Early growth response gene (Egr) 2 and 3 control inflammatory responses of tolerant T cells

A thesis submitted for the degree of Doctor of Philosophy by

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Declaration

I hereby declare that the work completed in this thesis is my own work unless otherwise stated, and has not been submitted for any other degree.

Becky Omodho

Abstract

This study investigated the role of tolerance induction in an inflammatory setting in regard to the early growth response genes Egr2 and Egr3. T cells robustly respond to pathogenic antigens during infection, but are tolerant to stimulation by self-antigens. The intrinsic mechanisms for self-tolerance in the periphery are still not clear. Egr2 and 3 are induced in tolerant T cells in response to antigen stimulation by NFAT-medicated tolerant signalling; however, their function in tolerant T cells is still unknown.

The study demonstrated that Egr2 and 3, induced in tolerant T cells, are not directly involved in defective proliferation and IL-2 production, the hallmarks of T cell tolerance. However, they are essential for preventing inflammatory response of tolerant T cells. In the absence of Egr2 and 3, tolerant T cells show impaired proliferation and production of IL-2, but produce high levels of IFN-γ, a key inflammatory cytokine. This phenotype resembles CD4 T cells from autoimmune diseases such as lupus which show poor proliferative response, but hyper-inflammation. Our study demonstrated, for the first time, a distinctive mechanism to control inflammation from proliferative tolerance regulated by Egr2 and 3, which may be an important mechanism for the control of autoimmune diseases.

Acknowledgements

Dedication

This is for my parents Rose and Wellington Omodho; thank you for everything you have done for me. Thank you for teaching me that respect is earned, honesty is appreciated, trust is gained, and happiness is a journey.

I am forever grateful for the both of you.

I love you.

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Abbreviations

AIRE	Autoimmune regulator
Ap-1	Activator protein 1
APC	Antigen presenting cell
APDF-1	Apoptosis death factor-1
APECED	Autoimmune polyendocriopathy candidiasis dystrophy syndrome
Blimp-1	B lymphocyte induced maturation protein 1
BSA	Bovine serum albumin
cDNA	copy-deoxyribonucleic acid
cTEC	Cortical thymic epithelial cells
CTLA-4	Cytotoxic T lymphocyte antigen 4
CsA	Cyclosporine A
DAG	Diacylglycerol
DCs	Dendritic cells
DED's	Death-effector domains
DGK-α	Diaglycerol kinase alpha
DP	Double positive
DN	Double negative
DNA	Deoxybibonucleic
DTX-1	Deltex-1
Egr	Early growth response
Egr2-/-Egr3-/-	Egr2 and 3 knockout
ELISA	Enzyme linked immunosorbent assay
EMSA	Electromobility shift assay
Emoes	Eomesodermin
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FBS	Foetal bovine serum
FOXP3	Foxhead box P3
GATA-3-	GATA binding protein 3
GEF's	Guanine nucleotide exchange factors
GMCSF	Granulocyte-macrophage colony-stimulating factor
HLA	Human leukocyte antigen
IL	Interleukin
IF	Interferon
IgSF	Immunoglobulin superfamily
INO	Ionomycin
iTreg	induced T regulatory cell
IPEX	Immunodysregulation, polyendrocrinopathy, enteropathy, x-linked syndrome
LAT	Linker for activation of T cells,
LPS	lipopolysaccharide
mTEC	medullary thymic epithelial cells
MHC I	Major histocompatibility complex class 1
MHC II	Major Histocompatibility complex class 2
NFAT	Nuclear factor of activated T cells
NFkb	Nuclear factor kappa-light-chain-enhancer of activated B cells
PAMPs	Pathogen associated molecular patters
PBS	Phosphate buffered saline
PMA	Phorbol 12-mysristate 1 acetate

PKC	Protein kinase C
PLCγ	Phospholipase Cγ
RASGRP-1	Ras guanyl-releasing protein 1
RAG	Recombination activating gene
RORγt	Receptor related orphan receptor gamma-theta
SEA	Staphylooccal enterotoxin A
STAT1	Activator of transcription 1
STAT-5	Activator of transcription 5
SOS	Son of seven less homologue
SOCE	Store-operated Ca2+-entry
SP	Single positive
SLE	Systemic lupus erythematosus
T-bet	T box transcription factor
TCR	T cell receptor
Th	T helper
TNF	Tumour necrosis factor
TRAs	Tissue restricted antigens
Treg	T regulatory
TSSP	Thymus specific sense protease
VDJ	Variable, Diversity, Joining
Vβ	Variable beta
WT	Wild type

Chapter 1.   Introduction

1.1 Governance of the immune system

The immune system is a group of specialised organs, tissues, cells and processes designed to protect the body against infection. This complex system is divided into two main functional groups: innate immunity, which takes place immediately following antigen encounter, and adaptive immunity which is triggered a number of days later (Delves and Riott, 2000).

1.2 Innate immunity

1.2.1 Cells of innate immunity

Macrophages play a key role as they are not only capable of engulfing and killing bacteria by phagocytosis, but also present the antigen to CD4+ T cells as antigen presenting cells, triggering the adaptive immune response (Aderem and Ulevitch, 2000). Neutrophils are short-lived cells and are only able to survive about 6 hours in circulation before undergoing apoptosis. They are a set of specialised killers that are able to destroy pathogens when recognised. Eosinophils and basophils are involved in the mediation of inflammation and remain responsive to cytokines produced by the adaptive immune system (Beutler, 2004). The innate immune system has adapted to the challenge of the recognition of a vast number of antigens by having receptors that are able to recognise conserved motifs. This is necessary as pathogens are able to mutate. The motifs are referred to as pathogen associated molecular patters (PAMPs). They include bacterial cell wall components such as lipopolysaccharide (LPS), yeast cell wall, and formulated proteins (Aderem and Ulevitch, 2000). These molecules are activators of the innate immune system. LPS is able to trigger the release of cytokines such as tumour necrosis factor (TNF), IL-1 and IL-6, causing symptoms such as hypotension, metabolic acidosis, organ failure and fever. LPS is recognised by toll-like receptors, which are adapters that help trigger an immune response. The humoral arm of the innate immune system includes components such as complement proteins, which are a set of proteolytic enzymes that cause inflammation, lysis of bacteria and phagocytosis, and antimicrobial peptides which are able to kill microbes and the acute phase response (Beutler, 2004).

1.2.2 Cytokines

Cytokine production by the innate immune arm provides an immediate response to inflammation and infection (Iwasaki and Medzhitov, 2010)(McGettrick and O'Neill, 2007)). Cytokines produced include IL-4, IL-10, IL-6, IFN-γ and TNF. Release of these cytokines can be

triggered by complement activation or by pathogens such as bacteria and fungus. For the system to be optimal, the release of cytokines is regulated. Initially cytokines mount allergic or inflammatory reactions which subside after the reaction is complete (Hu and Ivashkiv, 2009). Another set of cytokines is also able to activate T cells and allow for adaptive immune responses (Iwasaki and Medzhitov, 2010). Some of the cytokines released are shown in Table 1

Cytokine	Function	Pro/anti-inflammatory	Reference
TNF	Induces septic shock, recruits neutrophils and monocytes to inflammatory site	Pro-inflammatory	(Griffin *et al.*, 2012)
IL-1	Allows histamine production in mast cells. Increases cell adhesion expression on leucocytes	Pro-inflammatory	(Ben-Sasson *et al.*, 2009; Carmi *et al.*, 2009)
IL-6	Causes acute phase protein release in the liver. Activates cytotoxic T cells and encourages differentiation of B cells	Pro-inflammatory	(Arango Duque and Descoteaux, 2014)
IFN-γ	Secreted by natural killer cells and macrophages to elicit inflammatory response	Pro-inflammatory	(Schroder *et al.*, 2004)
IL-10	Suppresses macrophage activation, hinders production of inflammatory cytokines	Anti-inflammatory	(Fiorentino *et al.*, 1991)
TGF-β	Supresses Th1 and Th2 activity	Anti-inflammatory	(Travis and Sheppard, 2014; Josefowicz, Lu and Rudensky, 2012)

Table 1 Cytokine functions

1.2.2.1 Complement pathway

Complement is a host defence system developed within innate immunity that is triggered by the identification of pathogens via surface receptors (Janeway,Charles,A,Jr *et al.*, 2005). It can be activated in 3 ways, and thus has three specific pathways. Firstly, the classical pathway is activated through the activation of the molecule C1q. Secondly, the lectin pathway is activated through non-self-recognition using pattern-recognition receptors. Finally, the alternative pathway takes place as a result of spontaneous hydrolysis of the molecule C3 (Walport, 2001) (Janeway,Charles,A,Jr *et al.*, 2005).

The three main effects are: priming the immune response by generating anaphylatoxins, the direct lysis of surfaces that have been targeted for destruction and opsonisation, and clearance of the target including the recruitment of cells such as macrophages and neutrophils (Janeway,Charles,A,Jr *et al.*, 2005; Walport, 2001).

1.2.3 Adaptive immunity

The adaptive immune system is primed to be able to deal with invading pathogens in a timely manner. A broad repertoire of T cells must be generated in order to respond to the large number of pathogens (Schartner, Fathman and Seroogy, 2007). The adaptive immune system has evolved to maximise the response to non-self-antigens and reduce response to self-antigens.

1.2.3.1 Cells; general function

Adaptive immunity heavily relies on the ability of B and T lymphocytes to express a wide range of unique surface receptors created via somatic DNA arrangement and random chain pairing (O'Garra and Vieira, 2004). The adaptive immune response has a number of advantages. Firstly, it has a high diversity of antigen receptors meaning it has a high specificity for antigens, thus it is able to target a wide variety of antigens. Secondly, it has the capability of immunological memory due to the production of antibodies by B lymphocytes. Thirdly it allows for clonal expansion and specialisation to deal with district pathogens. In addition, the adaptive immune system has self- and non-self-discrimination functionality in order to avoid autoimmune disease development. The problem of self and non-self-discrimination is tackled by negative and positive selection in the thymus, which will be discussed later (Palm and Medzhitov, 2009).

1.2.3.2 Cell mediated immunity

Cell mediated immunity refers to the functions of T lymphocytes in the immune system; there are two main types, CD4+ and CD8+ T cells. The fate of the T cell during adaptive immunity depends on two major molecules: T cell receptors (TCR) and Major Histocompatibility Complexes (MHC) (O'Garra and Robinson, 2004). For T cells to be activated, these molecules must interact in the correct manner. Maintaining the right amount of these cells is crucial for optimal host defence. This works with the first step of the adaptive immune system to recognise where the infection is coming from and what kind of infection it is (Palm and Medzhitov, 2009).

The consequences of TCR interaction with peptide-MHC ligands depend heavily on the quality of the interaction. TCR engagement is essential for an immune response. The extent to which

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the TCR-ligand pair interacts with the CD4/CD8 co-receptor plays an important role in producing high quality signals (O'Garra and Robinson, 2004).

1.2.3.3 Humoral mediated immunity

Humoral immunity allows for the activation of B cells and thus antibody production. The antibody response that requires antigen specific T cell help are referred to as T cell dependant antigens (Janeway,Charles,A,Jr *et al.*, 2005). The signal for B cell activation can either be T cell dependant or independent according to the pathogen encountered. Activation of B cells leads to clonal proliferation to produce plasma cells and memory cells. The plasma cells then produce antibodies that circulate in the body while the memory cells remain creating immunological memory in case of re-encounter with the pathogen (Janeway,Charles,A,Jr *et al.*, 2005).

Figure 1: Innate and Adaptive Immunity
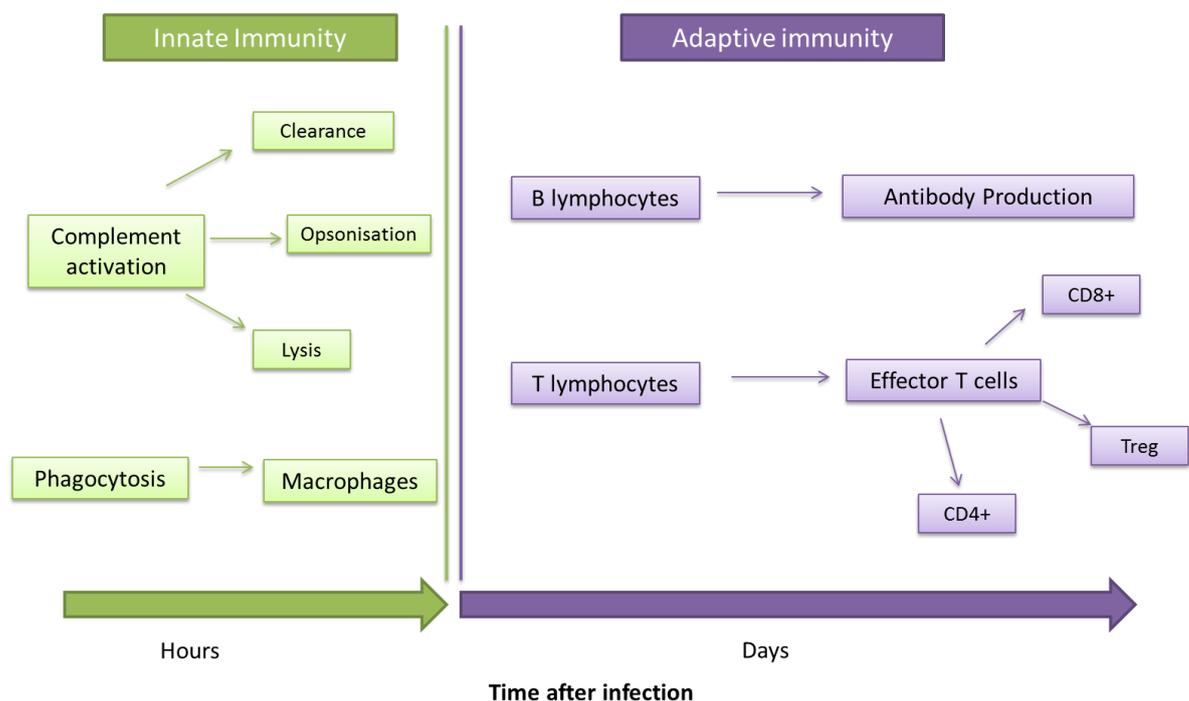
The immune system is made up of two main parts., innate and adaptive. Innate immunity which takes place just hours after infection. It includes complement pathway activation inducing pathogen lysis, opsonisation and clearance. Phagocytosis is also induced causing cells such as macrophages to engulf invading pathogens. Adaptive immunity includes B and T lymphocyte function; B lymphocytes are able to produce antibodies and aid in immunological memory and T lymphocytes differentiate into effector subsets such as CD4+,CD8+ and T regulatory cells (Treg). With each subset having a specific role in immunological defence.

1.3 Central tolerance

Central tolerance refers to the events in early life of a lymphocyte that focus the adaptive immune system against invading pathogens. Central tolerance is the process by which developing B and T cells are primed to be non-reactive to self. It includes the development of B cells within the bone marrow and that of T cells within the thymus. Central tolerance allows for the main development of self-tolerance, and includes all the mechanisms by which antigen-receptor recognition of self-antigens at specific sites result in the generation of tolerant B and T lymphocytes (Hogquist, Baldwin and Jameson, 2005).

Around 90% of DP thymocytes express TCRs that interact so poorly with the available self-peptide-MHC ligands that the signals required are not sustained, leading to cell death by neglect (Vieira, 2004). The hallmark of central tolerance is clonal deletion; this is the suicide of T cell progenitors that have a high affinity for self-peptides (Schietinger and Greenberg, 2014). Although central tolerance plays a large role in the development of self-reactive T and B lymphocytes, it is still possible for reactive lymphocytes to pass into other parts of the body because not all self-antigens are expressed within central tolerance sites. Thus the periphery is able to deal with these using peripheral tolerance mechanisms (Hogquist, Baldwin and Jameson, 2005).

1.3.1 T cell development

T cells originate from bone marrow haematopoietic precursors similar to B cells. They then lose their potential to become any other subset along the way. There are 4 main stages in the production of functional thymocytes: the entry of lymphoid progenitors into the thymus, the generation of double positive thymocytes in the outer cortex of the thymus, the selection of double positive (DP) thymocytes in the inner cortex and finally the interaction of positively selected thymocytes with medullary thymic epithelial cells.

1.3.1.1 Generation of CD4+ and CD8+ thymocytes

Lymphoid progenitors begin in the bone marrow and later migrate to the thymus; this is where T cell development takes place. The first step of T cell selection takes place in the thymic cortex. At this point thymocytes showing no affinity for peptide-MHC expressed by

cortical epithelial cells die (Xing and Hogquist, 2012; Palmer, 2003). Central tolerance depends on the presentation of self-antigens and is part of the thymic selection process. T cell differentiation takes place in a number of stages. In order for T cells to be active they must go from double negative to double positive T cells. Each T cell subset is identified by its surface receptors CD44 and CD25 (Germain Ronald N., 2002). There are 4 main double negative (DN) stages; (DN1, CD44$^+$CD25$^-$; DN2, CD44$^+$CD25$^+$; DN3, CD44$^-$CD25$^+$; and DN4, CD44$^-$CD25$^-$). Before T cells become double positive cells, they interact with MHC molecules to determine their fate, and this is referred to as active signalling.
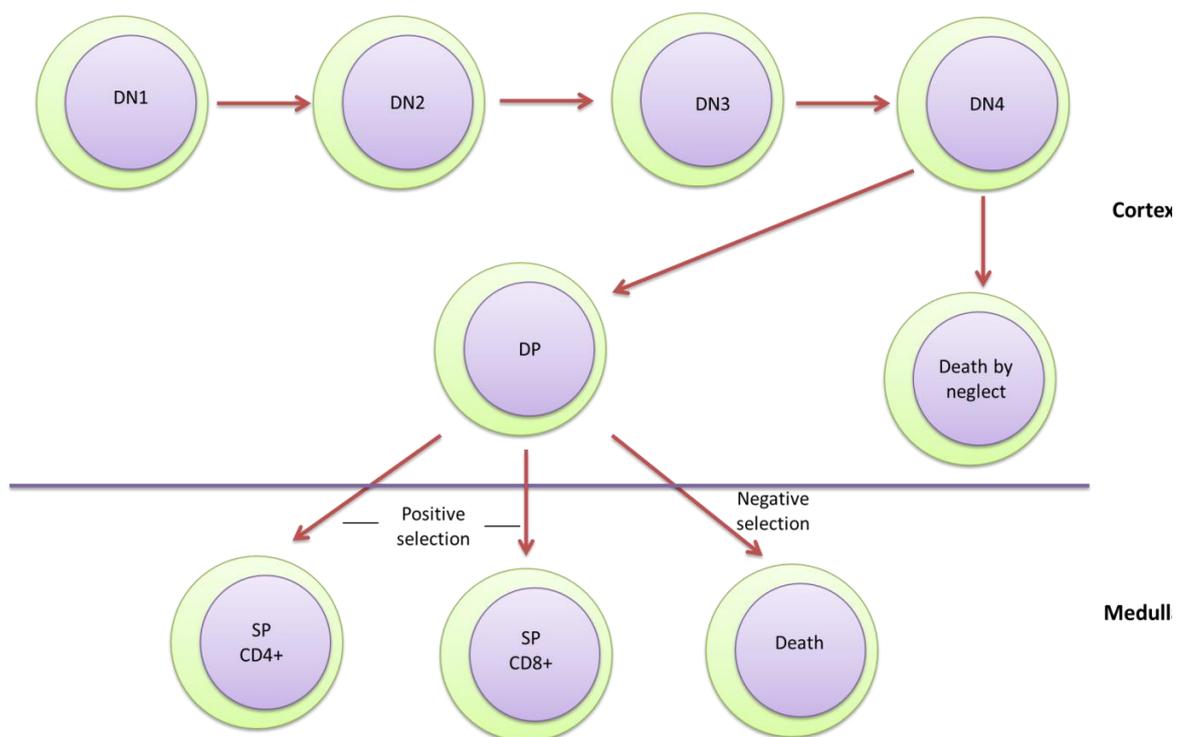
Figure 2: T cell development in the thymus

T lymphocyte development takes place in the thymus, which is a primary lymphoid organ. The development involves the maturation of T cells from the double negative (DN) stages to the effector single positive (SP) T cells. The DN stage has 4 main phases, DN1, CD44+CD25-; DN2, CD44+CD25+; DN3, CD44-CD25+; and DN4, CD44-CD25-, that take place into the cortex of the medulla. Following DN4, there is cells death due to neglect but surviving cells become double positive (DP) T cells. DP cells meet antigen presenting cells (APCs) that carry the antigen via major histocompatibility complexes (MHC's) in the medulla of the thymus. This allows for either positive selection or negative selection. Positive selection results in the production of effector SP T lymphocytes such as CD4+ and CD8+, while negative selection results in cells death via apoptosis. Surviving cells move out of the cortex into the periphery.

1.3.1.2 T cell receptor (TCR) and antigen receptor diversity

For a T cell to be functional it must have a TCR on its surface. These are transmembrane proteins that consist of heterodimers; α/β or γ/δ (Germain Ronald N., 2002); (Delves and Riott, 2000; Delves and Riott, 2000; Delves and Riott, 2000)). The development of the TCR begins at the DN2 stage in the thymus from the Pre-TCR formation to mature TCR. The T cell receptor is made up of two main chains with a constant domain and variable region. There are two main classes of T cell receptor; the α/β and the γ/δ. During T cell maturation we see the development of the α/β TCR. Each the α and β chains are joined using a disulphide bond and anchored within the cytoplasm of the cell (Brownlie and Zamoyska, 2013).
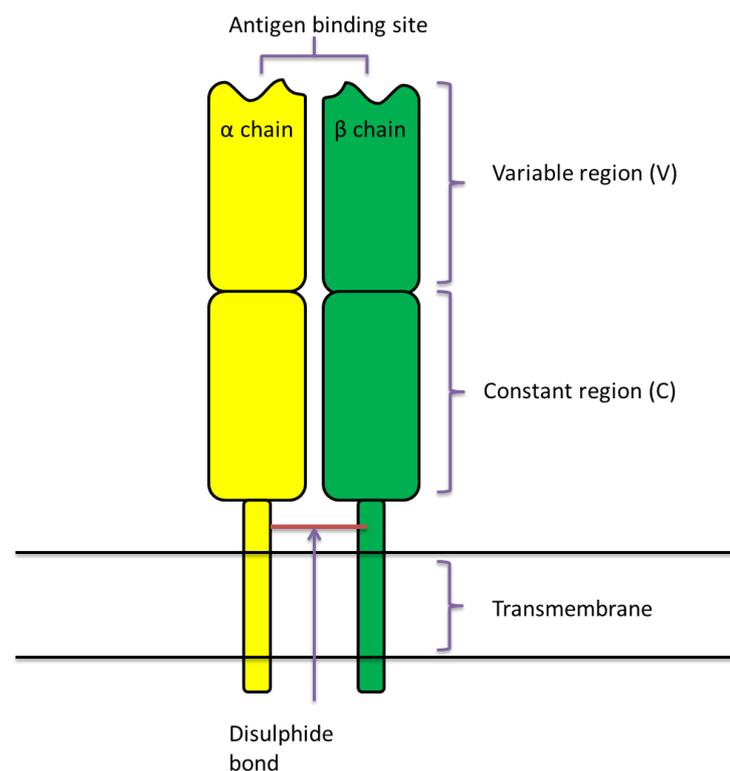
Figure 3: αβ T cell receptor structure

The α/β TCR is showing two main regions, the variable region (V) and the constant region (C). Each chain is anchored in the cytoplasm and α and β chains joined via a disulphide bond.

The variable domain in the α/β TCR recognises complexes formed by the peptide and MHC molecule. The TCR must be diverse in order to allow for the identification of a vast array of pathogens. To increase diversity, a recombination process takes place which results in the modification of the variable genes. There are four regions in T cell receptor gene organisation: Variable (V), Diversity (D), Constant (C) and Joining (J) regions. The recombination process involves these segments. In order for recombination to occur, Recombination Activating Genes (RAG-1 and RAG-2) must be present to control the enzymatic processes that need to take place (Delves and Riott, 2000). This process gives rise to TCR-β; this then meets pre TCR (expressed in stages DN2-DN4) and forms a mature TCRαβ. The replacement of the TCR isotypes gives rise to double positive (DP) thymocytes (αβ-TCR+CD4+CD8+) (Germain Ronald N., 2002). Thymocte development allows for specialised reactions to specific antigens. The resulting large number of DP thymocytes must undergo positive selection in order to determine what subset would be most necessary for function.

1.3.1.3   Antigen presentation

T cells require peptide presentation in order to recognise the antigen; this is done with the help of antigen presenting cells or APCs. In order to present an antigen to T cells APCs must process the antigen; that is, to degrade the protein into recognisable peptide fragments. APCs can be divided into two main categories: professional APCs which include mature dendritic cells, monocytes, macrophages and B cells, and non-professional APCs include fibroblasts and epithelial cells (Holling, Schooten and van Den Elsen, 2004).

In order for T cell maturation, APCs must present a peptide with the aid of a major histocompatibility complex or MHC. Only then can a T lymphocyte adequately become functional, as T cells are not able to recognise peptides that are not bound or loaded onto an MHC molecule (Holling, Schooten and van Den Elsen, 2004); (Janeway,Charles,A,Jr *et al.*, 2005).

Major histocompatibility complex (MHC)

The role of MHC molecules is to bind processed antigens (peptide fragments) to T lymphocytes via the TCR. There are two main types of MHC: MHC class I which present antigenic peptides to CD8+ T cells, and MHC Class II which present to CD4+ lymphocytes. MHC molecules are

glycoproteins and are part of the immunoglobulin superfamily. The MHC class I and II molecules are similar in structure and are made up of four domains. In the MHC class I three are α-chain domains and one is β, while MHC class II is made up of two α chains and two β chains. They each form a peptide binding cleft (Holling, Schooten and van Den Elsen, 2004) (see Figure 4).

Human MHC genes are known as human leucocyte antigen or HLA, while in the mouse they are known as H2 genes. MHC molecules have adapted to their function in a number of ways to prevent pathogens from invading the immune system. Firstly, they are polygenic, that is there are several different MHC class I and class II genes allowing for wider ranges of peptide-binding specificities; within humans, the MHC class I HLA (-A,-B and -C) and class II HLA (-DR,-DP and -DQ). Secondly, they are highly polymorphic, meaning there are increased variants of each gene, and in fact MHC genes are the most polymorphic genes found (Holling, Schooten and van Den Elsen, 2004); (Janeway,Charles,A,Jr *et al.*, 2005).
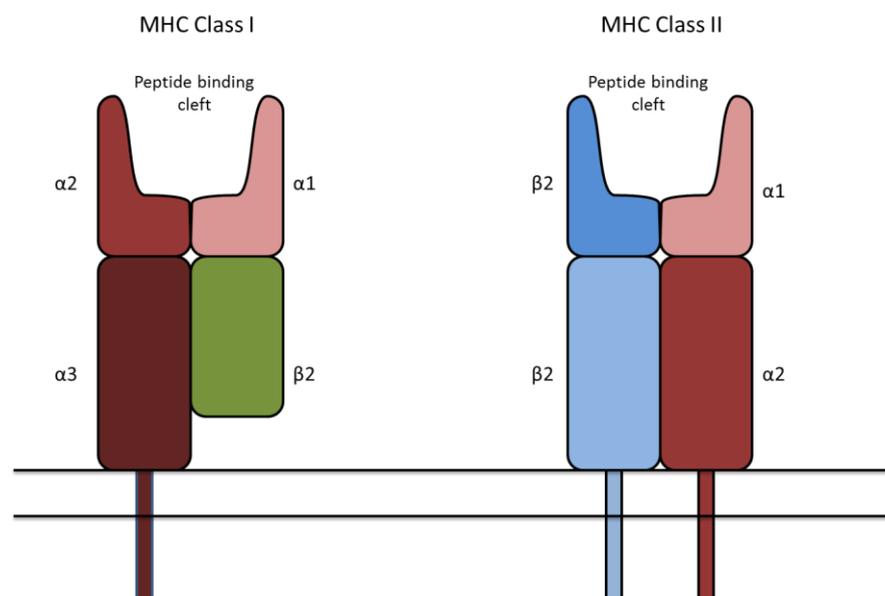
Figure 4: Major histocompatibility complex (MHC) Structure

Major histocompatibility complex (MHC) had two main types; MHC class I and MHC class II. Each are made from α and β chains. The MHC class I contains three α subunits and one β. While MHC class II contains two α subunits and two β subunits.

