCTCACAGATCCTCTTCTGAGATGAGTTTTTGTTCGCCGTTCTCC CGGAATCC-3'

PCR amplification of T-bet and Tbox

Tbet and Tbox fragments were amplified by PCR with Tbet-RV as a template. Reaction mixture composed of the following components; 10x Pfx amplification buffer, 10mM dNTP mixure, 50 mM $MgSO_4$, Primers 10 µM, mouse cDNA template, and platinum pfx DNA polymerase (Invitrogen) plus dH_2O to make up the final reaction volume). All PCR cloning steps were performed with Pfx polymerase. PCR reaction for Tbet; 94 °C 5 minutes and 30 cycles at 94 °C 15 seconds, 53 °C 30 seconds, 68 °C 120 seconds and upon completion of 30 cycles, 68 °C for 10 minutes. PCR reaction conditions for Tbox; 94 °C 5 minutes and 30 cycles at 94 °C 15 seconds, 53 °C 30 seconds, 68 °C 90 seconds and upon completion of 30 cycles, 68 °C for 10 minutes. PCR products were run on agarose gel and Tbet and Tbox specific bands were extracted from the gel using the QIAquick Gel Extraction Kit according to the manufacture's instructions. The concentration of the gel purified DNA was measured by NanoDrop (Thermo Scientific).

Cloning Tbet and Tbox into pcDNA3.1

PCR products for Tbet and Tbox were individually digested with XhoI and EcoRI (New England Biolabs) according to the manufactures instruction. The pcDNA3.1 vector was also digested with the same restriction enzymes. This will result in complementary ends of both vector and Tbet and Tbox fragments. Ligation reaction was set-up individually for Tbet and pcDNA3.1 and Tbox and pcDNA3.1. The digested products were mixed together with T4 ligase (New

England Biolabs) as per manufacture's instructions and incubated at 16 °C for one hour.

The ligation products for Tbox-pcDNA3.1 and Tbet-pcDNA3.1 were transformed into DH10α *Escherichia coli* bacteria by heat shock as described in section 2.2.8. Ten clones for Tbox-pcDNA3.1 and ten for Tbet-pcDNA3.1 were picked after 16-18 hours of incubation at 37 °C and further grown in 5 ml LB medium containing 100 µg/ml of ampicillin for plasmid extraction. Of the ten clones grown for Tbet-pcDNA3.1, only 4 grew in culture and of the ten clones picked for Tbox-pcDNA3.1, 7 grew in culture. Plasmids were isolated from these clones by mini-prep using the QIAquick spin mini-prep kit (Qiagen) according to the manufacturer's instruction. In order to determine the correct ligation of our desired DNA sequences, a further restriction digestion was carried out.

Tbet-pcDNA3.1 and Tbox-pcDNA3.1 were digested with XhoI and EcoRI (New England Biolabs) using the manufacturer's protocols. The digested product was separated by electrophoresis. For Tbet-pcDNA3.1 a product of 1.6 kb in size was expected after digestion with XhoI and EcoRI, which includes the T-bet sequence tagged with Myc. For Tbox-pcDNA3.1 a product of approximately 600 bp was expected after digestion with XhoI and EcoRI. The positive clones were subsequently sent for sequencing (Source Bioscience) to ensure no mutations have occurred prior to the plasmids being used for further experiments. Comparative analysis of sequencing data with sequences for both Tbet-pcDNA3.1 and Tbox-pcDNA3.1 in UGENE confirmed a match and the plasmid were used in future experiments. Table 2.5 presents all primers and table 2.6 presents all vectors used in this study.

Primer		Sequence 5'-3'
Myc Tagged T-bet	Sense	CTAGACTCGAGGCCACCATGGGCATCGTGGAGCCGGGC
	Anti-sense	TGGTGGGAATTCTCACAGATCCTCTTCTGAGATGAGTTTTTGTTCGCCGTTGGGAAAATAATTATA
Myc Tagged T-box	Sense	CTAGACTCGAGGCCACCATGAAGCTGAGAGTCGCGC
	Anti-Sense	GGTGGAATTCTCACAGATCCTCTTCTGAGATGAGTTTTTGTTCGCCGTTCTCCCGGAATCC
CNS-22	Sense	TACCGAGCTCCATGCAAGTATTAACATGC
	Anti-sense	CTTTTAGATCTGAAAATTAGACACCTAATTTGC
-468	Sense	TTTTCAGATCTAAAAGTTTGAAAAGGCTTCCCCC
	Anti-sense	ATGCCAAGCTTTGTCTTCTCTAGGTCAGCCG
Egr3	Sense	GATCCACCTCAAGCAAAAGG
	Anti-Sense	CGGTGTGAAAGGGTGGAAAT

Table 2.5 Primer sequences for constructs

DNA	Vector	Additional Information	GFP + or -
IFNγ promoter (-468) and CNS-22	pGL2-b IFNγ-pGL2-b	Reporter vector contains coding sequence for firefly luciferase.	-
Egr2 CCS*	pCDNA3.1 (-)	Tagged with Flag	+
Egr3 CCS		Tagged with Flag	+
Egr2 ΔZF		Egr2 CCS without ZF domain	+
T-bet CCS		Tagged with Myc	+
Tbox		Tbox DNA binding domain of T-bet only, Tagged with Myc	+

*Consensus Coding Sequence (CCS)

Table 2.6 Information on all constructs

2.7 Luciferase Assay

In 24 well plates, cells to be transfected for instance, HEK293 or HEK293-T-bet were seeded into each well the day before transfection, to ensure the cells had suitable confluency. Calcium phosphate method was used to introduce DNA into cells as described in 2.2.7. Cells were transfected with a total of 2.5 µg of total DNA per well with the vectors specified for individual experiments. In each experiment the concentration each vector relative to others, was kept the same with 0.5 µg of the reporter construct IFNγ-pGL2-b, keeping total DNA concentration at 2.5 µg per reaction. Experiments were done in triplicates each time.

The luciferase system is very sensitive and rapid to quantify luciferase activity. The principle is based on the production of light by converting chemical energy of luciferin oxidation through an electron transition forming the product molecule oxyluciferin. The enzyme firefly luciferase catalyses this formation of oxyluciferin using Adenosine Triphosphate and Magnesium^{2+} (ATP-Mg^{2+}) as a co-substrate. In practice, a flash of light is generated when enzyme and substrate are combined, which decays rapidly.

Prior to commencing the experiment, the luciferase assay reagent is prepared according to the manufacturer's instruction, normally stored at -80 °C and kept away from light. The HEK293-Tbet cells were first gently washed with 1 X PBS being careful not to dislodge the cells. To this 100 µl 1 X Passive Lysis Buffer (PLB) was added (Promega), prepared according to the manufacturer's instructions. The cells were agitated at room temperature for 15 minutes in PLB. Cells were then collected and vortexed for 30-60 seconds to further aid in the lysis. At this stage the cells should be lysed releasing protein content. Protein concentration was determined by Bradford assay to normalize luciferase readings to protein concentration. 10 µl of the lysate was added into a well of a 96 well plate, followed by 50-100 µl of the luciferase assay reagent. Luminescence was measured on KC4 luminometer (Bio-Tek Instrumentation Inc). Luciferase readings for each sample were normalized by protein concentration.

2.8 Statistical Analysis

Data presented within this study were expressed as mean +/- s.e.m or +/- s.d where specified. Student's unpaired two-tailed t-test was used to analyse the statistical significance of differences between groups. Differences with a $p < 0.05$ were considered significant. Statistical analysis was done on Microsoft Excel.

Chapter 3: Results

Generation of Cell Lines and Constructs

3.1 Generation of cell lines and constructs

3.1.1 Generating HEK293 cell line with stable expression of mouse T-bet

A stable T-bet expressing cell line was developed in order to carry out luciferase reporter assays. The HEK293 cell line was transfected with a mouse T-bet expression vector (pCDNA3.1-Tbet), where T-bet is tagged with Green Fluorescence Protein (GFP). A simple yet effective method of calcium phosphate transfection was used for transfecting the cells. Transfected cells were cultured and sorted for GFP positive cells (figure 3.1A). The GFP positive cells were cultured for 5-7 days until confluent and then analysed for T-bet expression by staining with T-bet PE-cy7 (Figure 3.1B). Cells were stained for anti-T-bet PE-cy7 to ensure only T-bet was detected, since a positive GFP signal may be indicative of a mutated construct. Although 9.8% of cells were GFP positive, only 3.7% were actually T-bet-PerCP positive (Figure 3.1B). Therefore the cells were sorted a total of four times for GFP. Finally, the 4 x sorted cells (figure 3.1C) were stained with an anti-T-bet PEcy7 antibody and the cells were analysed by flow cytometry (figure 3.1D). Approximately 80% of T-bet PE-cy7^{+} and GFP^{+} cells were detected without any prior stimulation. This cell line constitutively expressing T-bet was used in luciferase assay, where HEK293-T-bet cells were transfected with other constructs, without the need to transfect T-bet.

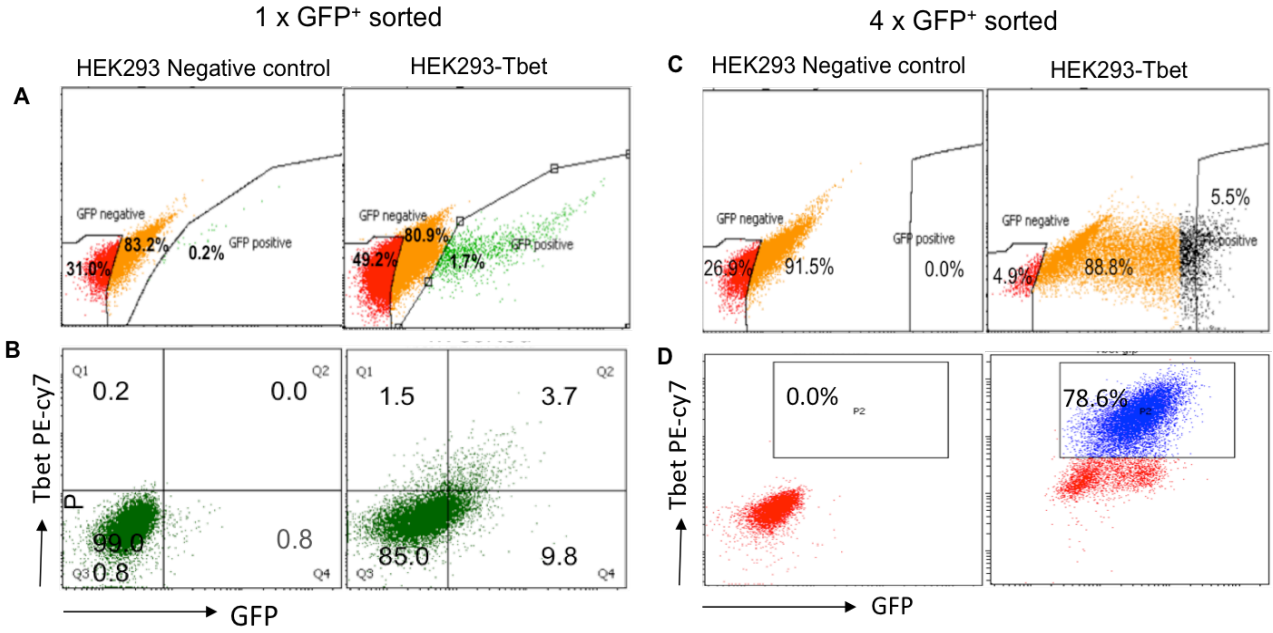

Figure 3.1: HEK293 cells transfected with Tbet-GFP were sorted by FACS and stained by T-bet PE-cy7 for analysis of T-bet positive cells.

HEK293 cells transfected with Tbet-GFP were sorted. Cells expressing low levels of GFP are avoided, only cells with high GFP expression are selected for sorting, which in this case represents 1.7% of the population (**A**). 1 x sorted GFP positive HEK293 cells were analysed for T-bet expression 5-7 days after sorting. Cells were not stimulated prior to analysis of T-bet expression by staining with anti-Tbet PE-cy5 (**B**). GFP positive cells were subsequently sorted a fourth time by gating on cell with high expression of GFP, which represented 5.5% of the population (**C**). The 4 x sorted cells were stained for T-bet PE-cy7 after culturing for 5-7 days and analysed for T-bet positive cells by flow cytometry (**D**).

3.2 Generation of IFNγ Luciferase reporter gene

3.2.1 IFNγ CNS-22 and -468 construct and primer design

To investigate the effect of Egr2 and Egr3 on T-bet mediated expression of IFNγ, we generated an IFNγ reporter construct, containing IFNγ promoter and enhancer regions upstream of luciferase. The construct was primarily designed on UGENE, which is a free open source bioinformatics software. UGENE has a multitude of functions but for the purposes of this project, it allows a user to create, edit and annotate nucleic acid and protein sequences. A vector of your

choice can be used as a basis into which your genes of interest can be cloned and annotated, indicating the relevant restriction sites. This then acts as a guide for the molecular biologist to generate the construct in the lab.

To generate the IFNγ reporter construct, we first investigated key enhancer regions of IFNγ expression that housed the T-bet binding site. The construct was finally adapted from Hatton and colleagues (2006), who investigated chromatin dynamics that regulated IFNγ gene expression. They compared approximately 150,000 kb of DNA sequence encompassing the *IFNγ* locus from multiple species. Cross-species sequence analysis of human, mouse, rat, chicken, opossum and cow revealed that, while the IFNγ gene is rather weakly conserved, some non-coding sequences that met the criteria of a conserved non-coding sequence (CNS) exist, upstream and downstream of the IFNγ gene. The criteria for conserved non-coding sequence, includes, greater than 70% sequence identity and greater than 100 bp long. CNS regions -55 kb, -34 kb and -22 kb upstream of the IFNγ start site were analysed for consensus transcription binding sites. Interestingly, CNS-22 contained an unusually high concentration of consensus sequences for transcription factors known to be involved in T cell development and cytokine gene expression. Among these were, GATA3, STAT1, STAT4, STAT6 NF-κβ, Ikaros and T-bet. Sequence comparison of CNS-22 between different species showed that these consensus-binding sequences are highly conserved.

Further into the investigation of CNS-22 by Hatton and colleagues by reporter assay, showed that in the presence of T-bet, CNS-22 reporter expression was enhanced and in the absence, almost complete loss of reporter activity. CNS-34 and CNS-55 also had enhanced reporter activity, albeit to a lesser extent than CNS-22, illustrating a regulatory role for CNS-22 in IFNγ expression. The -468 bp IFNγ promoter fragment also increased luciferase expression in the presence of T-bet, but markedly increased when

combined with CNS-22. We therefore, adopted this reporter strategy for our purposes to investigate the effect of Egr2 and Egr3 on T-bet mediated expression of IFNγ, by generating a reporter construct containing the IFNγ 468 bp promoter and CNS-22 (Figure 3.24).

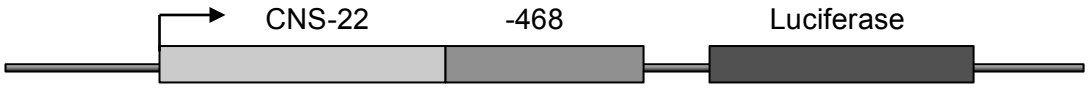

Figure 3.2: Diagrammatic representation of IFNγ reporter assay, with CNS-22 and -468 IFNγ promoter upstream of luciferase in pGL2-b vector.

3.2.2 Cloning -468 into pGL2-b

The pGL2-b luciferase reporter vector is a wonderful medium to investigate factors that potentially regulate gene expression. These vectors carry the coding region for firefly luciferase (*Photinus pyralis*). DNA sequences of interest are cloned upstream the luciferase coding sequence (Figure 3.2) and transfected into a suitable cell line or primary cells. The primer sequences for -468 and CNS-22 were designed with a combination of restriction sites for SacI, BglII and HindIII for cloning into pGL2-b vector and were designed using PerlPrimer (Box 2.1, Methods and Materials). These primers were used in PCR reactions to amplify the -468 promoter and CNS-22 regions from mouse genomic DNA. Gel images of the PCR products for - 468 IFNγ promoter (Figure 3.3) and CNS-22 (figure 3.4) are shown. PCR products for both -468 and CNS-22 were gel purified for ligation into pGL2-b.

CNS-22 and -468 IFNγ enhancer regions were ligated into pGL2-b separately. First the smaller of the two fragments -468, and vector pGL2-b were digested with KpnI and HindIII to generate compatible sticky ends. The digested vector and fragment were treated with shrimp alkaline phosphatase to prevent self-ligation of vector, followed by ligation of -468 into pGL2-b with T4 ligase. To ensure the ligation was successful, the now, pGL2-b-468 vector was digested with KpnI and HindIII, which should theoretically cut out the -468 fragment from

pGL2-b-468 (Figure 3.3C). Figure 3.3B demonstrate the KpnI and HindIII sites as present on the pGL2-b-468 vector on UGENE.

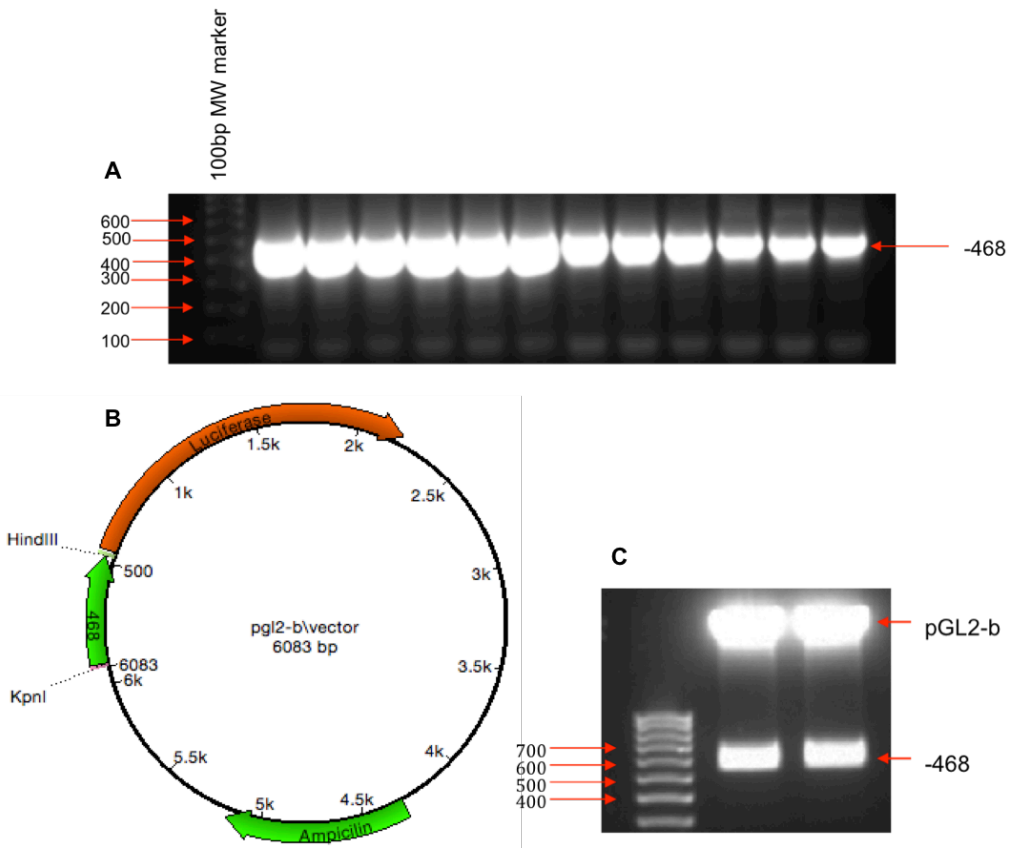

Figure 3.3: Cloning -468 IFNγ promoter into pGL2-b luciferase vector

The IFNγ -468 promoter region was amplified by PCR and the product separated by gel electrophoresis. PCR amplification should yield a 468 bp fragment. The red arrow on the right of the gel image A points to the expected product. Of the 10 PCR reactions (to maximize DNA for ligation reactions), all loaded into 12 wells with a 100 bp marker on the first left lane (**A**). The pGL2-b vector with the -468 fragment is represented in UGENE (**B**) with KpnI and HindIII restrictions sites indicated. pGL2-b-468 was digested with KpnI and HindIII and the digested fragments were separated on agarose gel. Digestion produced two products, one of which is the pGL2-b vector without the -468 fragment and the -468 fragment, both indicated by red arrows.

3.2.3 Cloning CNS-22 into pGL2-b-468.

To ligate the CNS-22 fragment into pGL2-b-46 vector, both CNS-22 and vector was digested with BglII and SacI to produce compatible sticky ends. The digested fragments ligated with T4 ligase after treatment with shrimp alkaline phosphatase to prevent self-ligation. To ensure ligation was successful, the vector now theoretically containing both CNS-22 and -468 in the correct orientation were digested with HindIII, of which there are 2 restriction sites (Figure 3.4B). Digestion with HindIII produced two fragments of the expected sizes (figure 3.4C). The vector now containing CNS-22 and -468 in the correct orientation will be identified as **IFNγ-pGL2-b**.