1.3.1.4 T cell fate

For tolerance to be maintained, selection of mature T cells must take place from an array of immature thymocytes within the bone marrow, and three outcomes are possible: negative selection, positive selection or death by neglect (see Figure 5) (Palmer, 2003). It has been reported that death by neglect is due to a lack of the TCR to bind to the MHC-peptide complex, thus the cells do not receive a signal. It has been shown that 90% of the thymocyte population dies this way, leaving the remainder for further selection.
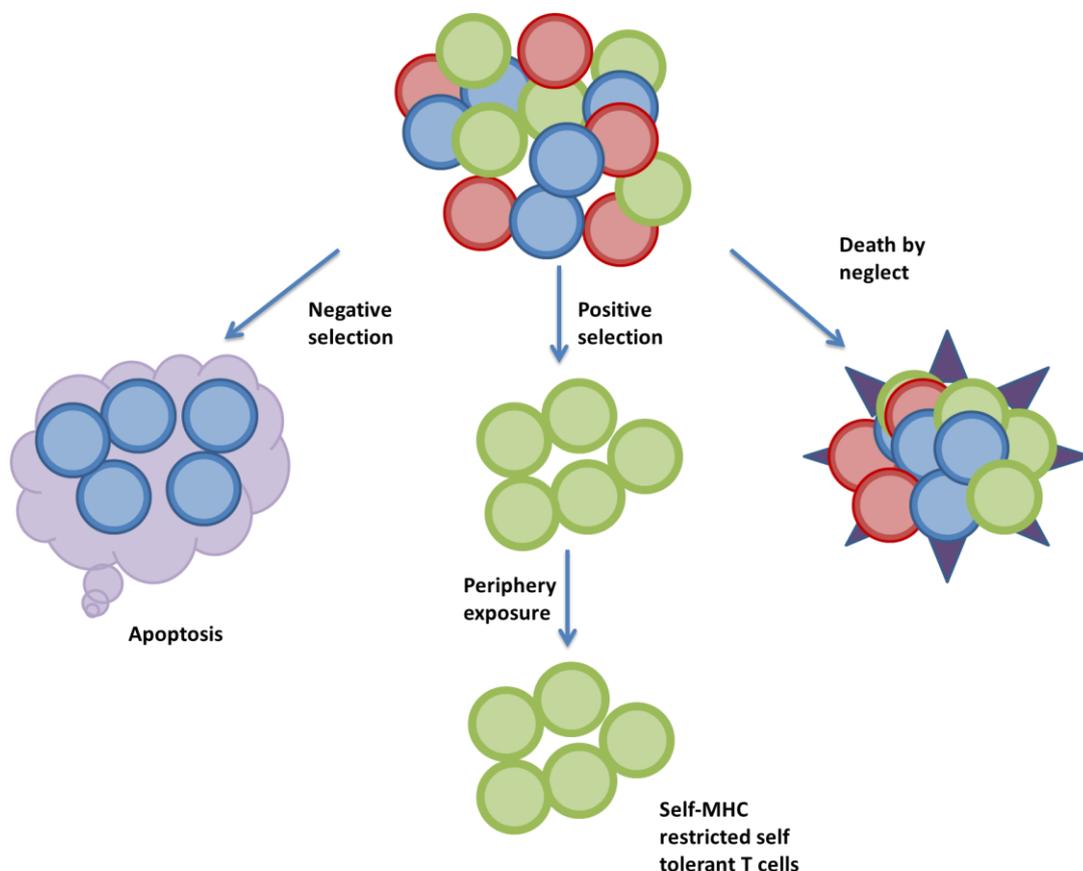
Figure 5: T lymphocyte fate

On selection T cells can undergo 3 possible fates: 1) Death by neglect, which happens to 90% of thymocytes, is due to a failure of the TCR to bind to/recognise the MHC-peptide complex. 2) Positive selection where double positive thymocytes (DP) mature into single positive (SP) thymocytes and takes place when the TCR recognises the self-peptide MHC complex producing self-MHC restricted self-tolerant T cells. 3) Negative selection when the TCR recognises the self-peptide MHC and self-antigen causing apoptosis.

T cell fate is highly dependent on the interaction between the TCR and the MHC-peptide complex. DP cells are able to interact through the mature TCR complex with peptide-MHC complexes that are expressed by stromal cells within the cortex (Takahama, 2006). Here the quality of interactions with these complexes dictates whether positive selection will take place. Once the TCR has recognised the MHC ligand at an adequate/low affinity and is able to interact with it, the DP thymocytes are able to receive signals for differentiation and survival giving rise to single positive (SP) lymphocytes (Takahama, 2006; Germain Ronald N., 2002; Germain Ronald N., 2002; Delves and Riott, 2000).

Reports have shown this is dependent on the 'affinity model', meaning the strength of the signal. Negative selection is activated when the molecules interact with high affinity whereas a low/intermediate affinity interaction results in positive selection. If there is lack of affinity at all, cells will die by neglect. In addition, it was found that the mechanisms within the body are able to allow high-affinity self-reactive T cells to become T regulatory cells (T regs) (Klein *et al.*, 2009). Together these mechanisms make up central tolerance between TCR and MHC-peptide complex determines the fate of the T cell. It has been reported that intermediate signals lead to the production of T reg cells (see Figure 6) (Klein *et al.*, 2014).
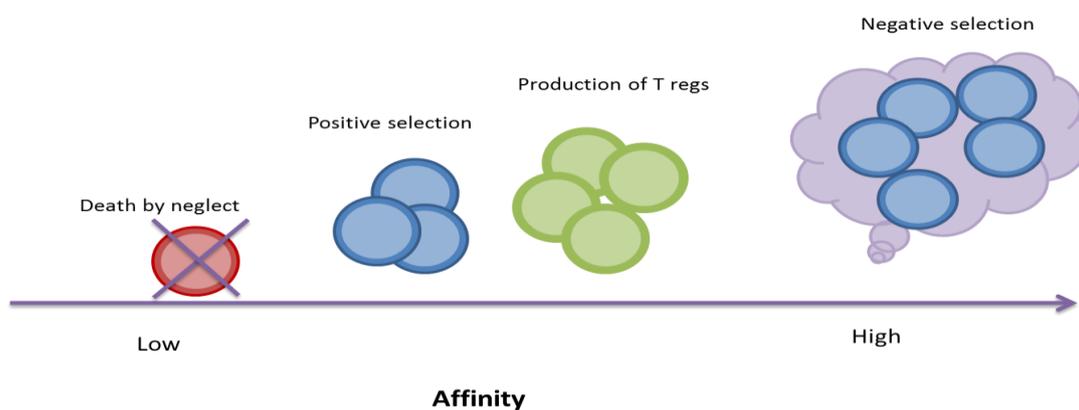
Figure 6: Affinity thresholds in T cell selection

The TCR can recognise the MHC-ligand at different affinities. Negative selection is activated when the molecules interact with high affinity whereas a low affinity interaction results in positive selection. High-affinity self-reactive T cells become T regulatory cells If there is lack of affinity at all, cells will die by neglect (Klein *et al.*, 2014).

1.3.1.5   Positive selection

Thymocyte differentiation involves the transition of double positive thymocytes (DP) into single positive (SP) thymocytes. (Takahama, 2006)(Germain, 2002). Positive selection takes place when the TCR meets the self-peptide MHC complex with a low affinity interaction (Figure 7) allowing for the survival of the cell (Nakagawa *et al.*, 2012). Reports have found positive selection to be dependent on the TCR and MHC-complex interaction within T cells but not in B cells (Baldwin *et al.*, 2005). Positive selection has been reported to primarily take place within the cortex of the thymus and is assisted by specialised antigen presenting cells called cortical thymic epithelial cells (cTECs) (Nakagawa *et al.*, 2012). cTECs were found to play an essential role in the processing of antigens and reports have suggested their role in multiple proteolytic pathways within positive selection (Nakagawa *et al.*, 2012).
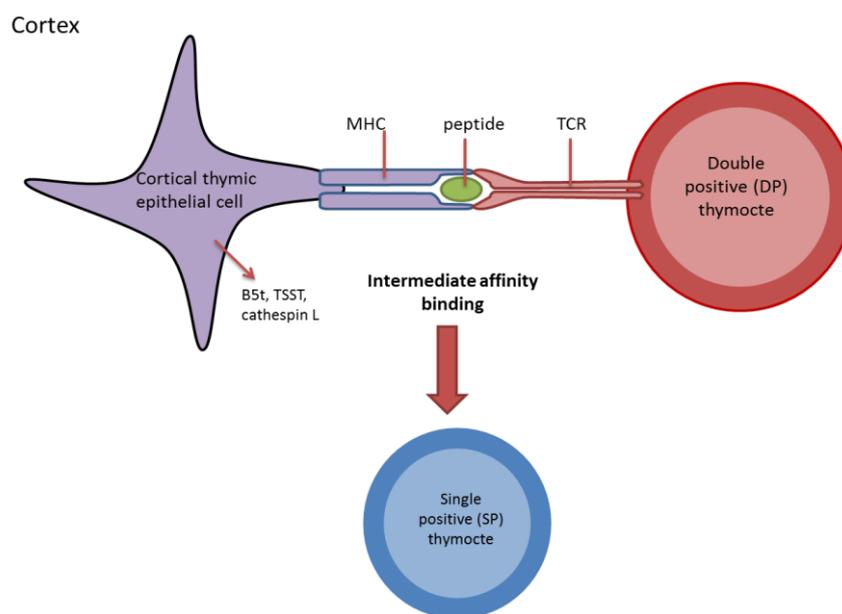
Figure 7 : Positive selection in T lymphocytes

Positive selection takes place in the cortex. It involves the selection of double positive thymocytes into single positive thymocytes. On the TCR recognising the MHC-self-peptide complex at an intermediate strength, the lymphocyte is allowed to survive and become a single positive lymphocyte. Cortical epithelial cells or cTECs are able to release proteases that aid in MHC stabilisation to encourage TCR binding.

Positive selection involves the generation of single positive thymocytes from double positive ones, that is the generation of CD4+ and CD8+ T cells (Figure 7) (Hogquist, Baldwin and

Jameson, 2005). Antigen processing via cTECs in regard to CD8+ commitment requires MHC class II presentation. In this case, cTECs express a proteasome called β5t (Florea *et al.*, 2010). In the case of the generation of CD4 T cells, with MHC class II interaction, cTECs express a different set of proteases, namely cathepsin L and thymus-specific sense protease (TSSP). Lack of cathepsin L results in inefficient CD4 T cell selection (Marrack and Kappler, 1987) with a reported 60-80% reduction in CD4 single positive T cell compartment within the thymus (Stritesky *et al.*, 2013b). Caspesins protect against the improper loading of MHC class II molecules making it imperative for efficient positive selection. TSSP has been found to be the key protease that cTECs use in distinct pathways. It has been suggested it has a role in the generation of MHC class II bound peptides (Stritesky *et al.*, 2013a). TSSP is encoded by Prss16 and within Prss16-/- mice, the positive selection of MHC class II restricted to TCR was significantly reduced (). Once positive selection has taken place, cells are able to translocate into the medulla.

1.3.1.6   Negative selection

The process of negative selection contributes to the deletion of self-reactive T cells thus helping to avoid autoimmunity. In the event of a TCR recognising the self-peptide-MHC complex and the self-antigen at a high affinity (see Figure 8), negative selection prevails (Starr, Jameson and Hogquist, 2003)(Palmer, 2003). A small fragment of immature T cells bear TCR that are able to bind very well to self-ligands. High avidity interactions elicit signals that lead to the deletion of thymocytes. The death of T cells, known as clonal deletion, is a hallmark of central tolerance (Kyewski and Klein, 2006). Apoptosis is the process best characterised as the mechanism of negative selection as signalling on engagement of these TCRs with self-peptide MHC ligands promotes rapid cell death. Cortical DP thymocytes fail to receive TCR signals and are also programmed to die at this stage (Goldrath and Bevan, 1999)(Hogquist, Baldwin and Jameson, 2005).

A large amount of research has gone into the studies of negative selection control, more specifically the importance of antigen presentation within this process. It has been reported that negative selection is able to take place both in the cortex and in the medulla of the thymus (Stritesky *et al.*, 2013b; Stritesky *et al.*, 2013b). However, the majority of reports state

the medulla is the main area for negative selection as it plays a crucial role in the antigen presentation via medullary thymic epithelial cells (mTECs).

The thymic medulla is able to express a large array of tissue restricted antigens or TRAs. These are antigens usually expressed within peripheral organs (Klein *et al.*, 1998). The expression of TRAs has been shown to play a major role in the deletion of self-reactive CD4 lymphocytes highlighting the medulla within central tolerance (Laufer, Fan and Glimcher, 1999; van Meerwijk and MacDonald, 1999). In fact, direct presentation of TRAs by mTECs has been shown to result in deletion of CD8+ T cells (Gallegos and Bevan, 2004).

Interestingly, mTECs have been suspected to have alternative methods in antigenic presentation as they have been reported to have decreased efficiency in MHC class II presentation of extracellular antigens (Klein, Roettinger and Kyewski, 2001). A number of mouse strains have been shown to have impaired characteristics within the medulla; for example, nuclear factor kappa-light-chain-enhancer of activated B cells (NFkb) (Kappler, Roehm and Marrack, 1987) and TNF-6 (Cho and et, 2003) deficient mice show disturbed medullary patterns. Importantly, mTECs express the transcription factor autoimmune regulator (AIRE). Defects in AIRE lead to the multi-organ syndrome known as autoimmune polyendocriopathy candidiasis dystrophy syndrome (APECED) (Peterson *et al.*, 1998). AIRE-deficient mice develop spontaneous multi-organ disease (Anderson *et al.*, 2002)(Peterson *et al.*, 1998). mTECs also express B7 family of co-stimulatory molecules and can be helpful in the induction of clonal deletion of T cells that respond to TSAs (Xing and Hogquist, 2012).
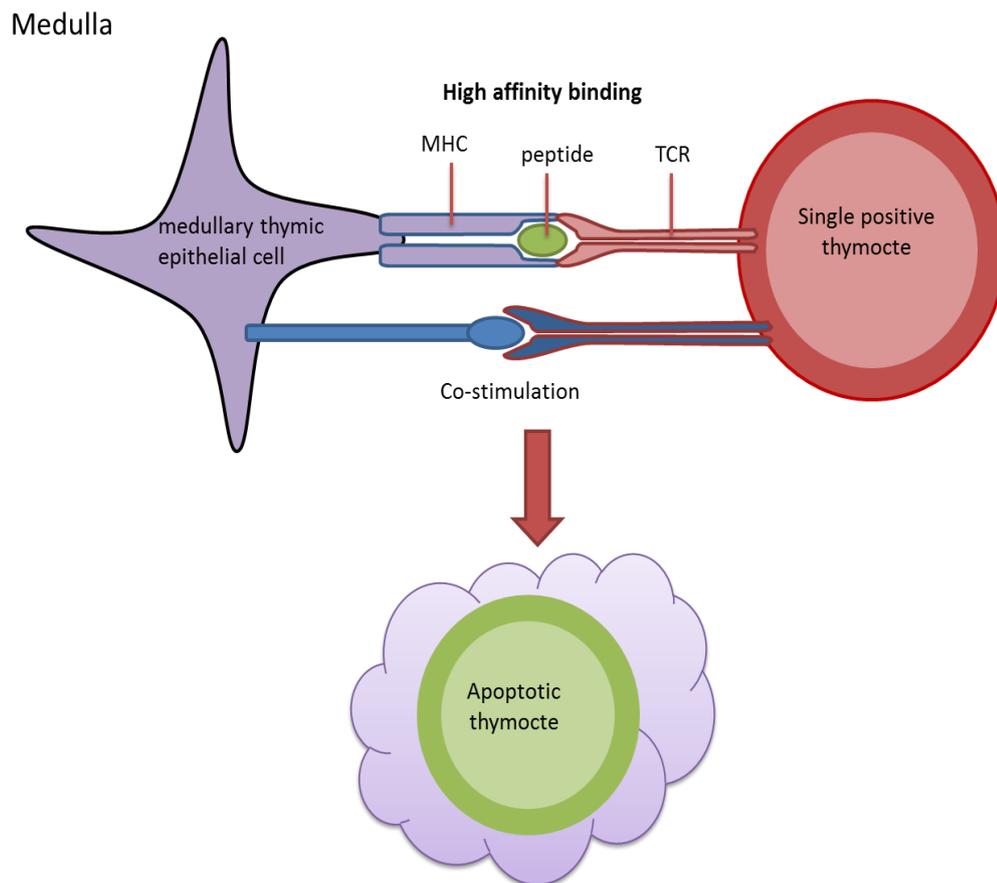
Figure 8: Negative selection

Negative selection takes place when the TCR recognises the self-peptide-MHC complex and the self-antigen at a high affinity leading to apoptosis. This takes place in the medulla of the thymus. mTECs are able to act as antigen presenting cells for this process and express tissue restricted antigens. Co stimulation is also provided by mTECs as they express the B7 co-stimulatory molecule that aids in deletion via apoptosis.

1.3.1.7   Molecular pathology of positive and negative selection

Central tolerance has been shown to be directly upregulated by a number of factors. The mechanisms that govern both positive and negative selection have been widely studied. Within positive selection, a number of signals have been shown to be important in function. Positive section is dependent on TCR interaction at a low affinity. Thus investigation into factors downstream of TCR have been studied. Importantly it has been shown that cyclosporine A and FK506 can impair positive selection (Wang *et al.*, 1995), meaning a calcium flux caused by TCR engagement plays a crucial role. It has also been found that NFAT (nuclear factor of activated T cells) activation is required for positive selection as it falls downstream of

the calcium flux. It was reported that in the absence of NFAT 4, positive selection was impaired (Oukka *et al.*, 1998). LCK, a molecule downstream of signalling, was shown by Hashimoto to be required for positive selection (Hashimoto *et al.*, 1996). Another such molecule is ZAP70; zap70-/- mice showed disturbed positive selection compared to wild type mice (Negishi *et al.*, 1995). This means that TCR activation in positive selection is essential.

The major factor in negative selection is that of apoptosis. There have been two apoptosis pathways suggested, and one is that of the Bcl-2 family. The Bcl-2 apoptosis pathway allows for the activation of caspase 9 after cytochrome c binds to APDF-1 (apoptosis death factor-1) (Adams and Cory, 2001). Cytochrome c thus allows for a number of cascades eventually leading to cell death. Significantly, the Bcl-2 family of genes plays an important role. For example, BIM deficient mice seem to have no negative selection mechanism in place (Bouillet *et al.*, 2002). Apoptosis initiation can involve cell surface death receptors. They are able to initiate caspases via their death-effector domains (DEDs) leading to cell death (Palmer, 2003). They include Fas and tumour necrosis factor (TNF) (Nagata, 1997) (Figure 9). The activation allows for caspase activation and eventually leads to apoptosis (Tait and Green, 2010).

Negative selection has also been found to link to the costimulatory signal. It was shown CD28-/- mice do not undergo anti-CD3 induced apoptosis (Noel *et al.*, 1998). In addition, it has been shown that CD28 can induce apoptosis (Palmer, 2003) suggesting that this signal is important. CD40 has also been shown to play a role as its deficiency results in blockade of negative selection (Foy *et al.*, 1995). CD40L is a member of the TNF family, and decreased expression leads to incomplete deletion in self-reactive cells. The map-kinase pathway (MAP-K) has been studied within positive and negative selection. The activation of this pathway seems to influence the kinetics of signalling during positive and negative selection (Palmer, 2003).
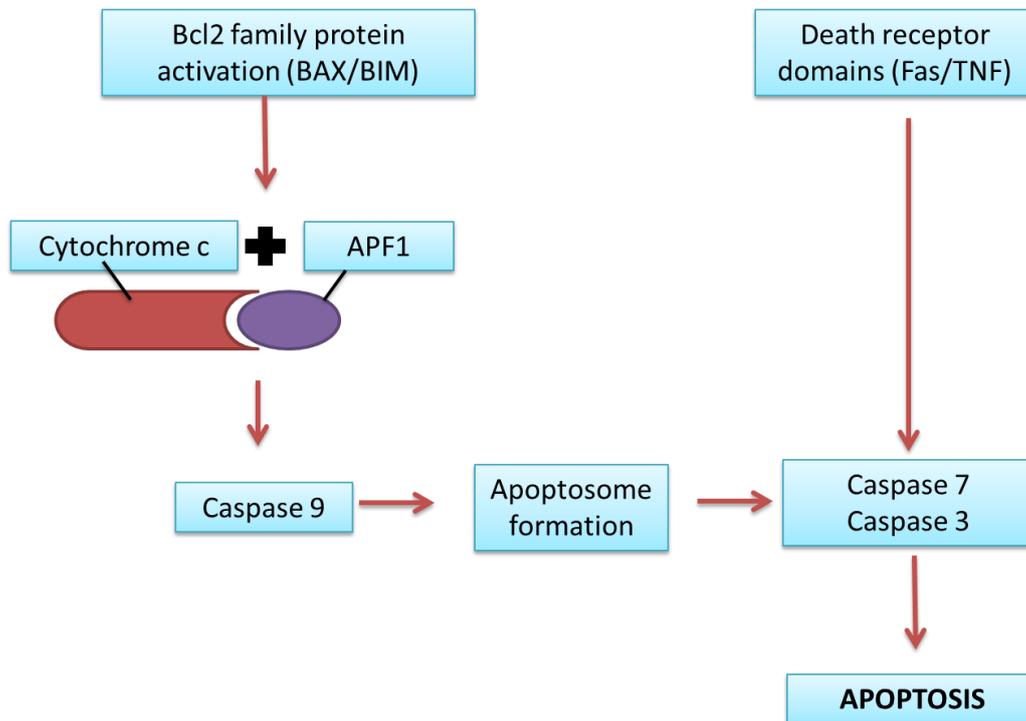
Figure 9: Bcl-2 apoptosis pathway

The Bcl-2 apoptosis pathway is triggered by the activation of Bcl-2 family such as BIM and BAX. This is able to activate cytochrome c within the cytoplasm. Cytochrome c then binds to apoptosis factor 1 or APF1 causing the activation of caspase 9. at this point the apoptosome is formed and leads to caspase 7 and caspase 3 activation to induce apoptosis. Death receptor domains within the cytoplasm can also cause apoptosis. They are able to trigger the activation of caspase 7 and caspase 3 and lead to apoptosis.

(Tait and Green, 2010)

1.3.1.8   Development of natural T regulatory (nT-reg) cells

T reg cells are also produced in the thymus. They are CD4+ CD25+ cells that function to control the differentiation of CD8 and CD4 effector T cells. They make up about 5-10% of CD4+ T cells in healthy adult mice and humans (Vieira, 2004). Their function or activation only takes place in the periphery. Their production in the thymus relies heavily on the expression of transcription factor forkhead box P3(FOXP3) (Hsieh, Lee and Lio, 2012; Josefowicz, Lu and Rudensky, 2012). This transcription factor is thought to programme the development of this subset. Loss or alteration in this gene in mice caused a phenotype in mice with symptoms that included early onset type 1 diabetes, thyroiditis and autoimmune endocrinopathy (O'Garra and Vieira, 2004). Humans with FOXP3 mutations develop IPEX (Immunodysregulation,

polyendrocrinopathy, enteropathy, x-linked syndrome) which is a severe multi-organ autoimmune disease that requires bone marrow treatment. Thus it is important to generate T reg cells as they help maintain immune tolerance and prevent autoimmunity (O'Garra and Vieira, 2004).

The development of this subset in the thymus is not fully understood. There are many mechanisms that give rise to these cells. Thymocytes receive signals based on TCR; affinity to the TCR was of greatest concern although it has been found that an intermediate signal allows for the induction of FOXP3 expression with low affinity leading to negative selection and high affinity to positive selection (Hsieh, Lee and Lio, 2012).

Downstream of the TCR there are a number of mechanisms that give rise to the development of T reg cells, including the activation of NFAT and NF-Kb. Among the many molecules activated it has been found that NF-Kβ plays the biggest role. Increased NF-Kβ expression via overexpression of IKKb was sufficient to bypass TCR stimulation as it results in the development of FOXP3+ T reg cells in the thymus of RAG deficient mice (Hsieh, Lee and Lio, 2012).

1.4 Peripheral T cell tolerance

1.4.1 T cell Activation

Once cells have reached the point of SP in the thymus they are still naïve. In order for CD8 and CD4 T cells to become functional a few things must take place. This process occurs in the periphery as these cells are transported out of the thymus into peripheral organs such as the spleen and lymph nodes where activation occurs.

There are three main signals that have to take place in order for a T lymphocyte to be activated and become functional (**Figure 10**). The first is the antigen must be appropriately presented on the surface of an MHC molecule allowing for the identification of the antigen by the TCR. The second is the co-stimulatory signal which is a second interaction between the APC (antigen presenting cell) and the T cell. On T cells the receptor is known as CD28 and APCs are able to use the B7 receptor. The coming together of these two receptors allow for the second signal to stabilise T cell activation.
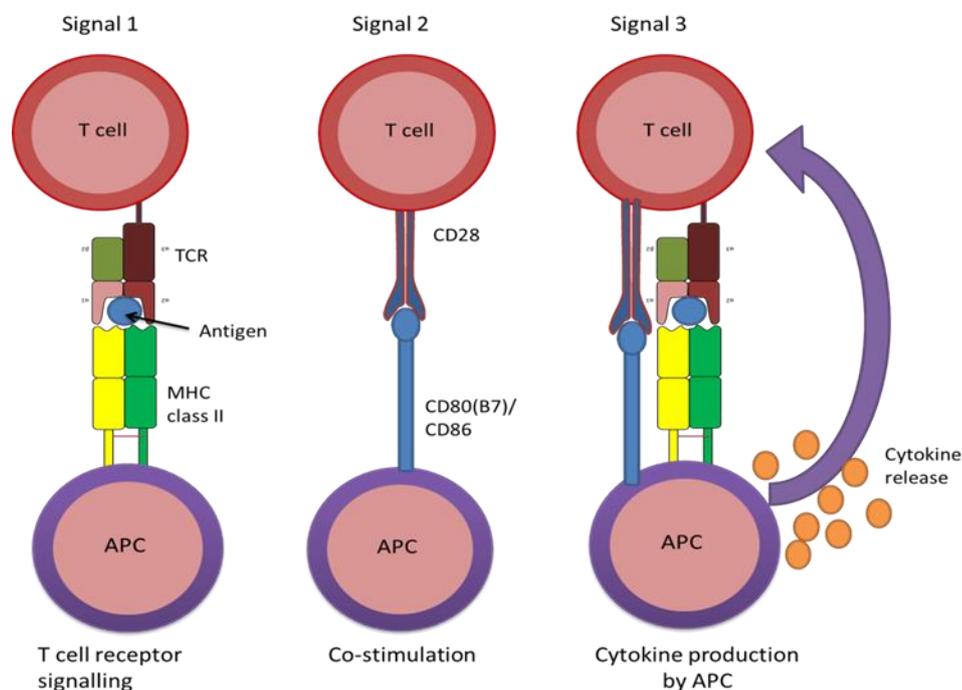
Figure 10: Signals required for T lymphocyte activation

In order for activation to take place T cells need three signals; 1) T cell receptor (TCR) signalling is required for antigen recognition via the major histocompatibility complex (MHC) class II molecule that lays on the antigen presenting cell (APC), 2) Co-stimulatory signalling through CD28 on the T cell and CD80/B7 on the APC, 3) the production of cytokines by antigen presenting cells which is able to aid in T cell differentiation.

(Gutcher and Becher, 2007).

Co-stimulation, which is discussed later, also plays a role in the development of this subset; it was found that without co-stimulation there was an 80% drop in the number of FOXP3 T reg cells. The primary role of the CD28 is to enhance the survival of thymocytes undergoing differentiation and CD40, another costimulatory molecule, plays a role in the expansion of T reg populations (Hsieh, Lee and Lio, 2012).

The third signal is the production of cytokines by the APC after the two initial interactions. The cytokine secretion is responsible for directing the differentiation of specific cells into the appropriate effector subtypes (Gutcher and Becher, 2007). The most important APC cells are the dendritic cells (DCs), and these are activated via the recognition of pathogenic antigens by cell surface pattern recognition receptors such as toll like receptors and intracellular pathogen sensing receptors which include nucleotide oligomerisation domain (NOD)-like receptors. It

has been reported that the priming of dendritic cells allows for the initiation of immune function. Mature DCs are able to allow the binding of an MHC class II molecule onto their surface in order for ample T cell activation. In addition, DCs have an enhanced ability to stimulate naïve T cells (Luckheeram *et al.*, 2012a). DCs consist of different subsets and aid with co-stimulation to activate the T cell, for example the interaction between CD28 on the TCR and CD80 on the DC (Luckheeram *et al.*, 2012b; Gutcher and Becher, 2007). DCs carry the ligands of CD28 that lay on the TCR that is CD80 (B7-1) and CD86 (B7-2) which are upregulated after DC activation. APCs such as dendritic cells are also the main source of cytokines following T cell activation. They are able to release different cytokines and aid T cell differentiation following successful activation which will be discussed later (Luckheeram *et al.*, 2012a). One of the main cytokines produced by CD4+ T cells is IL-2, although other subgroups are able to produce it, such as CD8+ T cells and thymic cells (Nelson, 2004)(Nelson, 2004)

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IL-2 is produced in the secondary lymphoid organs and production is strongly induced after T cell activation (Boyman and Sprent, 2012). IL-2 is responsible for clonal expansion of CD4 T cells into their functional subsets (Fathman and Lineberry, 2007). Its production allows for the activation of a number of transcription factors that in turn activate the production of cytokines that direct T helper expansion to the required subset.

1.4.1.1   TCR signalling

CD4 T cell activation requires signals from the antigen and the co-stimulatory molecule. Each signal produces a cascade that together allow for the production of IL-2 (Boyman and Sprent, 2012; Fathman and Lineberry, 2007). TCR signalling is essential for T cell activation (Abraham and Weiss, 2004). In order to recognise the antigen α and β dimers of the TCR are put into place (Abraham and Weiss, 2004). TCR activation allows for a cascade of events that in turn activate proliferation and differentiation. On TCR activation via antigens presented on MHC, it has been found that changes in cytoskeletal arrangement are induced along with transcription factor expression (Brownlie and Zamoyska, 2013). Activation via TCR causes recruitment of the SRC family of kinase members Fyn and Lck. These molecules are able to promote the survival of naïve T cells (Seddon and Zamoyska, 2002). In addition, Fyn and Lck are important for transportation within the nucleus. They are able to phosphorylate ITAMs allowing for ZAP70

activation. It has been reported ZAP-70 then undergoes conformational changes and phosphorylates LAT or linker for activation of T cells, and SLP76 (Deindl *et al.*, 2007).

LAT activates a number of mechanisms such as mobilisation of transcription factors and expression of T cell growth genes (Brownlie and Zamoyska, 2013). LAT activation also results in activation of Ras via guanine nucleotide exchange factors or GEF's, and phospholipase Cγ (PLCγ) on the Golgi apparatus (Quintana *et al.*, 2005)(Bivona *et al.*, 2003). RASGRP-1 or Ras guanyl-releasing protein 1 requires translocation to the Golgi membrane. Significantly, this is controlled by calcium (Ca^{2+}). When the TCR recognises an antigen, ZAP70 is activated and this allows for a calcium flux within the cell. Ca^{2+} levels increase following TCR activation and Ca^{2+} levels have been emphasised in the control of transcription factor translocation (Feske, 2007). Along with DAG (Diacylglycerol), increased calcium levels allow translocation of RASGRP-1 to the Golgi membrane (Bivona *et al.*, 2003). Activation of the Ras pathway activates a mitogen-activated protein kinase or MAPK cascade which eventually leads to the activation of nuclear factor of activated T cells (NFAT). Map kinase activation causes the activation of activator protein 1 (AP-1) which is a heterodimer of FOS and JUN; Ras pathway activation is reported to induce Fos (Quintana *et al.*, 2005). Increased calcium levels thus cause the activation and nuclear translocation of NFAT (Harris *et al.*, 2004; Su *et al.*, 1994b). These processes lead to proliferation and differentiation into effector T cells.

1.4.1.2 Co-stimulatory signalling

Full T cell activation requires help from co-stimulation. In the 1980s the two signal hypothesis where TCR signalling was found to be amplified by the CD28 co-receptor was described (Sharpe and Freeman, 2002). Signalling molecules fall into two main categories: the tumour necrosis factor superfamily (TNF) and the immunoglobulin superfamily (IgSF) (Chen and Flies, 2013). CD28 expressed on T cells and B7 on antigen presenting cells belong to the IgSF family of receptors (Gonzalez *et al.*, 2005; Sedy *et al.*, 2005).

CD28 has been reported to play a significant role in T cell activation with T cell responses being disturbed in CD28-/- mice and in CD80-/- and CD86-/- mice. CD80 and CD86 are ligands of CD28 (see Figure 11) (Sharpe and Freeman, 2002; Lenschow, Walunas and Bluestone, 1996). CTLA-4 or cytotoxic T lymphocyte antigen 4 is able to prevent interactions of CD28 and B7 that lays on the APC. Upregulation of CTLA-4 causes downregulation of CD28 following

endocytosis. CTLA 4 is only produced after T cell activation has taken place in order to suppress T cell immune responses (Chen and Flies, 2013). It was reported that lack of CD28 mediated co-stimulation resulted in decreased T cell proliferation (Lucas *et al.*, 1995; Green *et al.*, 1994). Unlike TCR signalling, it was reported that CD28 signalling is not sensitive to cyclosporine A (June *et al.*, 1987). CD28 signalling has been found to have many roles including cell cycle progression and clonal expansion. It also allows for the activation of downstream transcription factors such as NFAT, AP-1 and NF-kβ (Acuto and Michel, 2003).
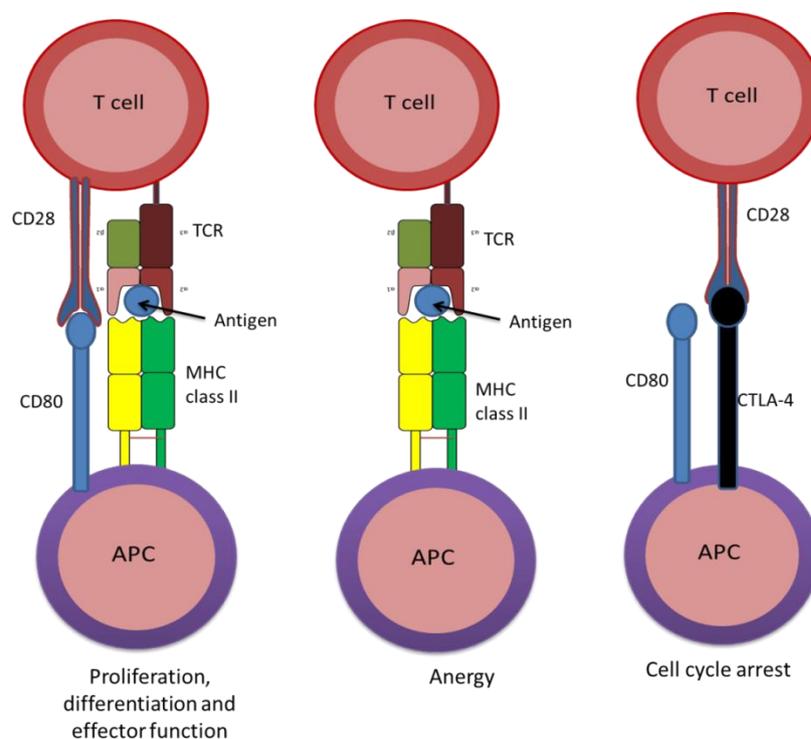
Figure 11: CD28 in T cell interactions

A T cell becomes active after the T cell receptor (TCR) meets a major histocompatibility complex (MHC) class II molecule loaded with and antigenic peptide. This allows for proliferation, differentiation and effector function. Anergy takes place when a T cells receives the signal from the T cell receptor (TCR) without that of the co-stimulatory (CD28). Cytotoxic T lymphocyte antigen 4 (CTLA4) has been shown to interact with the CD28 molecule on the T cell preventing the binding of CD80, this has been reported to cause cell cycle arrest to suppress the immune system.

(Chen and Flies, 2013)

1.4.1.3   Cytokine signalling

The production of IL-2 after TCR and co-stimulatory signalling allows for proliferation and differentiation. On T cell activation, cytokines are released to allow for the influence of T cell subset differentiation. Cytokines produced are able to influence differentiation via open receptors. In culture, T cells express IL-2; IL-2 receptors on T cells receive IL-2 and activate pathways that induce gene expression that aid in proliferation and differentiation into effector cells (Alberts, Johnson and Lewis, 2002). Cytokine medicated differentiation into multiple subsets is possible through similar mechanisms discussed later.

1.4.1.4   Transcription network

The three main transcription factors that are present following T cell activation are NFAT, NFKB and AP-1. Activation results in proliferation, differentiation and cytokine production as a result of transcription factor function. All the transcription factors are engaged downstream following TCR and CD28 stimulation (Gutcher and Becher, 2007).

NFAT has been reported to be the most directly linked to Ca^{2+} influx. The NFAT family is made up of NFAT 1, 2, 3, 4 and 5, four of which are regulated by calcium (Macian, 2005). As regulation of NFAT is important, the calcium mechanism within TCR control has been heavily studied. Following TCR-dependant antigenic biding, DAG results in binding of InsP3. When InsP3 meets its receptor on the endoplasmic reticulum (ER) it allows the release of Ca^{2+} from Ca^{2+} stores, although this increase is not sufficient to raise levels significantly. ER also allows for the activation of the SOCE (store-operated Ca^{2+} entry) pathway that opens CRAC (Calcium release-activated channel) pathways (Feske, 2007) in the plasma membrane, and the Ca^{2+} flux using these channels are what truly elevate intracellular calcium (Crabtree and Olson, 2002). This flux activates calcineurin which in turn dephosphorylates NFAT, which allows for NFAT activation and translocation of NFAT into the nucleus (Crabtree and Olson, 2002).

Once in the nucleus, NFAT is able to act with AP-1. CD28 has been implicated in AP-1 expression, however the bulk comes from the TCR (Acuto and Michel, 2003). CD28 was reported to activate cJUN, which activates AP-1 leading to the production of IL-2 (Su *et al.*, 1994a). NFkB expression has, however, been primarily linked to CD28 signalling. Activation of PKC-θ (Protein kinase C) leads to the activation of NFkB. AP-1 is able to be activated by CD28

via the activation of Ras and MAP kinase proteins which allow for c-Fos and c-Jun expression (dimers of AP-1) and thus cells that are CD28-/- have a defect in IL-2 production (see Figure 12) (Acuto and Michel, 2003).
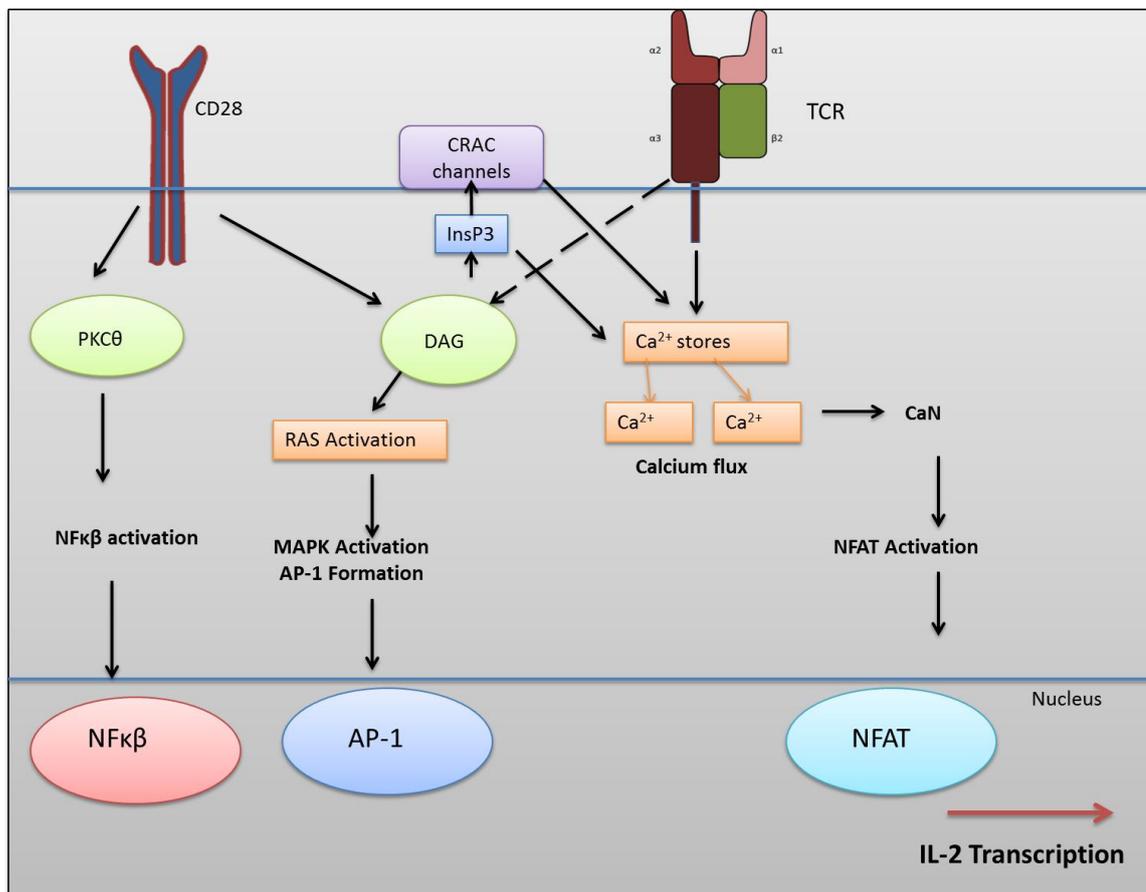
Figure 12: T cell activation transcription network

T cell receptor activation (TCR) is able to induce DAG expression and activate InsP3. Insp3 induces calcium ($Ca2+$) release from calcium stores and inducing calcium stores within the membrane (CRAC channels) to increase Ca2+ levels within the cell. Ca2+ release activates calcineurin (CaN) and activates nuclear factor of activated T cells (NFAT) and allows for its translocation into the nucleus. CD28 activation triggers RAS activation alongside TCR and activated the MAPK pathway and AP-1 formation and translocation into the nucleus. CD28 also activates protein kinase C (PKθ) allowing for NFκβ expression and translocation into the nucleus. NFκβ, NFAT and AP-1 in the nucleus initiate the transcription of IL-2 which favours proliferation and differentiation.

1.4.2 T cell differentiation

CD4 T cells are able to differentiate into a number of sub populations depending on cytokine production after stimulation. T helper (Th) subsets are distinguished by cytokine production and expression for specific transcription factors (Coomes, Pelly and Wilson, 2013). Each subgroup is specialised for a different purpose and a particular cytokine governs each subset. Previously it was reported that T helper cell differentiation is clear cut and divides into two main subsets, Th1 and Th2 (Mosmann *et al.*, 2005; Heinzel *et al.*, 1989). Since then other subsets have been described. It is important to note that some cytokines are produced by more than one subgroup. There are 5 main subgroups, they include Th1, Th2, Treg, Tfh (T follicular help) and Th17 (Zhu, Yamane and Paul, 2010). The first group, Th1, produces IFN-γ, IL-2, IL-3 and TNF-α. The governing cytokine is IFN-γ, thus these cells induce pro-inflammatory responses by activating macrophages, eliminating infection, and causing delayed type hypersensitivity. Th2 cells predominantly secrete IL-4 but also secrete IL-13 and IL-5. They are able to stimulate B cell activation and help humoral immunity activation. The Th17 subset mainly produces IL-17A and IL-17F while the T reg subgroup produces IL-10. This particular subgroup is referred to as induced T regulatory T cell (iTreg) which is distinct from the natural T reg cell. This subgroup is identified by its ability to produce IL-10. The final subset is that of T follicular helper cells. This is another B cell helper with the production of IL-6 and IL-21. These cells aid in immunoglobulin class switching within the germinal centre (Zhu, Yamane and Paul, 2010; Gutcher and Becher, 2007).

1.4.2.1 Transcription network for distinct T helper differentiation

Th1 differentiation results in the production of IFN-γ and is initiated by the production of IL-12 (Trinchieri, Pflanz and Kastelein, 2003). The most important and master regulator of Th1 differentiation is the T box transcription factor (T-bet). It was reported that the expression of T-bet is dependent on signal transducer and activator of transcription 1 (STAT1) (Chen, Laurence and O'Shea, 2007). IFN-γ is also able to activate STAT1; this signal is amplified as T-bet induces more IFN-γ expression and upregulates IL-12R expression (Afkarian *et al.*, 2002). STAT4 also plays a role in Th1 differentiation. STAT 4 is induced by IL-12 and also leads to the

production of IFN-γ causing a positive feedback loop for IL-12R and T bet expression (Luckheeram *et al.*, 2012a).

Th2 differentiation is governed by the transcription factor GATA binding protein or GATA-3, with IL-2 and IL-4 being essential for functional differentiation. STAT6 is induced by the production IL-4 (Zhu *et al.*, 2004; Kaplan *et al.*, 1996) (Luckheeram *et al.*, 2012a). GATA-3 deficiency leads to incomplete Th2 differentiation. STAT-5 has also been implicated in Th2 differentiation. IL-2 is able to activate STAT-5 independent of IL-4 and so without the activation of GATA-3, thus both GATA-3 and STAT-5 are required for full Th2 differentiation. It has been reported that GATA-3 and STAT-5 bind to the IL-4 locus at different sites (Luckheeram *et al.*, 2012a).

Th17 cell differentiation involves the master regulator retinoic acid receptor-related orphan receptor gamma-t or RORγt, and is governed by a number of cytokines, namely IL-6, IL-23 and TGF-β. It has been reported that the process can be divided into 3 parts: TGF-β and IL-6 in differentiation initiation, amplification of cells by IL-21, and the use of IL-23 in stabilisation (Luckheeram *et al.*, 2012a). TGF-β signalling allows for RORγt activation inducting the production of IL-17F and IL-17A (Ivanov *et al.*, 2006). RORγt expression is also governed by STAT-3 which is activated downstream of cytokine signalling (Bettelli *et al.*, 2006).

T reg cell differentiation is governed by FOXP3 expression and these cells are classified as CD4+CD25+. Following T cell activation, forkhead transcription factor (FOXP3) is expressed. T regs can be naturally occurring or induced (iTregs) in the periphery. IL-2 signalling allows for STAT-5 induction which in turn activates FOXP3 expression (Luckheeram *et al.*, 2012a; Burchill *et al.*, 2007). T follicular help cell differentiation has been reported to depend on the transcription factor Bcl6. IL-6 and IL-21 production allow for its activation (Luckheeram *et al.*, 2012a).
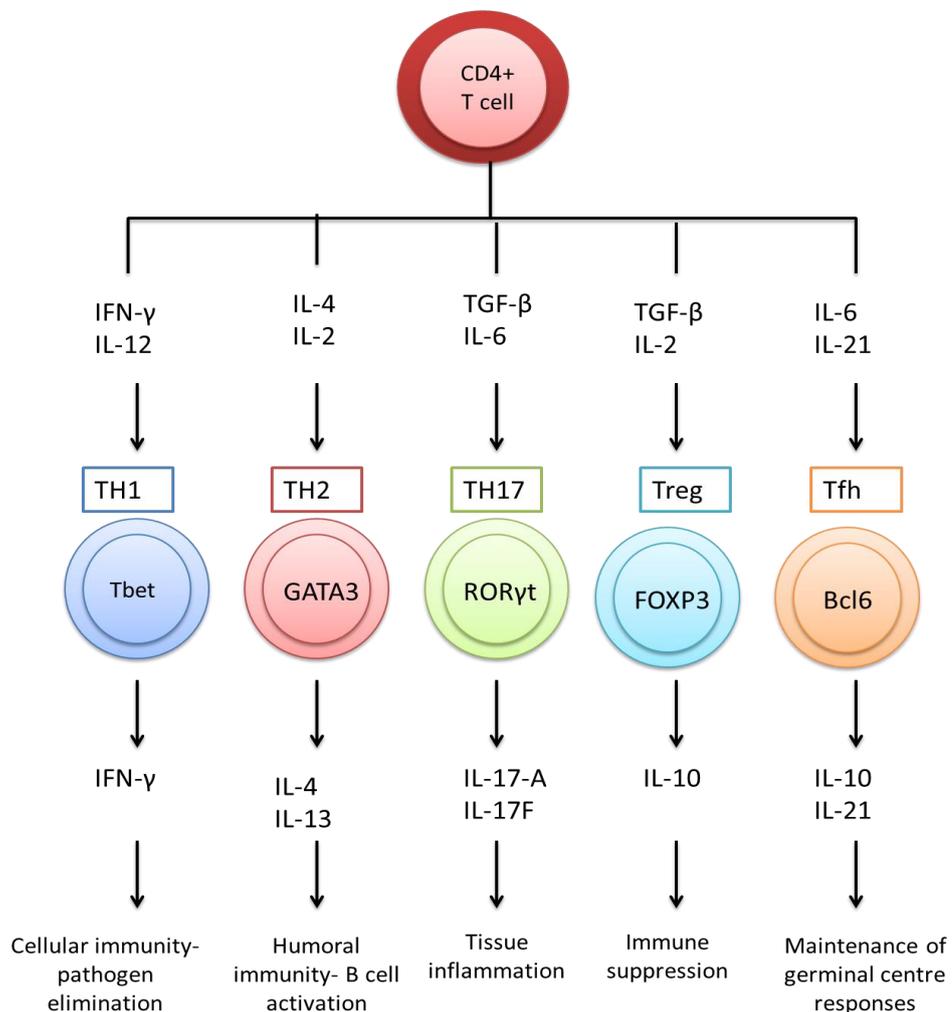
Figure 13: T helper cell subsets

CD4+ T cells are able to differentiate into 5 main T helper (Th) subsets: T helper 1 (Th1) subset is influenced by the production of IFN-γ and IL-12. These cytokines induce T bet expression and in turn Th1 cells produce IFN-γ. The T helper 2 (Th2) subset is influenced by the production of IL-4 and IL-2. this in turn causes the expression of transcription factor GATA-3 within the Th2 cells. Th2 cells produce IL-4 and IL-13 and play a role in humoral immunity. The T helper 17 (Th17) subset is influenced by the production of IL-6 and TGF-B by CD4+ T cells, this allows for RORgt expression and production of IL-17A and IL-17F to aid in tissue inflammation. The T regulatory (Treg) subset is also influenced by the production of TGF-B with IL-2, this allows for transcription factor FOXP3 expression and production of IL-10 by the cells. Treg cells paly a major role in immune suppression and tolerance. The T follicular help (Tfh) subset is produced after increased levels of IL-6 and IL-21. this allows for the expression of Bcl6 and production of IL-10 and IL-21 and play a role in the maintenance of germinal centre response.

1.4.2.2   T helper cell plasticity

CD4+T cells have plasticity to enable them to adapt quickly and deal with pathogens including bacteria and viruses. Different subsets are thus sometimes able to influence others in order to best deal with the pathogen and restore immunological balance. Some groups have been shown to have increased plasticity compared to others. This has been attributed to a number of mechanisms that influence this such as epigenetics, cytokine signals, transcription factor co-expression and the stability of the cell cycle as shown in Figure 14 (Coomes, Pelly and Wilson, 2013).

DNA methylation

Cell cycle stability

Mechanisms of T cell plasticity

Cytokine signalling

miRNA mediated regulation

Immune cells and receptors
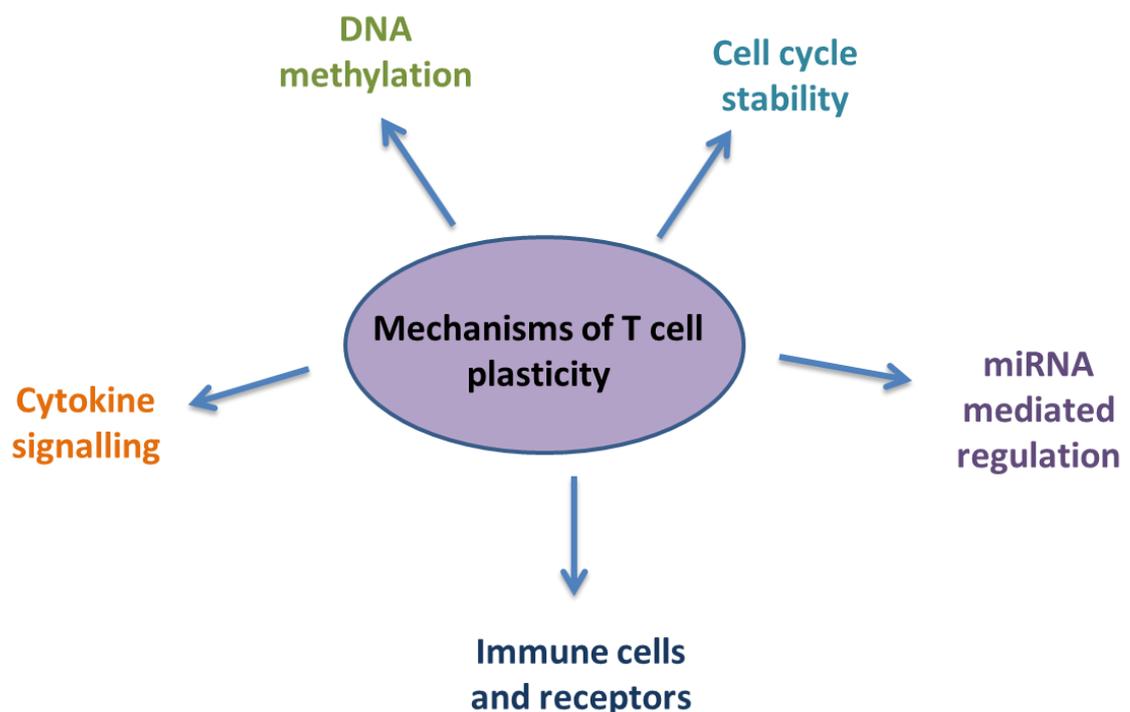
Figure 14: Mechanisms of T cell plasticity

There are a number of mechanisms that have been found to play a role in T helper cell plasticity. They include; cell cysle progression, miRNA mediated regulation, immune cell function, cytokine signalling and DNA methylation.

The relationship between Th1 and Th2 has been closely studied. The plasticity of these cells decreases with increased rounds of cell division (Grogan *et al.*, 2001). That is in the early stages Th1 and Th2 can convert into one another as shown by the groups Murphy *et al.* (1996)

and Zhu *et al.* (2006). They showed partly differentiated Th2 cells can produce IFN-γ after IL-22 induction while Th1 cells can also induce IL-14 (Zhu *et al.*, 2006; Murphy *et al.*, 1996).

Th17 cells have also been found to have great plasticity. It has been reported that its inhibition or suppression can be achieved by IL-4, IFN-γ and IL-2 (Coomes, Pelly and Wilson, 2013). Cells that secrete IFN-γ potentially stemmed from Th17 cells (Hirota *et al.*, 2011), and Th17 cells can become Th1 cells in vitro. Recent data collectively shows Th17 can influence Th1 secretion and T bet expression (Coomes, Pelly and Wilson, 2013).

It has also been reported that Th17 cells can become Th2 cells if programmed properly (Coomes, Pelly and Wilson, 2013). Th17 cells were found to produce IL-4 after culture in Th2 conditions (Panzer *et al.*, 2012). T reg cells have also been shown to be unstable and have high plasticity. It was shown that FOXP3+ T reg cells could become Th1 cells. FOXP3 deletion in T reg cells results in high IFN-γ and IL-2 production (Coomes, Pelly and Wilson, 2013) indicating the Th1 subset. It was also reported that TGF-β promotes both RORγt and Foxp3 (Zhou *et al.*, 2008). It was reported that the development of Th17 or T reg is dependent on the presence or absence of IL-6 (Xu *et al.*, 2007).

1.4.2.3   T helper subset functions in adaptive immunity

Each T helper cell subset plays an essential role in immunity. The effector T cell influences the type of immune response. Th1 cells play a big role in the elimination of pathogens (Del Prete, 1992). This is through the effector function of IFN-γ production leading to an inflammatory response. They are able to activate macrophages and thus kill pathogens. IFN-γ receptors on the macrophage surface bind to IFN-gamma produced by Th1 cells. Macrophages then express CD40 on the T cell resulting in phagocytosis (Murray *et al.*, 1985). CD8+ T cells can also be activated by the production of Il-2 from Th1 cells and encourage cytotoxic function. In addition, IL-2 promoted memory in CD8 T cells following infection (Williams, Tyznik and Bevan, 2006).

Th2 cells are able to produce a multitude of cytokines including IL-4, IL-9, IL-10, IL-25 and IL-5. They have been reported to stimulate B cells along with Th1 cells. Particularly, Th2 cells stimulate B cells during class switching of IgE via IL-4 (Luckheeram *et al.*, 2012b). IL-4 and IL-6 also allow for the inflammatory setting to be increased through activation of GMCSF

(granulocyte-macrophage colony-stimulating factor) (Doucet *et al.*, 1998). IL-10 functions to clear immune cells following a heightened immune response thus maintaining homeostasis (Couper, Blount and Riley, 2008). Th17 cells respond mainly to fungi and bacteria. Their production of IL-17A also contributes to the development of autoimmune disease via induction of pro-inflammatory cytokines such as IL-6 and TNF-α (Luckheeram *et al.*, 2012a). Th17 cells also produce IL-2 that is able to activate B cells to differentiate into memory cells. T follicular help cells function mainly in the germinal centre in the development of memory B cells (Fazilleau *et al.*, 2009).

1.4.2.4   Intrinsic mechanisms of tolerance

Ignorance

T cell ignorance takes place when a T cell presented to a self-antigen does not have a high enough affinity to result in stimulation, leaving the cell to remain 'ignorant' (Parish and Heath, 2008; Parish and Cooke, 2004). These cells thus exist within the T cell repertoire in the periphery and are able to become reactivated and contribute to autoimmune disease later, especially after viral infection (Ohashi *et al.*, 1991).

Senescence

Senescence is an irreversible state that relates directly to telomere shortening. It is a mechanism that results in the permanent cell cycle arrest which is due to the cells' inability to divide and grow. Senescence that has recently been added as one of the mechanisms of tolerance (Schietinger and Greenberg, 2013).

Exhaustion

Exhaustion takes place when a T cell is exposed to an antigen repeatedly resulting in the cell gradually losing its effector function. This is most commonly seen during chronic infection. This process has been closely studied within CD8 T cells compared to CD4 and is associated with the expression of B Lymphocyte induced maturation protein 1 (Blimp-1) and Eomesodermin (Emoes) (Shin *et al.*, 2009). This state is neither fixed or irreversible thus it lacks the ability to clear out the pathogen (Schietinger and Greenberg, 2014). It has been reported that there is a sustained expression of PD-1 within T cell exhaustion which is speculated to support tolerance

induction, and this has been found to be the case within chronic viral infection (Wherry *et al.*, 2007).

CD4 T cell anergy

T cell anergy is a tolerance mechanism wherein a lymphocyte is functionally inactivated following an antigen encounter in the absence of co-stimulation (see Figure 15). This is an active state, and the cell remains alive for an extended period of time, albeit in a hyporesponsive state. The state must be more than 24 hours long to exclude cells that have begun apoptosis (Shwartz, 2003). The hallmark of T cell anergy is the failure to synthesize the T cell growth factor IL-2 resulting in decreased proliferation (Wells, 2009). Anergic cells have been found to not only have a reduction in IL-2 secretion but in a number other cytokines including IFN-γ, TNF-α and IL-3 (Zheng, Zha and Gajewski, 2008). This state of clonal anergy was found to be reversible by the addition of IL-2 (Fathman and Lineberry, 2007).

It has been found that disruption of the cell cycle can also lead to anergy. Cell cycle progression blocks have been used to prove this theory. For example, when rapamycin is added to fully stimulated T cell clones, it leads to anergy and this is a similar case with hydroxyurea. Since rapamycin blocks the cell cycle at G1 and hydroxyurea blocks at early S phase, it is hypothesised that G1 to S phase is where co-stimulation exerts its greatest effect (Shwartz, 2003). Induction of CD4 T cell clonal anergy can be achieved not only be giving a cell part of stimulation, but by competition and co-inhibition. Cytotoxic T lymphocyte antigen (CTLA-4) is able to compete with B7 for CD28 binding (Shwartz, 2003). B cell anergy has been thought to result from defective B cell receptor signalling and chronic antigen receptor occupancy leading to the downregulation of surface IgM (Gauld *et al.*, 2005).

Maintenance of CD4 T cell anergy-transcription networks

The lack or absence of co-stimulation leads to anergy induction. This results in reduced efficiency of MAPK and other pathways, resulting in reduced AP-1 and NFKb activity within the nucleus and decreased IL-2 (Wells, 2009). A number of essential pathways have been proposed to maintain this state. In order for anergy to take place, a number of things must happen within the T cell: TCR activation leads to an increase in intracellular calcium via the molecule ZAP70; this calcium flux allows for NFAT translocation; and co-stimulation within the

T cell causes MAPK activation where the DAG which lies in the membrane activates RAS guanyl-releasing protein 1 (RASGRP-1) which phosphorylates RAS GTP to MAPK activation and AP-1 expression. Within tolerant cells the TCR activation is normal however with a lack of CD28, DAG is phosphorylated by diaglycerol kinase alpha (DGK-α) into phosphatidic acid. Thus there is lack of activation of RASGRP-1 and lack of MAPK activation and AP-1 formation (Shwartz, 2003).

NFAT signalling and CD4 T cell anergy

A very important part of T cell activation is the calcium flux caused by antigenic stimulation; this leads to the activation of NFAT more than any other signal. This particular signal has been found to be the most critical in the maintenance of CD4 T cell anergy; this must be able to take place even in anergic cells in order to allow NFAT mobilisation to the nucleus (Harris *et al.*, 2004).