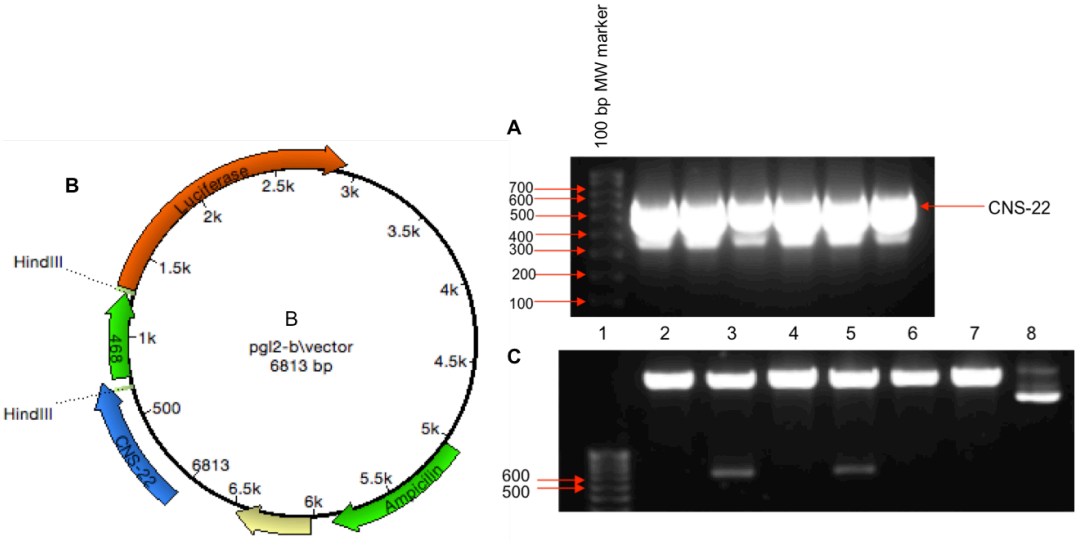

Figure 3.4: Cloning CNS-22 into pGL2-b-468

The IFNγ CNS-22 region, amplified by PCR and the product separated by gel electrophoresis. The size of this fragment is 739 bp. A thick band, sized between 500-700 bp indicated by a red arrow on the right of the gel image appears to be the correct PCR product. A smaller band beneath this band, the size of which is approximately 400 bp appears to be a non-specific PCR product. Each lane represents a separate PCR reaction (**A**). The CNS-22 fragment cloned upstream of -468 and luciferase in the pGL2-b vector is presented in UGENE (**B**) indicating the HindIII restriction sites. pGL2-b-468-CNS-22 were transformed into competent cells and 7 positive clones were selected by antibiotic resistance to ampicillin and digested with HindIII. Only two of these yielded expected fragment sizes of approximately 570 bp in lanes 3 and 4 (**C**).

The final step before this vector can be used in experiments was sequencing. These clones were sequenced by Source Bioscience and after comparative analysis of the sequencing results with the sequence on UGENE of IFNγ-pGL2-b revealed it to be correct. These clones were grown at a larger scale for MIDI preparations and normalized to give a concentration of 1 μg/μl for ease of use in experiments.

3.3 Generation of pcDNA3.1-Tbox and pcDNA3.1-T-bet

3.3.1 Cloning Tbox and T-bet into pcDNA3.1

Using a mouse T-bet-RV (Professor Kenneth Murphy) vector as a template, T-bet and Tbox coding region within T-bet were amplified by PCR. The PCR products for these were resolved in agarose gel. Mouse T-bet consensus coding sequence (CCDS) is 1593 nucleotides in length and the Tbox region 576 nucleotides. The primers were designed to house a Myc sequence for both T-bet and Tbox, which is 30 nucleotides in length, plus restrictions sites, therefore the expected PCR product sizes for T-bet and Tbox are 1648 bp and 641 bp, respectively (Figure 3.5A). The PCR products were subsequently gel purified and ligated into pcDNA3.1 vector comprising an internal ribosomal entry site (IRES), an ampicillin (Amp) resistance gene and Green Fluorescent Protein (GFP) (Figure 3.5B). The Tbox and T-bet sequences were inserted into the pcDNA3.1 vector by digestion with XhoI and EcoRI to generate compatible sticky ends. The ligation products were subsequently transformed into competent cells. Positive clones were further analysed to determine that they are correct by restriction digestion with XhoI and EcoRI, which should cut out the T-bet and Tbox fragments (Figure 3.5C).

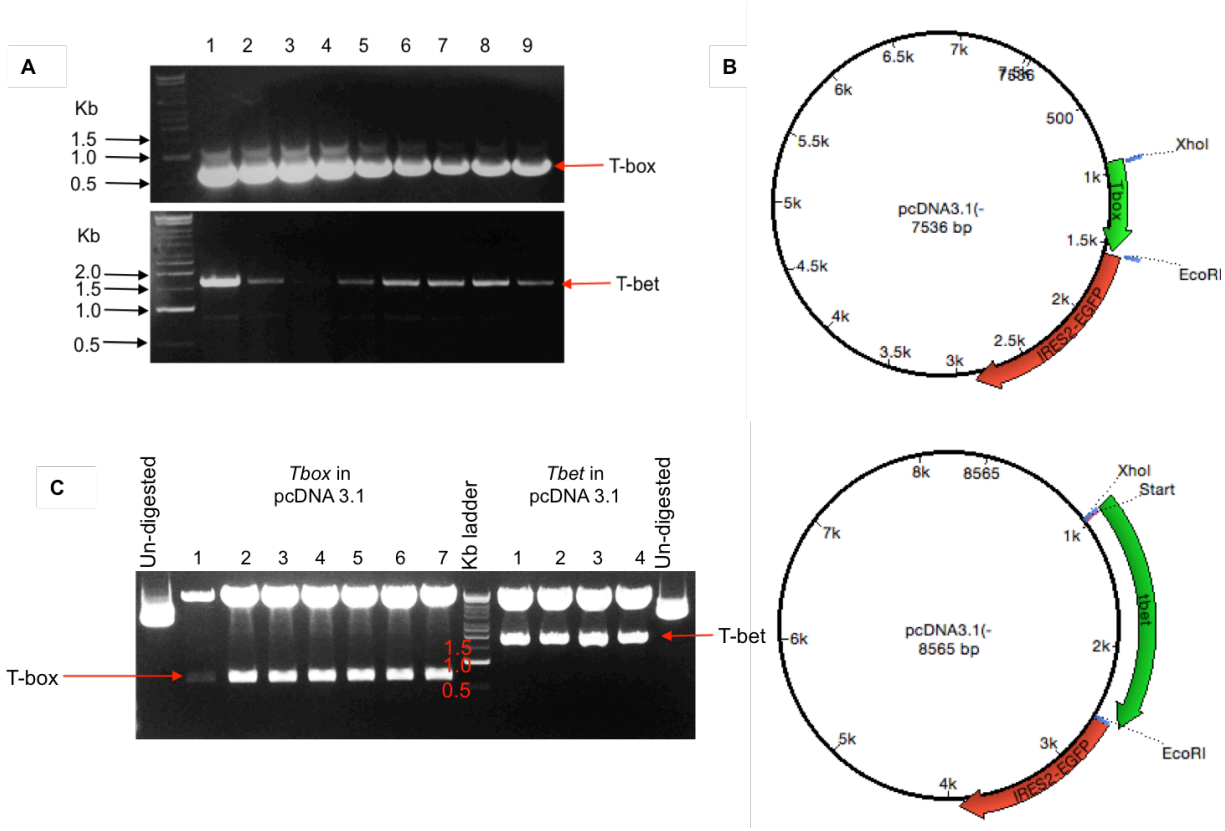

Figure 3.5: Cloning Tbox and T-bet into pcDNA3.1

Using Tbet-RV as a template, T-bet and Tbox specific primers tagged with Myc (see methods) were used to amplify these two genes. The PCR products were separated by electrophoresis and in image **A** (top) a band approximately 641 bp in size representing Tbox was visualized, indicated by red arrow. In image **A** (bottom), an approximate size of 1648 bp band representing T-bet, indicated by a red arrow on the right of the gel image. T-bet and Tbox were cloned into pcDNA3.1 on UGENE (**B**), respectively. For cloning these fragments into vector, restriction sites for XhoI and EcoRI were incorporated into the PCR primers for T-bet and Tbox. Digestion of Tbox in pcDNA3.1 and T-bet in pcDNA3.1 with Xho1 and EcoR1 was done to determine the genes have successfully been integrated into pcDNA3.1 in the correct orientation. Xho1 and EcoR1 digestion reveals a digested fragment of approximately 600 bp band as Tbox (left) and 1.6 kb band representing T-bet. These results indicate that the T-bet and Tbox were ligated into the pcDNA3.1 vector (**C**).

A restriction digestion of pcDNA3.1-Tbox and pcDNA3.1-Tbet by XhoI and EcoRI reveled the expected cut fragments from both these constructs. These positive clones were then sent for sequencing to Source Bioscience. Comparative analysis of the sequencing results with the sequences of constructs in the UGENE software indicated correct matching of sequences. These results revealed the constructs to contain the correct sequences without any mutations and therefore were used in further experiments.

Mechanism of Egr2 and 3 regulation of T-bet function

3.4 Egr2 controls T-bet mediated IFNγ production

3.4.1 Egr2 inhibits T-bet binding to consensus DNA binding sequence

Taken together, our results thus far demonstrate a role for Egr2 and 3 in controlling IFNγ production in effector T cells. To investigate the mechanism by which Egr2 and 3 do this, we first conducted experiments to examine whether Egr2 and 3 had the ability to antagonize T-bet function at the protein level. A possible mechanism could be that Egr2 and 3 can directly inhibit T-bet binding to its target sequence on the IFNγ promoter and/or enhancer regions. To investigate this possibility, a DNA Oligo was designed to house the T-bet binding consensus sequence, adapted from Szabo and colleagues (2001). Tbox is the DNA binding domain of T-bet, and the Tbox binding consensus sequence was incorporated into a 24 nucleotide sequence seen here; sense 5'-GACAGCTCACACTGGTGTGGAGCA-3' and labelled with Cyanine 5. Both sense and anti-sense sequences were labelled.

The probe was first incubated with protein lysates from un-transfected cells as a negative control (Figure 3.6A). In principle, a labelled probe housing the T-bet DNA binding consensus site once exposed to lysates containing the T-bet protein with result in a protein-DNA interaction. This complex will inevitably cause the migration of the probe to slow down and invariably cause a shift in the probe when compared to a free probe unburdened with protein. If Egr2 affects T-bet binding, the band intensity will reduce as T-bet is arrested by Egr2. Interestingly, in the presence of Egr2, the T-bet specific band fades considerably, indicating a loss of T-bet binding to its consensus sequence (Figure 3.6A). To ensure the shift is there as a result of T-bet binding, a super-shift with anti-T-bet is used to confirm this. Probe bound to both T-bet and anti-T-bet slows the migration of this complex further, causing the band to shift once more known as a super-shift. Hence, a super-shift in figure 3.6A confirms T-bet specific binding. This result demonstrates a novel mechanism by which Egr2 inhibits Tbet binding to its consensus binding sequence. It was interesting to explore the prospect of whether T-bet may have and equally antagonizing effect on Egr2 binding.

An Egr2 Cy5 labelled DNA oligo was adopted from Chavrier and colleagues (1990). ChIP data analysis of Egr2 DNA binding sequence revealed a binding sequence of TGTAGGGGGCGGGGGCGGGGTTA. Similar to the EMSA for T-bet, HEK293 cells were transfected with Egr2 with or without T-bet. Nuclear lysates extracted from transfected cells were used to examine interaction of Egr2 with its target DNA Oligo (figure 3.6B). As a negative control, the Egr2 DNA Oligo was first incubated with lysates from un-transfected HEK293 cells. Incubation with lysates from Egr2 transfected cells revealed an intense band indicating Egr2 binding to its target sequence. In the presence of T-bet, the band intensity remained unaltered in any way. Egr2 specific binding was confirmed by super-shift with anti-Egr2 antibody. These results demonstrate that although Egr2 prevents T-bet binding to its target sequence, T-bet does not affect Egr2 function. But, how this interaction renders T-bet unable to bind to its consensus binding sequence remains a mystery. An interaction may cause a conformational change in T-bet structure thereby disrupting the Tbox domain. Egr2 may interact with the Tbox domain and thus block this site. To answer these questions, the mechanism was explored further by protein-protein interaction experiments.

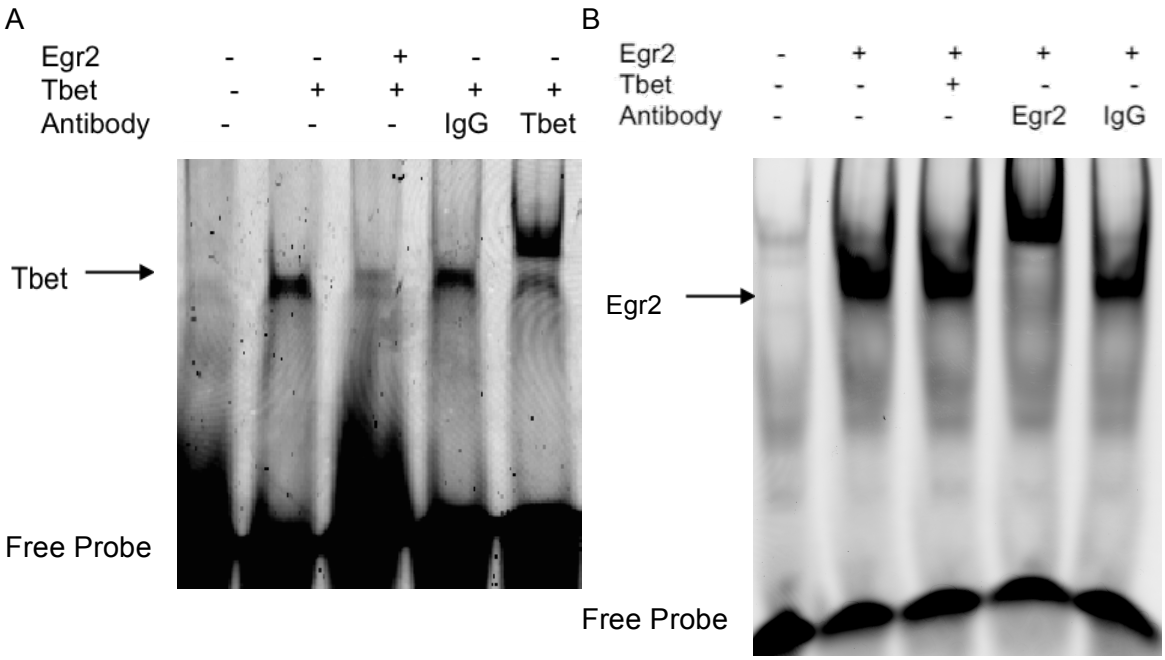

Figure 3.6: EMSA with Cy5 labelled Tbet and Egr2 consensus binding Oligos

HEK293 cells were transfected with T-bet with or without co-transfection of Egr2. Nuclear proteins were extracted and incubated with the Cy5 labelled Oligo probes housing the T-bet DNA binding site used in an EMSA assay for detection of the interaction of T-bet with its consensus DNA oligo. The specificity was confirmed by super-shift with an anti-T-bet antibody (**A**). HEK293 cells were transfected with Egr2 with or without co-transfection of T-bet. Nuclear proteins were extracted and incubated with the Cy5 labelled Oligo probes housing the Egr2 DNA binding site used in an EMSA assay for detection of the interaction of Egr2 with its consensus DNA Oligo. Specific binding was confirmed by super-shift with an anti-Egr2 antibody (**B**). The data are representative of three experiments. The data represent three independent experiments with similar results.

3.5 Egr2 inhibits T-bet mediated IFNγ expression

To assess a potential regulatory role for Egr2 and 3 in T-bet mediated IFNγ expression, a T-bet dependent IFNγ reporter, consisting of the proximal IFNγ promoter flanked by the -32kb enhancer region, was analysed. Adapted from Hatton and colleagues (2006), enhancer elements, conserved noncoding sequence -22 (CNS-22) and -468 IFNγ promoter upstream the IFNγ transcriptional start site (TSS) were cloned into a luciferase pGL2-b vector. CNS-22 is located 22 kb upstream of the IFNγ promoter and just like the - 468

promoter region contains T-bet binding sites. CNS-22 and -468 were cloned into the pGL2-b vector upstream the renilla luciferase gene. The IFNγ-pGL2-b reporter construct was then transfected into HEK293 cells together with T-bet with or without Egr2 and/or Egr3 to measure luciferase activity. Luciferase activity was measured 24 hours after transfection.

IFNγ-pGL2-b transfected into HEK293 cells without T-bet acted as a negative control, ensuring no endogenous transcription factors were capable of binding and inducing luciferase activity. Consistent with previous reports (Hatton *et al*., 2006), the high levels of reporter gene expression was T-bet dependent (Figure 3.7). T-bet mediated reporter activity was profoundly inhibited in the presence of Egr2 or 3 and completely abolished by co-transfection of both Egr2 and 3 (Figure 3.7). These findings demonstrate that although Egr2 and 3 are not involved in regulating the expression of T-bet; they inhibit T-bet mediated IFNγ expression.

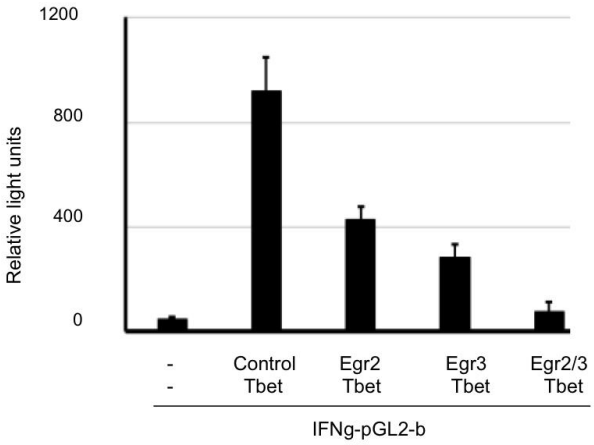

Figure 3.7: Egr2 and Egr3 inhibit IFNg-pGL2-b reporter expression by Tbet

CNS-22 IFNγ enhancer region and -468 IFNγ promoter region were linked to a firefly luciferase vector (IFNγ-pGL2-b). This IFNγ reporter vector was transfected in HEK293 cells by calcium phosphate, along with different combinations of T-bet, Egr2 and Egr3. 20 hours after transfection, luciferase activity was analysed by measuring luminescence while exposure of nuclear protein to luciferase substrate. Data are relative light units +/- s.e.m normalized with protein concentration and the experiment was done in triplicates.

3.6 Egr2 and 3 interact with T-bet

3.6.1 Egr2 interact with T-bet in T cells

To explore the mechanism of how Egr2 and 3 regulate T-bet mediated IFNγ expression, first the protein interaction was analysed in T cells. CD4 T cells isolated from WT and CD2-Egr2/3$^{-/-}$ mice were stimulated with anti-CD3 and anti-CD28 to induce both T-bet and Egr2. The nuclear lysates from knockout mice are used as a negative control. A high affinity Egr2 antibody was used to precipitate Egr2 from CD4 T cell nuclear lysates. Capturing Egr2 with high affinity Egr2 antibody will also capture any proteins complexed with it. The precipitate is then heated at 90 degrees Celsius to dissociate proteins in complex and separated in a SDS-polyacrylamide denaturing gel. The proteins separate according to size and an antibody specific for a particular protein can be used to detect your protein of interest. This technique is known as co-immunoprecipitation (Co-IP).

With this in mind, Egr2 was precipitated from nuclear lysates extracted from WT and CD2-Egr2/3$^{-/-}$ mice. Although CD2-Egr2/3$^{-/-}$ CD4 cells express T-bet after antigen stimulation *in vitro* and *in vivo*, they are used here as a negative control due to the absence of Egr2. Following a western blot on Egr2 immunoprecipitates for T-bet, in the WT sample, T-bet was detected (figure 3.8Ai), which is a 60 KDa protein. This shows that Egr2 and T-bet do indeed interact at the protein level in T cells. As an additional control, the expression of T-bet was also determined in both WT and CD2-Egr2/3$^{-/-}$ cells prior to immunoprecipitation (figure 3.8Aii), which shows that T-bet is expressed at a relatively similar level in both WT and CD2-Egr2/3$^{-/-}$ cells. To strengthen the validity of this interaction, the experiment was repeated in reverse, where in both WT and CD2-Egr2/3$^{-/-}$ nuclear lysates, T-bet was immunoprecipitated with anti-T-bet. This was followed by a western blot for Egr2 with CD2-Egr2/3$^{-/-}$ as a negative control. Egr2 was detected in the WT samples, proving this interaction in reverse (figure 3.8Bi).