Map kinase pathway and anergy

There have been multiple blocks in the map kinase pathway identified with anergy. It was found that the DNA binding of the activator protein-1 (AP-1) in anergic T cells was reduced in reporter assays. Studies of the signalling pathway found defects in the activation of the immediate upstream molecules of the mitogen activated protein kinase (MAP-K) families (DeSilva *et al.*, 1996; Li *et al.*, 1996). It was also found that in the lack of activation of MAPKs extracellular signal-regulated kinase 1 (ERK1) and or ERK2 in anergic T cells results from a defect in the GTP loading of RAS. Even though its guanine-nucleotide exchange factor (GEF) was activated normally via son of seven less homologue (SOS) (Fields, Gajewski and Fitch, 1996).
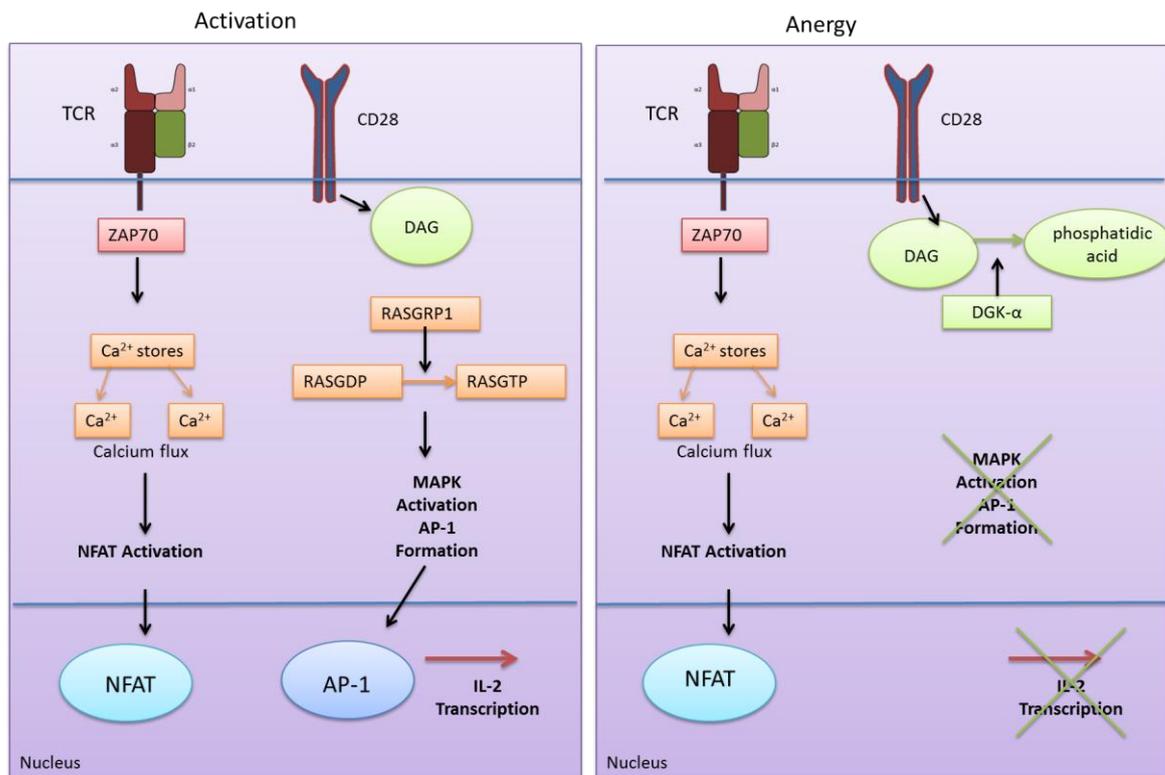
Figure 15: Mechanism of CD4+ T cell anergy

On activation of the T cell receptor (TCR) and costimulatory molecule (CD28), TCR is able to binding to activate ZAP70, this releases activated calcium (Ca^{2+}) into the cytoplasm. This calcium flux causes activation of NFAT and allows for its translocation into the nucleus. CD28 activation allows for diacylglycerol (DAG) activation and its activation of RAS guanyl-releasing protein 1 (RASGRP-1), RASGRP-1 phosphorylates RASGDP to form RASGTP and cause mitogen-activated protein kinase (MAPK) activation and formation of AP-1 through its dimers cFOS and cJUN. Its translocation into the nucleus on meeting NFAT allows for IL-2 transcription to take place leading to proliferation and differentiation. However during anergy there is lack of co-stimulation. DAG is phosphorylates by diacylglycerol kinase alpha (DGK-α) to form phosphatidic acid. Thus RASGRP-1 loading is not possible and there is a lack of MAPK activation and AP-1 formation leading to hindrance in IL-2 transcription.

RAS has been found to be defective in anergic cells, thus the defect is in RAS activation. Therefore, downstream MAPK activation could be overcome by the addition of diacylglycerol (DAG) analogue phorbol 12-mysristate 1 acetate (PMA) to cultures of T cells during re-stimulation (Fields, Gajewski and Fitch, 1996). Another pathway proposed for RAS activation in T cells is independent of SOS and activated by the protein kinase C (PKC) activator PMA (Downward *et al.*, 1990).

RASGRP-1 is recruited to the Golgi membrane by increases in the levels of DAG and intracellular calcium following T cell activation. Once there, the RSGRP1 promotes the conversion of GDP to GTP on RAS leading to the activation of the MAPK pathway. Defects in the RASGRP-1 pathway may lead to the induction of T cell anergy as RASGRP-1 deficient mice are deficient in proliferation and maturation. DGKs (diaglycerol kinases) phosphorylate DAG into phosphatidic acid, reducing the amount of DAG that would be able to activate RASGRP-1. Its inactivation would interfere with the MAP kinase-AP-1 pathway blocking T cell activation and lead to anergy (Zheng *et al.*, 2012).

Anergy reversal and inhibition

The need for a lack of co-stimulation for anergy induction is not the factor that dictates the avoidance of anergy. IL-2 is able to reverse the anergic state, meaning IL-2 signalling though the IL-2 receptor is the signal that allows the escape form anergy. Il-2 binding to the IL-2 receptor induces activation of the Jak-Stat and MAPK pathway allowing for the reversal of anergy (Wells, 2009).

NFAT translocation is controlled in a calcium dependant manner. The drug cyclosporine A has been reported to play a significant role in anergy inhibition. This immunosuppressive drug is able to inhibit the function of calcineurin, which is essential for NFAT translocation into the nucleus (Jenkins *et al.*, 1990)(Shwartz, 2003). Studies have shown that when cyclosporine A is added to culture with cells receiving only an antigenic stimulation, anergy is inhibited and cells are able to produce a normal amount of IL-2 (Yamamoto, Hattori and Yoshida, 2007)(Jenkins, Schwartz and Pardoll, 1988)(Fathman and Lineberry, 2007).

E3 ubiquitin ligases and anergy

Ubiquitin ligases have been studied in the anergy setting recently. The five major ones under study are Cbl-b, Itch, Grail, c-cbl and Nedd4. These proteins are responsible for flagging proteins for degradation and destruction.

Cbl-b expression is linked to calcium mobilisation and calcineurin activation during anergy induction (Heissmeyer *et al.*, 2004). It plays an important role in the downregulation of TCR expression during antigen recognition (Naramura *et al.*, 2002), with Cbl-b deficient mice developing spontaneous autoimmunity (Krawczyk *et al.*, 2000). There is increased C-blb

expression within anergic T cells, leading to the recycling and ubiquitination of TCR in endocytic vesicles (Mueller, 2004).

Grail expression is also increased within anergic T cells, and studies show increased mRNA levels in induction using calcium ionophore such as ionomycin (Mueller, 2004). Itch expression increases with anergy induced by ionomycin with levels similar to grail and Cblb.

1.4.3 Extrinsic mechanisms

1.4.3.1 T regulatory cells

T reg cells are able to function by providing protection in the periphery. There are two main subsets of T reg cells described. These are the naturally occurring T regs and the adaptive/IL-10 producing iT reg cells.

Naturally occurring T regulatory (nTregs) cells CD4+CD25+

Naturally occurring T reg cells were first described by Sakaguchi *et al.* (2005). These T reg cells develop in the thymus and are generated during early events of T cell development. Phenotypically they are CD4+CD25+ and also express high levels of cytotoxic T-lymphocyte antigen-4 (CTLA-4) and glucocorticoid-induced TNF receptor (GITR). These cells represent 5-10% of CD4+ T cells in healthy human adults (O'Garra and Vieira, 2004)(O'Garra and Vieira, 2004)

. In the periphery, natural T reg cells function in a contact-dependant manner to stop the development of self-reactive T cells. nTregs are able to bind directly to either the T helper cell or the antigen presenting cell, inhibiting the development of autoimmunity (Bluestone and Abbas, 2003).

Adaptive/inducible T regulatory cells (iTregs)

Adaptive T reg cells are generated form mature T cells populations following specific antigen stimulation (O'Garra and Barrat, 2003). Adaptive T reg cells originate from the thymus but are derived from classical T cell subsets (Bluestone and Abbas, 2003). They receive additional differentiation after further antigenic stimulation, and may not require co-stimulation through

CD28 to be functional. They have been found to be cytokine dependant and once activated release cytokines such as IL-10 and TGF-β (O'Garra and Barrat, 2003).

IL-10 producing T regs are known as T regulatory 1 (TR1) and function by inhibiting the production of TNF and IL-12 primarily produced by macrophages. CD8+ T regulatory t cells have also been identified. They are able to produce IL-10 or TGF-β- Figure 16(Bluestone and Abbas, 2003).
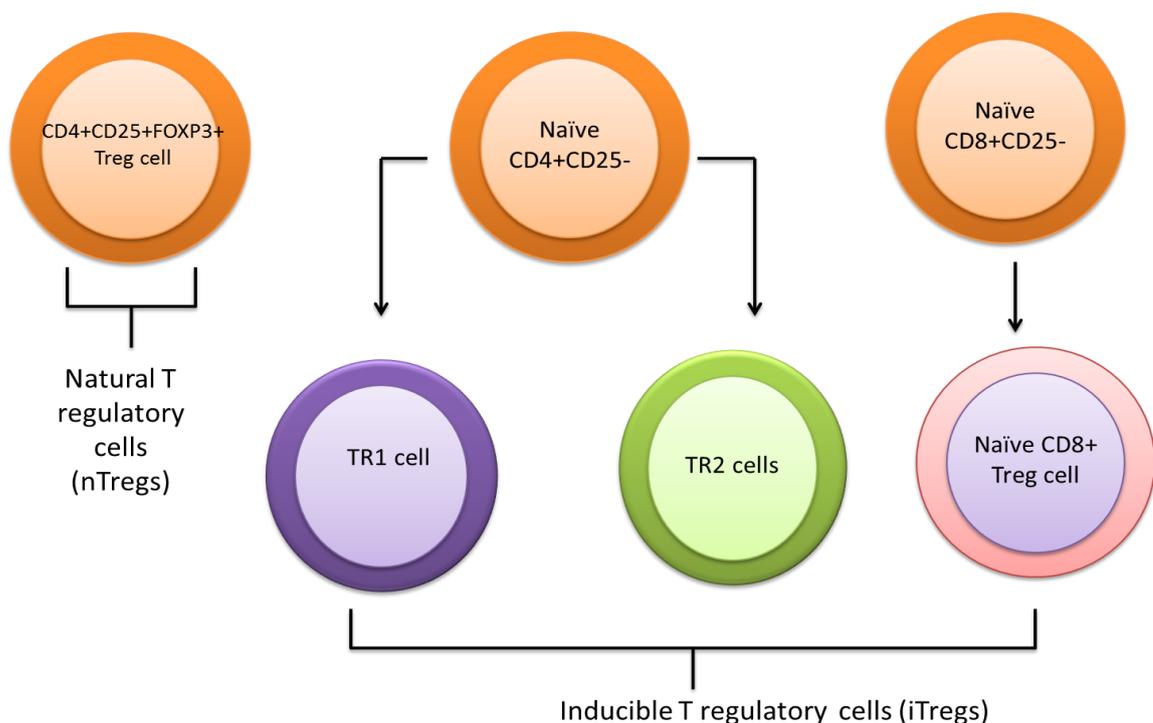
Figure 16: Production of T regulatory T cells

T regulatory cells (Treg) are first produced in the thymus. On meeting a double positive t cell meeting a self-antigen at an intermediate affinity, Natural Treg (nTreg) (CD4+CD25+FOXP3+) cells are produced. Naïve CD4+T cells are able to become inducible Treg (iTreg) cells within the periphery on meeting self or foreign antigens. iTregs include the TR1 subset and the TR2 subset. Naïve CD8+ T cells are able to produce CD8+ Treg cells following encounters with self or foreign antigens within the periphery.

1.5 Autoimmunity

One of the roles of the immune system is to effectively prevent attack on self by reactive self-antigens leading to autoimmunity. The main function of central tolerance being clonal deletion and peripheral to ensure self-reactive T cells are kept at bay. This system is robust although it occasionally breaks down. This breakdown can be due to a number of reasons that include altered apoptosis, decreased clearance of self-antigens, and reduced fitness of T reg cells that may in turn lead to the development of autoimmune disease (Wasterberg, Klein and Snapper, 2008). The main cytokines that play a role in the induction of autoimmunity include IFN-γ, IL-17 and IL-22, and if the productions of these cytokines are not monitored such as by the production of Treg cells, autoimmune disease ensues.

1.5.1 Mechanisms of tolerance breakdown

1.5.1.1 Environmental influence

Environmental triggers play a large role in autoimmune disease development. Triggers that lead to tolerance breakdown include exposure to UV radiation, smoking, infection, toxins and antibiotics (Dooley and Hogan, 2003) and they have effects that lead to the development of autoimmune disease. Dysregulation of the immune system plays the primary role, wherein Treg cell production is compromised (Vojdani, 2014). Toxins have been much studied as factors that causes autoimmune disease, working to either induce aberrant cell death or by the mechanism of xenobiotics which are organic compounds that are able to induce antibody production against tissue protein following binding. It has been reported that chemicals as whole play a very large role in that they are able to alter proliferation and stimulate release of reactive oxygen species (Vojdani, 2014). Infection with certain viruses or bacteria may also trigger an increase in autoreactive antibodies or T cells leading to periods of exaggerated immune responses (Wucherpfennig, 2001). The mechanisms that can be induced include induced cell death which causes release of toxic material leading to inflammation. Molecular mimicry takes place, in which foreign antigens have similar structure to self-antigens leading to activation of T cells that are cross-reactive with self-antigens (Kivity *et al.*, 2014). This can be seen in cases of herpes simplex virus antigen which cross-reacts with the cornel antigen leading to stromal keratitis. Epitope spreading can also take place where a protein changes

confirmation and later causes the induction of autoimmune disease as seen in the mouse model of encephalomyelitis (multiple sclerosis) (Dooley and Hogan, 2003) (Reeves *et al.*, 2009). Bystander activation and stimulation of pattern recognition receptors (PRRs) can also take place, where viral antigens stimulate PRRs, activation of these receptors leads to the release of inflammatory cytokines via the APC (Duke, 1989). Another factor is that of dietary components, and studies have shown that an increase in sodium intake can aggravate autoimmune disease by activation of Th17 cells (Kleinewietfeld *et al.*, 2013). Sex has also been found to play a role with more women being susceptible to autoimmune diseases such systemic lupus due to high levels of oestrogen (Tiniakou, Costenbader and Kriegel, 2013).

1.5.1.2 Genetics

Genetic predisposition is a well-studied factor contributing to the development of autoimmune disease. It has been found that a large amount of predisposition has been attributed to association with the human leukocyte antigen (HLA) which encode for MHC genes. Rheumatoid arthritis is attributed to the HLA-DR4 while Type 1 diabetes is attributed to HLA-DR3. T regulatory cells are also affected and it has been reported a genetic defect in T regs leads to X-linked recessive disorder IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) (Ray *et al.*, 2012).

1.5.1.3 Dendritic cells (DCs)

Dendritic cells have recently been found to play a role in autoimmune disease development. It is well known that DCs, which are professional antigen presenting cells, are able to promote and maintain tolerance and aid in adaptive immune system response, thus they are immunogenic. The two types of DCs that are implicated in autoimmunity are the myeloid DC (mDC) also known as the conventional DC (Cheong *et al.*, 2010) and plasmacytoid DCs which express CD45R and are generally quiescent (Reizis *et al.*, 2011). Tolerogenic DCs work to sustain self-specific tolerance and promote anergy, deletion and apoptosis of T cells. Within rheumatoid arthritis it has been reported that mDCs and pDCs are highly expressed and activated. Leading to their production of interferons (IFNs) and increased expression of MHC class II encouraging T cell activation (Santiago-Schwarz, 2004). In EAE the which is the animal equivalent of Multiple sclerosis, it has been found that mDCs are involved in disease development and pDCs which are usually able to increase the production of Treg cells produce

IFNs (Bailey-Bucktrout *et al.*, 2008). Clinical studies have shown increased frequency of MDCs within the central nervous system (CNS) which allows for reactivation of T cell responses to the myelin (Amodio and Gregori, 2012). In addition, the mDCs have been found to produce IFN-γ meaning the activated CD4 T cells are polarised toward Th1 responses by the activated mDCs (Vaknin-Dembinsky *et al.*, 2008).

1.5.1.4   Immune regulation

Immune regulation plays a large role in the development of autoimmune disease development. If regulation of the well-established tolerance mechanisms breaks down, autoimmune disease will ensue. This is seen in the disruption of mechanisms such as anergy, deletion and ignorance which lead to autoimmune disease development. Natural T reg cells express FOXP3 and it has been shown that mutations in FOXP3 lead to the autoimmune disease IPEX immune dysregulation polyendocriopathy X linked syndrome (Refaeli, Van Parijs and Abbas, 1999)

(Refaeli, Van Parijs and Abbas, 1999; (Gambineri, Torgerson and Ochs, 2003). Fas-FasL interaction allows for cell death within lymphocytes to take place, and mutations in Fas genes lead to the development of an autoimmune lymphoproliferative syndrome. CTLA-4, which is essential for T cell immunoregulation, has been found to play in a role in lupus development () and in type 2 diabetes (Chu *et al.*, 1996) caused by polymorphisms in gene. It has also been found that CTLA-4-/- mice develop a lymphoproliferative disease (Refaeli, Van Parijs and Abbas, 1999).

1.5.1.5   Inflammation

Inflammation may play one of the largest roles in the development of autoimmune disease and is assisted by the maturation of dendritic cells (Ray *et al.*, 2012). Danger signals can be presented by invading pathogens such as bacteria and viruses which result in inflammation. Inflammation induced maturation of DCs provides a pivotal role in autoimmune development following infection (Turley, 2002). T cells that escape tolerance against self-peptides in the thymus are possibly activated in the periphery when the peptides are altered due to outside stimulus, leading to T cell organ infiltration and inflammation (Skapenko, Lipsky and Schulze-Koops, 2006).
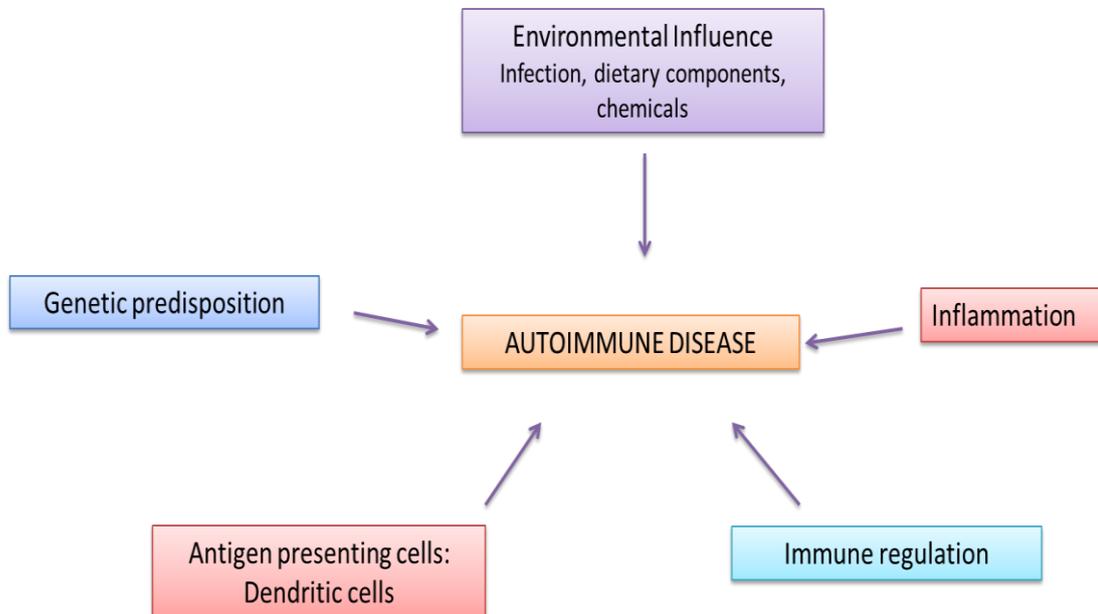
Figure 17: Tolerance breakdown mechanisms

Autoimmune disease can ensue due to a number of factors; environmental influence, Inflammation, Immune regulation, Genetic predisposition and alteration of dendritic cell function. These factors have been shown to aid in tolerance breakdown leading to autoimmune disease.

1.5.2   Systemic and organ specific autoimmune disease

Autoimmune diseases area particular problem in the world today as most lack a cure and are difficult to control. For this reason, a large amount of research is being carried out to better understand them (Rioux and Abbas, 2005). It has been reported that women are more affected by autoimmune disease than men, and show more severe symptoms (Whitacre, 2001). Autoimmune disease can be classified as either organ specific or multisystem/systemic. Organ specific autoimmune diseases are recognised by attack on one specific organ while in systemic autoimmune disease autoantibodies are directed against multiple organs or molecules in the body (Sinha, Lopez and McDevitt, 1990). In organ specific autoimmune disease auto-antibodies are directed toward a specific organ, as seen in Type 1 diabetes where anti-insulin antibodies are found. Systemic autoimmunity has antigens that are present within most cells in the body, as in SLE (systemic lupus erythematosus) where there are anti-chromatin auto-antibodies present which affect a wide range of cells (Janeway,Charles,A,Jr *et al.*, 2005).

1.5.3 Autoimmune diseases with inflammation

Inflammation plays a huge role in the development of autoimmune disease and has remained a hallmark in multiple disorders (Theofilopoulos and . Bona, 2002). Within rheumatoid arthritis the production of cytokines such as 1L-6, TGF-α and IL-17 are able to encourage tissue degradation by means of inflammation. In particular, IL-6 is able to call on neutrophils that greatly contribute to joint damage and inflammation by secretion of oxygen intermediates (Dayer and Choy, 2010). TNF-α causes increased monocyte activation and T cell apoptosis and TCR dysfunction (McInnes and Schett, 2007). Another inflammatory autoimmune disease is SLE, which is characterised by the production of auto-antibodies (Mok. C.C, 2003). Within systemic lupus self-reactive T cells which escape the thymus become activated. These T cells can then provide help to B cells for antibody production and secrete pro-inflammatory cytokines, both adding to tissue damage and release of self-antigens (Shlomchik, Craft and Mamula, 2001).

1.6 Experimental settings for tolerance induction

1.6.1 Induction of CD4 T cell clonal anergy in vitro

In vitro anergy was first described by Schwartz and Jenkins in the 1980's using CD4 + T cell clones. T cell clones were used and their reactions compared with chemically fixed APCs and normal APCs. They found that T cells reacting with the fixed APCs produced less IL-2 and proliferated poorly compared to the live APCs. Once they re-challenged them, they were found to be hyporesponsive (Jenkins *et al.*, 1987a)(Schwartz *et al.*, 1989) indicating an unresponsive tolerant state they named anergy.

There are a number of ways to induce anergy in vitro. The most common is the delivery of a strong TCR signal in the absence of co-stimulation (Wells, 2009) using the plate bound peptide CD3 specific antibody alone to stimulate CD4 T cells (Fathman and Lineberry, 2007). Another method of inducing T cells anergy is the use of ionomycin. This is a calcium ionophore that functions by inducing the release of intracellular calcium stores, leading to the activation of NFAT proteins much like the CD3 stimulation although using an intracellular pathway (Fathman and Lineberry, 2007).

1.6.2 In-vivo tolerance induction

Adoptive tolerance or in vivo anergy is described as the dysfunction of T cells induced by suboptimal in vivo stimulation (Schietinger and Greenberg, 2013)(Schietinger and Greenberg, 2014; Schietinger and Greenberg, 2013)

2014). There are a number of models that have been used to induce this state of unresponsiveness: the systemic delivery of superantigens; adoptive transfer of TCR transgenic T cells into hosts that express conjugate antigen as a self-antigen; and administration of soluble peptide antigen into TCR transgenic mice (Dubois *et al.*, 1998; Pape *et al.*, 1998; Kawabe and Ochi, 1990).

1.6.2.1 Superantigens

Superantigens are proteins that are able to bridge the surface of MHC class II molecules and make use of the TCR Vβ domain that lies outside the antigen recognition sites (see Figure 18) (Fathman and Lineberry, 2007; Kawabe and Ochi, 1990). Examples include staphylococcal enterotoxin A and B which are bacterial superantigens. The mechanisms of viral superantigens have not been as closely studied as bacterial, although have a similar mode of binding to cause T cell stimulation (Janeway,Charles,A,Jr *et al.*, 2005). Unlike other proteins, superantigens recognised by T cells without the need for processing into peptides which are otherwise captured by MHC molecules because they are able to bind to the Vβ regions and the MHC class II molecules away from the complementary site formed by TCR and MHC molecules (Fraser, 2011). It has been reported that superantigens are able to stimulate 2-20% of all T cells.

Stimulation via superantigens causes a massive production in cytokines by CD4 T cells, which in humans causes diseases such as food poisoning and toxic shock syndrome (Fraser, 2011). Bacterial superantigens are now being used within the tolerance mechanism to induce tolerance. It has been reported that multiple injections of bacterial superantigens are able to induce a state resembling that of in vitro anergy characterised by decreased cytokine production and decreased proliferation (Shwartz, 2003). Initial superantigen stimulation results in a large portion of T cells being activated resulting in increased expression of cytokines such as IL-2, IFN-γ and TNF-α. Once there is a surge, it is followed by a sudden decrease in T cell numbers over a period of about three days apparently due to the

mechanism of apoptosis. Huang *et al*. (2007) showed that the remaining T cells are hyporesponsive by a mechanism similar to that of anergy (Huang *et al.*, 2007; Kawabe and Ochi, 1990). Multiple groups have used superantigens to maintain this form of tolerance that required continuous administration of stimulation in vivo (Huang *et al.*, 2007).
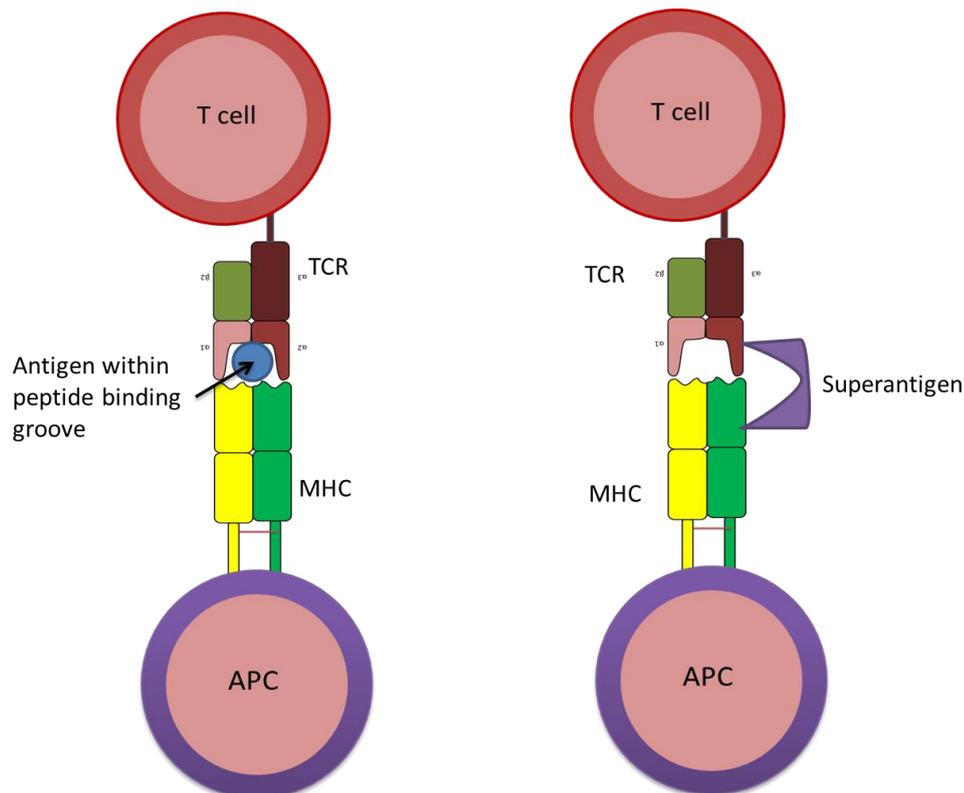
Figure 18: Comparison of TCR binding and superantigen binding

TCR signalling takes place when the MHC Class II molecule carrying an antigenic peptide on the APC meets the TCR on the T cell. Superantigen binding is able to bind of outside of the peptide binging groove to the TCR and the MHC class II molecule in the absence of antigen recognition

1.6.2.2 Repeated antigenic stimulation

The use of peripheral models has become the hallmark for tolerance induction in vivo and TCR transgenic mice have been used as a major tool in these processes. It has been found that repeated antigenic stimulation leads to the induction of in vivo tolerance (Shwartz, 2003). The administration of soluble peptides has been used with cells from transgenic mice to induce anergy (Pape *et al.*, 1998). Cells transferred from transgenic mice into normal hosts and repeatedly challenged with soluble peptides have been shown to firstly result in T cell death.

However, the remaining cells have been shown to be unresponsive to further stimulation, express perturbed IL-2 production and are less proliferative. On lack of constant peptide administration cells regained their ability to proliferate and produce IL-2 at normal levels (Rocha, Grandien and Freitas, 1995)(Shwartz, 2003).

1.6.2.3 Oral tolerance induction

Oral tolerance takes place when an orally administered antigen, such as OVA or anti-CD3, is able to supress immune responses, either locally and systemically. This is caused with the use of food antigens at different doses that are able to cause decreased proliferation when administered on a TCR transgenic (Tg) model which allows all T cells to have a common TCR. This results in antigen-specific tolerance (Pabst. and Mowat., 2012). Oral tolerance induction typically results in the reduction of cytokine production, T cell-proliferation, and systemic delayed-type hypersensitivity. This process involves the mucosal system that is the gut-associated lymphoid tissue (GALT). Oral tolerance depends on this system for functionality. The mechanisms of the antigen intake play a significant role using sites such as Peyres's patch (PP) and the gut-draining mesenteric lymph nodes (mLNs) for induction and effector sites such as the lamina propria (LP) and intestinal epithelium. Orally administered antigens are first recognised by dendritic cells (DC) in the mLNs. Studies have shown that uptake through the intestine depends on molecular weight, with low molecular weight material such as polysaccharides cross directly through the epithelium via diffusion while larger molecules pass via apical membrane transportation. Dissemination of the antigen has been speculated to take place via the liver (Pabst. and Mowat., 2012). There have been multiple forms of oral tolerance described and it has been reported that one of the main determinants is the antigenic dose. Studies have found that at low doses of antigenic feed the induction T regulatory cells are triggered while at high doses anergy is induced. Induction of oral tolerance has been found to result in the production of T regulatory cells such as IL-10 producing Tr1 cells, natural Treg cells and Th3 Treg cells (Pabst. and Mowat., 2012). Reports have shown oral tolerance requires β-7 integrin gut homing of iTregs (Hadis *et al.*, 2011).

1.6.2.4 Adoptive transfer

Like antigenic stimulation, adoptive transfer relies on transgenic models, especially those within a Rag 2-/- background. Mice that lack Rag 2 have been found to have no mature

lymphocyte population due to defects in VDJ arrangements; this allows them to act as a neutral background for immune response studies (Shinkai *et al.*, 1992). Tanchot *et al*. (2001) first described this method. They took CD4 T cells from specific for pigeon cytochrome c (PCC) and transfected TCR transgenic T cells on a rag 2-/- background into CD3-/- mice in order to induce tolerance. They reported decreased levels of IL-2 by 10 fold and proliferation in remaining T cells along with decreased IFN-γ and IL-10 IL-4, IL-3 (Shwartz, 2003; Tanchot *et al.*, 2001). The use of Rag2-/- mice within oral tolerance can be seen in studies with antigens such as OVA. Where hypo responsiveness to a fed antigen is caused due to re-challenge with the same antigen; the Rag2-/- background allows for a neutral setting to monitor tolerance induction via proliferation (Faria and Weiner, 2006). Rag2-/- mice have been used in conjunction with other transgenic models in a similar mechanism to oral tolerance induction. Safford *et al.* adopted the use of 6.5 TCR transgenic (Tg) mice that are specific for the hemagglutinin antigen and C3-HA mice which express hemagglutinin as a self-antigen. These mice were backcrossed in the TCR Tg Rag2-/- B10.D2 background. To induce tolerance, they took T cells from the 6.5 TCR tg mice and adoptively transferred them into the C3-HA mice on the TCR Tg Rag2-/- B10.D2 background. This was successful as the self-peptide within the C3-HA mice was able to recognise the hemagglutinin (homologous antigen) as it is an adjuvant resulting in antigen-specific tolerance. This allowed for the monitoring of gene upregulation and downregulation within a neutral tolerant background (Safford *et al.*, 2005).

1.7 Early growth response (Egr) genes

1.7.1 Egr family

The early growth response gene family is made up of four members: Egr1 (Krox24 and nerve growth factor inducible (NGFI)-A), Egr2 (Krox20), Egr3, and Egr4 (NGFI-C). They are a family of DNA-binding zinc finger transcription factors which are induced when T cells are activated (Eva L. Decker, 2003). All Egr members share a highly conserved zinc finger motif; they all contain three cys2his2 that bind to a GC rich consensus motif GCGGGGGCG (Carleton *et al.*, 2002). Their DNA binding zinc finger domains are very closely related with about 82% homology, but their flanking regions are what differentiate them. This region is able to indicate the specific function of each EGR protein (Eva L. Decker, 2003; Carleton *et al.*, 2002). These genes are

expressed in a large number of cells including B cells, neurons and thymocytes. A number of binding elements for EGR proteins have been identified in cytokines such as IL-2 and cell surface receptors such as FasL (Eva L. Decker, 2003).

1.7.2 Egr function

The early growth response genes have been studied closely, with each gene found to have a specific function within the body. Early studies of these genes were in relation to neurobiology with the Egr1 taking the lead in research (Beckmann and and Wilce, 1997). Egr1 was the most heavily studied with its functions linked to neurobiology. It was reported to be expressed after activation from injury, stress and even multiple growth factors (Pagel and Deindl, 2011). It has also been reported to be essential in the development of memory (Jones *et al.*, 2001) with Egr1 deficient mice being unable to develop long term memory (Jones *et al.*, 2001).

Egr2 had been reported to have primary functions that lay in peripheral nerve myelation, where it has been found to be essential (Topilko *et al.*, 1994). It also plays a role in bone formation (Levi, 1996) and hindbrain segmentation (Nonchev *et al.*, 1996). Egr3 is reported to play a role in muscle spindle formation with Egr3-/-mice showing defects in this process (Tourtellotte and Milbrandt, 1998a).

Egr1, 2 and 3 have all been linked to lymphocyte development; Carleton *et al*. (2002) first reported the transcription factors are upregulated during thymocyte differentiation once they are past the β selection stage in the DN4 stage. Egr4 however was not detected within this population. Li *et al*. (2011) also reported the upregulation of Egr1, 2, and 3 within thymocyte development. Egr4 has been reported to interact with NF-kβ and play a role in the production of inflammatory cytokines (Wieland *et al.*, 2005).

1.7.3 Egr2 knockout model

Egr2 and 3 have become the main focus within the study of immunity. Thus mouse models for these genes have been generated and have allowed for better understanding of their function within B and T lymphocytes which are the main arms of the immune system.

In 2008 Zhu *et al.* used a CD2-specific Egr2 knockout mouse to more clearly understand the role of Egr2. This group showed that Egr2 plays an important role in the prevention of autoimmune disease development. On deletion of Egr2 within lymphocytes, they found the

population of CD44 high T cells became hyper-responsive exhibiting increased proliferation and activation. This led to the accumulation of this population and with that, increased production of inflammatory cytokines IL-17 and IFN-γ both of which are associated with autoimmune disease SLE (Zhu *et al.*, 2008). In order to further confirm the role of Egr2 within the development of T cells, Li *et al.* (2011) used a CD2 specific Egr2 knockout mouse and an Egr2 transgenic to investigate this. They reported the expression of Egr2 influences the development of B and T cells. They showed within developing thymocytes in transgenic mice, there is an increase in double positive cells that are able to mature into single positive cells compared to the WT equivalent.

Miao *et al.* (2013) further looked at Egr2 expression in the control of inflammatory cytokine production. As it was previously reported that Egr2 knockout mice produce IL-17, they concentrated on the differentiation of Th17. They reported Egr2 as able to negatively regulate the expression of IL-17, As IL-17 expression is controlled Batf in CD4 T cells. They considered that Egr2 is able to control Th17 differentiation by inhibiting Batf.

1.7.4 Egr2 and Egr3 knockout model

Li *et al.* (2012a) carried out analysis on CD2-Egr2-/-Egr3-/- mice. The mice presented with early onset systemic autoimmunity, accompanied by increased by lymphocytic infiltration in multiple organs. IL-2 production and proliferation of B and T cells from CD2 Egr2- and Egr3-deficient mice was impaired on stimulation in vitro, showing that Egr2 and Egr3 are essential for efficient proliferation of naïve B and T cells. Production of inflammatory cytokines IFN-γ, IL-21 and IL-17 was increased in Egr2-/-Egr3-/- CD4+ T cells. It was also found that Egr2 and Egr3 induce SOCS-1 and SOCS-3 expression with activation of STST-1 and STAT-3 and there was reduced expression of SOCS-1 and SOCS-3 in Egr2/Egr3 Knockout mice. It has recently been reported that Egr2 and 3 are able to regulate T follicular help cell differentiation, and Ogbe *et al.* (2015) found that Egr2 and Egr3 are able to regulate the expression of Bcl6 (B cell lymphoma 6) which is required for the differentiation of Tfh cells. Egr2 and 3 absence results in defective expression of BCL6. The roles of Egr2 and 3 within T cells are shown in Figure 19.
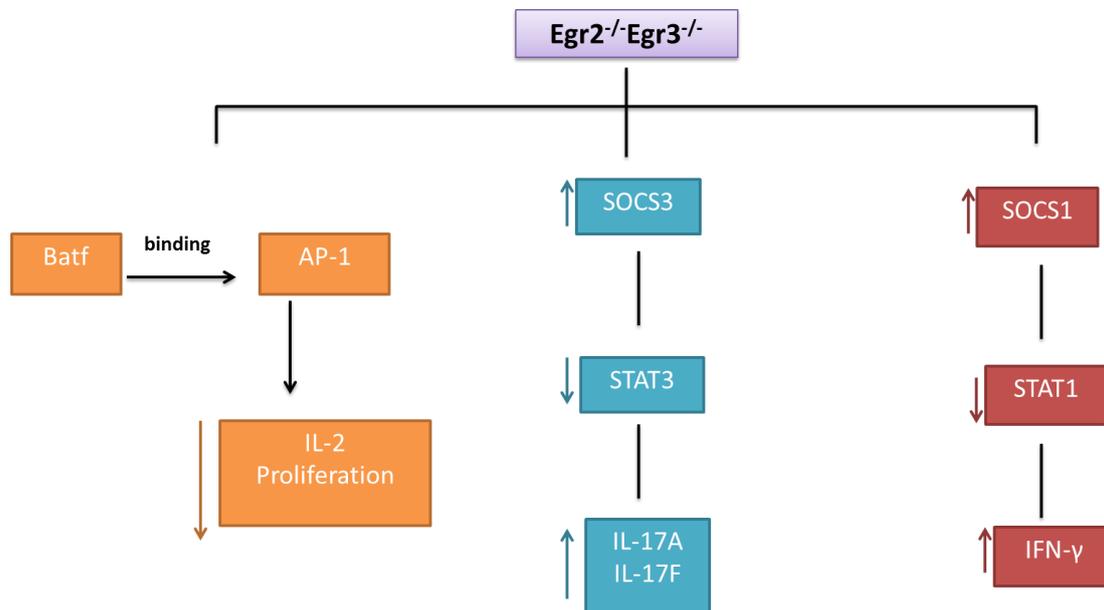
Figure 19: The role of Egr2 and Egr3 in T cells

The Egr2-/-Egr3-/- knockout mouse model first described by Zhu *et al.* has been shown to have decreased IL-2 and proliferation levels due to transcription factor Batf binding to AP-1 in the absence of Egr2, whereas in presence of Egr2, Batf would bind to Egr2. In addition, the Egr2-/-Egr3-/- mouse model has been reported to have increased levels of SOCS-3 and SOCS-1 expression, this resulted in decreased expression STAT-3 and STAT-1 respectively, this lead to increased production of pro-inflammatory cytokines IL-17 (A and F) and IFN-γ respectively. (Sumitomo *et al.*, 2013)

1.7.5 Egr2 in autoimmune disease

Egr2 has recently been studied within autoimmune disease development. Egr2 knockout mice were found to have an increased production of inflammatory cytokine production, coupled with development of autoimmune disease in the later stages of life (Zhu *et al.*, 2008). Miao *et al.* (2013) studied patients with multiple sclerosis and identified Egr2 as one of the factors that influenced differentiation. MS is primarily driven by Th17 mediated inflammation and the TH17 T cell subset also plays a major role with the production of IL-17 shown to contribute to tissue destruction during inflammation and found to be involved in the maintenance of chronic inflammatory disease in patients (Romagnani, 2008). Miao *et al.* (2013) found decreased Egr2 expression in patients with MS compared to healthy patients, showing Egr2 expression plays a heavy role in mediation of inflammation. Egr2 has also been implicated within systemic sclerosis (SSC) which is characterised by autoimmune disease. There have been reports of increased expression of Egr2 from patients with SSC and within mouse models

(Fang *et al.*, 2011). Similarly, it has been shown within the Japanese population that Egr2 was associated with susceptibility to systemic lupus erythematosus (SLE) (Myouzen *et al.*, 2010). Systemic lupus has also been studied in regards to Egr2 the absence of Egr2 results in the increased production of inflammatory cytokines coupled with symptoms such as glomerulonephritis (Li *et al.*, 2012b). There have also been genetic links to egr2 and the development of SLE, with polymorphisms in Egr2 identified in patients (Myouzen *et al.*, 2010).

1.7.6 Egr2 and 3 in tolerance

The presence of Egr2 in anergic cells has recently been closely studied. Harris *et al.* (2004) found that Egr2 is required for full anergy induction. They looked at the expression of Egr2 in the cells and found anergic T cells maintain long term expression and this was the case following re-stimulation. Later studies by Zheng *et al.* (2012) have also proposed that Egr2 is needed for T cell anergy induction in vitro and in vivo. Anti-CD3 induction was used to induce anergy in vitro, however in vivo induction was measured after a single dose of superantigen administration. Diaglycerol kinases (DGKs) have been found to play a role in anergy induction. It has been demonstrated that DGK-α is upregulated following anergy induction and may be due to Egr2 expression (Zheng *et al.*, 2012).

Reports have shown an immediate target of the NFAT-mediated transcription following calcium flux in T cells is the EGR- early growth response transcription factors (Shwartz, 2003). T cell clones screening analysis showed Egr2 and 3 expression during anergy may be dependent on the activity of calcineurin and PKC (Safford *et al.*, 2005).

1.8 Aim of study

Early growth response genes (Egr) 2 and 3 have been found to be important for T cell development. Previously, we have shown that CD2-specific Egr2 and Egr3 knockout mice developed autoimmune diseases with hyper-inflammatory phenotypes. Recent studies have shown Egr2 and Egr3 play an important role in T cell tolerance, which allows for protection against autoimmune disease development. However, it is still unknown how Egr2 and 3 are able regulate T cell tolerance. This project aims to investigate the mechanism of Egr2 and 3 in T cell tolerance. The main objectives of the project fall under three areas.

Firstly, it was important to investigate the induction of T cell tolerance in vitro in the absence of Egr2 and Egr3 through the use of antigenic stimulation without that of co-stimulation. This led to studies to examine the role of Egr2 and Egr3 induction in an NFAT dependant manner. In addition, well known markers of T cell tolerance such as ubiquitin ligase gene expression were assessed within the tolerant setting.

Secondly we investigated in vivo tolerance induction in the absence of Egr2 and Egr3 using superantigens. IL-2 and proliferation studies were used and T cells populations within the tolerant setting assessed.

Finally, we investigated the function of Egr2 and 3 in the control of inflammatory response within tolerant T cells; by looking at cytokine production following tolerance induction within the inflammatory setting provided by T cells from the Egr2/Egr3 knockout model.

The hypothesis of this study is that Egr2 and 3 induced in tolerant T cells have distinct roles in controlling TCR signalling and inflammatory responses.

Chapter 2. General Methods and Materials

2.1 Generation of Egr2-/-Egr3-/- mice

2.1.1 Animal housing and care

All mice used in the study were housed in Brunel University according to established institutional guidelines under the authority of the UK Home Office project licence (Guidance on the Operation of Animals, Scientific Procedures Act 1986) in the Biological Services Unit. Weights and animal wellness were recorded during all experiments carried out in accordance with the project licence guidelines registered to Dr. Su-Ling Li. All mouse controls were done using WT mice within each experiment. In-vitro stimulations used PBS (phosphate buffered saline) as controls.

2.1.2 CD2 specific Egr2 knockout mice (CD2-Egr2-/-)

Egr2 has been shown to be a crucial player in hind brain development and myelation of the peripheral nervous system (Tourtellotte and Milbrandt, 1998a). Systemic knockout of Egr2 results in multiple defects leading to early death (Tourtellotte and Milbrandt, 1998a)(Nonchev *et al.*, 1996).

A conditional knockout of Egr2 was thus carried out in lymphocytes using the CD2 promoter and the cre-loxp system (Zhu *et al.*, 2008). Cre is a recombinase enzyme, which was found in the P1 bacteriophage. The cre (cyclisation recombination) gene is attached to the CD2 promoter in lymphocytes. Cre is able to recombine DNA substrates where lox-p (locus of X over P1) sites are present. Lox-p sites are 34 base pairs in length, and are usually placed on the ends of the target gene; this is called flanking.
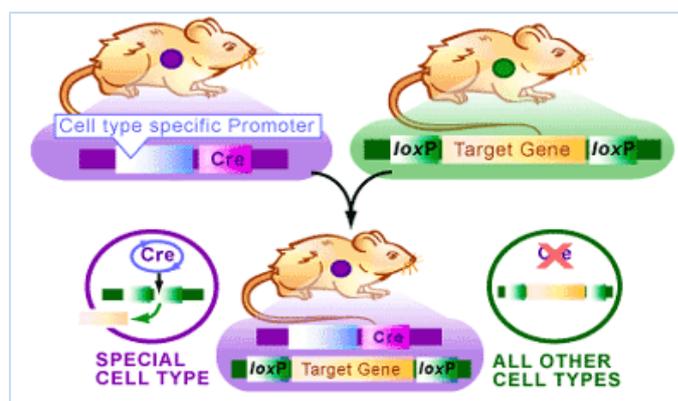
Figure 20 - Cre-Loxp system
The cre loxp system is a method used for the targeted deletion of genes. Loxp is able to 'flank' the gene. When cre meets loxp p, it is able to 'splice' the regions the loxp sites have marked and result in the deletion of said gene. Diagram from (415 Sauer, Brian 1998).

Mice were generated with Egr2 flanked by lox-p sites and other mice were generated to express CD2 specific cre. These mice were crossed resulting in the inactivation of egr2 in the lymphocytes. Once this had been done the mice were then crossed in a C57BL/6 background to purify. The expression of cre and loxp was verified via polymerase chain reaction (PCR) (Zhu *et al.*, 2008).

CD2 specific Egr2^{-/-}Egr3^{-/-} mice.

Egr3 -/- was carried out systemically using a neomycin cassette (Tourtellotte and Milbrandt, 1998a). A vector containing Egr3 was flanked using Loxp and a neomycin resistant positive selection cassette was added to create a sequence of homology. The egr3 sequence was then replaced by neomycin. This vector was linearised and transferred in embryonic stem cells. The cells were then cloned and injected into blastocysts and then inserted into female mice. The offspring were backcrossed with C57BL/6 mice until a stable germline was established (Tourtellotte and Milbrandt, 1998a).

In order to create CD2-Egr2-/-Egr3-/- mice, CD2-Egr2-/- mice (Zhu *et al.*, 2008) and Egr3-/- (Tourtellotte and Milbrandt, 1998a) interbred on the non-autoimmune disease prone C57BL/6 background (Li *et al.*, 2012a).

2.2 Genotyping

In order to confirm Egr2 and 3 knoclout mice, genotyping was carried out. DNA extraction was carried out as follows. Mouse tails were collected into Eppendorf tubes and labelled. In each tube, 20µl of extraction buffer (Sigma Aldrich, Gillingham, Dorset, UK, E-7526) was added. Samples were vortexed and 5µl of tissue preparation solution (Sigma Aldrich, Gillingham, Dorset, UK T-3073) was added. Samples were vortexed for not less than 30 seconds, centrifuged at 14,462g for 1 minute, and incubated for 30 minutes at 55°c. Samples were then heated at 95°c for 3 minutes for enzymatic denaturation. 20µl of neutralisation buffer (Sigma Aldrich, Gillingham, Dorset, UK, N-3910) was added to the solution in the tubes, vortexed and centrifuged to mix contents. Distilled/autoclaved water was added to each sample: 150µl to knockout samples and 300µl to transgenic samples.

The principle of Polymerase Chain Reaction (PCR) is to generate huge numbers of copies of a specified gene. This process incudes three major steps; denaturation, annealing and extension,

PCR was carried out as follows: Each PCR tube was prepared with master mix (Thermo scientific, Loughborough, UK) solution with primers. For all mixes: with 7.5µl master mix, 0.25µl sense primer and 0.25µl antisense primer For Egr3 KO samples however we used 7.5µl master mix with 0.5µl Egr3 sense primer, 0.5ul Egr3 antisense primer and 0.5ul Neo primer. 1.5µl of tissue (tail) sample was added into each PCR mix and ran on the required program. Programs are shown in Table 2.

Genotyping Sample	Primer sequence	PCR protocol	Band Size (bp)
Egr2 Knockout LoxP	Sense: GTG TCG CGC GTC AGC ATG CGT Antisense: GGG AGC GAA GCT ACT CGG ATA CGG	95°C for 5min 94°C for 40sec} 65°C for 40sec} 30 Cycles 72°C for 40sec} 72°C for 5min 4°C for 10min	Upper Band = 210bp Lower band = 195bp
Egr2-/- Cre	Sense: CCA ACA ACT ACC TGT TCT GCC G Antisense: TCA TCC TTG GCA CCA TAG ATC AGG	95°C for 5min 94°C for 40sec} 56°C for 40sec} 40 Cycles 72°C for 40sec} 72°C for 5min 4°C for 10min	150bp
Egr3-/-	Sense: CTA TTC CCC CCA GGA TTA CC Antisense: TCT GAG CGG GCT GAA ACG Neo: GAT TGT CTG TTG TGC CCA GTC	95°C for 5min 94°C for 40sec} 57°C for 40sec} 35 Cycles 72°C for 40sec} 72°C for 5min 4°C for 10min	Upper band = 700bp Lower band = 350bp

Table 2: Polymerase chain reaction programmes, primers and band sizes

Gels were made using 2g agarose powder (Invitrogen, cat. Number-15510-027) dissolved in 100ml of 0.5% TAE buffer. This was heated until it dissolved and cooled for 2 minutes before adding 10µl ethidium bromide solution. The solution was poured into the moulding blocks and left to solidify. The samples were loaded into a container filled with 0.5TAE buffer with 1.5µl 100 base pair ladder: 100µl 6X loading dye (Fisher scientific, R0611, LE115RG,UK) and 50µl 100bp ladder (Invotrogen, cat.no-15628-019, PA49RF,UK) and ran at their respective volts. Visualisation was via a Syngene Genesys:G-Box.

2.3 Organ collection and preparation

Mouse organs were collected and fixed overnight with 4% paraformaldehyde (PFA) (at least 3 times the amount of the sample) and then changed to 70% ethanol (Fisher scientific, LE115RG,UK- E/0650DF/17) until tissue processing. Samples were processed in a 22-hour process as follows:

Solution	Immersion Time (Hours)	Drain Time (Seconds)
70% Ethanol	2	15
90% Ethanol	2	15
95% Ethanol	2	15
95% Ethanol	2	15
95% Ethanol	2	15
95% Ethanol	2	30
100% Xylene	2	15
100% Xylene	2	30
Wax	2	60
Wax	2	120

Table 3: Tissue processor program

Organs were then placed into cassettes (Thermo scientific, Loughborough, UK, Shandon-41743) and embedded in wax. Sample cassettes were left to harden for at least 6 hours at room temperature and then stored at -20 °C. Cutting of each sample was done using a microtome and samples cut to thickness between 5 and 7 microns and immediately placed in a water bath at 45°C and then transferred on slides and heated at 60°C for 8 minutes to allow the paraffin section to adhere to the slide, before putting slides at 37°C overnight. Frozen sections were snap-frozen in liquid nitrogen following tissue collection. Embedding was done in Tissue-Tek™ CRYO-OCT Compound (Thermo scientific) and sectioned with the use of a cryostat. 8µm-thick sections were cut and left at room temperature for 30 minutes. This was then followed by fixation in 1:1 acetone: methanol solution at -20˚C for 10 minutes. Sections were allowed to air dry for another 10 minutes before storing in -80˚C freezers.

2.4 Haematoxylin and Eosin (H&E) staining

This is the most common form of staining. The affinity of dyes allows us to visualise different structures. We used a regressive method where we overstrained the tissue with haematoxylin and then washed and stained with eosin. The procedure used was follows.

Slides were treated with 100% Xylene (Fisher scientific, LE115RG,UK- X/0200/17) for 3 minutes. This was repeated twice, followed by treatment with 50% xylene and 50% ethanol for 3 minutes. Slides were then treated with 100% ethanol (Fisher scientific, LE115RG,UK-E/0650DF/17) for 3 minutes twice, and then 90% Ethanol for 3 minutes twice. The slides were rinsed with distilled water and treated with haematoxylin for 6 minutes. They were then washed under running water for 5 minutes; dipped in acid alcohol until samples turned pink; washed again and treated with 0.2% ammonium solution for 40 seconds and washed again; treated with eosin for 45 seconds and washed again under running water; treated with 95% ethanol for 2 minutes three times; 100% ethanol for 2 minutes three times; 50-50 ethanol-xylene for 2 minutes; and finally 100% xylene for 2 minutes 3 times. Slides were then mounted with histomount (National Diagnostics- HS-103) and cover slipped. Photos were captured with an Axiscope microscope

2.5 Immunohistochemistry of frozen sections

Immunohistochemistry was carried out on frozen sections incubated at room temperature for one hour with the directly labelled primary antibody Texas Red conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Lab, UK) at a 1:200 dilution. Slides were then washed with 1xPBS for 10 minutes and left to air dry. Mounting was done using Vectashield with DAPI (Vector laboratories, H-1200) and viewing done with a Zeiss fluorescence microscope with Smart capture software (Digital Scientific Cambridge, UK).

2.6 Isolation of CD4 T cells

Fresh spleen and lymph nodes were collected and homogenised in PBS (phosphate buffered saline) (Fisher Scientific Loughborough, UK) using a 40µm nylon cell strainer or mesh (Falcon, Stafford, UK). The cells were centrifuged at 124g and re-suspended in 0.8% ammonium chloride for 5 minutes at 37°C to lyse the red blood cells. Lysis was stopped by adding PBS and

centrifugation at 124g for 5 minutes. Cells were then re-suspended in beads buffer, a mixture of 50ml PBS and 3ml RPMI for adequate suspension of the magnetic beads.

Using a positive selection kit, CD4 positive cells were then isolated by Magnetic Activated Cell Sorting (MACS) (Miltenyi Biotech, Almac House, Bisley. UK). Briefly, cells were suspended in beads buffer and 20 µl of CD4 beads per WT spleen and 25µl per Egr2-/-Egr3-/- spleen was added. The mix containing anti-CD4 antibodies (L3T4) attached to magnetic beads was incubated at 37°C for 20 minutes with shaking every 5 minutes with the labelled CD4 T cells present in the suspension. Beads buffer was added up to 10ml to allow washing of unlabelled anti-CD4, and cells were centrifuged at 124g for 5 minutes at room temperature. The supernatant was discarded and the cells re-suspended in 500µl beads buffer. The column was washed and 500µl of cells were added. This allowed for CD4+ T cells to be retained within the column and unlabelled cells to pass through the column by washing with 1.5ml beads buffer. The column was filled to the top with RPMI with 10% Foetal bovine serum (FBS), which contains basic nutrients, hormones and growth factors that cells require to survive (Thermo Fisher Scientific, Life technologies, Paisley UK), and plunged out to elute CD4 + T cells into a separate 15ml tube.

2.7 Tolerance induction

2.7.1 In-vitro CD4+ T cell anergy induction

CD4+ T cells were cultured in RPMI 1640 medium (500ml) stored at 4°C (Gibco-Life technologies, Paisley, UK) supplemented with 50ml 10% FBS, 500µl 50µm β-mecaptoethanol, 10ml sodium pyruvate, 500µl 50µg/ml gentamicin and 5ml 300mg/L L-glutamine all obtained from Gibco-Invitrogen, Life technologies, Paisley UK. To induce a state of anergy anti-CD3 (eBioscience, clone 145-2C11) was used; plates were coated with 1µg anti-CD3/ml at 300µl/well and activated plates coated with 1µg/ml anti-CD3 and 2µg/ml anti-CD28 (eBioscience, clone 37.51). Control wells were coated with 300µl PBS. The coated plates were incubated for more than 60 min at 37°C to allow antibodies to adhere to the surface of high binding plates. Coating solution was discarded after an hour and 1ml PBS was added to each well to remove unbound antibody and immediately discarded. 2 x 10^6 cells per well in 300µl medium was added to the plate before 24-hour incubation. This was for anergy induction and control. Cells were re-suspended and transferred to a 96-well plate coated with 1µg/ml anti-

CD3 and 2µg/ml anti-CD28, then re-stimulated for a further 72 hours. Alternatively, cells were transferred to an uncoated plate and left to rest overnight before re-stimulation.

To test multiple concentrations of re-stimulation, plates were coated with anti-CD3 and anti-CD28; 96 well flat bottom plates were coated with titration of anti-CD3 at 1µg/ml, 0.5µg/ml and 0.25µg/ml all with 2µg/ml anti-CD28 at 50µl/well. The plate was incubated for more than 60 min at 37°C before seeding cells. Re-stimulated cell medium was collected after 48 hours for ELISA. After 72 hours, 3H-Tdr was added to each well and incubated overnight.

2.7.2   In-vivo tolerance induction

Age and sex matched CD2-Egr2-/-Egr3-/- mice were injected 6-8 times, twice a week with superantigen to induce in vivo tolerance. For each in vivo sample 6-8 mice were used, where 2 were pooled together to make one sample. Superantigens are able to stimulate T cells outside of the peptide binding groove allowing stimulation of a larger array of the T cell population. Staphylococcal enterotoxin A (SEA) (Sigma Aldrich, Gillingham, Dorset, UK) was administered 8 times with 20µg/ml SEA diluted in 100ml PBS per injection per mouse (fourteen mice were used on average per experiment) for anergy induction with a WT and CD2-Egr2-/-Egr3-/- sample injected once as an 'active' sample positive control (two mice used per experiment), and non-injected or PBS injected as negative controls (2 mice used per experiment). All mice were sacrificed on the same day and CD4 T cell isolation carried out. Mice weights were recorded before each injection.

2.7.2.1   Ex-vivo culture

CD4 T cell isolation was carried out after organ collection. Each sample had 2 mice pooled together, i.e. cells from two WT mice treated once with SEA made one (active) sample; cells from 2 WT mice treated with SEA multiple times made one (tolerant) sample and similarly with Egr3-/-Egr3-/- mice. WT CD4 negative cells were used as antigen presenting cells (APCs). Briefly, cells were collected and rested in RPMI medium for 30 mins at 37°C. Antigen presenting cells were collected and treated with mitomiycin for 30 minutes at 37°C to stop intracellular DNA production, thus stopping cell function. Prior to culture at 37°C, APCs were loaded with SEA for 3 hours at a concentration of 10µg/ml and washed twice to remove

unbound SEA with PBS before culturing with CD4+ T cells (APCs from 'active' samples were used for active cell culture and APCs from tolerant samples were used for tolerant culture). Culture was done in a 50-50 ratio with 50% APC with 50% CD4+ T cells for 48-72 hours. CD4 T cells from each sample were stained with the appropriate cell surface markers immediately after the culture was complete (refer to chapter on flow cytometry). All other cells were cultured with APCs overnight in RPMI with loaded APC (controls were cultured without SEA).