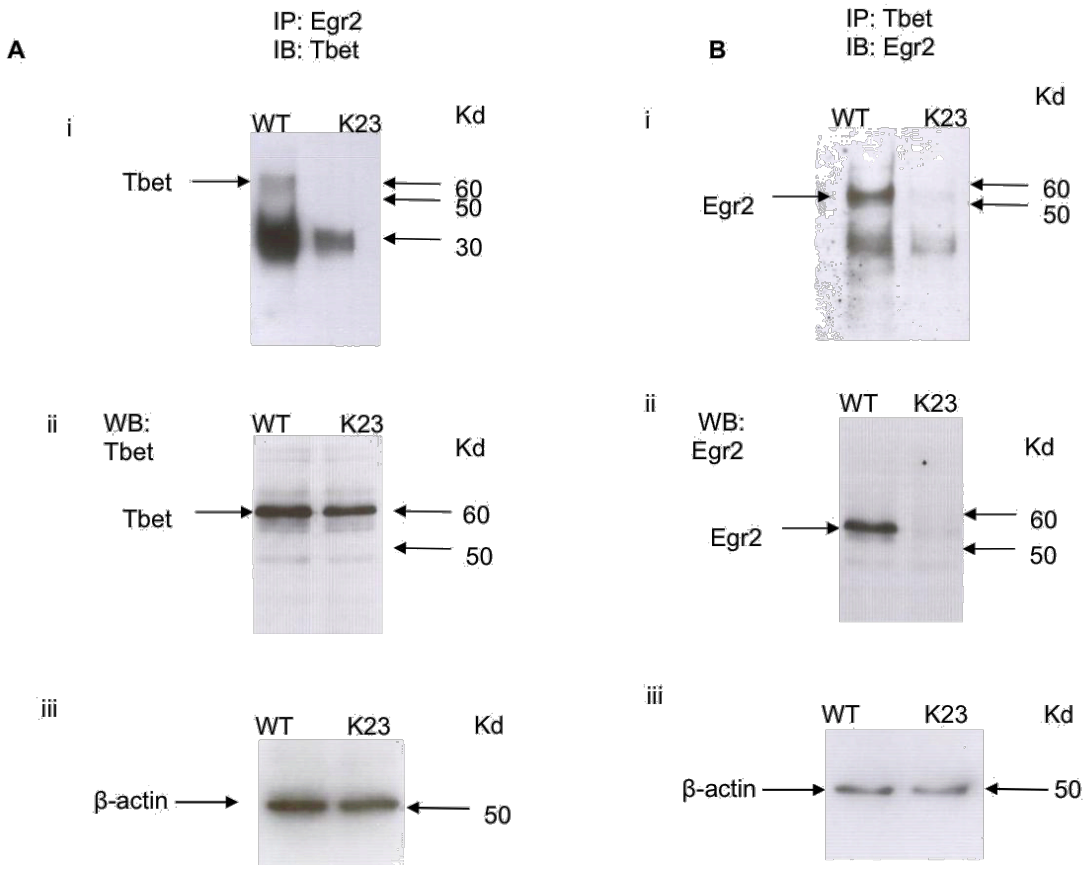

Figure 3.8: Co-immunoprecipitation for Egr2 and Tbet in CD4 T cells

CD4 T cells were isolated from wild type and CD2-Egr2/3$^{-/-}$ mice and stimulated *in vitro* with anti-CD3 and anti-CD28 for 16 hours to induce T-bet and Egr2. Nuclear protein was extracted and Egr2 (**A**) and T-bet (**B**) were precipitated by anti-Egr2 and anti-Tbet, respectively. Precipitated proteins were blotted and stained for T-bet (A) and Egr2 (B). Loading controls for T-bet (Aii) and Egr2 (Bii) expression were determined by nuclear lysates blotted prior to immunoprecipitation with antibody including β-actin loading control (Aiii and Biii). The data represent three independent experiments with similar results.

3.6.2 Egr2 and Egr2ΔZF interact with T-bet and Tbox

The results so far demonstrate an interaction between T-bet and Egr2 in T cells but no indication of which regions are necessary for this interaction to occur. First, we wanted to eliminate the zinc finger (ZF) DNA binding domain of Egr2. From Egr2 EMSA data, it was apparent that T-bet does not have any affect on Egr2 binding to target consensus sequence (figure 3.6B). It seems reasonable therefore to assume that this interaction does not affect the Egr2ΔZF domain. A

construct was developed, whereby the Egr2 gene without the segment coding for the ZF domain was cloned into a pcDNA3.1 vector. Co-immunoprecipitation experiments revealed the Egr2ΔZF domain was not necessary for Egr2 and T-bet protein interaction (figure 3.9A). Further to this, we decided to test the notion of Egr2 and 3 potentially blocking the Tbox DNA binding domain of T-bet as a mechanism to block T-bet function. To do this, the Tbox region of T-bet was tagged with Myc and cloned into pcDNA3.1 vector. Transfection of the vector into HEK293 cells along with flag-tagged Egr2 and Egr2ΔZF and co-immunoprecipitation with appropriate antibodies on nuclear lysates, revealed Egr2 was able to bind to Tbox (figure 3.9B). In addition, the Egr2ΔZF domain was not required for Egr2 to interact with the Tbox domain.

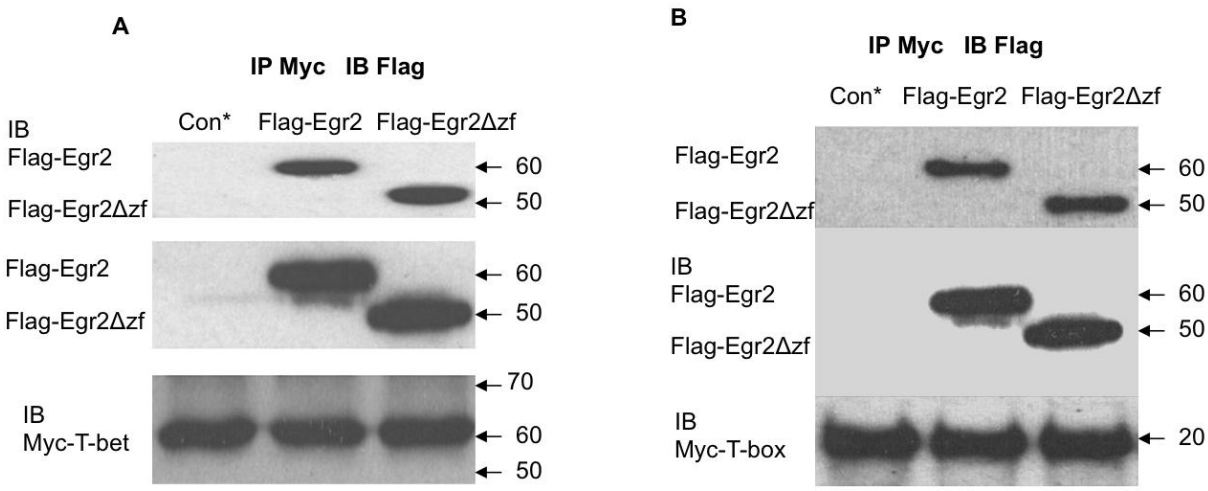

Figure 3.9: Egr2 and Egr2ΔZF interacts with T-bet (A) and Tbox (B)
HEK293 cells were co-transfected with Flag-tagged Egr2, Flag-tagged Egr2ΔZF, Myc-tagged T-bet and Myc-tagged Tbox. Nuclear lysates were precipitated with anti-Myc antibody after which they were blotted with anti-Flag for Egr2 and Egr2ΔZF. For controls, to ensure similar expression nuclear lysates were blotted with anti-Flag prior to precipitation for both blots (middle panel for A and B) and anti-Myc for T-bet and Tbox expression (bottom panel for A and B). *Control; precipitation with anti-Flag with nuclear lysates from un-transfected HEK293 cells as a negative control. The data represent three independent experiments with similar results.

3.6.3 Egr3 interacts with T-bet and Tbox

Co-IP experiment were conducted to prove the overlapping function of Egr2 and to determine a similar binding pattern for Egr3 with T-bet. An antibody for Egr3 is yet to be developed, therefore to investigate Egr3 and T-bet interaction, Flag-tagged Egr3 cloned into pcDNA3.1 was transfected into HEK293 cells along with Myc-tagged T-bet and Tbox to obtain nuclear protein lysates. Egr3 was immunoprecipitated by anti-Flag followed by Immunoblot to detect the 60 KDa T-bet and 20 KDa Tbox. We found that Egr3 directly interacted with T-bet and more importantly, with the Tbox domain (figure 3.10). This provides the evidence of the mechanism by which Egr3 also can inhibit T-bet function. Collectively, these results demonstrate a novel mechanism by which Egr2 and Egr3 can antagonize T-bet function in activated T cells.

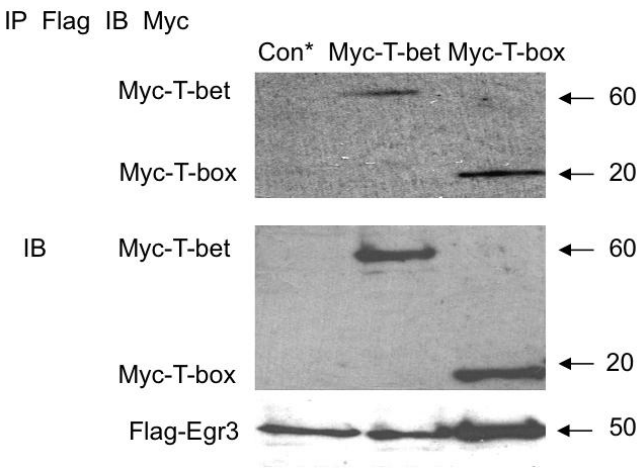

Figure 3.10: Egr3 interacts with T-bet and its DNA binding domain Tbox

HEK293 cells were co-transfected with Flag-tagged Egr3 and Myc-tagged T-bet and Myc-tagged Tbox. Nuclear lysates were precipitated with anti-Flag antibody after which they were blotted with anti-Myc (top panel). To ensure similar expression nuclear lysates were blotted with anti-Myc prior to precipitation for Myc-Tbet and Myc-Tbox expression (middle panel) and anti-Flag for Flag-Egr3 expression (bottom panel). *Control; precipitation with anti-Flag with nuclear lysates from un-transfected HEK293 cells as a negative control. The data represent three independent experiments with similar results.

3.6.4 Egr2ΔZF inhibits T-bet mediate IFNγ expression

Although Egr2 without the ZF domain was able to interact not only with T-bet but also the Tbox domain, it still poses the question as to whether it has the capability to have any regulatory effect on T-bet function with respect to IFNγ expression. To test whether Egr2ΔZF can inhibit T-bet mediated IFNγ expression the luciferase reporter assay was repeated with IFNγ-pGL2-b reporter gene containing the IFNγ promoter and CNS-22 enhancer regions. Analogous to Egr2, Egr2ΔZF had a similar inhibitory effect on T-bet mediated IFNγ expression, seen in figure 3.11 by a considerable reduction in luciferase activity, when IFNγ-pGL2-b was co-transfected with T-bet and Egr2ΔZF.

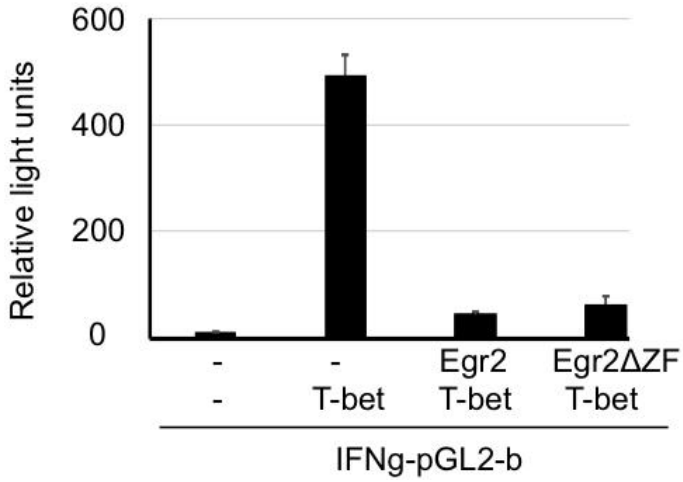

Figure 3.11: Egr2ΔZF inhibits Tbet mediated IFNg expression

CNS-22 IFNγ enhancer region and -468 IFNγ promoter region were linked to a firefly luciferase vector (IFNγ-pGL2-b). IFNγ-pGL2-b was co-transfected with T-bet only (second bar from left) and with T-bet and Egr2 or Egr2ΔZF (third and fourth bar from the left) in HEK293 cells. The first bar on the left represents a negative control where IFNγ-pGL2-b was transfected into HEK293 alone. 16-18 hours after transfection, cells were harvested for protein extraction followed by assessment of luciferase activity. Data are relative light units +/- s.e.m normalized with protein concentration and the experiment was done in triplicates.

3.7 Egr2 and 3 expressions are reciprocally regulated by antigen stimulation and effector cytokines.

3.7.1 Egr2 expression suppressed by IL-12

Although T-bet was expressed in CD4 T cells cultured under different T helper differentiation conditions, the percentage of Egr2 positive cells in wild type T_H1 conditions was significantly reduced compared to T_H2 and T_H17 conditions suggesting that T_H1 cytokines may repress Egr2 and/or 3 expression, which may be important for T_H1 differentiation. To test this notion, we conducted a rather simple experiment, where CD4 and CD8 T cells from WT mice were isolated and stimulated with anti-CD3 and anti-CD28 with or without rIL-12, a T_H1 inducing cytokine. After stimulation 78% of Egr2 was induced in $CD4^+$ T cells and 62% in CD8. When stimulated in the presence IL-12, Egr2 expression plummeted to 32% in CD4 and 5% in CD8. IL-12 on its own was unable to induce Egr2 expression. Indeed, the induction of Egr2 by TCR stimulation was inhibited by IL-12 (Figure 3.12).

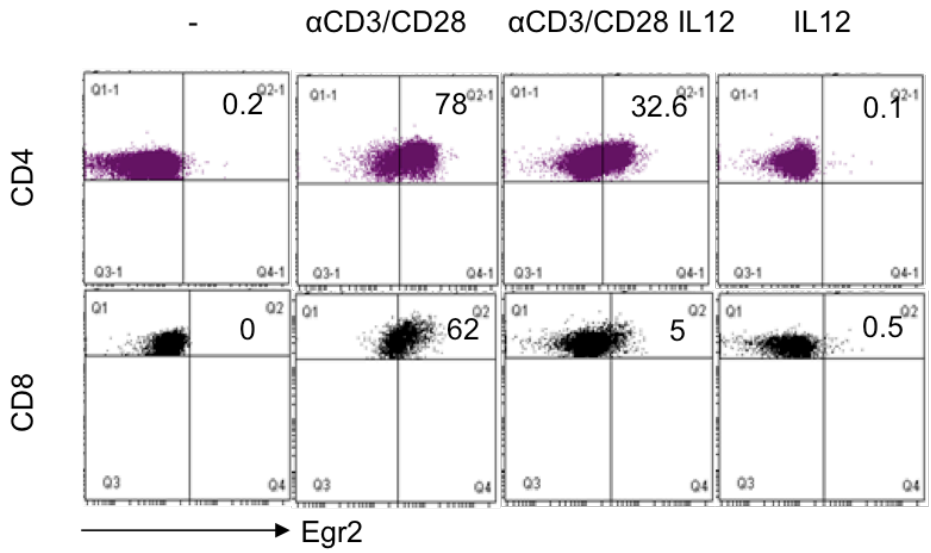

Figure 3.12: Expression of Egr2 in CD4 and CD8 T cells suppressed by IL-12

Naïve CD4 and CD8 T cells from WT mice were stimulated with the indicated stimuli for 16 hours before analysis of Egr2 expression by flow cytometry. Egr2 expression was analysed in un-stimulated as a negative control (first panel from left). This was followed by stimulation with anti-CD3 (5μg/ml) and anti-CD28 (2 μg/ml) for 16 hours in the absence (second panel) and presence of IL-12 (third panel). Finally, CD4 and CD8 cells were cultured with recombinant IL-12 alone. The data are from pooled cells of four mice in each group and represent three independent experiments with similar results.

3.7.2 Egr2 and Egr3 expression suppressed by IFNγ

In addition to the inhibitory effect of IL-12, we also examined the effect of IFNγ on Egr2 and 3 expression in a similar fashion. CD4 T cells were activated in the presence and absence of recombinant-IFNγ (Figure 3.13). CD4 cells were stimulated with 1 μg/ml anti-CD3 only for 16 hours, instead of the conventional combination of anti-CD3 and anti-CD28. This concentration of anti-CD3 induced approximately 20 % of Egr2. This concentration was close to the degree of Egr2 expression in physiological conditions. The cells were then stimulated in the presence of varying concentrations of recombinant IFNγ, namely 50 ng/ml and 200 ng/ml and the expression of Egr2 determined by flow cytometry. Expression of Egr2 was reduced by 50 ng/ml IFNγ and even more so by 200 ng/ml to 8%

and 2.9%, respectively. This was also the case for Egr3 as determined by RT-PCR (figure 3.13C). Repression of Egr2 by exogenous IFNγ resulted in a gradual increase in endogenous IFNγ (Figure 3.13B). These results demonstrate a feedback mechanism by which T_H1 signature cytokine IFNγ and T_H1 inducing cytokine IL-12 can abolish Egr2 and 3 expression in effector T cells.

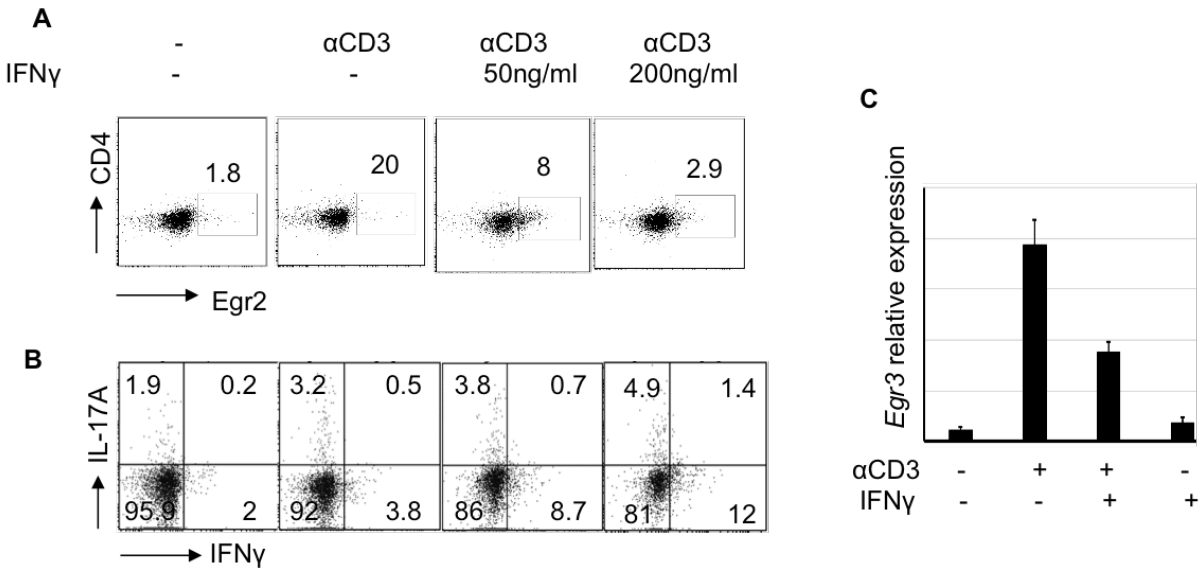

Figure 3.13: Expression of Egr2 and Egr3 in CD4 and CD8 T cells suppressed by exogenous IFNγ

CD4 T cells were stimulated with anti-CD3 (1μg/ml) with or without IFNγ at the indicated concentrations for 16 hours and then analysed for Egr2 (**A**), IL-17A and IFNγ (**B**) expression by flow cytometry and Egr3 expression was measured by RT-PCR. The data are from pooled cells of four mice in each group and represent three independent experiments with similar results.

3.7.3 IFNγ signalling can directly inhibit Egr2 expression

To further investigate the mechanism by which IFNγ can negatively regulate Egr2 expression, we designed a Egr2 reporter construct. The -3Kb to +1bp promoter region of the mouse Egr2 gene was cloned from a BAC clone and inserted into the luciferase reporter vector pGL3 basic. The Egr2 reporter gene was transfected into EL4 cells, which is a mouse T lymphocyte lymphoma cell line from the C57BL/6N strain. The cells were stimulated in the presence and

absence of recombinant IFNγ. Interestingly, we found that IFNγ signalling was able to significantly reduce Egr2 expression indicated by reduced luciferase activity (Figure 3.14). Together these results demonstrate a feedback loop mechanism by which IFNγ and IL-12 are able to negatively regulate Egr2 and Egr3 expression in T cells.

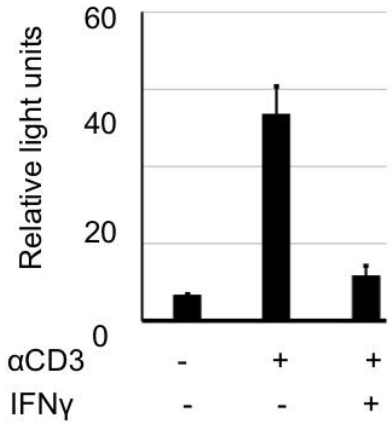

Figure 3.14: Egr2 reporter gene activity reduced by IFNγ
Egr2 reporter gene was transfected into EL4 cells, which were then stimulated with anti-CD3 (1µg/ml) in the presence or absence of IFNγ (50ng/ml) for 16 hours. Protein lysates were obtained and luciferase Egr2 reporter gene activity measured. Data are relative light units +/- s.e.m normalized with protein concentration and the experiment was done in triplicates.

3.7.4 Egr2 and Egr3 expression is exclusively dependent on TCR stimulation

To establish the interplay between these transcription factors and effector cytokines in T cells during T cell responses, we dissected their expression profiles during T cell activation and rest *in vitro*. To do this, CD4 T cells from WT mice were stimulated with anti-CD3 and anti-CD28 for 5 hours followed by 16 hours of rest. This cycle of stimulation and rest was repeated three times all together. During the rest phase, cells were transferred into new wells without any plate bound antibodies and the medium in which they were cultured was unchanged throughout the entire experiment. This ensured that any effector cytokines released by T cells would be maintained during each phase.

The results from this experiment were very interesting indeed. First, we found that Egr2 and 3 was induced by TCR stimulation very early during T cell activation (Figure 3.15 B and D). After 16 hours of rest, the level of Egr2 and 3 expression reduced significantly and re-stimulation induced both Egr2 and 3 once again. Interestingly, The IFNγ and T-bet expression profiles were different. T-bet was induced after CD4 T cell stimulation with anti-CD3 and anti-CD28, but the expression was not reduced during each rest phase, in fact the expression of T-bet continued to increased regardless of whether the cells were stimulated or resting. This expression profile was parallel to that of IFNγ, which also increased after subsequent stimulation and rest phases (Figure 15 A and C).

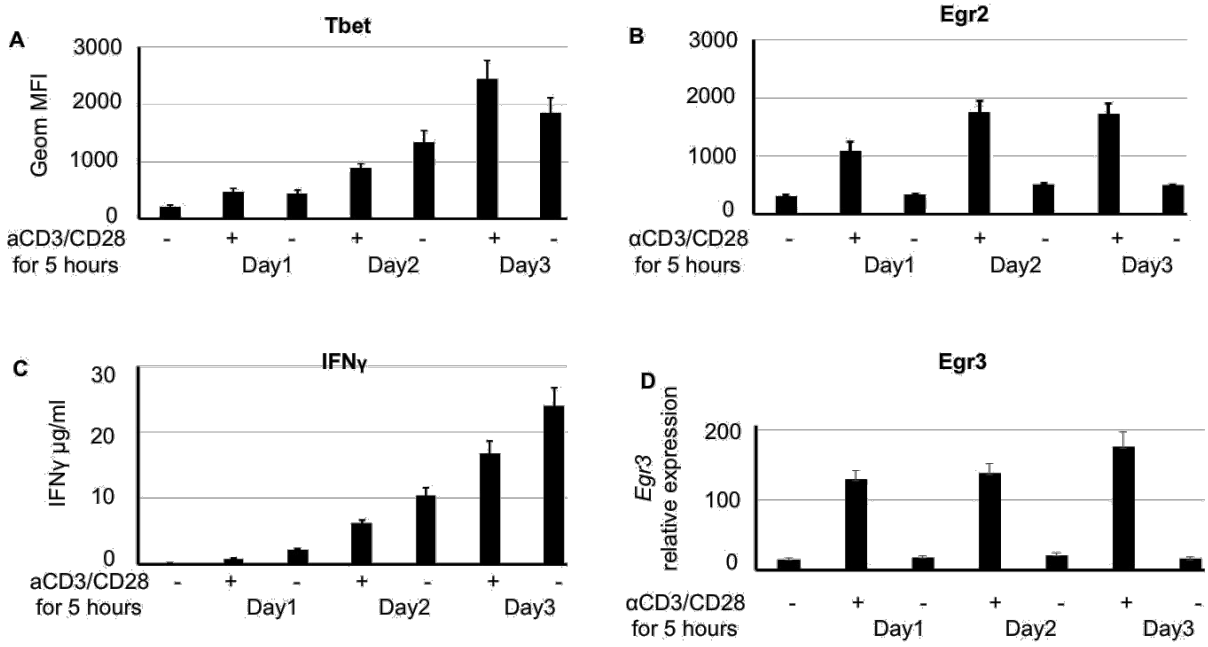

Figure 3.15: Kinetic expression of T-bet, Egr2, Egr3 and IFNγ in CD4 T cells during *in vitro* antigen stimulation and rest.

Naïve CD4 T cells were repeatedly stimulated with anti-CD3 and anti-CD28 for 5 hours with 16 hour rest intervals over a three day period. The cells were cultures in the same medium over the course of three days with no fresh medium added at any stage. Egr2 (**B**), T-bet (**A**), Egr3 (**D**) and IFNγ (**C**) expressing cells were determined before and after each interval by flow cytometry, RT-PCR and ELISA, respectively. The data are representative of three independent experiments.

Although a simple experiment, it demonstrates some interesting points regarding the regulation of Egr2 and Egr3 in T cells. First, in this context Egr2 and Egr3 expression appears to be dependent on TCR stimulation and in the absence of TCR stimulation, both Egr2 and Egr3 expression are abolished. Since the medium in which the cells were maintained remained the same during each interval, any effector cytokines produced by activated T cells were maintained in the culture. Therefore, during the rest phase in the absence of TCR stimulation, the effector cytokines can shut down Egr2 and Egr3 expression, and therefore boost T cell differentiation by promoting T-bet expression. Interestingly, since T-bet and Egr2 and 3 expression are all induced by TCR stimulation, it seems reasonable to assume that during these early stages of T cell activation, Egr2 can inhibit T-bet activity, preventing differentiation. The accumulation of effector cytokines can consequently shut down Egr2 expression and lead to the differentiation of effector cells to counter a viral infection.

Therefore, in conclusion the function of Egr2 and 3 in is vital for controlling T cell activation and differentiation. Egr2 and 3 deficient T cells also displayed impaired proliferation in response to viral infection. In fact, our group have recently discovered an important role for Egr2 and 3 in promoting T cell clonal expansion during viral infection (Miao, *et al*., 2016 see appendix for paper). Egr2 and 3 expression is induced early during T cell activation and our results demonstrate that it is essential to control IFNγ expression and potentially T_H1 differentiation by regulating T-bet function.

Function of Egr2 and Egr3 in T cells during viral infection

3.8 Egr2 and Egr3 deletion in CD2-Egr2/3$^{-/-}$ CD4 and CD8 T cells

3.8.1 Egr2 and Egr3 expression analysed by FACS and PCR to confirm deletion in CD2-Egr2/3$^{-/-}$ mice.

CD2-Egr2/3$^{-/-}$ mice were identified by genotyping. The deletion of Egr2 and Egr3 was further illustrated by FACS analysis for Egr2 expression and PCR for Egr3 expression in CD4 and CD8 T cells. These results demonstrate the successful deletion of Egr2 and 3 T cells. CD4 and CD8 cells were isolated and stimulated *in vitro* with anti-CD3 and anti-CD28 for 16 hours, which induces Egr2 and Egr3 expression in T cells. For Egr2, cells were stained with fluorescent antibodies, and analysed by flow cytometry. Egr2 expression was determined by analyzing mean fluorescence intensity (MFI) (figure 3.16A) and revealed that in CD2-Egr2/3$^{-/-}$ mice, its expression was negligible after stimulation. A fluorescently tagged antibody is not yet available for Egr3. Therefore, cells were analysed for Egr3 expression by PCR (figure 3.1B), and the expression pattern was similar to that of Egr2. In WT, Egr3 expression was induced but remains undetectable in knockout mice. These results were used to confirm the successful deletion of both Egr2 and Egr3 in T cells in CD2-Egr2/3$^{-/-}$ before mice were used for any further experiments

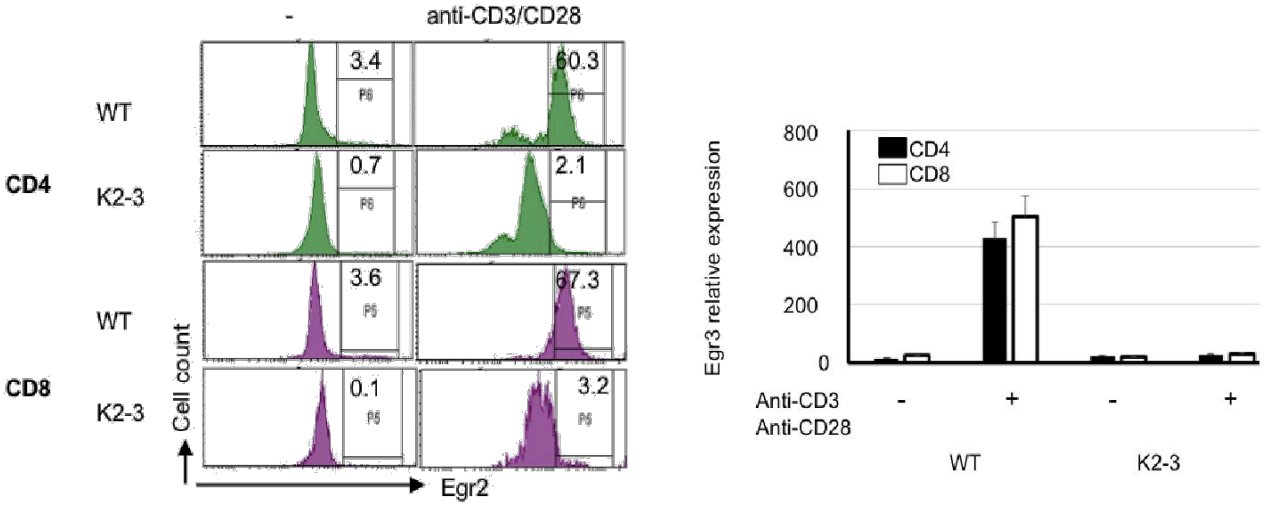

Figure 3.16: Egr2 and Egr3 expression in CD4 and CD8 cells

CD4 and CD8 cells from WT and CD2-Egr2/3$^{-/-}$ were stimulated with anti-CD3 and anti-CD28 for 16 hours *in vitro*. Egr2 expression (**A**) was measured by flow cytometry and presented as a histogram of mean fluorescence intensity (MFI). Egr2 induction was seen as a shift to the right of MFI for WT cells but not for CD2-Egr2/3$^{-/-}$ CD4 and CD8 T cells. Relative expression of Egr3 against β-actin in WT also induced after *in vitro* stimulation but not in CD2-Egr2/3$^{-/-}$ (**B**). Data in A represents three independent experiments with similar results. B is mean +- s.d. from triplicated data and represents three experiments with similar results.

3.9 Abnormal cytokine production and impaired proliferation of Egr2 and 3 deficient T cells

3.9.1 IL-2 and IFNγ production and proliferation of T cells in vitro

CD2-Egr2/3$^{-/-}$ mice developed a late onset of systemic autoimmune disease and in general the illness is characterized by abnormal lymphocyte activation and production of effector cytokines (Li *et* al., 2012, O' Shea *et al*., 2001). IL-2 production can be attributed to CD4 and CD8 T cells following activation, although IL-2 production by CD8 T cells is comparatively weak and mice deficient in IL-2, CD25 or CD122 develop systemic autoimmunity. Activated T cells secrete IL-2, which stimulates growth and differentiation of effector T cells and have high CD25 expression to increase responsiveness to IL-2 (Boyman

and Sprent., 2012). We therefore isolated T cells from CD2-Egr2/3$^{-/-}$ mice before the onset of autoimmune symptoms and WT mice to analyse the expression of IL-2 after stimulation with anti-CD3 and anti-CD28 *in vitro*. Figure 3.17A/B show that Egr2 and 3 deficient T cells had impaired production of IL-2 in response to antigen stimulation *in vitro* compared to their WT counterparts. To investigate the function of Egr2 and 3 in T cell proliferation *in vitro*, naïve CD4 T cells (CD44^{low}CD4^{+}CD62L^{+}) were isolated from WT and CD2-Egr2/3$^{-/-}$ mice, labelled with Carboxyfluorescein succinimidyl ester (CFSE) and stimulated with anti-CD3 and anti-CD28 for 72 hours. CFSE is a protein dye and this amino reactive dye forms stable covalent bonds with cell proteins. Cell division is measured as successive halving of the fluorescent intensity of CFSE by flow cytometry. The cells were analysed for CD25 expression and proliferation by flow cytometry. CD25 is the IL-2 receptor α-chain, which is up regulated in response to antigen receptor stimulation by IL-2. CD25 expression is indicative of an activated T cell with increased responsiveness to IL-2 (Boyman and Sprent., 2012). Figure 3.2C shows the proliferation status, Egr2 and 3 deficient CD4 and CD8 T cells having impaired proliferation in response to TCR stimulation compared to WT, demonstrated by successive cell division by flow cytometry and dilution of CFSE. To our surprise, in contrast to the impaired IL-2 production, antigen receptor stimulation induced high amounts of the inflammatory cytokine IFNγ in CD4 T cells. These results demonstrate, a potential role for Egr2 and Egr3 in promoting T cell proliferation and for the control of IL-2 and IFNγ cytokine expression (figure 3.17A).

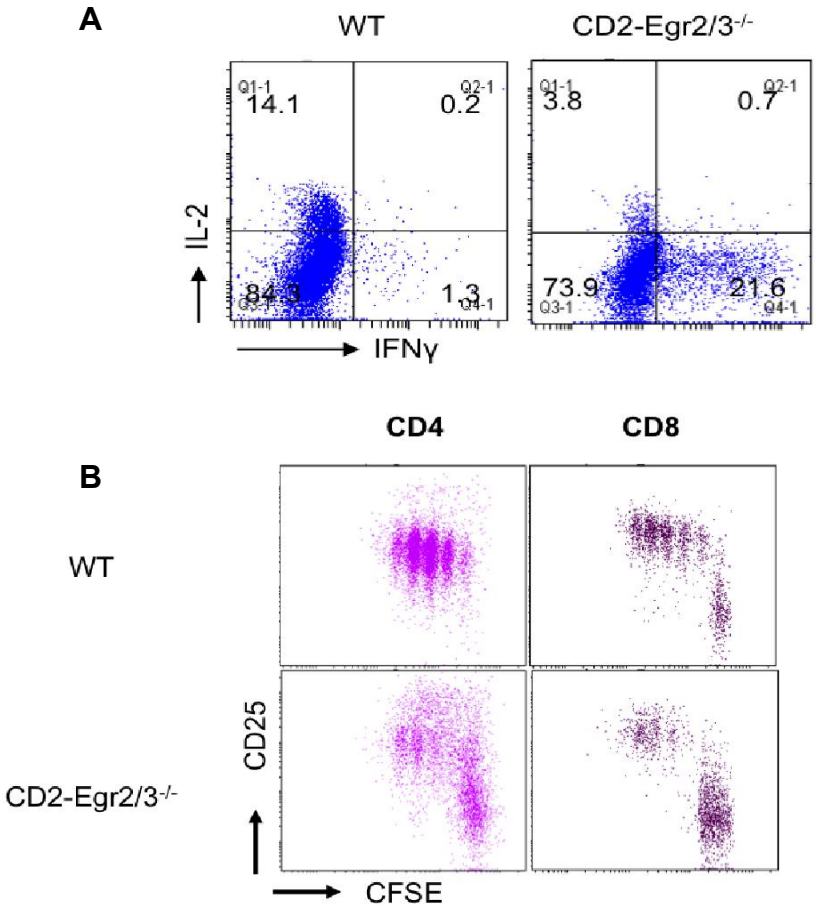

Figure 3.17: IFNγ and IL-2 expression and *in vitro* proliferation of CD4 and CD8 T cells from WT and CD2-Egr2/3$^{-/-}$ mice.

CD4 T cells were isolated from 8 week old CD2-Egr2/3$^{-/-}$ and WT mice, by CD4 magnetic beads and stimulated *in vitro* for 16 hours with anti-CD3 and anti-CD28 followed by cytokine expression analysis by flow cytometry. The cytokines were stained with fluorochrome labelled antibodies. Representative flow cytometry analysis of IFNγ^{+} and IL-2^{+} CD4^{+} cells (**A**). Naïve CD4 (CD44^{low}CD4^{+}CD62L^{+}) and CD8 (CD44^{low}CD8^{+}CD62L^{+}) cells from 8-week-old WT and CD2-Egr2/3$^{-/-}$ mice were labelled with CFSE, stimulated *in vitro* for 72 hours with anti-CD3 and anti-CD28. Cells were gated on CD4^{+}CD25^{+}or CD8^{+}CD25^{+} and CFSE dilution was analysed (**B**). The data represent three independent experiments with similar results.

3.9.2 IFNγ and TNFα production in T cells during OVA-VV_{WR} infection

Our group has previously demonstrated that CD2-Egr2/3$^{-/-}$ T cells expressed increased levels of pro-inflammatory cytokine mRNAs after TCR engagement *in vitro* indicating that Egr2 and 3 are required to control inflammatory cytokine expression (Li *et al*., 2012). In figure 3.17A we demonstrated an increased production of IFNγ expression at the protein level after CD4 TCR engagement *in vitro*. To determine whether this reflects increased levels of inflammatory cytokine expression during viral infection WT, CD2-Egr2/3$^{-/-}$ and CD2-Egr2 Tg mice were infected with OVA-VV_{WR} and 7 days post infection, splenic CD4 and CD8 cells were incubated with virus infected LB27.4 cells for 16 hours before cytokine analysis. As naïve CD4 T cells differentiate into effector cells, they gain the ability to produce anti-viral cytokines such as IFNγ. CD8 T cells in a similar fashion differentiate into effector CTL and gain the ability to produce cytokines such as, IFNγ and tumor necrosis factor alpha (TNFα) and cytotoxic molecules such as granzymes and perforins, to eliminate infectious agents (Kaech and Ahmed., 2001). Therefore, we analysed IFNγ and TNFα expression by CD4 and CD8 cells (figure 3.18).

In 8 week old OVA-VV_{WR} infected CD2-Egr2/3$^{-/-}$ mice, $CD4^+$ T cells displayed a marked increased in the percentage of IFNγ expression which was ~ 29% compared to WT virally infected CD4 cells with a ~ 13% increase after infection. In a similar fashion, CD8 T cells from OVA-VV_{WR} infected CD2-Egr2/3$^{-/-}$ mice displayed a considerable increased in TNFα expression of ~ 70.8% compared to ~ 31.7% of age matched WT cells. IFNγ expression in Egr2 and 3 deficient CD8 T cells was ~ 48.1% compared to ~ 22.4% in WT. This progressive increase in the percentage of IFNγ and TNFα expression parallels the progressive accumulation of $CD44^{high}$ cells in virally infected CD2-Egr2/3$^{-/-}$ mice. OVA-VV_{WR} infected CD2-Egr2 Tg mice displayed an opposite picture with regard to cytokine expression. CD2-Egr2 Tg CD4 and CD8 cells had low levels of IFNγ and TNFα expression. CD4 Egr2 Tg mice one displayed a ~ 3% IFNγ expression compared to ~ 1% of un-infected Tg mice and CD8 Egr2 Tg mice had ~ 6.1% and ~ 7.4% of IFNγ and TNFα expression after OVA-VV_{WR} infection, respectively (figure 3.18).