2.8 Cell viability

Trypan Blue staining was used to assess cell viability. Briefly, 10µl of CD4 T cell suspended in RPMI was mixed with 10µl of trypan blue and cells counted. The following equation was used to determine cell viability using a countess automated cell counter (Invitrogen):

Cell Viability (%) = total viable cells (unstained) ÷ total cells (stained and unstained) × 100 (http://www.sigmaaldrich.com/technical-documents/articles/biofiles/cell-viability-and-proliferation.html#sthash.UdUP75eK.dpuf).

2.9 Enzyme Linked Immunosorbent Assay (ELISA)

Enzyme Linked Immunosorbent Assay or ELISA is a widely used technique useful for determination of antigen and antibody concentration (Gan and Patel, 2013). In this case we carried out a sandwich ELISA, which takes place when an antigen is detected between two specific antigens (Gan and Patel, 2013), for the detection of IL-2 and IFN-γ levels. Both were carried out using kits: IL-2 (eBioscience, Altrincham, Cheshire. UK) and IFN-γ (BD, Oxford Science Park, Oxford, UK). The following reagents were prepared: Wash buffer made from PBS + 0.05% tween-20, 1x Coating buffer made from 1ml of 10x coating buffer + 9ml Deionised water, 1x assay diluents for dilutions or blocking buffer made from 1 part 5x assay diluents + 4 parts water and Stop solution: 2N H2SO4.

2.9.1 Interleukin 2 (IL-2) ELISA

IL-2 ELISAs were carried out according to manufacturer's instruction and all reagents obtained from the kit unless otherwise stated. Briefly, plates were coated by adding 100µl/well of capture antibody at a dilution of 250X per well in coating buffer on a high binding plate (Granier Bio One, Stonehouse, UK) and incubated overnight at 4°C to allow adherence of antibodies. Dilution for IL-2 standard was calculated from certificate of analysis provided with

the kit. The next day, wells were washed 5 times with at least 250μl of wash buffer for 5 minutes, on a shaker. Wells were blocked with 200μl per well of 1x assay diluents (blocking buffer) for at least 2 hours at room temperature to avoid unspecific binding, washed 5 times with 250μl wash buffer for 5 minutes, and thoroughly blotted and 100μl of sample added per well. The standard was added using serial dilution with the highest concentration of 200pg/ml down to 0pg/ml in blocking buffer. Plates were incubated overnight at 4°C. Wells were washed 5 times with at least 250μl of wash buffer on shaker for minutes, the next day. 100μl of the detection antibody was added at a concentration of 250x per well and the plate incubated at room temperature for 1 hour. Wells were washed and 100μl of Avidin enzyme HRP at a concentration of 250x to each sample well for 1 hour. Wells were washed 7 times for 5 minutes on a shaker, the plate was properly blotted, and 100μl of 1xTMB solution added to each sample well. Readings were taken after 15 minutes, 30 minutes and 1 hour after development in the dark.

2.9.2   Interferon gamma (IFN-γ) ELISA

In the case of IFN-γ, plates were coated with 100μl capture antibody at a concentration of 250x in coating buffer at 4°C overnight. The next day wells were washed with no less than 250μl wash buffer (0.05% Tween 20 in PBS) for 1 minute each. This was repeated 5 times and 100μl/well blocking buffer (10% FBS in PBS) added for 1 hour at room temperature. Wells were then washed with wash buffer 5 times for 1 minute each and 100μl samples added; the standard was diluted according to certificate of analysis with the highest dilution of 2,000pg/ml to 0pg/ml and incubated for 2 hours at room temperature on a tightly sealed plate. The plate was washed with wash buffer 5 times for 1 minute each and 100μl of detection and Avidin enzyme HRP at a concentration of 250x was added for 1 hour before washing 10 times with wash buffer for 1 minute per wash. Plates were properly blotted and 100μl TMB of solution added. Plates were read after 30mins incubation at 450nm and the results analysed according to manufacturer's instruction.

2.10 Flow cytometry

Flow cytometry is a technique used to investigate multiple parameters simultaneously within a cell population or a single cell. Single cell suspensions were obtained from homogenised spleen tissues. Surface staining with fluorescent marked antibodies was carried out first. The

flow cytometry kit was obtained from eBioscience, Altrincham, Cheshire, and staining carried out according to the manufacturer's instruction. Cells were centrifuged at 1,844g for 5 minutes and supernatant discarded. Pellets were re-suspended in a solution with 2μl/antibody diluted in wash/permiabilisation buffer and incubated for 20 minutes In the dark. Cells were then fixed with fixation buffer from the kit.

For IFN-γ, measurement cells were first stimulated with 200ng/ml PMA and Ionomycin per well in 300μl RPMI in order to bypass barriers into the nucleus and simulate the cells, and Golgi stopper (BD Bioscience, Oxford Science Park, Oxford, UK) was added at a 1/100 dilution per well to prevent the escape of intracellular cytokines. The cells were incubated for 3 hours at 37 degrees. Nuclear staining followed where, cells were collected and centrifuged at 1844g for 5 minutes and supernatant discarded and fixed using Fixation/permiabilisation solution for 20 minutes at 4°C. Samples were centrifuged and 300μl permiabilisation buffer added for 10 minutes at room temperature. 300μl permiabilisation/wash buffer was added to the same tube and immediately centrifuged at 1,844g for 5 mins. Antibody dilutions were prepared and pellets re-suspended and incubated in the dark for 20 minutes. Pellets were washed with 300μl wash buffer and centrifuged for 5 minutes at 1,844g and finally re-suspended in PBS ready for flow cytometry analysis.

Samples were run through a 6 channel flow cytometer on a LSRII or Canto (BD Immunocytometry Systems) by Professor Ping Wang at the London School of Medicine and Dentistry, Queen Mary University of London. For flow cytometry analysis, single cell suspensions were analysed using FlowJo (Tree Star).

2.10.1 Flow cytometry antibodies

The following antibodies were used: fluorescein isothiocyanate (FITC)-conjugated antibodies to CD4, Vβ3; IFN-γ Phycoerythrin (PE)-conjugated antibodies to, CD4, CD62L, CD69, IL-2, Vβ3, Egr2; allophycocyanin (APC)-conjugated antibodies to CD44, Egr2, CD69, IFN-γ; and PEcy7-conjugated antibodies to CD44, T-bet, IFN-γ. All antibodies were obtained from E-bioscience.

2.11 Proliferation measurement

Proliferation was assessed using thymidine (3[H]-TdR). It is able to metabolically integrate into the DNA of each new cell, allowing for proliferation to be monitored. To carry out proliferation

studies, CD4 T cells were isolated and the respective treatment administered. Cells were then plated on a 96-well plate at 2.5×10^5 cells/well and stimulated with 1μg/ml anti-CD3 and 2μg/ml anti-CD28 for 72 hours. Following this, 0.5μCi of per well of 3[H]-TdR Tritium diluted in RPMI was added, and the plate further incubated at 37°C for 8 hours. Plates were read using a scintillator; tritium is able to emit radiation that in turn excites a fluid during scintillation. This allows for the production of light which is measured when detected (Madhavan, 2007).

2.12 Electronic mobility shift assay (EMSA)

The electronic mobility shift assay or EMSA is a method used to detect protein-nucleic acid interactions. All EMSA were completed at Queen Mary University London by Dr. Miao. EMSA represents the binding of a transcription factor with the promoter region of a gene. Definition of binding sites allows for probe designs, and probes were designed as published by Li *et al.* (Li *et al.*, 2012a). All probes were marked with a fluorescent marker, known as the hot probe, and one unlabelled probe (cold probe) was also used as a control. Prior to carrying out EMSA, CD4 T cells were stimulated with 1μg/ml anti-CD3 and 2μg/ml anti-CD28 for 3 hours or with 1μg/ml anti-CD3 overnight followed by re-stimulation with 1μg/ml anti-CD3 and 2μg/ml anti-CD28 for 3 hours.

2.12.1 EMSA protein extraction

Protein extraction was performed as follows. 1ml of cytoplasm lysis buffer (Sigma Aldrich, Gillingham, Dorset, UK) was added to each sample for 15 minutes and inverted every 3-5 minutes. Samples were centrifuged at 14,462g for 1 minute and supernatant discarded. 150μl lysis buffer and 1.5μl proteinase buffer was added to each sample pellet and placed on a shaker at 4°C for 30 minutes. Samples were then sonicated for 2 minutes 30 seconds and centrifuged at 124g for 10 minutes, and the concentration of protein was assessed using bovine serum albumin (BSA) assay.

2.12.2 Bradford assay-protein quantification

Protein quantification using a BSA assay was performed using 100XBSA reagent (New England Biolabs). BSA is able to form a complex when bound to protein lysate, and this allows for a shift in absorbance. Serial dilution was carried with BSA was prepared on a 96-well plate from a concentration of 5mg/ml down to 0mg/ml at 200μl per well. A similar amount of protein

extraction diluted in water and Bradford reagent was added to other wells on the same plate. The plate was incubated at room temperature for 5 minutes before being passed through a KC4 Luminometre (Bio Tech Inc.). A standard curve was produced with BSA readings and protein concentrations.

Acrylamide gels were made using 1ml 30% acrylamide (Flowgen Bioscience, Nottingham, UK), 300µl 10x TBE (Amresco, Cleveland, USA), 100µl 10% APS (Sigma Aldrich, Gillingham, Dorset, UK), 20µl TEMED and 2.6ml water. Gels were pre-run for 1 hour at 100V in 0.5x TBE.

2.12.3 EMSA Binding reactions

For binding reaction steps, 10x binding buffer was used containing 100nM Tris, 500mM KCl, 35mM DDT, pH 7.5, 2.5% Tween20, Poly-Di-dc 0.5µg/µl in TE, pH 7.5 (Sigma Aldrich, Gillingham, Dorset, UK). Binding reactions were made with 2µl of 10x reaction buffer, 1µl polyDidc, 2µl of 50% glycerol (Sigma Aldrich, Gillingham, Dorset, UK), 1µl of labelled probe, 5µg of nuclear extract and water for a total volume of 20µl per sample. After mixing, orange dye was added to samples and the samples loaded on the gel and run at 100V. Gels were scanned using a Typhoon 9400 imager (Amersham Biosciences).

Probes used were as follows:

AP-1   5'-CGCTTGATGACTCAGCCGGGA-3'   3'-GCGAACTACTGAGTCGGCCTT-5'.

NFAT   5'-CGCCCAAAGAGGAAAATTTGTTTCATA'-3'   3'-GCGGGTTTCTCCTTTTAAACAAAGTAT-5' (Sigma Aldrich, Gillingham, Dorset, UK).

2.13 RNA extraction

Cells were collected from their respective samples, pelleted and suspended in 500µl Trisol (Tri) reagent (Sigma Aldrich, Gillingham, Dorset, UK) to allow for the extraction of total RNA. Trisol is able to aid the precipitation of DNA and RNA from cells (Chomczynski, 1993). Cells were vortexed for 30 seconds and 400µl chloroform to aid homogenization. Samples were vortexed and incubated at room temperature for 15 minutes, allowing the protein to denature and

become soluble in the organic phase. Samples were vortexed for a further 30 seconds and spun down at room temperature for 10 minutes at 12,470g to separate the phases. These are the lower red organic phase, interphase (which is in the middle) and the aqueous upper phase. The RNA dissolves into the aqueous phase while protein and DNA remains in the interphase and the organic phase. The upper aqueous phase was then transferred to a new tube and 300µl of isopropanol added to precipitate, and the samples incubated at room temperature for 10 minutes. Samples were then spun down at 4°C for 10 minutes at 124g and the supernatant discarded. Pellets were washed with 80% ethanol and spun down for 5 minutes at 4,150g. Supernatant was discarded and the pellets left to air dry for 5-8 minutes. Finally, samples were re-suspended in RNase-free DEPC.

The RNA quality and quantity was assessed using a spectrophotometer nanodrop 2000c.

2.14 cDNA production

After RNA concentration was assessed, cDNA synthesis was carried out. RNA was converted to cDNA using the Superscript TMIII Reverse transcriptase enzyme. This allows for the formation of a cDNA strand from a complementary RNA template. To do this, random primers are used together with total RNA primers. Each sample was heated at 70°C to denature the RNA which was collected and cooled at 4°C to allow binding of random primers. 1µl dNtTP and 4µl of $MgCl_2$ were added to each sample along with 4µl 5x running buffer and 1µl of the reverse transcriptase enzyme. Samples were spun and treated at 25°C for 5 minutes for denaturation, 42°C for 1 hour for annealing, and 70°C for 15 minutes for the elongation step. This resulted in multiple copies of cDNA. Once ready the cDNA was diluted ready for the RT-PCR mix.

2.15 Primer design

The length of the average primer should be 18-24 nucleotides. To produce the programme, PerlPrimer was used. 20-23 nucleotide primers were designed with the sense and antisense designed to anneal to the exons of the sequence of interest to allow differentiation between amplification of cDNA and potential contaminating genomic DNA by melting curve analysis. The 5' (forward) or sense primer included the recognition site of the required restriction enzyme, while the 3' (backward) or antisense primer included the stop codon if needed. To deduce the melting temperature of each primer we used the Wallace rule (Tm=4x (G+C) +2X (A+T)) and the melting temperature set to Tm - 2°C and altered as necessary. A blast search

was then carried out to check each primer was correct. For the endogenous gene, mouse β-actin was used (Table 4).

Primer	Sequence
Egr2	TCAATGTCACTGCCGCTGAT
	AGAAATGATCTCTGCAACCAGAA
IFN-γ	CCATCAGCAACAACATAAGC
	AGCTCATTGAATGCTTGGCG
Il2	GCATGTTCTGGATTTGACTC
	CAGTTGCTGACTCATCATCG
Egr3	GATCCACCTCAAGCAAAAGG
	CGGTGTGAAAGGGTGGAAAT
Tbet pcr5	CATTGCAGTGACTGCCTACC
	CACTCGTATCAACAGATGCG
Batch 2	GCTAACCTCAGAACAGTTGAA
	GTGATTCCTCTCCGACAACAG
Tcf1	ATAGACAGCACTTCCCTGCC
	TTCTCTGCCTTGGGTTCTGC
Bhlhe40	ACGTTGAAGCACGTGAAAGC
	GAAGTACCTCACGGGCACAA
Itch	GCTCTAGTCGCTGTAGTCGG
	CAAACCTGAAGTTCTCAATGGCT
Cblb	GAGCTTTTTGCACGGACTAAGA
	GGTCCGACTGCATCCTGAAT
Dgk alpha	CTGGGCACTGGAAATGATCT
	AATCTTTCTCAAATTCTCACCTTCATA

Table 4: RT-PCR primer sequences

2.16 Real Time PCR

Real time PCR (RT-PCR) has the ability to monitor the progress of DNA/RNA amplification. It works on the same principle as the PCR, although this particular technique is able to use cDNA in order to determine relative gene expression or RNA levels within a sample. RT-PCR is able to use a 'housekeeping gene' that is not expected to change under experimental conditions as an internal standard or reference in comparison to the target gene. Quantification is made possible by the use of fluorescent probes. In this case we used SYBR Green I, which is able to bind to the minor groove of double stranded DNA and emit 1,000-fold greater fluorescence than when it is free in solution (Morrison, Weis and Wittwer, 1998).

After each round of synthesis, the amount of fluorescence was measured. We used a QuantiTect SYBR Green PCR kit (Quiagen, Manchester, UK). As per manufacturer's instruction, each sample had 1µl cDNA and a primer concentration of 5µM of each sense and anti-sense primer in a 1xSYBR Green master mix. A Rotor-Gene system (Corbett Robotics) was used to carry out the Real Time PCR for a total of 40 cycles

- Denaturation: 95°C for 15 seconds

- Annealing: 60°C for 20 seconds

- Elongation:72°C for 30 seconds

RT-PCR also has its limitations, such a SYBR green unspecific binding, thus it is important to produce a melting curve, and analysis must be performed using the Tm (the temperature at which 50% of a particular DNA duplex dissociates and becomes a single stand DNA) to show any unspecific products such as primer dimers.

2.17 RT-PCR data analysis

Rotor Gene software was used for RT-PCR analysis. RT-PCR products are measured at each cycle. The use of fluorescent dyes allows for the yield of increasing fluorescent signals. Each result has an exponential phase, a linear phase and a plateau phase reached when reactants are consumed. The monitoring of the reactions can be seen during the exponential amplification phase, allowing for the determination of the initial quantity of the target. During the exponential phase the amount of fluorescence emitted is higher than that of the background fluorescence and this is known as the Ct (cycle threshold) value. It represents the starting copy number of the original template. The linear phase of the reaction is the phase in which the optimal amplification is achieved and in an ideal reaction, the PCR product doubles after each cycle. The final phase is the plateau that indicates the end of a reaction; the components of the reaction are consumed, meaning the reaction is slowing down and the products are degrading (Wong and Medrano, 2005). Following this result after RT-PCR, a threshold value was determined after samples were run in the exponential phase. The numbers of cycles (Ct) from the samples were compared with those of the reference gene in this case, mouse gapdh was used. This was done in order to normalise the data. The following formula was used:

Ct (gapdh)-Ct (target gene)x10000.
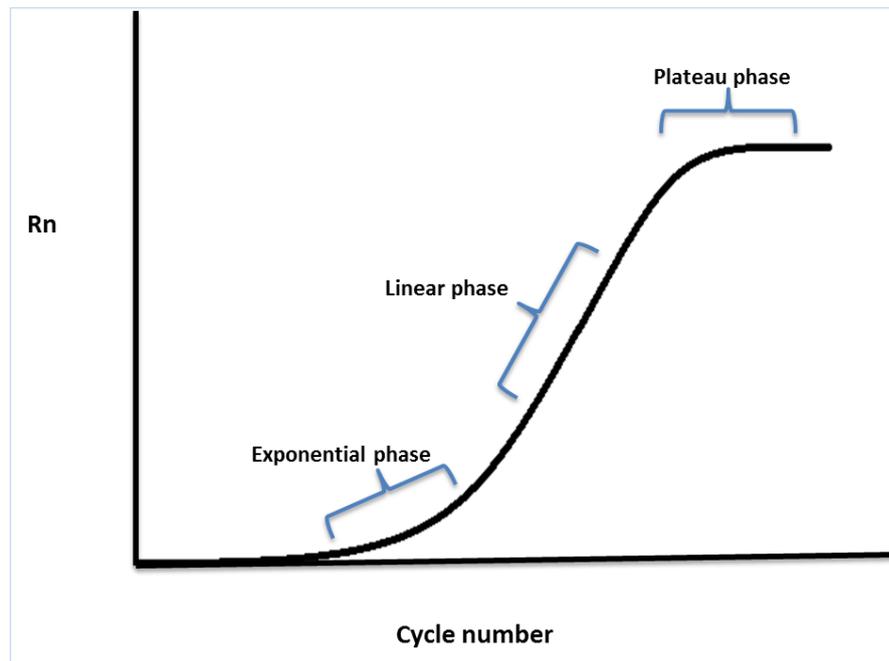
Figure 21: Phases of PCR amplification curve

There are 3 main phases of the PCR amplification curve: the exponential phase which allows for the quantification of the cycle threshold where the fluorescence emitted is higher than that of the background fluorescence; the linear phase which is the optimal point of PCR amplification with product doubling after each cycle; and finally the plateau phase where the reaction comes to an end.

Derived from (Wong and Medrano, 2005).

2.18 Statistical analysis

Student's T test was used to calculate significance to determine tolerance induction. P value of <0.05 was considered to be significant.

Chapter 3. In-vitro tolerogenic induction in Egr2 and Egr3 knockout CD4 T cells

3.1 Introduction

Immunological tolerance is described as the lack of immune response to a foreign antigen (bacteria, viruses) or to a self-antigen, which prevents the induction of autoimmune disease. Tolerance is divided into central and peripheral mechanisms; central tolerance embodies the generation and maturation of T lymphocytes within the thymus which plays the role in the T cells being able to distinguish self-antigens from foreign antigens. This results in the production of CD4+, CD8+ and T regulatory cells (Xing and Hogquist, 2012).

Peripheral tolerance takes place within secondary lymphoid organs such as the spleen. There have been a number of mechanisms described within peripheral tolerance. Exhaustion, which is often confused with anergy, takes place when CD8+ T cells become gradually unreactive due to persistent antigenic stimulation (Schietinger and Greenberg, 2013). Ignorance takes place when self-reactive T cells are unaware of antigen due sometimes due to low level expression. T cell anergy, which has mainly been studied within CD4 T cells, is induced by the activation of a T cell through antigenic stimulation in the absence of co-stimulation (Jenkins *et al.*, 1987b). It is described as an unresponsive state characterised by a decrease in IL-2 and proliferation and expression of T cell markers such as CTLA4 and is the cetral mechanim studied in this chapter (Jenkins *et al.*, 1987b)(Shwartz, 2003).

Egr (early growth factor response genes) 2 has been reported to be necessary for T nerve myelation (Topilko *et al.*, 1997). In addition, it has been found that Egr2 is necessary for T cell development. Mouse model Egr2-/-Egr3-/- has shown that Egr2 and 3 play a role in the development of autoimmune disease, with Egr2-/-Egr3-/- mice exhibiting lupus-like symptoms coupled with increased inflammatory cytokine production (IL-17A,IL-17F and IFN-γ) (Li *et al.*, 2012b). More importantly Egr2 has been studied within the peripheral mechanism of CD4+ T cell anergy. It has been found to play an important role within the induction of anergy (Safford *et al.*, 2005) and it has been reported that the mechanism of anergy is accompanied by increased expression of a number of genes namely cblb and itch. Cblb-/- and itch-/- mice develop autoimmune disease similar to Egr2-/-Egr3-/- (Safford *et al.*, 2005; Mueller, 2004). Within this chapter we investigate; the role of Egr2 and Egr3 within the tolerance setting,

starting with the induction of tolerance within the Egr2-/-Egr3-/- mouse model and the functionality of tolerance induction within the high inflammatory cytokine environment provided by the Egr2 and Egr3 knockout CD4 T cells in vitro.

3.2 Methods

To investigate the mechanisms of Egr2 and Egr3 in in vitro tolerance we used well established modes of tolerance induction. In order to do this, we used a number of techniques.

3.2.1 Organ collection, preparation and staining

Mice were housed in the animal unit in Brunel University as described in Section 2.1.1. WT and Egr2-/-Egr3-/- mice were culled and organs were collected at 4 weeks and 8 weeks for each. Spleens and lymph nodes were weighed and mouse weights recorded. Graphs were generated according to number of mice used per organ, and statistical analysis performed using Student's T test.

Kidneys were treated with 4% PFA overnight before organs were processed in a 22-hour process. Organs were embedded in wax and slides cut to between 5-7 microns thick. Organs underwent Haematoxylin and Eosin staining and were dewaxed with xylene three times treated with descending concentrations of Ethanol at 100% and 90% three times and treated with haematoxylin and then eosin before passing through ascending concentrations of Ethanol and 100% xylene.

Frozen sections were incubated at room temperature for one hour with the directly labelled primary antibody (Texas Red conjugated donkey anti-mouse IgG), washed with 1xPBS for 10 minutes and left to air dry. Mounting was done using Vectashield with DAPI (Vector laboratories, H-1200) and viewing with a Zeiss fluorescence microscope with Smart capture software (Digital Scientific Cambridge, UK).

3.2.2 CD4 T cell isolation

For this project it was important to isolate T cells directly from WT and Egr2-/-Eg3-/- mice for full use of primary cells. Cells were collected and red blood cells removed by the addition of 0.8% ammonium chloride for 5 minutes at 95°C. Cells were then treated with CD4 beads for 20 minutes in 1ml beads buffer (50ml PBS and 3ml RPMI) and using a positive selection kit, CD4

positive cells were then isolated by Magnetic Activated Cell Sorting (MACS) according to manufacturer's instruction.

3.2.3   Cell stimulation

In order to induce tolerance in vitro, we used plate bound anti-CD3 simulation alone at 1µg/ml. For comparison we used samples treated with anti-CD3 at 1µg/ml and anti-CD28 at 2µg/ml, and for negative controls we stimulated cells with 1xPBS. Cells were first incubated with plate bound antibodies for 24 hours at 37°C, washed and re-stimulated with plate bound anti-CD3 at 1µg/ml and anti-CD28 for 72 hours. IL-2 quantification was carried out using ELISA after 48 hours of incubation, and proliferation was carried out using a beta counter after addition of 3[H]-TdR 72 hours following re-stimulation. RT-PCR was carried out after collection of cells following re-stimulation and flow cytometry similarly (refer to main methods chapter).
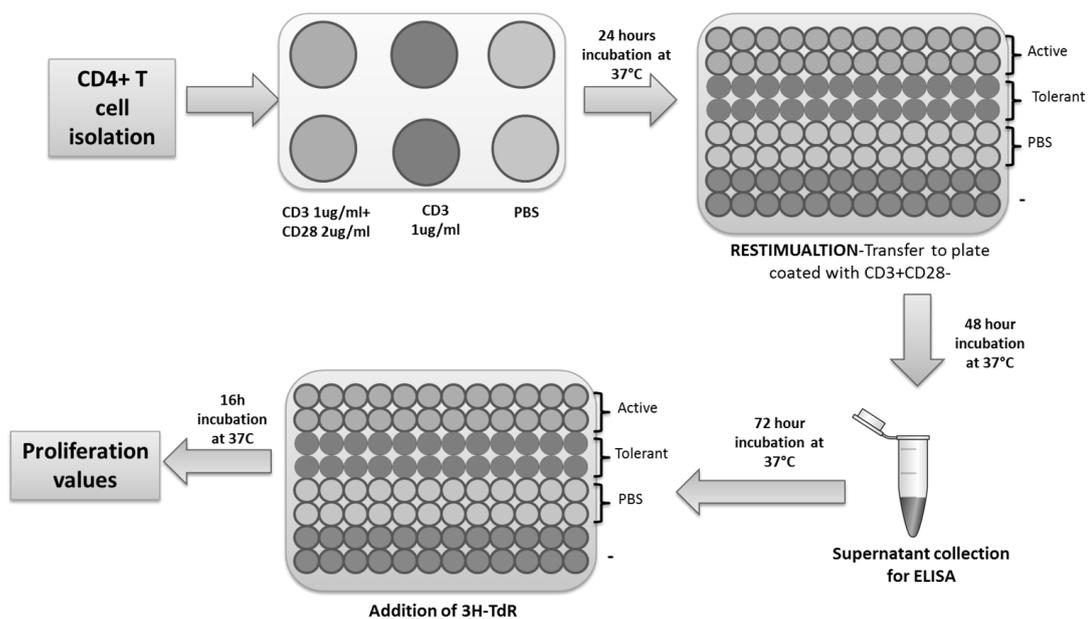
Figure 22: In-vitro tolerance induction

CD4+ T cells were isolated form Egr2-/-Egr3-/- and WT mice and stimulated with anti-CD3 1µg/ml and anti-CD28 2µg/ml (active), anti-CD3 alone at 1µg/ml (tolerant) or PBS (control). Cells were then incubated at 37°C for 24 hours to induce tolerance. cells were the re-stimulated with anti-CD3 and anti-CD28 for a further 48 hours at 37°C after which supernatant was collected for IL-2 and IFN-γ analysis via ELISA. The plate was then incubated for a further 24 hours and 3H-TdR/tritium was added. The plate was further incubated at 37°C and proliferation values assessed.

3.2.4 EMSA-electronic mobility shift assay

In order to determine the presence of transcription factors we used EMSA. Protein extraction was done first before quantification using a BSA assay and nanodrop. EMSA relies on the basis of the transcription factor binding to the promoter region. Once the binding probe is designed within the known promoter region, it is marked with a fluorescent marker or hot probe. Once the probe is added to the sample it binds to the desired promoter. Acrylamide gels were prepared and pre-run for 1 hour before samples were loaded. The consensus probes for AP-1 (5'-AGCTTCGCTTGATGAGTCAGCCG-3') and NFAT (5'- CTGTATCAAACAAATTTTCCTCTTTGG-3') were labelled and used in binding reactions with nuclear extracts from lymphocytes stimulated with anti-CD3 and anti-CD28, for CD4+ T cells and run on polyacrylamide gels.

3.3 Results

3.3.1.1 Confirmation of Egr2 and Egr3 Knockout Mice

In order to generate Egr2 and Egr3 knockout mice for this project, our group first developed Egr2 knockout mice in lymphocytes using the CD2 promoter (CD2-Egr2$^{-/-}$)(Zhu *et al.*, 2008). This was done because Egr2 deficient mice have a number of abnormalities including problems with peripheral nerve myelination (Topilko *et al.*, 1994). Once CD2-Egr2$^{-/-}$ mice were developed, they were crossed with Egr3 knockout mice (Egr3$^{-/-}$) mice to yield Egr2$^{-/-}$ Egr3$^{-/-}$ mice. For this study only Egr2$^{-/-}$ Egr3$^{-/-}$ mice were used unless stated otherwise.

Determination of CD2-Egr2-/-/Egr3-/- mice

Deletion of Egr2 using CD2 specific promoter was established using the Cre/ Lox-p system and confirmed using PCR. PCR results for CD2-Egr2$^{-/-}$ mice showed Cre at 195 base pairs and Lox- P homozygous at 210 base pairs.

In order to generate Egr3-/- mice, embryonic stem cells were used to carry out targeted mutagenesis by homologous recombination. Briefly, the Egr3 transcript was cloned into a pBluescript vector and disrupted using a fragment that contained a neomycin resistance selection cassette. The cassette deleted a fragment of the gene that encoded for the entire zinc finger binding domain resulting in Egr3-/- mice (Tourtellotte and Milbrandt, 1998b).