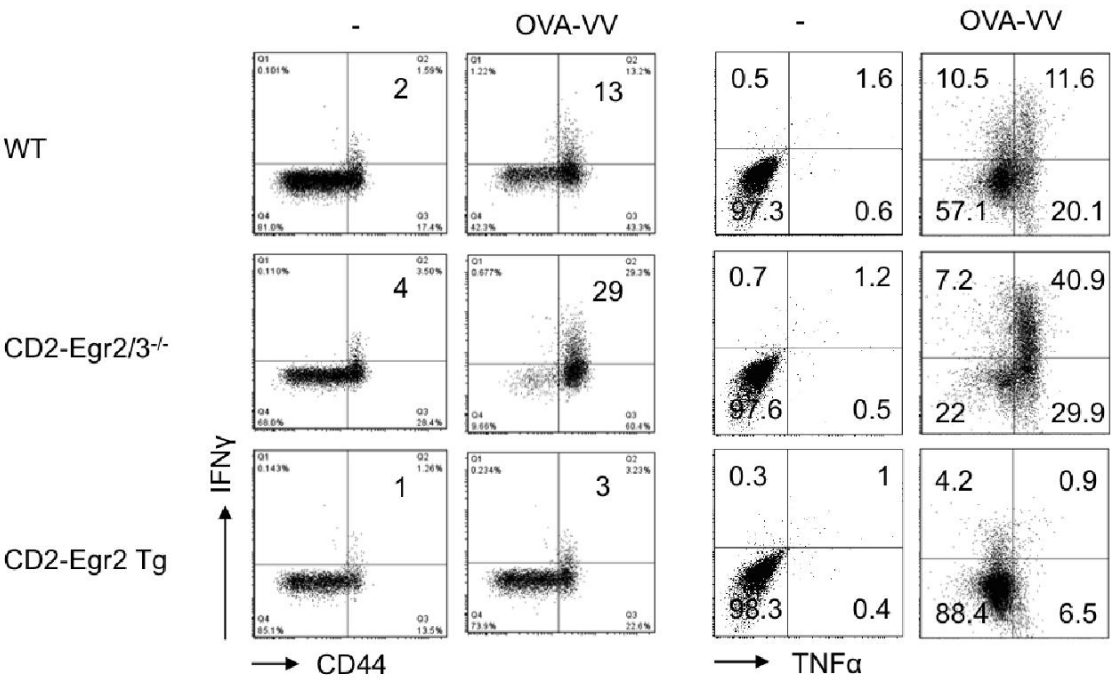

Figure 3.18: IFNγ and TNFα expression in CD4 and CD8 cells 7 days post OVA-VV_{WR} infection.

To determine the differentiation state of T cells 7 days after infection with OVA-VV_{WR} infection, the expression of IFNγ and TNFα was measured. Splenic CD4 and CD8 cells from uninfected and OVA-VV_{WR} infected were isolated from WT, CD2-Egr2/3$^{-/-}$ and CD2-Egr2 Tg mice and incubated with OVA-VV_{WR} infected LB27.4 cells for 16 hours before analysis of CD44, IFNγ and TNFα producing cells by flow cytometry. The data are from pooled cells of four mice in each group and represent three independent experiments with similar results.

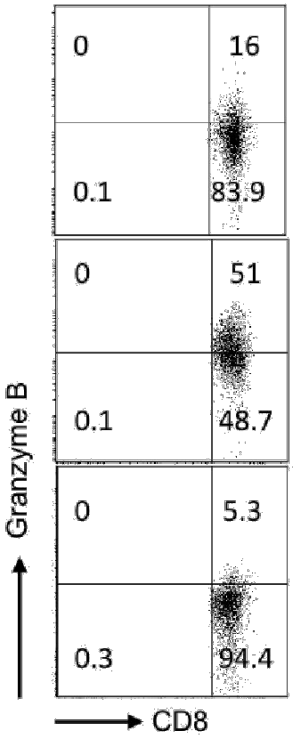

Figure 3.19: Granzyme B producing CD8$^+$ cells
Splenic CD8$^+$ cells were further examined for Granzyme B production by flow cytometry. In the same experiment described for figure 3.3, CD8$^+$ cells from infected WT, CD2-Egr2/3$^{-/-}$ and CD2-Egr2 Tg mice were isolated and incubated for 16 hours with OVA-VV$_{WR}$ infected LB27.4 cells, followed by Granzyme B analysis in CD8$^+$ cells. The data are from pooled cells of four mice in each group and represent three independent experiments with similar results.

CD8 cells from OVA-VV$_{WR}$ infected mice were analysed for granzyme B expression and we found that in Egr2 and 3 deficient T cells, there is a considerably high percentage of granzyme B production, which is ~ 51% compared to age matched WT infected cells of ~ 16%. T cells with forced Egr2 expression had a marked reduction in granzyme B production of ~ 5.3% compared to WT cells (figure 3.19). Cytotoxic molecules such as granzyme B are produced by CTLs to eliminate infections agents (Kaech and Ahmed., 2001).

3.9.3 IFNγ and TNFα in antigen specific T cells

So far, we have found Egr2 and 3 deficient cells to have an increased differentiated state into effector cells with elevated levels of IFNγ and TNFα and granzyme B, including a considerably high population of CD4 and CD8 CD44^{high} cells during viral infection. CD2-Egr2 Tg cells has the complete opposite phenotype to CD2-Egr2/3$^{-/-}$ T cells with decreased cytokine production and low CD44^{high} cell numbers (figure 3.6). To further determine whether this reciprocal pattern reflects the phenotype of H2-Kb SIINFEKL specific CD8$^+$ T cells, we infected mice with OVA-VV$_{WR}$ and analysed cytokine production in antigen specific CD8$^+$ T cells (figure 3.5). Compared to WT, CD2-Egr2/3$^{-/-}$ OVA-VV$_{WR}$

infected mice had a reduced numbers of antigen specific $CD8^+$ cells of ~ 1.1% compared to ~ 3.5% in WT. CD2-Egr2 Tg mice on the other hand showed a considerable increase in antigen specific $CD8^+$ cells of ~ 7.4%. The expression of IFNγ and TNFα was also analysed and a reciprocal relationship between CD2-Egr2/3$^{-/-}$ and CD2-Egr2 Tg was apparent. CD2-Egr2/3$^{-/-}$ antigen specific cells displayed a marked increase in TNFα and IFNγ expression of ~ 67.3% and ~ 48.0%, compared to ~ 24.1% and ~ 17.5% in WT, respectively. Antigen specific $CD8^+$ splenic cells from CD2-Egr2 Tg infected mice had a reduced expression of both TNFα and IFNγ of ~ 6.1% and ~ 2.6%, respectively, compared to WT (figure 3.20).

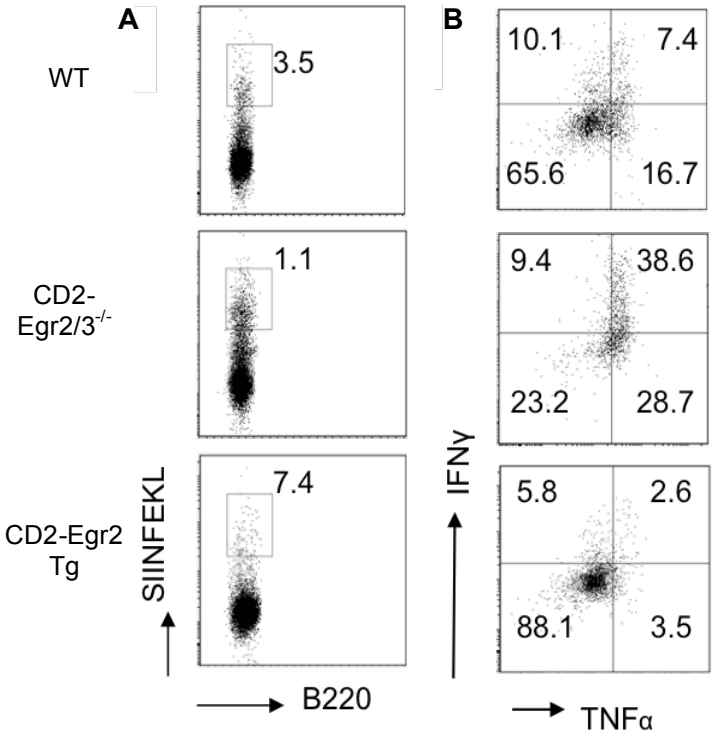

Figure 3.20: H2-Kb SIINFEKL specific T cells analysed for IFNγ and TNFα expression

Antigen specific $CD8^+$ cells were isolated from OVA-VV_{WR} infected mice 7 days post infection and incubated with OVA-VV_{WR} infected LB27.4 cells for 16 hours *in vitro*. Cells were stained for CD8 and B220 (to exclude B cells) and H2-Kb SIINFEKL tetramer. Cells were gated on $CD8^+B220^-$SIINFEKL $tetramer^+$ and further for analysis of IFNγ and TNFα producing antigen specific cells (**A**) and (**B**). The data are from pooled cells of four mice in each group and represent three independent experiments with similar results.

Previously our group discovered increased mRNAs of cytokines in naïve T cells after antigen challenge *in vitro* by RT-PCR, which included, IL-17A, IL-21, IL-4 GM-CSF, IL-6 and IFNγ (Li *et al*., 2012). We now show that in the absence of Egr2 and 3, T cells from virally infected mice have an increased expression of IFNγ and TNFα, which is paralleled by a rise in $CD44^{high}$ cells. Mice with forced expression of Egr2 displayed a suppressed state with respect to cytokine production and activation since a considerably reduced

numbers of $CD44^{high}$ cells were detected compared to WT (figure 3.5). IFNγ and TNFα producing cells along with secretion of cytotoxic molecules as granzyme B indicate the presence of virally responding T cells in a differentiated state. It seems reasonable therefore to conclude that Egr2 and 3 may have a prominent role in controlling T cell differentiation of virally responding T cells by controlling effector cytokine production. This statement however requires further experimental analysis.

3.10 Egr2 and Egr3 deficient T cells are hyperactive with impaired proliferation during viral responses

3.10.1 $CD44^{high}$ T cells during OVA-VV_{WR} infection

As seen previously, CD2-Egr2/$3^{-/-}$ mice when infected with OVA-VV_{WR} and analysed for CD44 expression, have high proportion of $CD44^{high}$ cells compared to WT. Activated T cells during viral infection up-regulate the expression of the cell surface molecule CD44, recognized as $CD44^{high}$ cells. CD44 is widely distributed cell surface glycoprotein expressed on lymphoid and non-lymphoid cells alike. Its function has been attributed to cell adhesion and migration, lymphocyte homing, formation of memory T helper 1 cells (Baaten *et al*., 2010) and it is used as a marker for activated T cells and memory cells. It would be interesting to find out whether Egr2 is prominently expressed in activated $CD44^{high}$ cells. We therefore analysed the percentage of $CD44^{high}$ cells in our mouse model after viral infection and the expression of Egr2 within this population.

Splenic CD8 cells from OVA-VV_{WR} infected mice were analysed for the activation marker CD44 expression by flow cytometry. Compared to ~ 51.5% of $CD44^{high}$ cells in WT mice post infection, Egr2 and 3 knockout mice showed a considerable increase in $CD44^{high}$ cells, which was approximately 81.8% (figure 3.21). $CD44^{high}$ cells peaked 7 days after infection with OVA-VV_{WR} in CD2-Egr2/$3^{-/-}$ mice and WT alike, but the proportion of $CD44^{high}$ cells are considerably higher in CD2-Egr2/$3^{-/-}$ mice during viral infection. Egr2 expression was analysed by flow cytometry in WT T cells from infected and un-infected

mice, and of the ~ 51.5% of CD44^{high} cells, ~ 18.6% were Egr2 positive. In contrast, only 1.3% of Egr2 positive cells were CD44^{low}. A mouse Egr3 specific antibody is yet to be developed, therefore Egr3 expression was analysed by PCR and after infection, Egr3 expression was highly induced in WT CD8^{+} T cells (figure 3.21). It appears that Egr2 and 3 are required to control T cell activation during T cells response to viral infection. We were very interested at this stage to further investigate the activation state and the proliferative potential of Egr2 and 3 deficient T cells by examining CD69 and Ki67 expression on T cells during infection

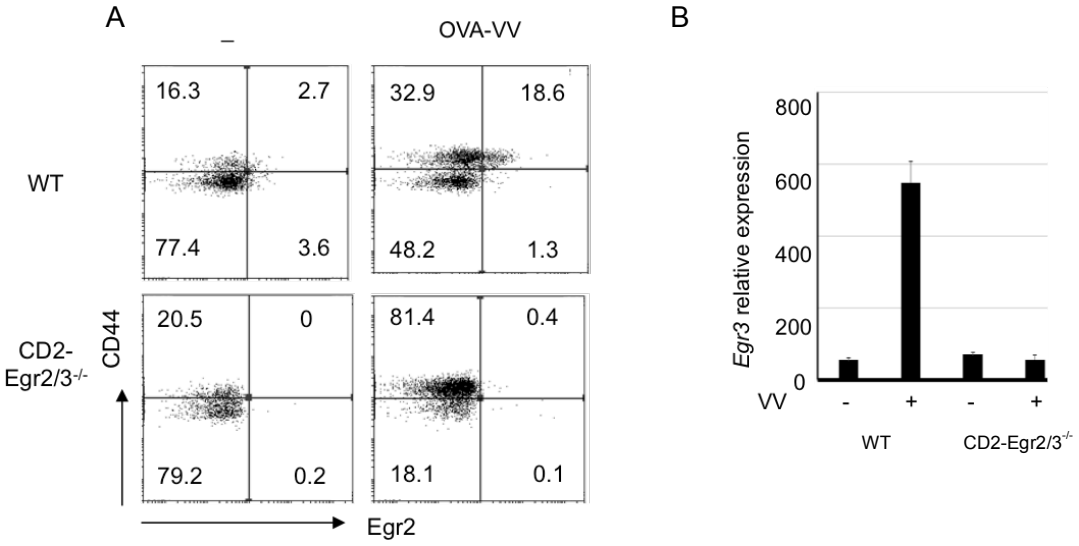

Figure 3.21: CD44^{high} cells high in CD2-Egr2/3$^{-/-}$ after OVA-VV infection
CD8 T cells isolated 7 days post OVA-VV infection from WT and CD2-Egr2/3$^{-/-}$. CD8 T cells were analysed for Egr2 and CD44 expression before and after OVA-VV infection by flow cytometry (**A**). Relative expression of Egr3 against β-actin was determined by PCR (**B**). The data are from pooled cells of four mice in each group and represent three independent experiments with similar results.

3.10.2 CD69$^+$ T cells during OVA-VV$_{WR}$ infection

CD69 is a C-type lectin receptor and it is the earliest activation antigen on lymphocytes. CD69 induces TGF-β expression and affects the migration of immune cells (Radulovic *et al*., 2013). Studies have shown CD69 to play an essential role in the regulation and migration of lymphocytes, which is expressed very early in T cells upon activation along with CD44. To further investigate the role of Egr2 and 3 in T cell activation, CD2-Egr2/3$^{-/-}$ T cells were analysed for CD69 expression to detect activated cells, 7 days after mice were infected with OVA-VV$_{WR}$. After infection, CD69 was induced in CD4$^+$ and CD8$^+$ T cells as expected, but the percentage differed radically between WT, knockout and transgenic mice. In WT, ~ 18.3% CD4$^+$ cells and ~ 24.6% CD8$^+$ were CD69$^+$. Egr2 and 3 deficient T cells had considerably higher percentages of both CD4$^+$CD69$^+$ and CD8$^+$CD69$^+$ cells which were ~ 34.7% and ~ 42.5%, respectively (figure 3.22). In addition, Egr2 was induced in approximately half of CD4$^+$ and CD8$^+$ CD69$^+$ cells. In contrast forced expression of Egr2 in CD2-Egr2 Tg mice, had considerably low levels of CD69$^+$, CD4$^+$ and CD8$^+$ T cells, which were ~ 9.7% and ~ 12.7%, respectively. The reciprocal pattern of high and low proportion of CD4$^+$CD69$^+$ and CD8$^+$CD69+ in CD2-Egr2/3$^{-/-}$ and CD2-Egr2 Tg during viral infection, respectively, illustrates a role for Egr2 and 3 in controlling T cell activation. This seems like a reasonable conclusion, since in the absence of Egr2 and 3 T cells appear to break activation thresholds and potentially differentiate into effector cells more readily. This is further strengthened with a reduced activation state of T cells with forced Egr2 expression.

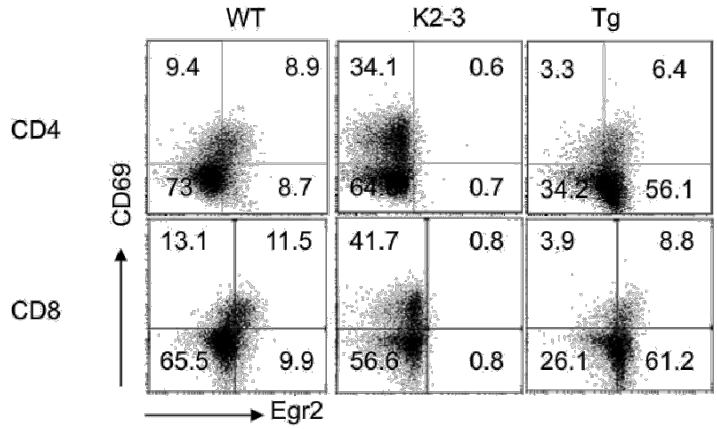

Figure 3.22: Activation marker CD69 expression on T cells 7 days post OVA-VV$_{WR}$ infection*

CD4^{+} and CD8^{+} T cells from OVA-VV$_{WR}$ infected WT, CD2-Egr2/3$^{-/-}$ and CD2-Eg2 Tg, were analysed for CD69 and Egr2 expression by flow cytometry (**A**). The data are from pooled cells of four mice in each group and represent three independent experiments with similar results.

*CD2-Egr2/3$^{-/-}$ mice are represented as K2-3 and CD2-Egr2 Tg are represented as Tg

3.10.3 CD62L^{+}CD44^{low}T cells during OVA-VV$_{WR}$ infection

We next analysed the naïve T cell population in mice during OVA-VV$_{WR}$ infection. CD62L (L-slectin) is a cell surface molecule expressed on naïve and activated/memory T cells. It has been demonstrated that naïve T cells express a CD62L^{hi}CD44^{low} phenotype, whereas memory T cells exhibit a CD62L^{low}CD44^{high} phenotype (Gerberick *et al* 1997). Therefore, we analysed CD62L and CD44 expression to examine the naïve T cell population after infection with OVA-VV$_{WR}$ in WT, CD2-Egr2/3$^{-/-}$ and CD2-Egr2 Tg mice. Interestingly, after OVA-VV infection, compared to WT mice, CD2-Egr2/3$^{-/-}$mice had a considerably low percentage of naïve CD4 and CD8 T cells of ~ 4.3% and ~ 8.9%, compared to WT which was ~25.2 % and ~ 47.8%, respectively.

Naïve T cell population in Egr2 transgenic mice as expected was increased for both CD4 and CD8 cells with ~ 53.0% and ~ 64.4%, respectively (figure 3.23). In parallel with high activated $CD4^{+}CD69^{+}CD44^{high}$ and $CD8^{+}CD69^{+}CD44^{high}$ T cells during viral infection in CD2-Egr2/3$^{-/-}$ mice (figure 3.6 and 3.7), the percentage of $CD4^{+}CD62L^{+}CD44^{low}$ and CD8+$CD62L^{+}CD44^{low}$ naïve T cells was lower in CD2-Egr2/3$^{-/-}$ mice when compared WT mice. In contrast naïve T cell numbers were high in CD2-Egr2 Tg mice during OVA-VV_{WR} infection compared to WT mice. Collectively in figures 3.21, 3.22 and 3.23, the results demonstrate a key role for Egr2 and 3 in controlling T cell activation during viral infection.

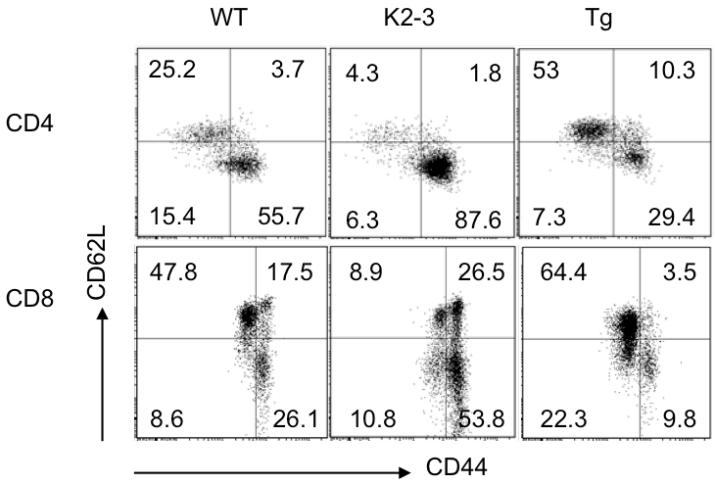

Figure 3.23: CD62L and CD44 expression in WT, CD2-Egr2/3$^{-/-}$ and CD2-Egr2 Tg mice 7 days post OVA-VV infection.

Mice were infected with OVA-VV and 7 days post infection, CD4 and CD8 T cells were isolated from spleen and analysed for CD62L and CD44 expression by flow cytometry to determine the percentage of naïve T cells before and after infection. The data are from pooled cells of four mice in each group and represent three independent experiments with similar results.

3.10.4 Ki67^{+} T cells during OVA-VV$_{WR}$ infection

Although T cells from CD2-Egr2/3$^{-/-}$ mice during viral infection, displayed increased activated states with a high proportion of CD4^{+}CD69^{+}CD44^{high} and CD8^{+}CD69^{+}CD44^{high} cells, IL-2 production and *in vitro* proliferation is severely impaired. To further assess proliferation of CD4 and CD8 cells during viral infection, we analysed T cells for Ki67 expression. Ki67 is a nuclear protein that plays a role in the regulation of cell division and it has been used in cancer biology to reveal tumour cell proliferation. Ki67 is present during all phases of cell cycle (G1, S, G2, mitosis) but it is absent in resting cells (G0). These characteristics make it an excellent marker for detecting proliferating cells (Scholzen and Gerdes 2000).

In parallel to impaired *in vitro* T cell proliferation of Egr2 and 3 deficient T cells, these cells also had reduced percentage of Ki67^{+} cells compared to WT. As expected, in WT CD4^{+} and CD8^{+} T cells, a large proportion of CD44^{high} cells were also positive for Ki67, indicative of activated and proliferating cells. To our surprise, in CD2-Egr2/3$^{-/-}$ mice, a very low proportion of CD4^{+}CD44^{high} and CD8^{+}CD44^{high} cells were actually positive for Ki67. This was highly unusual, since Egr2 and 3 deficient mice had a significantly higher proportion of CD4^{+}CD44^{high} and CD8^{+}CD44^{high} T cells compared to WT. The percentage of CD8^{+}CD44^{high} cells in WT mice was 44.2%, of which 36% were Ki67 positive, which is approximately 70%. For CD4^{+} T cells in WT mice, 47.6% were CD44^{high}, and 67% were also positive for Ki67. Calculated in a similar fashion, we found that only 30% of CD44^{high} cells were positive for Ki67 for both CD4^{+} and CD8^{+} cells in CD2-Egr2/3$^{-/-}$mice. In contrast, over 90% of all CD4^{+}CD44^{high} and CD8^{+}CD44^{high} cells in Tg mice were also positive for Ki67 (figure 3.24). Results from figure 3.24 illustrate a prominent role for Egr2 and 3 in promoting T cell proliferation during viral infection.

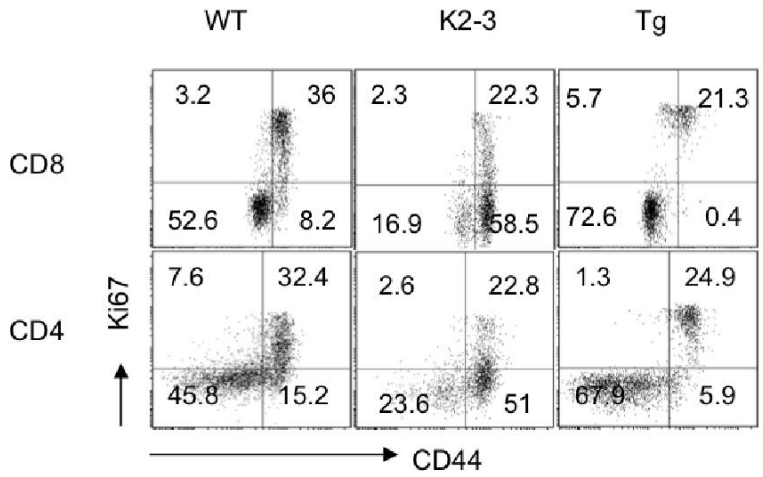

Figure 3.24: WT, CD2-Egr2/3$^{-/-}$ and CD2-Egr2 Tg T cells analysed for CD44 and Ki67 expression*.

Mice infected with OVA-VV$_{WR}$ were analysed for Ki67 and CD44 expression 7 days post infection by flow cytometry illustrating the percentage of CD4^{+}CD44^{high} and CD8^{+}CD44^{high} and CD4^{+}Ki67^{+} and CD8^{+}Ki67^{+} cells. The data are from pooled cells of four mice in each group and represent three independent experiments with similar results.

*CD2-Egr2/3$^{-/-}$ mice are represented as K2-3 and CD2-Egr2 Tg are represented as Tg

3.11 Egr2 and 3 controls T helper 1 (T_H1) differentiation but do not regulate T-bet expression

During adaptive immune responses, activated T cells undergo clonal expansion and differentiation into effector and memory cells. CD4 T helper cells, so called because they help modulate the immune response, differentiation into several subsets, each with unique qualities to target specific pathogens. CD4 T helper cells can differentiate into T helper 1 (T_H1), T_H2 and T_H17 subsets. Other subsets include, T regulatory cells (T_{Reg}), T follicular helper (T_{fh}) and memory cells. The T helper differentiation program is driven by effector cytokines, such as IFNγ for T_H1, IL-4, IL-5 and IL-13 for T_H2 and IL-17 for T_H17 (Zhu *et al*., 2010). Experiments so far have revealed that Egr2 and 3 deficient virally responding T cells acquire a hyper differentiated state, with elevated levels of IFNγ and TNFα expression. In contrast, T cells with forced Egr2 expression have a significantly reduced expression of these effector cytokines. We therefore wanted to investigate the role of Egr2 and 3 in $CD4^+$ T helper differentiation. To do this, naïve ($CD44^{low}CD62L^+CD4^+$) T cells were isolated from WT mice and cultured *in vitro* under T_H1, T_H2 and T_H17 conditions and the expression of cytokines unique to each lineage analysed accordingly.

3.11.1 CD4 T helper 1 (T_H1), T_H2 and T_H17 differentiation in vitro

The process of T helper differentiation can be driven *in vitro* with the aid of recombinant effector cytokines and antibodies. T_H1 differentiation is regulated by T-bet and during initial polarization T-bet is induced synergistically via TCR and IFNγ signalling. (Lighvani *et al*., 2001). Subsequent T-bet expression is induced by IL-12-STAT4 signalling in the absence of TCR signalling (Schulz *et al*., 2009). Therefore, recombinant-IL-12 (rIL-12) was used in combination with TCR stimulation to induce T_H1 cells *in vitro*. IL-4 signalling in turn antagonizes T_H1 differentiation by inhibiting IFNγ expression (Wurtz *et al*., 2004). Therefore, to induce *in vitro* differentiation into T_H1 lineage, naïve CD4 T cells were stimulated in the presence of rIL-12 and anti-IL4 to inhibit IL-4 mediated signalling. T_H2 lineage differentiation is regulated by the transcription factor GATA3, the expression of which is induced by IL-4 mediated STAT6 signalling (Scheinman and Avni 2009). Therefore, naïve T cells were cultured with IL-4 to

induce STAT6 and anti-IFNγ to prevent IFNγ mediated signalling events. The master regulator of T_H17 lineage differentiation is the transcription factor RORγt. TGF-β and IL-6 mediated STAT3 signalling is essential for RORγt expression and T_H17 differentiation (Yang *et al*., 2007). Therefore, T_H17 lineage commitment was established by culturing naïve T cells in the presence of TGF-β and IL-6 to induce STAT3 mediated RORγt expression and anti-IFNγ and anti-IL4 to inhibit alternative STAT1 and STAT4 signalling pathways, respectively.

The results posed a rather interesting scenario, with hyper differentiated states for $CD4^+$ T cells under T_H0, T_H1 and T_H17 conditions. T cells cultured under T_H0 conditions were stimulated with anti-CD3 and anti-CD28 along with rIL-2. Egr2 and 3 deficient $CD4^+$ T cells under these conditions displayed an increase in IFNγ expression compared to its WT counterpart. Interestingly, $CD4^+$ T cells cultured under T_H1 had a considerable increase in IFNγ expression in Egr2 and 3 deficient T cells, which was almost ~ 60% compared to the ~ 36.8% in WT cells. Surprisingly, IL-4 production in Egr2 and 3 deficient $CD4^+$ T cells cultured under T_H2 conditions was similar to WT. Finally, IL-17A production in Egr2 and 3 deficient $CD4^+$ cells also increased compared to WT from ~ 12.0% to ~ 20.1%. With the exception of T_H2, it seems that T helper differentiation of T_H1 and T_H17 is enhanced in Egr2 ad 3 deficient T cells compared to WT cells with increased production of effector cytokines unique to each lineage. When we examined IFNγ expression by T cells cultured under T_H2 and T_H17 conditions, we found that a considerable percentage of cells became IFNγ producing cells. For instance, in WT $CD4^+$ T_H2 cultured cells there is ~ 1.1% IFNγ expression, but Egr2 and 3 deficient $CD4^+$ T cells cultured under T_H2 conditions had ~11.8% of IFNγ producing cells. Likewise WT $CD4^+$ T cells cultured under T_H17 conditions had ~ 2.0% IFNγ expression but Egr2 and 3 deficient cells had ~ 13.3%, which is a considerable increase. Interestingly, Egr2 and 3 deficient cells do not appear to be skewed into becoming IFNγ producing cells from IL-4 and IL-17A secreting cells. Instead it seems a portion of $CD4^+$ T cells cultured under T_H2 or T_H17 conditions differentiate into IFNγ producing cells (figure 3.10A). These results demonstrate an important role for Egr2 and 3 in

controlling cytokine production in T_H1 and T_H17 cells, but most prominently in T_H1 cells.

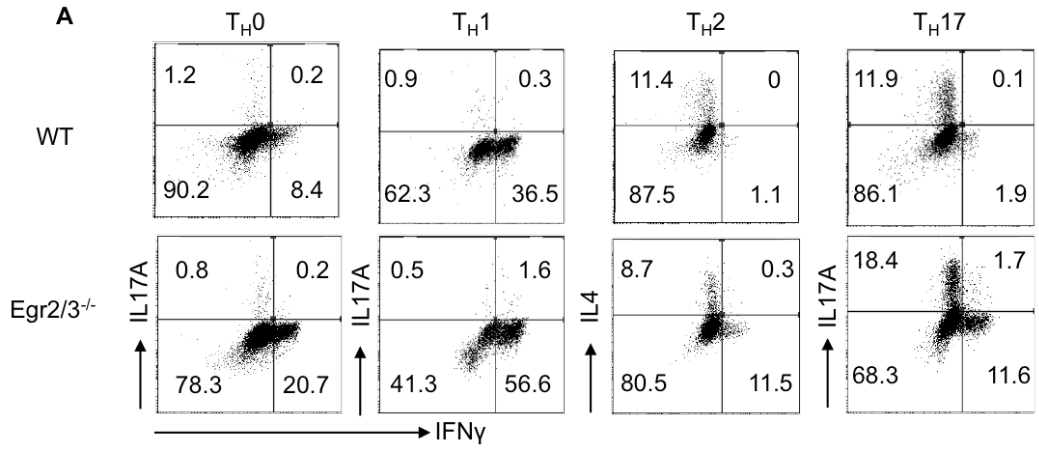

Figure 3.25: Naïve CD4 T cells from WT and CD2-Egr2/3$^{-/-}$ cultured under T_H0, T_H1, and T_H17 conditions *in vitro.*

$CD4^+CD62L^+CD44^{Low}$ cells isolated from wild type WT and CD2-Egr2/3$^{-/-}$mice were cultured *in vitro* with anti- CD3 and anti-CD28 under the indicated T helper conditions for 5 days. The cells were further stimulated by PMA and Inomycin (200 ng/ml for both) for 3 hours in the presence of Golgi stop. Cytokine expression was analysed by intracellular staining and detected by flow cytometry. The data are from pooled cells of four mice in each group and represent three independent experiments with similar results.

3.11.2 T-bet expression in T_H1, T_H2 and T_H17 cells in vitro

To further investigate the mechanism of how Egr2 and 3 control IFNγ expression, we first analysed the expression of T-bet in T_H1, T_H2 and T_H17 cells. T-bet is a master regulator of the T_H1 differentiation programme and directly regulates IFNγ expression in T cells (Szabo *et al*., 2000). We also analysed the expression of Egr2 along with T-bet in different T helper subsets from the experiment in figure 3.25. T-bet expression was pronounced in all T helper subsets, but interestingly the expression level was not altered in Egr2 and 3 deficient cells since an increase in IFNγ expression may have paralleled an increase in T-bet also. Although analysis of T-bet and Egr2 co-expressing T cells posed some interesting questions.

Under T_H1 conditions, T-bet expression was ~ 95.1 % in WT cells and ~ 78.2% in Egr2 and 3 deficient T cells. Although T-bet is induced in both WT and knockout cells under T_H1 conditions, only ~ 3.4 % were co-positive for both Egr2 and T-bet in WT. T-bet expression under T_H2 conditions was ~ 45.9% in WT and ~ 39.6% in Egr2 and 3 knockout cells of which ~ 32.9% were positive for both in WT. T-bet analysis in a similar fashion in T cells cultured under T_H17 conditions was ~ 13.8% in WT and ~ 20.7% in Egr2 and 3 knockout cells of which ~ 8% being co-positive for both Egr2 and T-bet in WT.

The percentage of T-bet and Egr2 co-positive cells is considerably low in T_H1 cells compared to T_H2 and T_H17, which poses an interesting question. IFNγ expression was highly induced in Egr2 and 3 deficient T_H1 cells, illustrating an important role for Egr2 and 3 to control IFNγ expression. T-bet, an important inducer of IFNγ in T cells has unaltered expression in Egr2 and 3 knockout cells, but its co-expression with Egr2 in $CD4^+$ T_H1 cells is considerably low. Therefore in T_H1 cells, only cells with very low or negligible Egr2 expression are able to induce IFNγ expression. And in contrast, IFNγ expression is low or negligible in WT T_H2 and T_H17 (figure 3.25) cells with a higher proportion of T-bet and Egr2 co-positive cells, particularly in T_H2 cells. This illustrates a possible role of Egr2 in antagonizing T-bet function since the percentage of T-bet and Egr2 co-expressing cells are more prominent in T_H2 and T_H17 lineages.

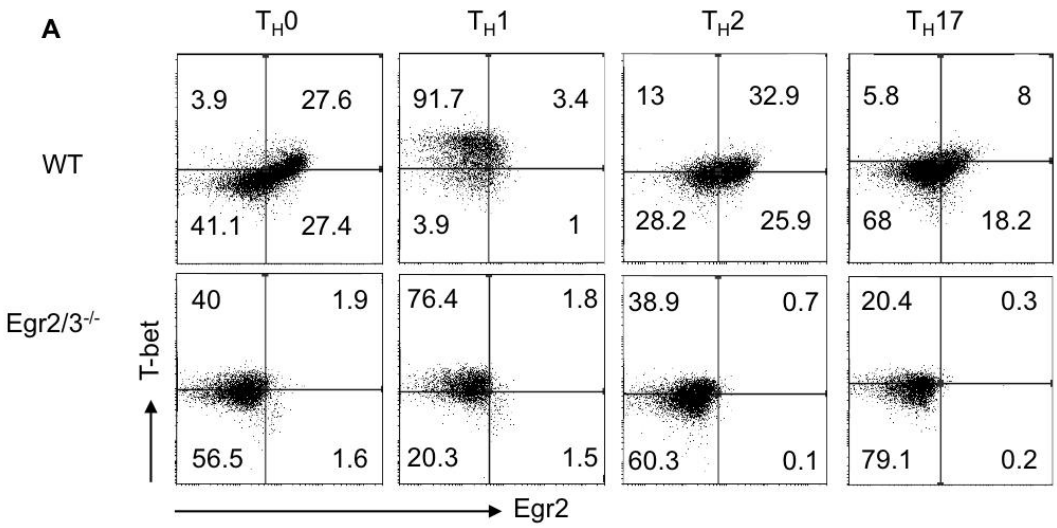

Figure 3.26: Expression of Tbet in T_H1, T_H2 and T_H17

Naïve $CD4^+CD62L^+CD44^{Low}$ cells cultured for 5 days under T_H1, T_H2 and T_H17 conditions followed by nuclear staining for T-bet and Egr2 analysed by flow cytometry. T cells co-expressing T-bet and Egr2 are represented in the top right quadrant of flow cytometry data. The data are from pooled cells of four mice in each group and represent three independent experiments with similar results.

Due to an alarming increase in IFNγ production by Egr2 and 3 deficient T_H1 cells, it seemed reasonable to assume the expression of T-bet would be enhanced in the absence of Egr2 and 3. But analysis of T-bet expression in the T helper lineages presented in figures 3.26, revealed that Egr2 and 3 do not appear have any influence on T-bet expression in effector T cells. We therefore decided to further investigate T-bet expression in T cells in both *in vitro* and *in vivo* conditions.

3.11.3 Egr2 and 3 do not regulate T-bet expression in T cells during viral responses

To further delineate the expression profile of T-bet in Egr2 and 3 deficient T cells, the expression was analysed *in vitro* and *in vivo* conditions. To assess the role of Egr2 and 3 in IFNγ production, naïve CD4 T cells were stimulated *in vitro* and the expression of T-bet and IFNγ was analysed. We found that Egr2 was co-expressed with T-bet in response to TCR stimulation *in vitro*. However, a single defect in Egr2 or Egr3 as well as Egr2 and 3 double deficiency did not alter the expression of T-bet. Although T-bet expression was not significantly altered in Egr2 and 3 deficient T cells, the proportion of IFNγ producing T cells in Egr2 and 3 deficient T cells was profoundly increased. To further assess the expression of Egr2 and 3 versus T-bet in effector T cells in response to viral infection, WT and CD2-Egr2/3$^{-/-}$ mice were infected intranasally with OVA-VV$_{WR}$ virus. Seven days after infection, the expression of T-bet and IFNγ production in T cells was analysed. Co-expression of Egr2 and T-bet was detected in both CD4^{+} and CD8^{+} T cells (Figure 3.27B). Consistently, deficiency of Egr2 and 3 did not reduce the expression of T-bet in T cells in response to viral infection (Figure 3.27C). Taken together, these data demonstrate that Egr2 and 3 are not involved in the regulation of T-bet expression but control the production of IFNγ by effector T cells. Since Egr2 and 3 do not appear to be regulating T-bet expression, there is a possibility that they may regulate T-bet function at the protein level, which is a notion we felt required further investigation.

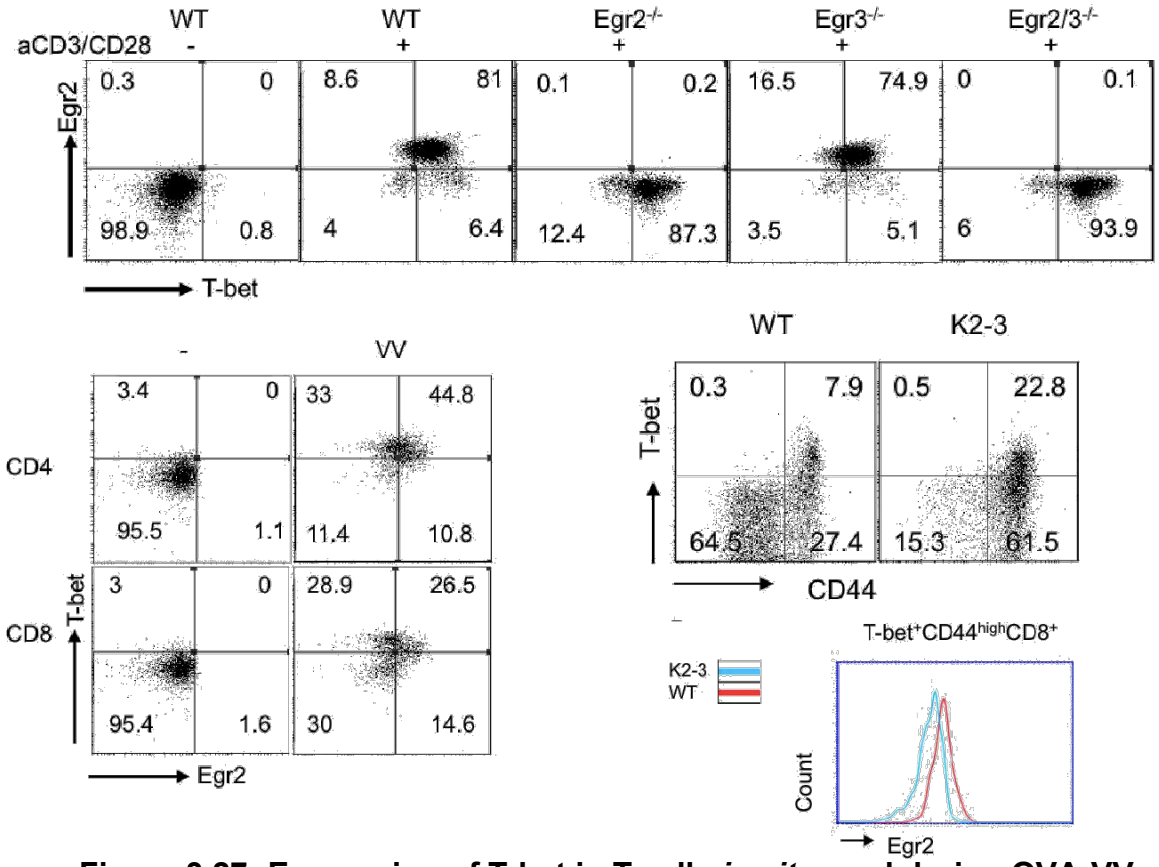

Figure 3.27: Expression of T-bet in T cells *in vitro* and during OVA-VV infection

Egr2 is co-expressed with T-bet in effector T cells and, together with Egr3, controls the production of IFNγ. Expression of T-bet and Egr2 in $CD4^+$ T cells from wild type (WT), CD2-specific Egr2 deficient ($Egr2^{-/-}$), Egr3 deficient ($Egr3^{-/-}$) and CD2-specific Egr2 and 3 deficient ($Egr2/3^{-/-}$) mice after stimulation with anti-CD3 and anti-CD28 for 16 hours (**A**). Wild type and CD2-$Egr2/3^{-/-}$ mice were infected with OVA-VV_{WR} intranasally for 7 days. T-bet and Egr2 expressing $CD4^+$ and $CD8^+$ T cells in wild type mice before and after infection (**B**). T-bet and CD44 expression in $CD8^+$ T cells from viral infected WT and CD2-$Egr2/3^{-/-}$ mice. Egr2 expression was analysed on gated T-$bet^+CD44^{high}CD8^+$ cells (**C**). The data in figure A is representative of three independent experiments. Data in figures B and C are from cells pooled from five mice in each group and are representative of three experiments. The data are from pooled cells of four mice in each group and represent three independent experiments with similar results.