To create Egr2-/-/Egr3-/- mice, the Egr2-/- were crossed with Egr3-/- mice. We crossbred to from the first generation and used littermates to form the second generation. At this stage, mice that were homozygous for Egr3, Lox-p heterozygous for Egr2 and cre positive were selected for further breeding the C57BL background (Li *et al.*, 2012a). PCR results showed Egr2$^{-/-}$/Egr3$^{-/-}$ mice with a positive Cre band, homozygous Lox-p band and a homozygous Egr3 band (Figure 23).
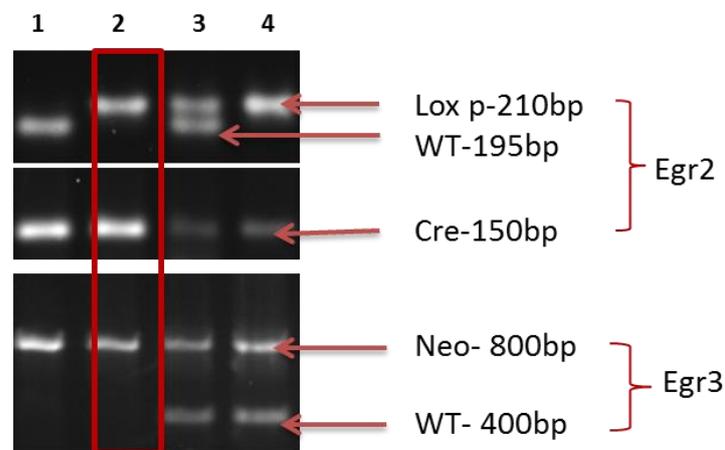
Figure 23: PCR verification of CD2-Egr2-/-Egr3-/- Mice

To create CD2-Egr2-/-Egr3-/- Mice Egr2-/- and Egr3-/- mice were crossed. PCR results show Egr2 knockout bands for lox-p allele at 210bp, WT allele at 195 bp and cre at 150bp. Egr3 knockout result shows the Neo allele at 800bp and the WT at 400bp. Lane 1 shows Egr2+/-Egr3-/-, Lane 2 shows Egr2-/-Egr3-/-, Lane 3 shows Egr2-/+Egr3-/+ and lane 4 shows Egr2-/-Egr3-/+. All mice used in this project were Egr2-/-Egr3-/- in lane 2 (indicated in red rectangle) unless stated otherwise.

In order to confirm protein expression of Egr2 Splenocytes were collected from Egr2$^{-/-}$Egr3$^{-/-}$ mice and WT mice were stimulated and stained with CD4 FITC and Egr2 APC (Figure 24). Flow cytometry was carried out by Professor Ping Wang.
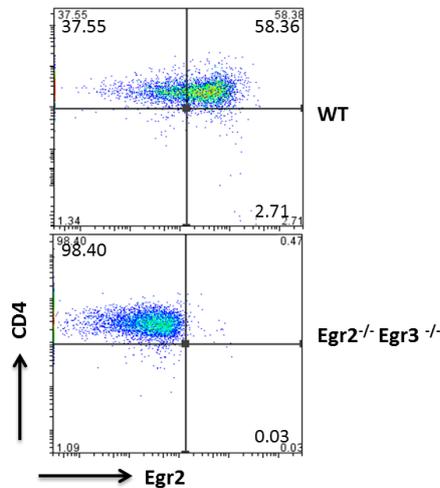
Figure 24: Confirmation of Egr2 Protein WT and expression in Egr2-/-Egr3-/- mice

Flow cytometry confirmation of Egr2 Protein WT and expression in Egr2-/-Egr3-/- mice Results show decreased Egr2 expression in Egr2-/-Egr3-/- compared to WT

3.3.1.2 Induction of anergy in Egr2 and 3 deficient T cells

Egr2 and 3 have been reported to be important for T cell tolerance and knocking them down renders T cells resistant to anergy induction in vitro (Zheng *et al.*, 2012). Previously, we discovered that Egr2 and 3 knockout mice develop severe autoimmune diseases suggesting that Egr2 and 3 deficient T cells lose self-tolerance (Li *et al.*, 2012a). The previous results are from single deficiency of Egr2 or 3 (Zheng *et al.*, 2012). We adopted the use of Eg2-/-Egr3-/- mice developed as previously described by Li *et al.* (Li *et al.*, 2012a). Within this chapter; in order to confirm the tolerogenic function of Egr2 and 3 in T cells, naïve CD4 T cells with deficiency of both Egr2 and 3 were assessed for the induction of anergy in vitro.

Egr2 and Egr3 are not involved in the induction of T cell anergy

T cell anergy is described as a tolerance mechanism that induces a state of functional underactivity recognised by decreased IL-2 and proliferation levels (Shwartz, 2003). It is largely due to the lack of 'second signal' or co-stimulatory signal after antigen stimulation (Shwartz, 2003). The role of Egr2 in this mechanism has recently been closely studied. Transfection of

Egr2 in Th1 cell line in vitro renders cells resistant to T cell receptor (TCR) stimulation by measuring proliferation and IL2 production (Zheng *et al.*, 2012)(Chai and Lechler, 1997)

 Chai and Lechler, 1997 which are the hallmarks for T cell anergy (Jenkins *et al.*, 1987a).

Using well-established protocols for induction of anergy in vitro, naïve CD4 T cells from WT and CD2-Egr2$^{-/-}$Egr3$^{-/-}$ mice were treated with anti-CD3 at different doses in the absence of costimulatory signals. The induction of anergy was compared to the T cell activation induced by both anti-CD3 and anti-CD28. Consistent with previous observations, Egr2 and 3 deficient CD4 T cells respond to anti-CD3 and anti-CD28 poorly in both proliferation and production of IL2 (Li *et al.*, 2012a). The proliferation was 2.5-fold less than wild type counterparts (Figure 25), together with the impaired IL-2 production in response to anti-CD3 and anti-CD28 stimulation (Figure 26). This observation is consistent with reported findings from other groups in previous Egr2 knockdown experiments (Zheng *et al.*, 2012).
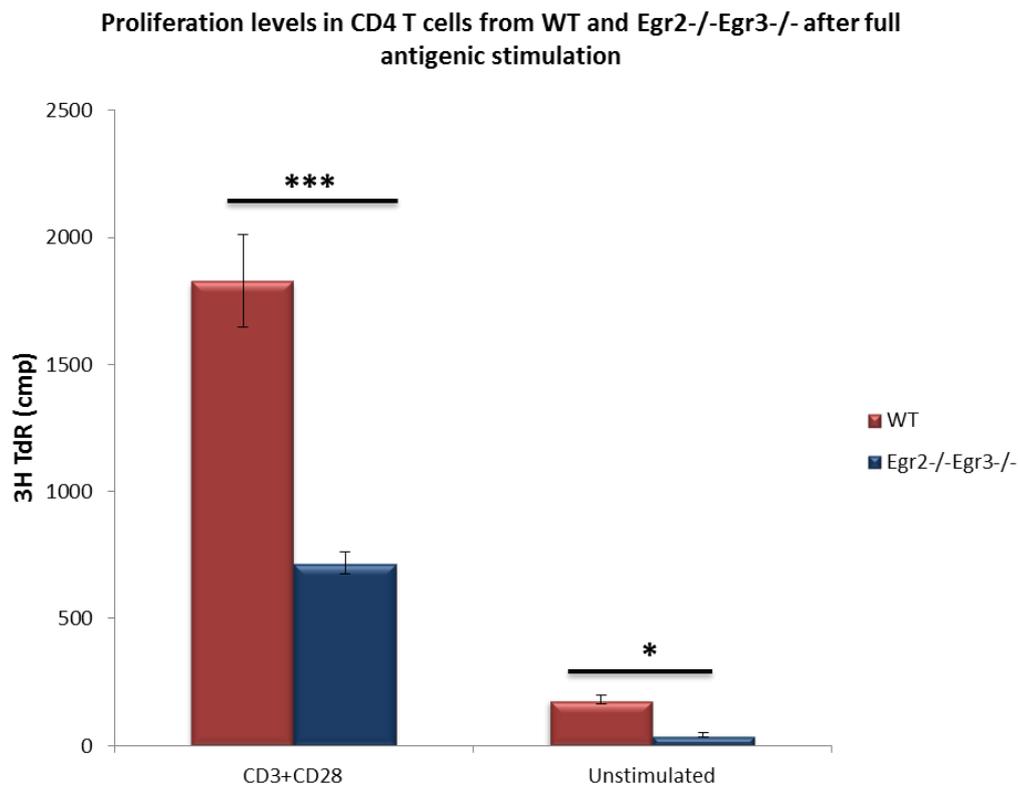
Figure 25: Deficiency of Egr2 and Egr3 impairs proliferative responses of CD4 T cells

CD4 T cells isolated for WT and Egr2-/-Egr3-/- mice were stimulated with or without anti-CD3 (1µg/ml) and anti-CD28 (2µg/ml) for 48 hours before pulsing with 3H-Trd for 8 hours. Proliferation was measured by β-counter. The data presented is a mean of four replicates from four mice and represent three independent experiments with similar results. Error bars calculated from standard error values. Statistical analysis was calculated using students T-test and p values set as follows –P<0.05 *, P<0.01 **, P<0.001***, NS-not significant.
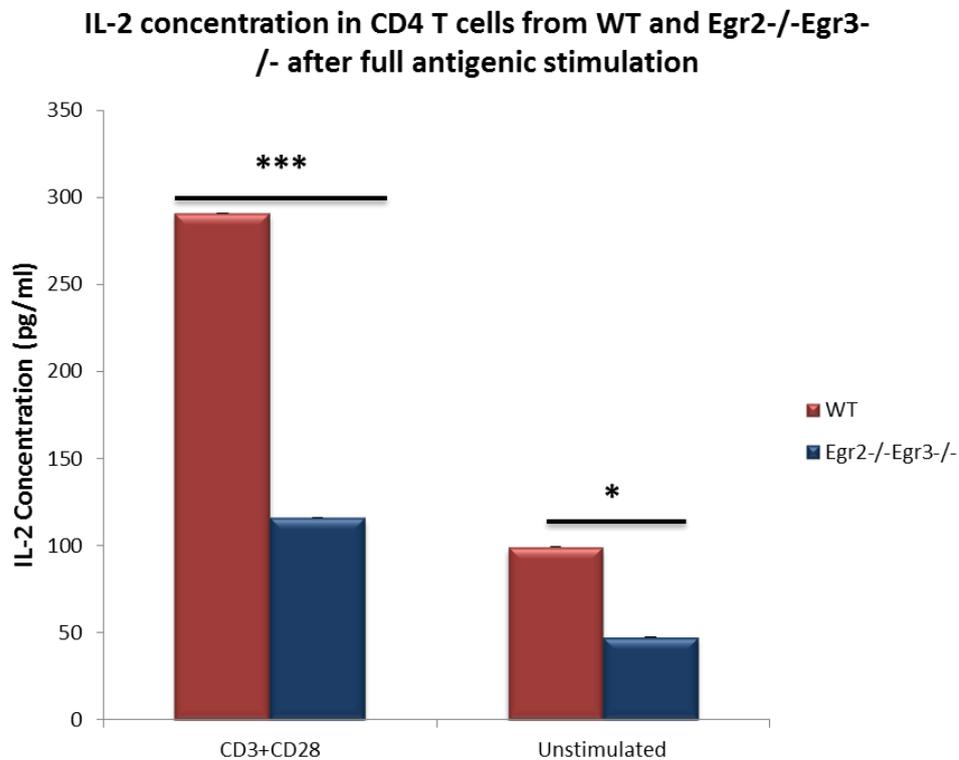
Figure 26: Deficiency of Egr2 and Egr3 impaired IL-2 production of CD4 T cells

CD4 T cells isolated for WT and Egr2-/-Egr3-/- mice were stimulated with or without anti-CD3 (1μg/ml) and anti-CD28 (2μg/ml) for 48 hours. IL-2 production in supernatant was assessed by ELISA. The data presented is a mean of four replicates from four mice and represent three independent experiments with similar results. Error bars calculated from standard error values. Statistical analysis was calculated using students T-test and p values set as follows –P<0.05 *, P<0.01 **, P<0.001***, NS-not significant

To induce anergy, naïve CD4 T cells from wild type and CD2-Egr2-/-Egr3-/- mice were stimulated with different doses of anti-CD3 in the absence of anti-CD28. Following anergy induction, we viewed the cells; we found 'clusters' of cells that we thought to be indicative of the cells proliferative ability thus we called them 'proliferation clusters'. The proliferation clusters had reduced in number within samples treated with anti-CD3 alone compared to those treated with anti-CD3 and anti-CD28, indicating reduced proliferation (Figure 27).
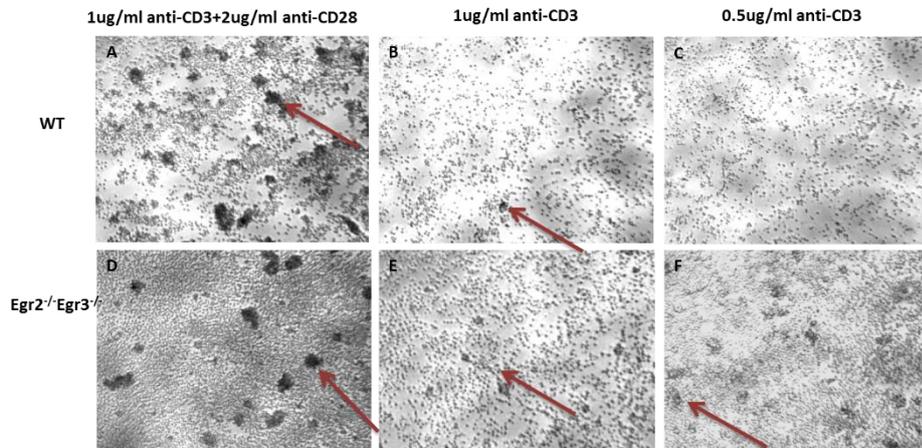
Figure 27: Visualisation of impaired proliferation levels in naïve CD4 T cells from wild type and CD2-Egr2$^{-/-}$Egr3$^{-/-}$ mice in response to anergic stimulation

CD4 T cells from WT and Egr2-/-Egr3-/- mice were stimulated with anti-CD3 at (A,D)1μg/ml, (B,E) 0.5μg/ml (C,F) in the absence of anti-CD28 for 24 hours for anergy induction, then re-challenged for a further 48 hours with anti-CD3 (1μg/ml) and anti-CD28 (2μg/ml). Cells were captured under x10 magnification. The data represent three independent experiments with similar results -Red arrows point to proliferation clusters.

To confirm this, we measured the proliferation using a beta counter and found the proliferation of wild type CD4 T cells was severely reduced, as was the production of IL-2 in comparison to the T cells activated by both anti-CD3 and anti-CD28. To our surprise, the proliferation of Egr2 and 3 deficient naïve CD4 T cells was also significantly reduced in comparison to the activation condition induced by both anti-CD3 and anti-CD28 (Figure 28). Consistent with the reduced proliferative response, the production of IL-2 was also impaired (Figure 29), indicating tolerance induction similar to that within the WT sample. This finding challenges the importance of Egr2 and 3 in anergy induction.
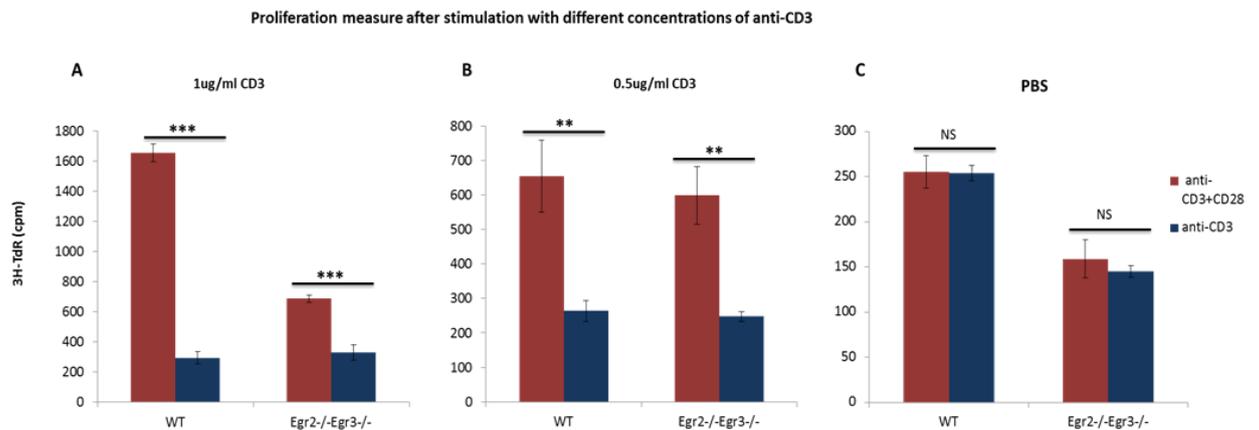
Figure 28: Impaired proliferative response in WT and Egr2-/-Egr3-/- CD4 T cells in response to anergic stimulation

Naïve CD4 T cells from WT and Egr2-/-Egr3-/- mice were stimulated with or without anti-CD3 at (A)1µg/ml, (B) 0.5µg/ml (C) and PBS in the absence of anti-CD28 for 24 hours for anergy induction, re-challenged for a further 48 hours with anti-CD3 (1µg/ml) and anti-CD28 (2µg/ml) before pulsing with 3H-Trd for 8 hours. Proliferation was measured by β-counter. The data presented is a mean of four replicates from four mice and represent three independent experiments with similar results. Error bars calculated from standard error values. Statistical analysis was calculated using students T-test and p values set as follows –P<0.05 *, P<0.01 **, P<0.001***, NS-not significant.
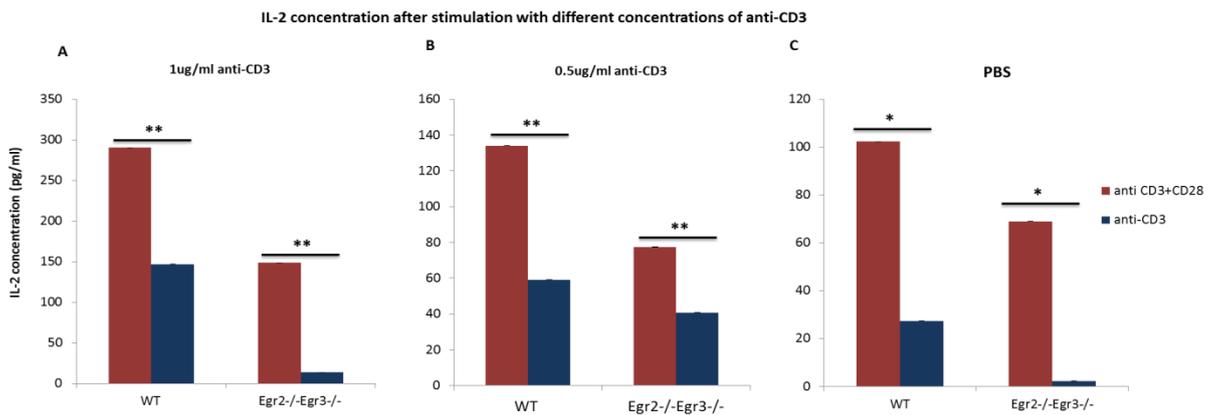
Figure 29: Impaired IL-2 production in WT and Egr2-/-Egr3-/- CD4 T cells in response to anergic stimulation

Naïve CD4 T cells from WT and Egr2-/-Egr3-/- mice were stimulated with anti-CD3 at (A)1μg/ml, (B) 0.5μg/ml (C) and PBS in the absence of anti-CD28 for 24 hours for anergy induction, washed and re-challenged for a further 48 hours with anti-CD3 (1μg/ml) and anti-CD28 (2μg/ml). IL-2 presence was measured using ELISA using culture medium collected after 48 hours. The data presented is a mean of four replicates from four mice and represent three independent experiments with similar results. Error bars calculated from standard error values. Statistical analysis was calculated using students T-test and p values set as follows –P<0.05 *, P<0.01 **, P<0.001***, NS-not significant.

To exclude the possibility that the reduced IL-2 and proliferation was not due to increased cell death in Egr2 and 3 deficient cells, the percentage of live and dead cells were compared. The reduced cell number in anergy cells compared to activated cells in wild type similar to Egr2 and 3 deficient CD4 T cells further indicated that Egr2 and 3 deficient T cells can undergo anergy induction at a similar level as the wild type counterpart (Figure 30).

The Induction of anergy in Egr2/3 deficient CD4 T cells is mediated by the NFAT pathway

The mechanism for induction of anergy in vitro with lack of secondary signal is due to partial activation of TCR mediated by the NFAT pathway (Fathman and Lineberry, 2007). Therefore, anergy is reversible if the NFAT pathway is blocked (Shwartz, 2003)(Fathman and Lineberry, 2007). Early studies have shown that for in vitro anergy to take place there is over activation of NFAT compared to transcription factors such as NFκβ. In addition, anergy induces the upregulation of a number of genes however the upregulation of these genes is not seen within NFA-/- mice and neither is anergy induction. Chai and Lechner (1997) showed the induction of

anergy can be blocked by the addition of cyclosporine A, which blocks NFAT dependant signalling (Jenkins, Schwartz and Pardoll, 1988).
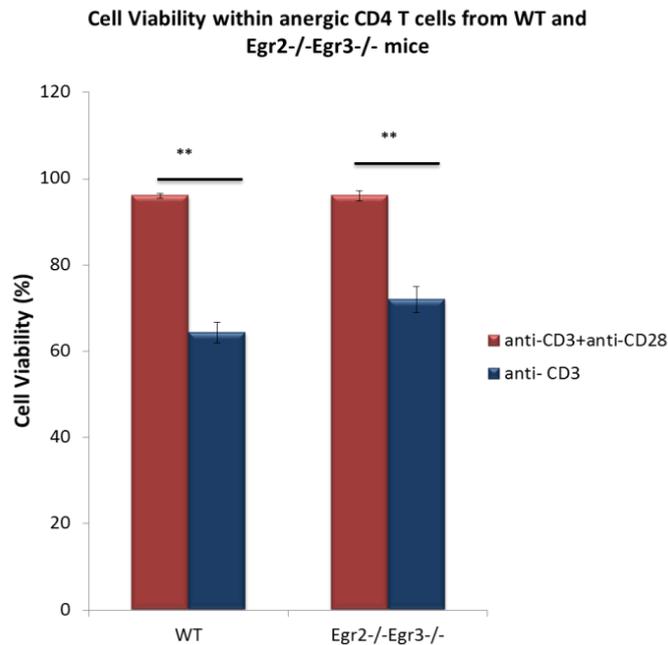
Figure 30: Negligible cell death within anergic WT and Egr2-/-Egr3-/- CD4 T cells

Naïve CD4 T cells from WT and Egr2-/-Egr3-/- mice were stimulated with anti-CD3 (1µg/ml) in the absence of anti-CD28 for 24 hours for anergy induction, washed and re-challenged for a further 48 hours with anti-CD3 (1µg/ml) and anti-CD28 (2µg/ml). Cells were diluted with trypan blue at a ratio of 1:1 and viability assessed using a countess automated cell counter to establish cell viability. The data presented is a mean of four replicates from four mice and represent three independent experiments with similar results. Error bars calculated from standard error values. Statistical analysis was calculated using students T-test and p values set as follows –P<0.05 *, P<0.01 **, P<0.001***, NS-not significant.

To assess the mechanisms for the induction of anergy in wild type vs Egr2 and 3 deficient CD4 T cells, we used cyclosporine A to block the NFAT pathway. Cyclosporine A is an undecapeptide isolated form the fungus *Hypocladium inflatum gams* and has been used for the treatment of allograft rejection (Borel *et al.*, 1977). It has been reported that CsA is able to inhibit T cell activation and prevent in vitro anergy by blocking the NFAT pathway (Shwartz, 2003). Safford *et al.* (2005) found that the addition of CsA to cells under anergy condition results in the production of increased levels of IL-2 similar to normal stimulation.

To assess this, naïve CD4 T cells from wild type and CD2-specific Egr2-/Egr3- mice were stimulated with activation or anergy conditions by anti-CD3 and anti-CD28 or anti-CD3 alone.

In another sample treated with anti-CD3 alone, CsA was added to investigate its effect on the induction of anergy. In the absence of CsA, anergy was induced in both wild type and Egr2 and 3 deficient CD4 T cells which exhibited reduced IL-2 concentrations coupled with decreased proliferation. However, addition of CsA effectively prevented anergy in both wild type and Egr2 and Egr3 deficient CD4 T cells (Figure 31 and Figure 32). Consistently, both proliferation and IL-2 production were reversed to similar levels compared to levels after a full antigenic stimulation using anti-CD3 and anti-CD28 in both wild type and Egr2 and 3 deficient CD4 T cells. These results not only confirmed the induction of anergy in Egr2 and 3 deficient CD4 T cells but also indicated that NFAT pathway activated by partial activation of TCR is the mechanism for both wild type and Egr2 and 3 deficient T cells. Thus, we have now discovered that Egr2 and 3 are involved in TCR mediated activation, but not in TCR mediated anergy.
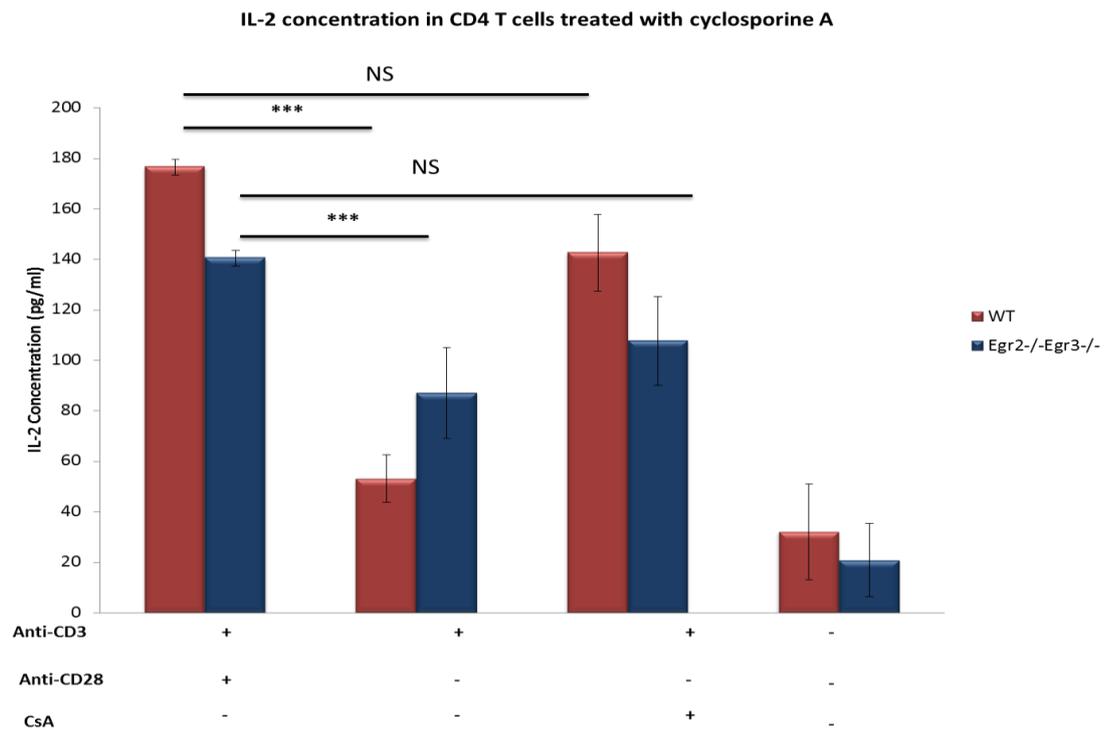
Figure 31: Cyclosporine A rescues IL-2 production in WT and Egr2-/-Egr3-/- anergic CD4 T cells

Naïve CD4 T cells from WT and Egr2-/-Egr3-/- mice were stimulated with either anti-CD3 (1μg/ml) in the absence of anti-CD28, anti-CD3 (1μg/ml) and 0.01μM CsA in the absence of anti-CD28 or with anti-CD3(1μg/ml) and anti-CD28 (2μg/ml) for 24 hours for anergy induction, re-challenged for a further 48 hours with anti-CD3 (1μg/ml) and anti-CD28 (2μg/ml). IL-2 presence was measured using ELISA using culture medium collected after 48 hours. Cells without stimulation served as negative control. Error bars calculated from standard error values. The data presented is a mean of four replicates and represent three independent experiments with similar results.
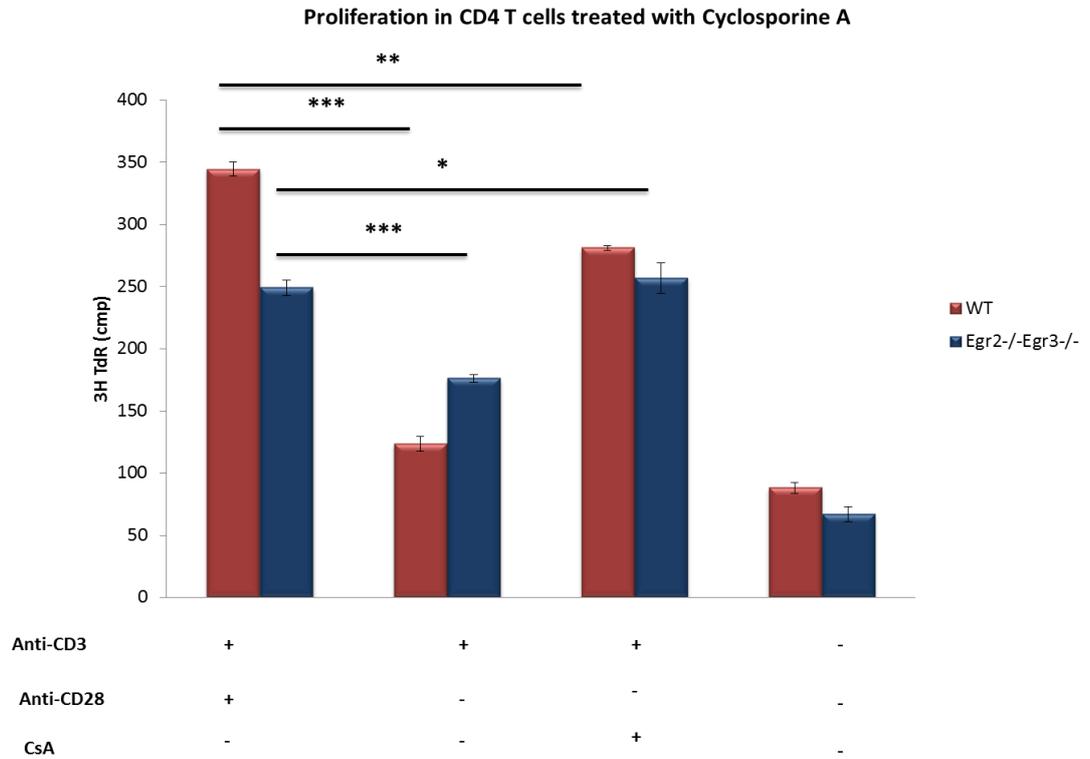
Figure 32: Cyclosporine A recovers proliferative responses of WT and Egr2-/-Egr3-/- anergic CD4 T cells

Naïve CD4 T cells from WT and Egr2-/-Egr3-/- mice were stimulated with either anti-CD3 (1µg/ml) in the absence of anti-CD28, anti-CD3 (1µg/ml) and 0.01uM CsA in the absence of anti-CD28 or with anti-CD3(1µg/ml) and anti-CD28 (2µg/ml) for 24 hours for anergy induction, then re-challenged for a further 48 hours with anti-CD3 (1µg/ml) and anti-CD28 (2µg/ml), Before pulsing with 3H-Trd for 8 hours. Proliferation was measured by β-counter. The cells without stimulation served as negative control. Error bars calculated from standard error values. The data presented is a mean of four replicates and represents three independent experiments with similar results.