Chapter 4: Discussion

4.1 Summary of findings

T-bet is a member of the Tbox family of transcription factors, that has an important function in the differentiation of CD4 T_H1 and cytotoxic CD8 effector cells, in response to viral infection, and in regulating the expression of IFNγ. A T_H1 mediated response is elicited in response to intracellular microorganisms, where the production of IFNγ mediates in activation of macrophages among other functions. T-bet does not only induces IFNγ expression and T_H1 differentiation, but also coordinates the T_H1 migratory programme. However, T_H1 mediated immune response must be stringently regulated in order to prevent the development of autoimmune disease. Therefore, it is not a surprise that the elements involved in the regulation of T-bet have been extensively studied since its initial discovery. Research has found that the function of T-bet is regulated by co-operators and repressors such as Runx2 and Runx3 and GATA3 and Bcl6 respectively, established on the plasticity of effector T cells induced by signals from the microenvironment during infection. It is however unknown whether T-bet mediated differentiation of effector T cells is controlled in adaptive immune responses. Findings in this thesis present the discovery of a novel mechanism where the transcription factors Egr2 and 3 have a direct repressive effect on T-bet function in both CD4 and CD8 effector T cells. This regulation of T-bet function is important to control immunopathology or effector T cells and plasticity of T_H2 and T_H17 differentiation. In addition, Egr2 and Egr3 were not required for, but control T cell activation and proliferation.

4.2 CD2-Egr2/3$^{-/-}$ develop systemic autoimmune disease

Egr2 and 3 are zinc finger transcription factors belonging to the early growth response gene family of transcription factors, that have been shown to have critical functions in hindbrain development (O'Donovan *et al*., 1999). Egr2 and 3 have been shown to play important roles in T cell function, for instance, our group previously found that Egr2 was expressed in CD44^{high} T cells under homeostatic conditions, and controls their proliferation and activation. The accumulation of CD4^{+}CD44^{high} T cells lead to the development of a late onset lupuslike autoimmune disease in CD2-specific Egr2 deficient (CD2-Egr2$^{-/-}$) mice. This was characterized by the accumulation of IFNγ and IL-17 producing CD4 T cells with a high degree of T cell infiltration in multiple organs. The mice also developed glomerulonephritis and loss of tolerance to nuclear antigens. However, in response to TCR stimulation, CD2-Egr2$^{-/-}$ did not display a hyper-proliferative state, which indicated a potential functional compensatory role attained by Egr3 (Zhu *et al*., 2008). At 3 months, CD2-Egr2$^{-/-}$ mice had a normal population of CD44^{high}CD62L^{low} cells, characteristic of effector phenotype T cells, which increased at 8 months compared to age-matched WT mice. At 15 months, all CD4^{+} T cells from CD2-Egr2$^{-/-}$ mice were CD44^{high}CD62L^{low}. Systemic autoimmunity developed in CD2-Egr2$^{-/-}$mice in later life, whereas Egr3$^{-/-}$ mice displayed no autoimmune symptoms. Therefore, in order to study the function of the transcription factors Egr2 and 3, conditional CD2-Egr2/3$^{-/-}$ mice were developed (Li *et al*., 2012).

4.3 Egr2 and Egr3 are essential to control IFNγ in effector T cells

CD2-Egr2/3$^{-/-}$ mice developed severe systemic autoimmune disease at 2 months of age with lymphocytic infiltration in multiple organs, such as the lungs, kidneys, spleen and lymph nodes. At 8 months, CD2-Egr2/3$^{-/-}$ became moribund with high levels of serum inflammatory cytokines, such as, IL-6, IL-17A, granulocyte macrophage colony stimulating factor (GM-CSF) and IFNγ (Li *et al*., 2012). CD2-Egr2/3$^{-/-}$ mice displayed increased production of inflammatory cytokines with hyper-activated phenotypes with impaired proliferation and IL-2 production *in vitro*. Upon analysis of IFNγ expression in response to antigen stimulation *in vitro,* we discovered that mice as young a

4 weeks old, displayed high levels of IFNγ production, before the onset of autoimmune disease. This indicated a crucial role for Egr2 and Egr3 in the control of IFNγ expression in T cells during adaptive responses. With this in mind, we set out to establish the mechanism by which the expression of IFNγ is regulated by Egr2 and 3 in T cells.

Previously our group discovered hyperactivation of STAT1 and STAT3 in CD2-Egr2/3$^{-/-}$ T cells. STAT proteins are well established as having essential roles in conveying cytokine-mediated signalling, particularly in CD4 T helper cell differentiation (Schindler *et al*., 2007). IFNγ signals through the Jak-STAT pathway and upon ligand binding STAT1 is activated. Song and Shuai, (1998) showed SOCS-1 exhibited inhibitory activity towards STAT1 and thus blocking IFNγ mediated antiviral and anti-proliferative activity. STAT3 signalling is important for T_H17 differentiation and cytokines that are important in inducing IL-17 include, IL-6, IL-21 and IL-23, which activate STAT3 (Korn *et al*., 2009). The function of STAT3 is not primarily associated with T_H17 differentiation, but STAT3$^{-/-}$ mice display detrimental IL-17 expression with a decreased severity of autoimmune disease. Suppressor of cytokine signalling (SOCS) 3 is essential for the control of STAT3 activation. Chen and colleagues (2006) showed that SOCS-3 is a major regulator of IL-23 mediated STAT3 phosphorylation and T_H17 differentiation. In CD2-Egr2/3$^{-/-}$ mice, expression of SOCS-1 and SOCS-3 was impaired following anti-CD3 and anti-CD28 stimulation *in vitro*. Subsequent experiments revealed a direct function of Egr2 and 3 in controlling SOCS-1 and SOCS-3 expression. Regulation of these two molecules seem vital in controlling IL-17A and IFNγ expression, but more stringent mechanism may be at work involving Egr2 and 3 in regulating these cytokines. Recently our group discovered that Egr2 interacts with Batf, a transcription factor that regulates IL-17A expression and T_H17 differentiation. Egr2 interaction with Batf and renders it unable to bind to DNA sequences derived from the IL-17A promoter (Miao *et al*., 2013, Schraml *et al*., 2009). The aim of this project was to study the mechanism by which Egr2 and 3 regulated IFNγ expression in T cell during adaptive immune responses.

Egr2 and 3 deficient T cells produced excessive IFNγ in response to anti-CD3 and anti-CD28 stimulation *in vitro*, indicating Egr2 and 3 have an important function in regulating IFNγ regulation in T cells. We examined IFNγ and TNFα expression in CD8 cells from CD2-Egr2/3$^{-/-}$ mice 7 days post infection with OVA-VV_{WR}. Analysis of antigen specific T cells, with the aid of SIINFEKL tetramers demonstrated an increased production of IFNγ and TNFα by antigen specific T cells. Differentiation of CD4 and CD8 T cells into effector cells such as T_H1 and CTL, is characterized by their ability to produce IFNγ and TNFα (Zhu *et al*., 2010, Kaech and Cui, 2012). Egr2 and 3 deficient T cells displayed a hyper differentiated state during antiviral responses with increased production of IFNγ and TNFα in antigen specific T cells, compared to age matched WT. Although this was the case, Egr2 and 3 deficient T cells had impaired proliferation in response to antigen stimulation *in vitro* and in response to OVA-VV_{WR} infection. As previously described, our group discovered an inhibiting effect of Egr2 on Baft, which is required for the differentiation of T_H17 cells. In fact, recently we have found that Egr2 and 3 are essential for the clonal expansion of antigen specific T cells during viral responses (Miao *et* al., 2016 – see paper in appendix). Our findings are supported by a group that recently, discovered that Batf is important for sustaining clonal expansion by preventing cell death of proliferating T cells (Kurachi *et al*., 2014).

Both Egr2 and 3, and T-bet are induced by antigen stimulation, but Egr2 and 3 expression is inhibited by effector cytokines, particularly IL12 and IFNγ, while T-bet is induced and promoted by cytokines. The differential expression in response to antigen and effector cytokines of Egr2, potentially limits T-bet mediated differentiation at early stages of T cell response to antigens. This may be essential for expansion of antigen specific T cells, as indicated by our findings that Egr2 and 3 deficient T cells are impaired in proliferative responses to TCR stimulation. T-bet is important for differentiation of effector T cells (Szabo *et al*., 2000). Therefore, inhibition of Egr2 and Egr3 expression by effector cytokines provides a feedback mechanism to release suppression of T-bet. Recently, we discovered that cytokine mediated suppression of Egr2 and

Egr3 expression is essential for coupling clonal expansion and differentiation (Miao *et al*., 2016)

4.4 Egr2 and 3 controls CD4 T_H1 differentiation

Egr2 and 3 deficient T cells displayed a hyper differentiated state, with excessive production of IFNγ by CD4 cells *in vitro* and IFNγ and TNFα by antigen specific CD8 T cells. Based on this phenotype we decided to investigate CD4 T helper differentiation *in vitro*. Both T_H1 and T_H17 cells exhibited a hyper differentiated state with high levels of IFNγ and IL-17A expression, respectively. Interestingly, the expression of IL-4 under T_H2 conditions remained similar to its wild type counterpart. Unexpectedly, a completely separate group of Egr2 and 3 deficient cells under T_H2 and T_H17 conditions also became IFNγ producing cells. These IFNγ producing cells under T_H2 and T_H17 conditions were not co-expressing IL-4 and IL-17A therefore indicating a role for Egr2 and 3 in controlling IFNγ expression in T cells but not for skewing the T helper lineages towards a T_H1 biased. However, in each of these conditions, only a proportion of cells are differentiated into T_H2 or T_H17 cells based on the production of IL-4 and IL17A, respectively. We have now demonstrated that Egr2 and 3 do not skew T helper lineages T_H2 or T_H17 towards a T_H1 phenotype, nor induce IFNγ production in T_H2 and T_H17 cells, but inhibit undifferentiated cells to produce high levels of IFNγ.

Along with cytokine expression under these T helper conditions, the expression of T-bet was also analysed with some interesting results. In WT cells, T-bet was co-expressed with Egr2 under all T helper conditions, however, under T_H1 conditions the proportion Egr2 positive cells along with Egr2 and T-bet co-expressing cells was considerably lower compared to the other lineages. The reduced Egr2 expression suggested that during T_H1 differentiation, the expression of Egr2 is reduced in order for activated CD4 T cells to differentiate into T_H1 effector cells during adaptive immune responses. This data suggests a potential role for Egr2 and 3 in controlling T_H1 differentiation by regulating IFNγ expression. Although we do not know if Egr2 and 3 play synergetic function with GATA3 and RORγt in T_H2 and T_H17 differentiations, minimized IFNγ production

in T_H2 and T_H17 cells differentiated from Egr2 and 3 deficient CD4 T cells indicate that inactivation of IFNγ locus in T_H2 or T_H17 plasticity is not regulated by Egr2 and 3.

It has been reported that T_H2 and T_H17 cells have remodelled IFNγ locus at T-bet regulatory sites, which prevent access of T-bet interaction (Balasubramani *et al*., 2010). Egr2 and 3 deficiency did not result in acquisition of T_H1 effector function by T_H2 and T_H17 cells demonstrating that T-bet in Egr2 and 3 deficient T_H2 and T_H17 cells plays limited effects on activation of IFNγ locus, suggesting in the absence of Egr2 and 3, the function of T-bet is suppressed by T_H2 or T_H17 plasticity programs such as GATA3 and RORγt (Zhu *et al*., 2006, Diehl *et al*., 2000). We have now demonstrated that Egr2 and 3 prevent undifferentiated T cells under T_H2 and T_H17 conditions to produce IFNγ and differentiate into T_H1 cells, which provides additional control of T_H1 differentiation under non-T_H1 conditions. In contrast to GATA3 and RORγt to control T-bet mediated T_H1 like T_H2 and T_H17 differentiation, respectively, Egr2 and 3 are not directly involved in the plasticity of T_H2 and T_H17 differentiation, but suppress T_H1 differentiation or T-bet mediated IFNγ production unconditionally. Egr2 and 3 expression is significantly low in T_H1 WT cells in comparison to T_H2 and T_H17 cells, suggesting that the feedback inhibition of Egr2 and 3 expression by cytokines mediating T_H1 differentiation is important for optimal T_H1 differentiation, which is essential for anti-viral responses. Importantly, although low expression levels of Egr2 and 3 benefits T_H1 differentiation, excessive T_H1 development of Egr2 and 3 deficient CD4 T cells under T_H1 condition demonstrates that Egr2 and 3 are essential to control T_H1 response for limiting immunopathology.

4.5 Egr2 and 3 are required to regulate IFNγ expression by inhibiting T-bet function

Multiple transcription factors have been identified that regulate the T_H1 lineage and IFNγ expression. Regulatory DNA elements, which tend to be conserved among species, have been identified in the *Ifng* locus. Nine conserved non-coding sequences (CNS) that contribute to regulatory functions have been identified thus far (Balasubramani *et al*., 2010). These CNS regions are situated

both up and downstream of the *Ifng* gene. Hatton and colleagues (Hatton *et al*., 2006) identified CNS-22, located 22 kb upstream of the transcriptional start site on the *Ifng* gene. They found that this region contained clustered consensus binding sequences of transcription factors essential for T helper differentiation, such as; T-bet, STAT4, STAT1, GATA3 and NF-κB. They also found CNS-22 to be accessible and associated with histone modifications in both T_H1 and T_H2 cells. Deletion of CNS-22 in the context of an *Ifng* reporter transgene abolished TCR dependent and independent *Ifng* expression in T_H1 effector cells. Based on the findings from our T helper experiment, we were inclined to investigate the effect of Egr2 and 3 on T-bet mediated expression of IFNγ. We devised a luciferase reporter gene into which the *Ifng* promoter and CNS-22, both of which contain T-bet binding sites, was cloned to investigate the influence of Egr2 and 3 on T-bet mediated IFNγ expression. We found, although Egr2 and 3 did not affect T-bet expression, they appeared to affect T-bet function by inhibiting T-bet mediated IFNγ expression. These results demonstrate a novel function of Egr2 and 3 to regulate T-bet function in T cells to regulate IFNγ and potentially influence T_H1 differentiation during viral infection. T-bet's function is not limited to the expression of IFNγ, it is also a master regulator of the T_H1 differentiation programme along with important function in mediating CD8 differentiation into effector cytotoxic T cells during viral infection (Szabo *et al*., 2000, Intlekofer *et al*., 2005). Although T-bet mediated differentiation of effector cells is essential for immune responses to infection, its function is balanced to limit immunopathology of effector T cells and to allow the development of memory T cells (Lazarevic and Glimcher, 2011).

A number of mechanisms that regulate the function of T-bet for differentiation of effector T cells have been discovered such as Id3 and Tcf-1 that counteract the effector differentiation of CD8 T cells. Yang and colleagues found Id2 or Id3 deficiency resulted in loss of distinct CD8 effector and memory populations (Yang *et al*., 2011). Jeannet and colleagues similarly demonstrated the importance of T cell factor 1 (Tcf-1) in establishing CD8 T cell memory (Jeannet *et al*., 2010). Xin and colleagues in addition demonstrated an overlapping pathway of effector differentiation of CD8 cells, directed by Blimp1 and T-bet

(Xin *et al*., 2016). In T helper differentiation, T-bet function is repressed in T_{fh}, T_H2 and T_H17 cells by Bcl6, GATA3 and RORαt mediated programs, respectively (Zhu and Paul, 2010), while Runx2 and Runx3 are co-factors that promote T-bet mediated IFNγ production in CD4 T cells (Djuretic *et al*., 2007, Wang *et al*., 2014). These counter regulatory mechanisms drive lineage plasticity under specific differentiation conditions. However, it is unknown whether there is general repressive mechanism for the control of T-bet mediated effector T cell differentiation. We have now discovered a direct repressive function of Egr2 and 3 for inhibition of T-bet function in both CD4 and CD8 effector T cells. This repressive function is essential for the control of immunopathology of effector T cells and plasticity of T_H2 and T_H17 differentiation. The regulation of such repressive function resulted from differentiation expression of Egr2 and 3, and T-bet in response to antigen and cytokine stimulation.

4.6 Mechanism of Egr2 and 3 in inhibition of T-bet function

In adaptive immune responses, activated T cells undergo clonal expansion and differentiation into effector cells (Kaech and Ahmed, 2001). The mechanisms that regulate differentiation of naïve T cells into effector cells involve a combination of TCR signal, balance of co-stimulatory and co-inhibitory and innate signals from the environment but more prominently the pattern of cytokine production determines the lineage commitment. The production of T helper lineage specific cytokines is regulated by master regulators of each specific lineage, for instance T-bet for T_H1, GATA3 for T_H2, RORγt for T_H17 and Bcl6 for T_{fh} (Zhu, Yamane and Paul, 2010). T-bet was discovered initially to be a master regulator of the T_H1 differentiation programme with the ability to inhibit the differentiation other T helper lineages, such as T_H2 and T_H17, but it is also important for the differentiation of cytotoxic CD8 cells (Intlekofer *et al*., 2005). The expression of T-bet is induced in response to antigen and effector cytokines such as IFNγ (Lighvani *et al*., 2001). T_H1 is the primary source of IFNγ in the immune system and T-bet directly induces its expression (Szabo *et al*., 2000, Balasubramani *et al*., 2010). An increase in T-bet expression in Egr2 and 3 deficient T cells was initially anticipated to correlate with the increase in

IFNγ, however, analysis of T-bet expression in Egr2$^{-/-}$, Egr3$^{-/-}$ and Egr2/3$^{-/-}$ T cells after antigen stimulation *in vitro* displayed no change when compared with WT.

Higher expression of T-bet has been reported in autoimmune diseases such as, Crohn's disease and chronic intestinal inflammation (Neurath *et al*., 2002, Matsuoka *et al*., 2004). Polymorphisms in *Tbx21* (gene encoding T-bet) have also been linked in humans to a greater risk of developing type I diabetes. Sasaki and colleagues found a high frequency of His33Gln substitution within the coding region of T-bet in patients with type I diabetes, where Gln33 T-bet displayed an enhanced transcriptional activity of the IFNγ gene (Sasaki *et al*., 2004). This is apparent in T-bet deficient nonobese diabetic mice (NOD) who are fully protected from type I diabetes (Esensten *et al*., 2009). In addition to inducing the T_H1 developmental program, T-bet also inhibits the differentiation of T_H2 cells by directly binding to and inhibiting GATA3 activity (Hwang *et al*., 2005). Indeed mice with a targeted deletion of the T-bet gene developed a pathological state in the lungs characteristic of patients with chronic asthma, which is a condition largely associated with infiltration by T_H2 lymphocytes (Finotto *et al*., 2002). Lazarevic and colleagues (2010) demonstrated a suppressive effect of T-bet on T_H17 lineage development, where T-bet was found to interact with the transcription factor Runx1 and inhibit Runx1 mediated transcription of *Rorc*. In light of these findings, T-bet deficient mice have displayed a higher frequency of T_H17 cells in several disease models.

In OVA-induced allergic lung inflammation model, T-bet$^{-/-}$ mice showed increased inflammation and infiltration of neutrophils and eosinophils compared to control mice. Interestingly, an increased level of IL-17 was detected instead of T_H2 cytokines, indicating IL-17 as a key mediator of airway inflammation in the absence of T-bet (Durrant *et al*., 2009). Therefore, T-bet's role is extended in not only in inhibiting T_H2 differentiation but also controlling T_H17 differentiation in the lungs. In proteoglycan-induced arthritis (PGIA) mouse model, which is an IL-17A independent model, T-bet$^{-/-}$ mice produced reduced levels of IFNγ but high concentrations of IL-17 (Doodes *et al*., 2010). In collagen induced arthritis

(CIA) mouse model, IFNγ-receptor deficient mice displayed an accelerated onset of disease and more severe CIA compared to control mice (Manoury-Schwartz *et al*., 1997). Taken together, these results indicate an immunomodulatory effect exerted by IFNγ and T-bet on the development of arthritis by limiting the degree of T_H17 responses.

In patients with relapsing Multiple Sclerosis (MS), the expression of T-bet was analysed in $CD4^+$ and $CD8^+$ T cells from peripheral blood and found to be up-regulated compared with healthy subjects and patients in remission (Frisullo *et al*., 2006). A study by Panitch and colleagues (1987), found that treatment of patients with MS with recombinant-IFNγ had substantial increase in pathological symptoms and relapses. In contrast, treatment with monoclonal antibodies to IFNγ for patients with MS, showed clinical benefits (Skurkovich *et al*., 2001). T-bet deficiency in EAE mice showed resistance to the development of EAE (Nath *et al*., 2006). T_H17 plasticity have shown to give rise to IL-17A and IFNγ double producing cells that have been implicated in the development of autoimmune disease, such as EAE. Wang and colleagues (2014) found that the development of IL-$17A^+IFN\gamma^+$ cells did not depend on the co-expression of RORγt and T-bet in T_H17 cells, rather the expression of T-bet and Runx1 and Runx3 proteins. T-bet interacts with Runx1 and Runx3 for maximal induction of IFNγ (Table 4.1), and IL-12 mediated induction of T-bet in T_H17 is required to induce epigenetics changes in the IFNγ locus, which are normally silenced in T_H17 cells and thus increasing Runx1 binding to these regions. Therefore, T-bet expression in $CD4^+$ T cells is essential in delicately balancing T_H1, T_H2 and T_H17 responses and control of IFNγ production as a contributing factor to the pathogenesis of autoimmune disease. Therefore, T-bet has to be delicately regulated in T cells in order to optimize immune response and prevent autoimmune disease. Although we did not find any evidence of Egr2 and 3 directly regulating T-bet expression, we sought to discover the functional significance of T-bet in the absence of Egr2 and 3.

To characterize the mechanism by which Egr2 and 3 regulate T-bet function, we conducted co-immunoprecipitation experiments. First, we demonstrated that Egr2 interacted with T-bet in T cells. The interaction of Egr3 could not be demonstrated in a similar fashion in T cells since an Egr3 specific antibody is yet to be developed, therefore T-bet and Egr3 interaction was shown in *in vitro* studies. To further illustrate how this interaction may contribute to loss of T-bet function with regards to IFNγ expression, we devised an EMSA experiment, where we first showed that in the presence of Egr2, T-bet was unable to bind to its target consensus sequence on the *Ifng* promoter. Mutational studies were conducted to elucidate the precise region/s that was important in this interaction. Interestingly, we found that Egr2 and 3 directly interacted with the Tbox DNA binding domain of T-bet, which demonstrated a mechanism of how Egr2 and 3 elicit a dysfunctional state in T-bet in binding to target DNA sequences. Further more, to investigate whether this interaction renders both transcription factors inactive, we found that Egr2 did not require its zinc finder DNA binding domain for this interaction and T-bet had no effect on Egr2 binding to its DNA consensus binding sequence. These findings present a novel mechanism by which T-bet function is regulated in T cells during adaptive immune responses by Egr2 and 3. Figure 4.2 and 4.3 illustrates the function of Egr2 and Egr3 in regulating SOCS1 and SOCS3 expression and Batf function based on our previous findings (Li *et al*., 2012) and the regulation of T-bet function based on the findings in this thesis.

T-bet is directly regulated at protein level by different mechanisms for enhancing or repressing its function (see table 4.1). These regulations are either promoting or suppressing T_H1 differentiation during specific T helper conditions. However, inhibition of T-bet function by Egr2 and 3 is less conditional. Therefore, regulation of T-bet mediated function is based on the regulation of Egr2 and Egr3 expression. Egr2 and Egr3 transcription factors do not express in naïve T cells (Miao *et* al., 2012, Li *et al*., 2012). It is known that T-bet is induced and enhanced by both antigen stimulation and effector cytokines to sustain its expression through adaptive immune responses

(Afkarian *et al*., 2002, Schulz *et al*., 2009). The regulation of Egr2 and 3 mediated suppression of T-bet is dependent on the regulation of Egr2 and 3 expression, reciprocally (Figure 4.1), by antigen and effector cytokines, which provide a balanced mechanism for controlling the levels of effector differentiation of CD8 and T_H1 T cells. We found that Egr2 and 3 deficient T cells are hyperactive and inflammatory in homeostatic conditions and produce high levels of IFNγ, indicating that the repressive function of Egr2 and 3 for T-bet function may also be key factor for the development of autoimmune diseases.

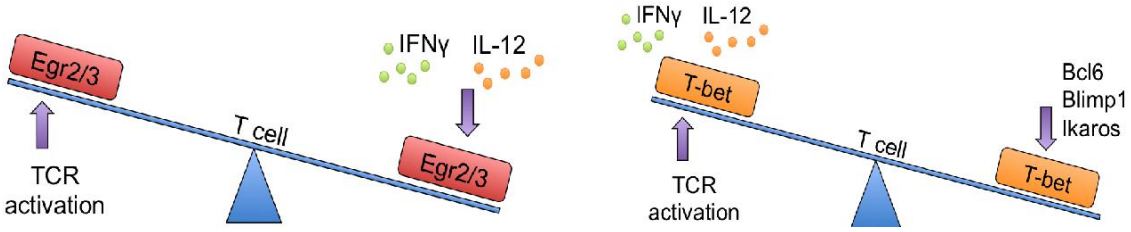

Figure 4.1: Diagrammatic representation of the reciprocal regulation of Egr2 and 3 expression by antigen and effector cytokines IL-12 and IFNγ (**A**) and the induction of T-bet by antigen stimulation and subsequent maintenance of expression by IFNγ and IL-12 and suppression of T-bet expression by Ikaros, Bcl6 and Blimp1 during T helper differentiation (**B**) (Thomas *et al*., 2009, Yu *et al*., 2009, Cimmino *et al*., 2008).

4.7 Conclusions

Although Egr2 and Egr3 were initially found to suppress T cell activation and production of IL-2, the overlapping function of Egr2 and 3 is to promote antigen mediated proliferation of naïve T cells, but repress activation state and production of effector cytokines. This is achieved by increasing AP-1 activation while suppressing activation of STAT1 and STAT3. (Li *et al*., 2012). In fact, recently we have found that Egr2 and 3 regulated the clonal expansion of antigen-specific T cells during viral responses (Miao *et al*., 2016- see appendix for paper). Recent findings also showed that Egr2 directly induces expression of T-bet in CD8 T cells in response to viral infection and in the absence of Egr2, CD8 T cells failed to be activated, and unable to proliferate and produce IFNγ

(Du *et al*., 2014). We could not explain the reasons for the conflicting results, but the expression of T-bet in Egr2 and 3 single deficient T cells as well as Egr2 and 3 double deficient T cells clearly demonstrate that Egr2 and 3 are not required for T-bet expression. Interestingly, in a similar Egr2 deficient model, the activation and response of T cells are normal in response to infection (Ramón *et al*., 2010), indicating an additional mechanism rather than Egr2 deficiency resulting in impairment of effector T cell function.

Egr2 and 3 are found only expressed in effector phenotype T cells and defects lead to severe autoimmune disease and excessive production of IFNγ (Li *et al*., 2012). Therefore, it is possible that T-bet is over functioning in effector phenotype T-cells in autoimmune diseases in the absence of Egr2 and 3. Glucocorticoids (GCs) have been used in the treatment of autoimmune disease due to their ability to induce an anti-inflammatory state in cells. For instance, T-bet and pSTAT-1 expression showed substantial increase in $CD4^+$ and $CD8^+$ T cells from peripheral blood of patients with relapsing MS (Frisullo *et al*., 2006). In a study conducted by Liberman and colleagues (2007) to investigate the effect of GCs on the regulation of T-bet, they found that GC inhibited T-bet transcriptional activity by soluble glucocorticoid receptors directly interacting with T-bet and blocking its DNA binding activity. Indeed, Frisullo and colleagues (2007) showed that treatment of relapsing MS patients with high doses of glucocorticoids reduced pSTAT-1 and T-bet expression and provides short-term beneficial effect on functional recovery. Therefore, targeting T-bet function in MS patients could be therapeutically beneficial. However, this approach is non-selective and targets all cells expressing GC receptors and silencing of T-bet may render patients immune-compromised due to T-bet's expression in a cascade of immune cell types. Therefore, Egr2 and 3 provide a potential target to modulate T-bet function in effector T cells. Previously our group found that Egr2 induction was reduced in T cells from treatment-naïve MS patients (Miao *et al*., 2012). This indicates an impaired expression of Egr2 in activated T cells may contribute to MS development. Our results have thus
far demonstrated that Egr2 and 3 are only induced by antigen stimulation and therefore the expression of Egr2 and 3 in effector phenotype T cells may

indicate that these cells are auto-reactive to self-antigens. Therefore new therapy can consider to modulate Egr2 and 3 expression in auto-reactive T cells to achieve therapeutic efficacy.

Egr2 expression was found to be important for T cell tolerance. Harris and colleagues (2004) found that silencing of Egr2 in T cells deemed them resistance to anergy induction. Safford and colleagues (2005) found that Egr2 and Egr3 were key regulators of T cell activation and over expression of Egr2 and 3 was associated with increased E3 ubiquitin ligase Cbl-b. Exposure of activated T cells to monoclonal αCD3 antibodies (αCD3 mAB) results in functional anergy (Smith *et al*., 1997). *In vivo* studies showed that αCD3 therapy promotes tolerance by depleting pathogenic T cells by inducing apoptosis and preserving T_{Reg} cell populations (Penaranda *et al*., 2011). Treatment of T cells with αCD3 results in the increased production of TGFβ by phagocytes exposed to apoptotic T cells (Perruche *et al*., 2008). TGFβ has immunoregulatory effects, including; inhibition of T cell activation and proliferation, inhibition of dendritic cell maturation and the induction of $FoxP3^+$ T_{Reg} cells (Travis and Sheppard, 2014). Currently there are several humanized αCD3 mABs developed and have undergone clinical trials with positive clinical responses (Kuhn and Weiner, 2016). Egr2 and 3 have been shown to have critical roles in T cell tolerance, therefore a possible mechanism is to induce high levels of Egr2 and 3 in auto-reactive T cells as a potential therapeutic approach to treat autoimmune diseases.

4.8 Future experiments

In this study we have established Egr2 and 3 as potent inhibitors of T-bet function in T cells. In addition to the differentiation of CD8 and T_H1 cells, T-bet has been found to be involved in innate immunity and also the development of immune memory. It would be interesting to further delineate the effect of Egr2 and 3 in these functions. T-bet is also a potential target for the development of anti-inflammatory therapy. To further define the molecular mechanisms for the interaction of Egr2 and/or 3 with T-bet can provide a system for anti-T-bet drug discovery for applications in the pathogenesis of autoimmune diseases. We

would like to further identify T-bet target genes that are regulated through Egr2 and 3, and validate the genes involved in driving the pathogenesis of autoimmune disease.

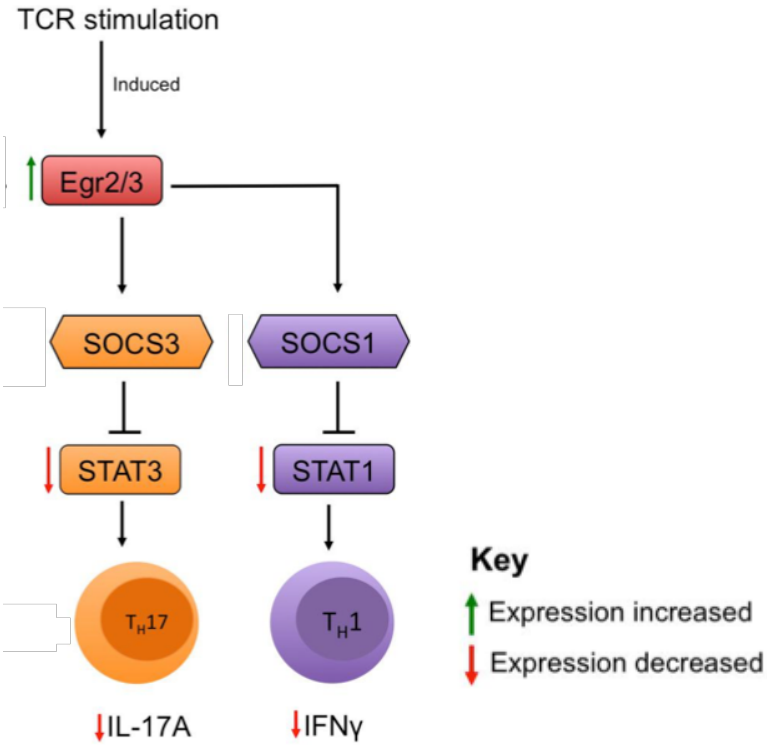

Figure 4.2 Egr2 and Egr3 in regulating SOCS1 and SOCS3 expression in T cells

Egr2 and 3 regulate the expression of SOCS1 and SOCS3, which are suppressors of STAT1 and STAT3, respectively (Li *et al*., 2012).

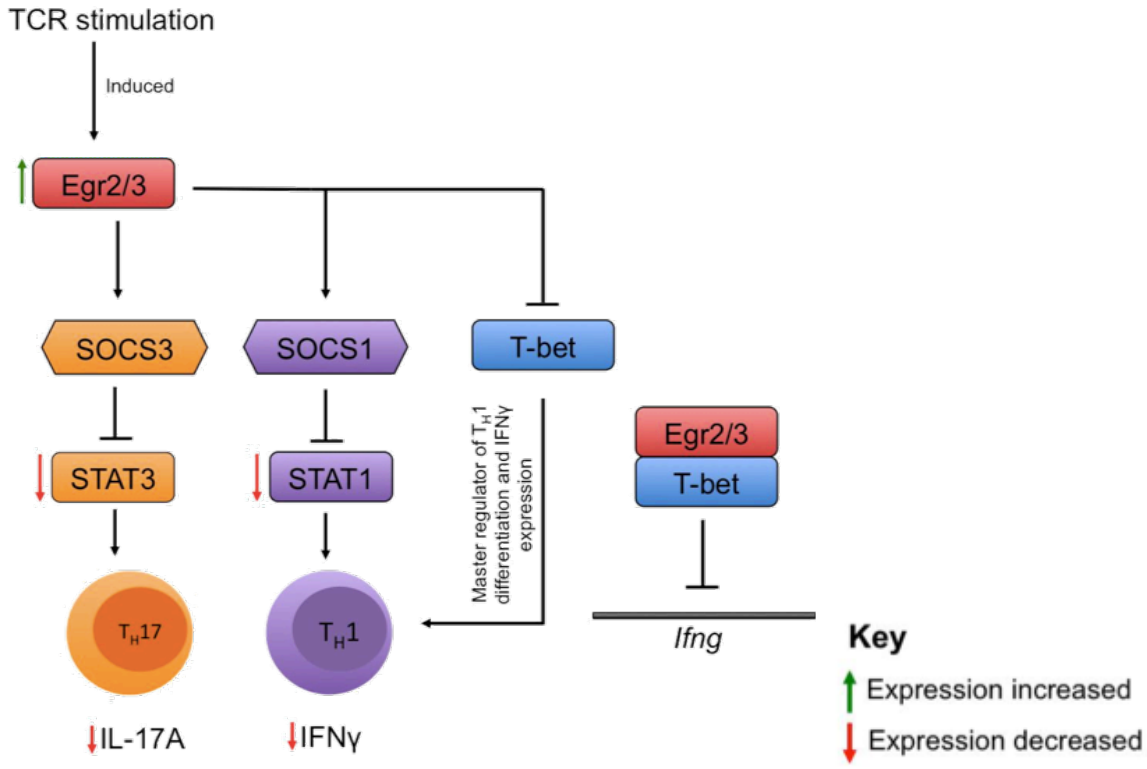

Figure 4.3: Function of Egr2 and Egr3 in regulation of T-bet in activated T cells

The findings of this project illustrate a mechanism by which Egr2 and Egr3 regulate T-bet activity by blocking the Tbox DNA binding domain and thus preventing T-bet binding to target sequence on the *Ifng* gene. T-bet function is also essential for modulating the T_H1 differentiation programme.

Protein	Phenotype	IFNγ High/Low	Relationship with Tbet	Reference
Ikaros	Short hairpin (sh) RNA mediated knockdown of Ikaros in T_H2 resulted in increased T-bet and IFNγ expression	High	Ikaros directly targets *tbx21* promoter to suppress T-bet expression	Thomas *et al*., 2009
Bcl6	$Bcl6^{-/-}$ mice displayed increase IFNγ and IL-17A expression one week after immunization with SRBCs (Sheep red blood cells)	High	Bcl6 directly binds to T-bet to suppress its expression to inhibit IFNγ production	Yu *et al*., 2009
Blimp1	Conditional T cell deletion of Blimp1 causes fatal colitis in mice	High	Blimp1 binds at multiple regulatory regions in *tbx21*and *ifng* genes to repress expression.	Cimmino *et al*., 2008
STAT1	$STAT1^{-/-}$ T cells have reduced T-bet expression levels.	50% reduction in IFNγ production compared to WT	Induction of T-bet is dependent on IFNγ-STAT1 signaling.	Afkarian *et al*., 2002
STAT5	STAT6 and IL4Rα deficient T cells produce STAT5 mediated IL-4 production without increase in GATA3 expression.	Decreased IFNγ in T cells primed under T_H1 conditions	T-bet expression was significantly inhibited in cells primed under T_H1 conditions and a retroviral transfection with a constitutively active form of STAT5A mutant.	Zhu *et al*., 2003
T cell factor-1 (TCF-1)	TCF-1 deficient mice have low T_H2 cytokine IL-4 in response to OVA-allergen (Asthma)	High IFNγ in activated CD4 T cells	TCF-1 is induced in activated T cells and directly induces GATA-3	Yu *et al*., 2009
RORγt	Cells treated with IL-12 experience STAT4 inactivation and suppression of T-bet expression	Low IFNγ expression in RORγt high T cells	No mechanism established, but a direct correlation with high RORγt expression results in reduced T-bet expression	Mukasa *et al*., 2010
Runx1	Runx1 deficient T cells have inhibition of $IL\text{-}17A^{+}$ $IFN\gamma^{+}$ T_H17 during EAE	IFNγ expression low	T-bet and Runx1 cooperate to induce IFNγ producing T_H17 cells and contribute to autoimmune disease.	Wang *et al*., 2014
Runx3	$Runx3^{-/-}$ T cells fail to produce adequate levels of IFNγ during T cell activation	IFNγ expression low	During T_H1 differentiation, Runx3 and T-bet co-operate to activate *Ifng* and silence *Il4*	Djuretic *et al.,* 2007
Egr2 and Egr3	A hyper differentiated state of T_H1 cells	High IFNγ in Egr2 and 3 deficient T cells	Egr2 inhibits T-bet function by binding to its DNA binding domain Tbox.	Singh *et al*., 20016

Table 4.1: Regulation of T-bet in T cells

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Conferences

The work from my thesis was presented at the following conferences:

College of Health and Life Sciences conference 2013
Egr2 regulates IFNg expression by suppressing T-bet function in T cells. Singh, R., Miao, T., Symonds, A. J., Ogbe, A., Omodho, B., Li, S., Wang, P.
Poster Presentation – Winner of prize for best poster

William Harvey Day, Queen Mary University of London 2014
Egr2 regulates T-bet mediated IFNg expression in T cells. Singh, R., Miao, T., Symonds, A. J., Omodho, B., Li, S., Wang, P.
Poster Presentation

4th European Congress of Immunology, Vienna, Austria 2015
Egr2 and 3 regulate IFNg expression in activated CD4 T cells by controlling T-bet activity. Singh, R., Miao, T., Symonds, A. J., Omodho, B., Li, S., Wang, P
Poster Presentation

Publications

1. Singh, R., Miao, T., Symonds, A. L., Omodho, B., Li, S., Wang., P. 2016 Egr2 and 3 inhibit T- bet mediated IFNγ production in T cells. ***J Immunol*** (Manuscript currently under revision for re-submission).

2. Miao, T., Symonds, A. L., Singh, R., Symonds, J. D., Ogbe, A., Omodho, B., Zhu, B., Li, S., Wang, P. 2016. Egr2 and 3 control adaptive immune responses by temporally uncoupling clonal expansion from T cell differentiation. ***J Exp Med*** (Manuscript currently under revision for re-submission).

3. Ogbe, A., Miao, T., Symonds, A. L., Omodho, B., Singh, R., Bhullar, P., Li, S., Wang, P. 2015 Early Growth Response gene 2 and 3 regulate the expression of Bcl6 and differentiation of T follicular helper cells. ***J Biol Chem*** 14 (33) 20455-65.