3.3.2   The effect of anergy induction in Egr2-/-Egr3-/- mice

3.3.2.1   The production of inflammatory cytokines in anergic Egr2 and Egr3 deficient CD4 T cells

The activation of T cells with TCR and co-stimulatory signals plays a crucial role in their eventual fate, however an additional signal is required, which is the cytokine influence ((Shwartz, 2003)). CD2 specific Egr2 deficient CD4 T cells were already reported to have accumulations of cells which produced high levels of inflammatory cytokines such as IL-17A (Miao *et al.*, 2013)(Li *et al.*, 2012a) Zheng *et al.*, 2012). Zheng *et al.* (2012) confirmed a similar result in CD4 T cells from Egr2 and 3 deficient mice. Egr2 and 3 deficient CD4 T cells treated with anti-CD3 and anti-CD28 for a full antigenic stimulation express much higher levels of IFN-γ compared to naïve wild type CD4 T cells (see Figure 33). Li *et al.* (2012a) found the CD2-Egr2-/-Egr3-/- mice presented with early onset autoimmune disease development with symptoms that included splenomegaly (Figure 34) and glomerulonephritis (Figure 35). Spleen and lymph node weights were taken from WT and Egr2-/-Egr3-/- mice at 4 and 8 weeks of age with lymph nodes and spleen weights at 8 weeks increasing at least two fold in Egr2-/-Egr3-/- mice (Figure 34). Kidneys from WT and Egr2-/-Egr3-/- mice were assed using haematoxylin and eosin staining and immunohistochemistry; we found increased cellularity in Egr2-/-Egr3-/- kidneys along with IgG deposits (Figure 35).
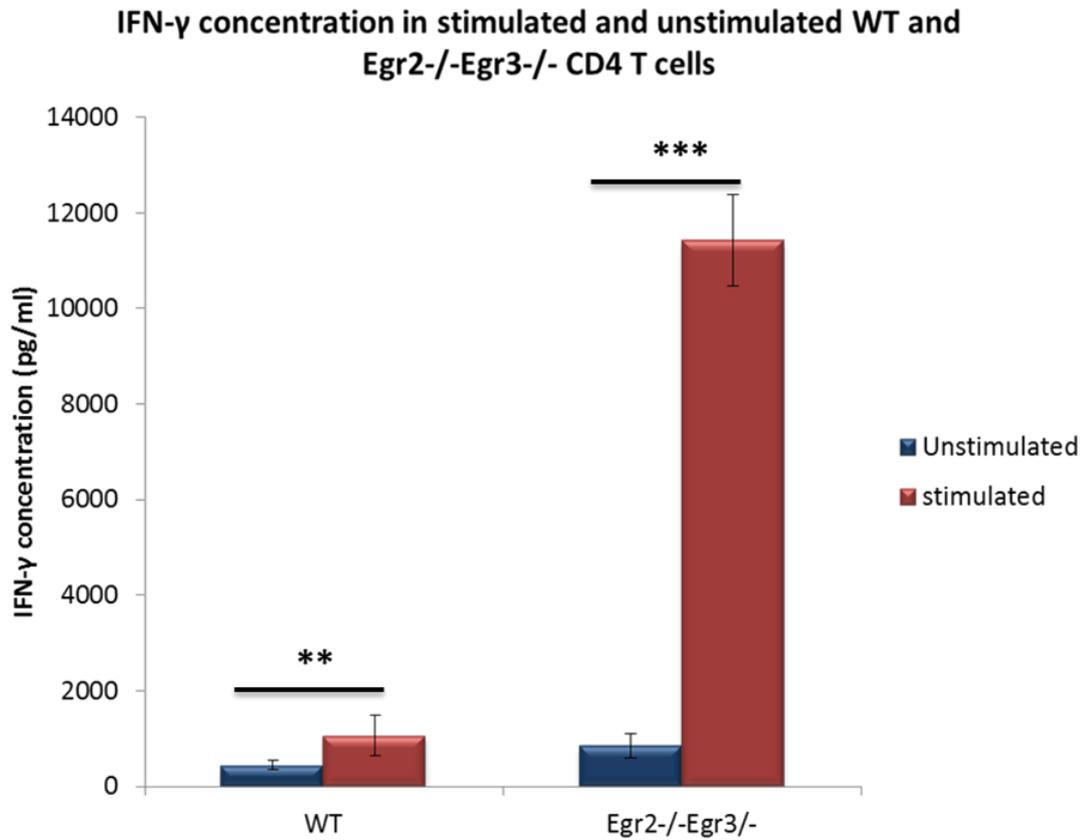
Figure 33: Deficiency of Egr2 and Egr3 results in increased IFN-γ production in CD4 T cells

CD4 T cells isolated for WT and Egr2-/-Egr3-/- mice were stimulated with anti-CD3 (1µg/ml) and anti-CD28 (2µg/ml) or left unstimulated for 48 hours and supernatant collected. IFN-γ production was measured in supernatant by ELISA. The data presented is a mean of four replicates from four mice and represent three independent experiments with similar results. Error bars calculated from standard error values. Statistical analysis was calculated using students T-test and p values set as follows –P<0.05 *, P<0.01 **, P<0.001***, NS-not significant.
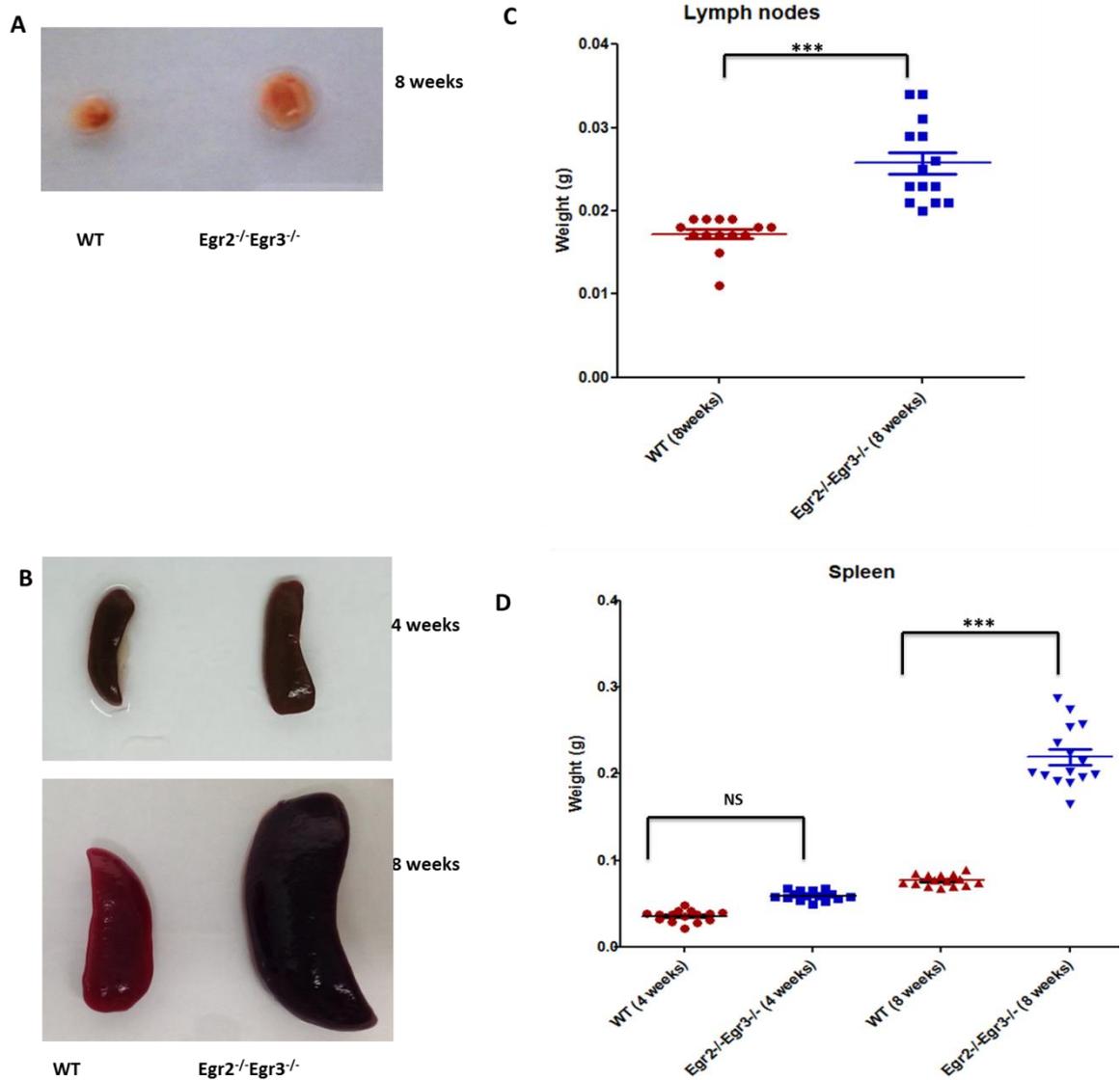
Figure 34: Egr2-/-Egr3-/- mice exhibit splenomegaly

A, C; Increased size and weight of lymph nodes and spleen in Egr2-/-Egr3-/- mice. B, D; Increased spleen size and weight in Egr2-/-Egr3-/- mice at 4 and 8 weeks compared to WT. The data represent 15 mice examined at each time points (4 weeks and 8 weeks). Probability was calculated using Student's T test, p values set as follows –$P<0.05$ *, $P<0.01$ **, $P<0.001$***, NS-not significant.
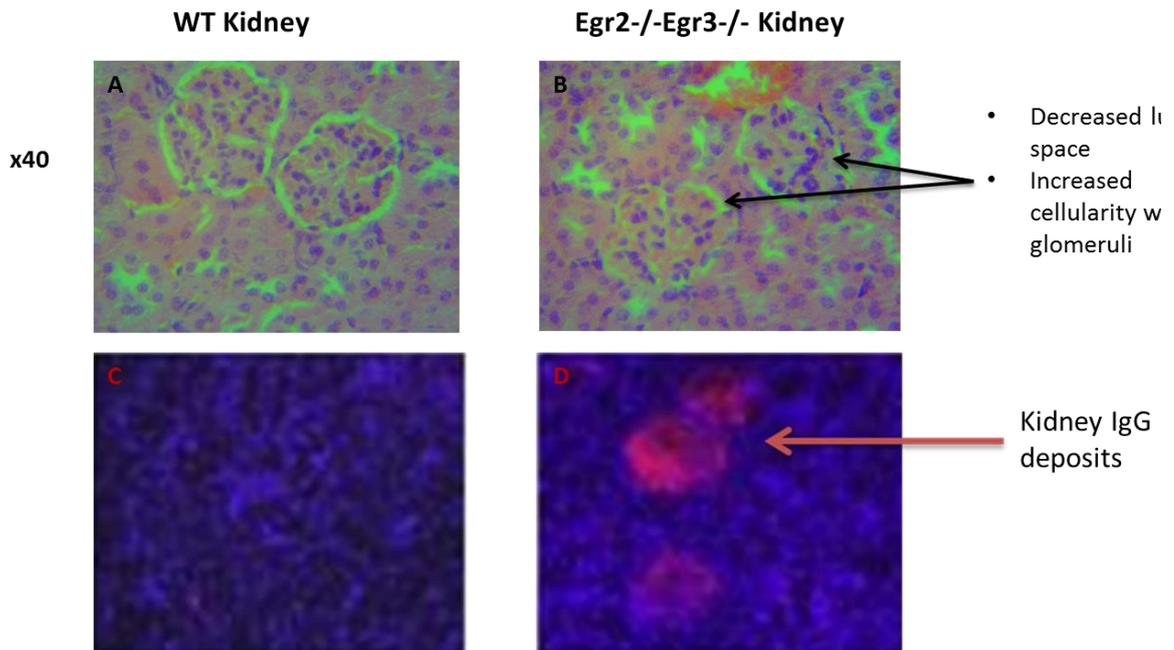
Figure 35: Glomerulonephritis in Egr2-/-Egr3-/- mice kidneys

Kidneys collected were stained with haematoxylin and Eosin; Egr2-/-Egr3-/- kidneys showed increased cellular deposition compared to WT kidneys (B). Immunostaining revealed IgG deposits within Egr2-/-Egr3-/- kidneys (D).

A hallmark of anergic CD4 T cells is the decreased expression of IL-2 compared to CD4 T cells treated with both antigenic and costimulatory signals. Evidently, IL-2 is the most important cytokine with its measure determining the state of the cells. Inflammatory cytokine production has not been investigated in the anergy setting, and the role of tolerance induction within an inflammatory or autoimmune disease environment has yet to be studied. In order to address these questions, we used Egr2-/-Egr3-/- mice as CD2-Egr2-/-Egr3-/- CD4 T cells have increased inflammatory cytokine production on full stimulation, we have shown anergy is inducible in Egr2 and Egr3 null CD4 T cells, we were curious about the effect anergy induction would have on inflammatory cytokine production. IFN-γ was shown to have the most dramatic increase in expression in CD2-Egr2-/-Egr3-/- CD4 T cells compared to other cytokines such as IL-17A after receiving full antigenic stimulation (Li *et al.*, 2012a).

To examine the expression of IFN-γ in anergic conditions, CD4 T cells from naïve WT and CD2-Egr2-/-Egr3-/- deficient mice were thus treated with anti-CD3 at a dose of 1µg/ml over 24 hours and re-stimulated over 48 hours before assessment of IFN-γ levels via ELISA. In

accordance with previous results, the Egr2-/-Egr3-/- CD4 T cells treated with anti-CD3 and anti-CD28 had increased expression of IFN-γ compared to the WT. To our surprise, IFN-γ levels within anergic CD4 T cells from Egr2 and 3 deficient CD4 T cells remained increased (Figure 36) Compared to those treated with anti-CD3 and anti-CD28. Interestingly as we earlier attempted to reverse anergy using cyclosporine A, we had found a similar result in IFN-γ. Addition of CsA allowed for high IFN-γ levels similar to samples treated without CsA in the anergy setting (Figure 37).
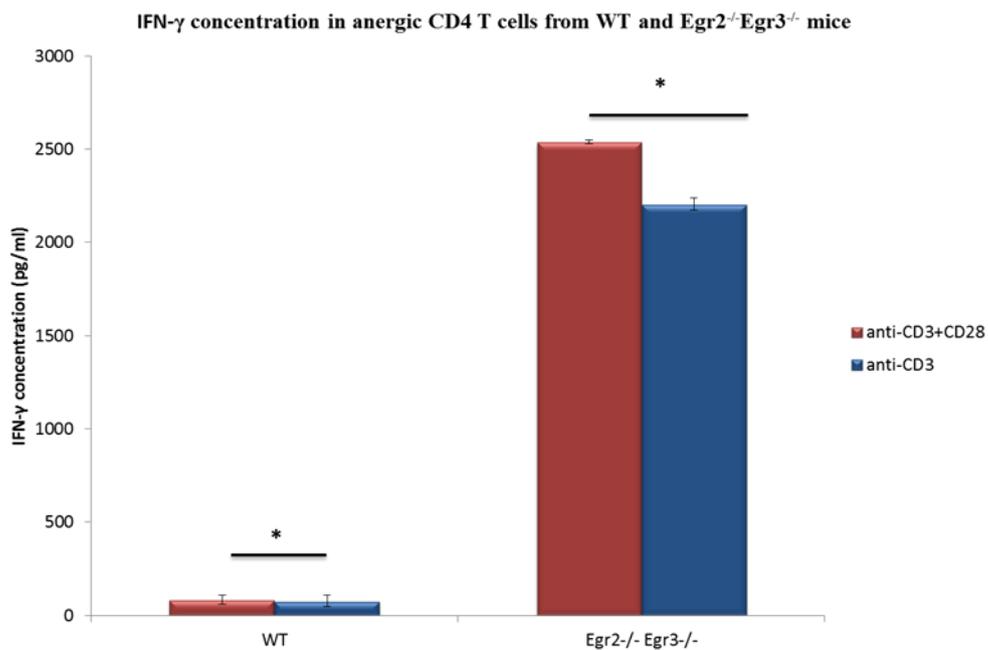
Figure 36: Increased IFN-γ concentration in anergic Egr2-/-Egr3-/- CD4 T cells

Naïve CD4 T cells from WT and Egr2-/-Egr3-/- mice were stimulated with anti-CD3 (1µg/ml) in the absence of anti-CD28 for 24 hours for anergy induction, washed and re-challenged for a further 48 hours with anti-CD3 (1µg/ml) and anti-CD28 (2µg/ml). IFN-γ production was measured using ELISA using supernatant. The data presented is a mean of four replicates from four mice and represent three independent experiments with similar results. Error bars calculated from standard error values. Statistical analysis was calculated using students T-test and p values set as follows –P<0.05 *, P<0.01 **, P<0.001***, NS-not significant.
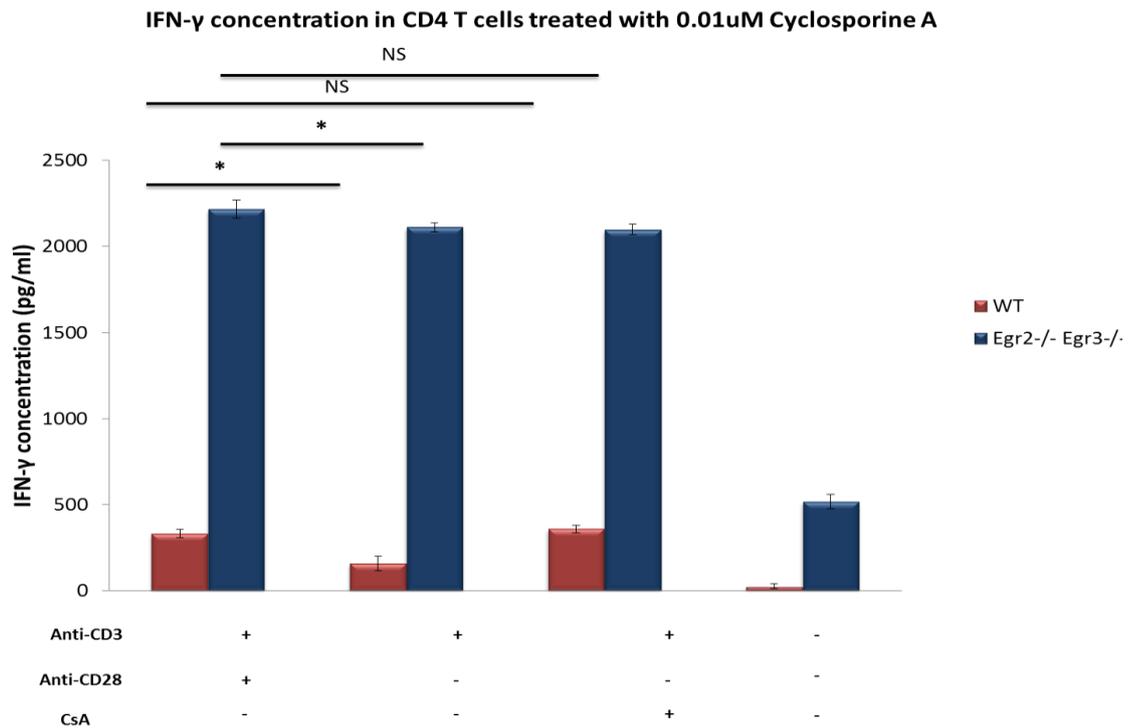
Figure 37: CsA addition results in high IFN-γ production in naïve CD4 T cells from wild type and CD2-Egr2-/Egr3- mice

CD4 T cells from WT and Egr2-/-Egr3-/- mice were stimulated with either anti-CD3 (1µg/ml) in the absence of anti-CD28, anti-CD3 (1µg/ml) and 0.01uM CsA in the absence of anti-CD28 or with anti-CD3(1µg/ml) and anti-CD28 (2µg/ml) for 24 hours for anergy induction, washed and re-challenged for a further 48 hours with anti-CD3 (1µg/ml) and anti-CD28 (2µg/ml). IFN-γ presence was measured using ELISA using culture medium collected after 48 hours. The data presented is a mean of four replicates from four mice and represent three independent experiments with similar results. Error bars calculated from standard error values. Statistical analysis was calculated using students T-test and p values set as follows –P<0.05 *, P<0.01 **, P<0.001***, NS-not significant.

These results, along with our previous findings, highlight the role of Egr2 and Egr3 in control of inflammatory cytokine production. As IFN-γ levels are still increased in Egr2 and 3 knockout CD4 T cells, including those in the anergic setting, the results show that anergy induction has little to do with Egr2 and Egr3 expression (as shown by Jenkins et al in 1987 during the first experaiments with anergy induction). Rather, it highlights the possible role of Egr2 and 3 in effector T cell differentiation and in turn, inflammatory cytokine production within the tolerant setting.

3.3.3 The impact of TCR signalling on Egr2 expression in the tolerance setting

3.3.3.1 Egr2 expression within mitogenic conditions

Egr2 is not expressed in naïve T cells, but is induced in response to TCR stimulation in vitro or in vivo (Li *et al.*, 2011). In contrast to previous reports (Safford *et al.*, 2005; Harris *et al.*, 2004), we have now demonstrated that Egr2 and 3 are involved in promoting TCR medicated proliferation, but dispersive for anergy induction. It has been reported that Egr2 and 3 are induced by TCR stimulation in both naïve and tolerant T cells (Zheng *et al.*, 2012). However, the differences in Egr2 and 3 expression in partial or mitogenic TCR stimulation is unknown, which may explain their function in naïve, but not tolerant T cells for regulation of proliferation.

To assess if the expression of Egr2 in tolerant T cells is solely based on the absence of co-stimulatory signal, naïve CD4 T cells from wild type mice were stimulated with different doses of anti-CD3 or combined stimulation of anti-CD3 and anti-CD28. Egr2 was rapidly induced in naïve CD4 T cells in response to anti-CD3 stimulation (Figure 38). However, the level of expression was dose dependent and we see the lower concentration the bigger the difference in Egr2 expression (Figure 39).
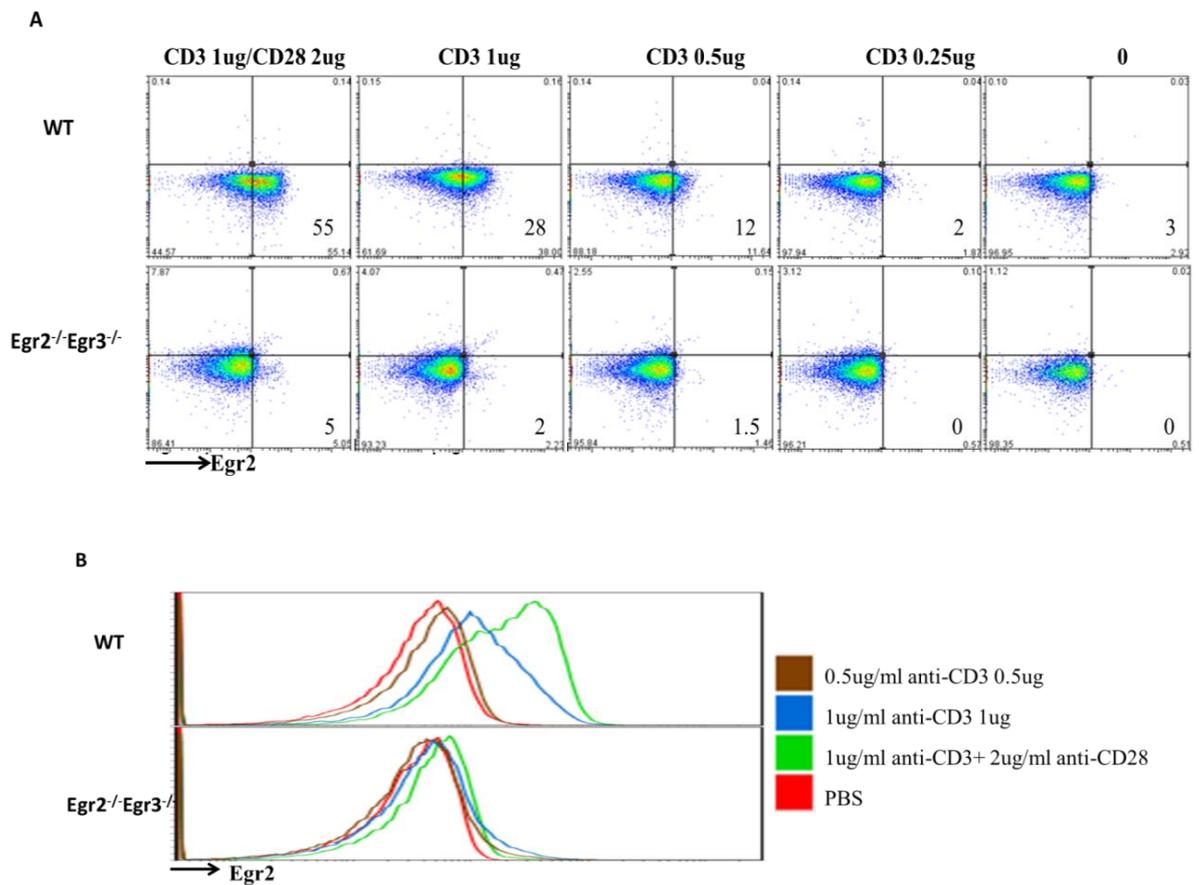
Figure 38: Induction of Egr2 expression in WT CD4+ T cells by TCR stimulation is dose dependent

Naïve CD4 T cells from four wild type mice were stimulated with different doses of anti-CD3 (1μg/ml, 0.5μg/ml, 0.25μg/ml) or combined stimulation of anti-CD3 (1μg/ml) and anti-CD28 (2μg/ml) for 24 hours. CD4 T cells were stained for Egr2 and expression monitored using Flow cytometry (A) Dot plot showing Egr2 expression correlated with anti-CD3 concentration within the WT CD4+ T cells. (B) Egr2 in WT with varying TCR stimulation Flow cytometry histogram, showing the kinetic shift of different samples treated with different concentrations of anti-CD3 in the naïve WT and Egr2-/-Egr3-/- CD4 T cells. Cells were gated on the CD4+ population. Data are representative of three individual experiments.

The levels of TCR signalling directly affect the proliferative responses of naïve T cells (Figure 40). The results further demonstrate the importance of Egr2 in promoting naïve T cell proliferation justified to the levels of TCR stimulation. The association of Egr2 with strength of TCR signalling in promoting proliferative responses is further supported by increased expression of Egr2 when naïve CD4 T cells were stimulated with a combination of anti-CD3 and CD28. These results also indicate that Egr2 is induced at high levels in naïve T cells by both TCR and co-stimulation and at low levels in the absence of co-stimulation.
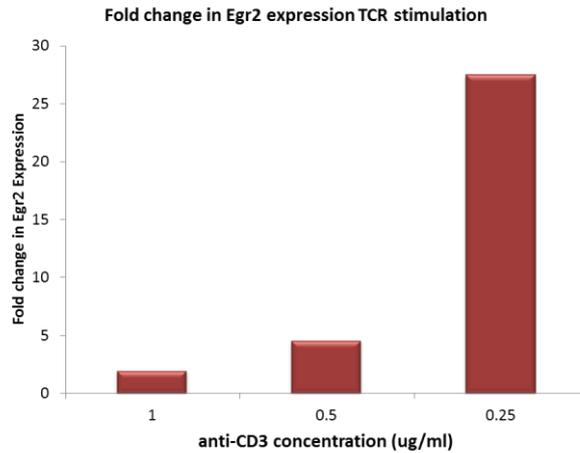
Figure 39: Change of Egr2 expression in response to different doses of anti-CD3 stimulation

Fold change from naïve CD4 T cells from wild type mice were stimulated with different doses of anti-CD3 (1µg/ml, 0.5µg/ml, 0.25µg/ml) or combined stimulation of anti-CD3 (1µg/ml) and anti-CD28 (2µg/ml) for 24 hours. CD4 T cells were stained for Egr2 and expression monitored using Flow cytometry. Fold change was calculated in comparison to samples treated with anit-CD3+anti-CD28. Cells without stimulation served as control. The figure shows increasing fold change with decreasing anti-CD3 concentration.
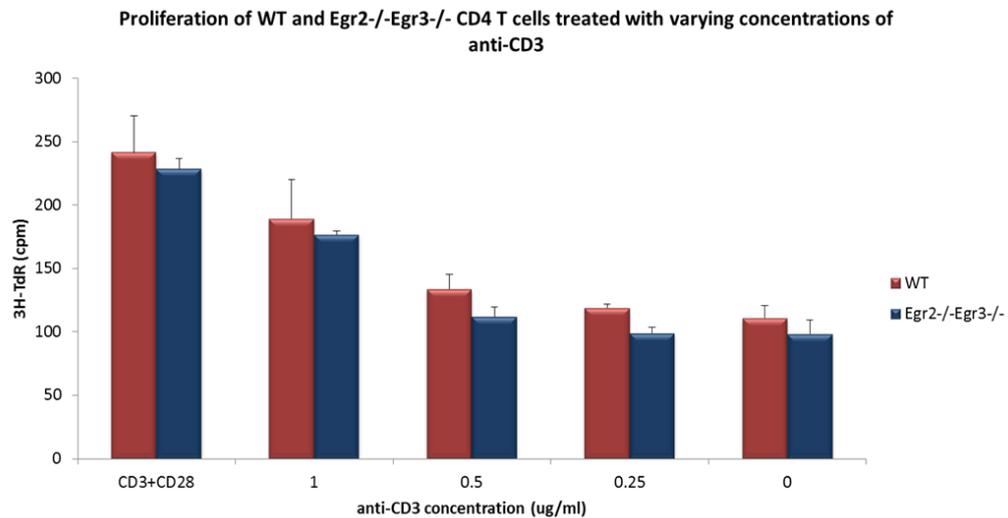
Figure 40: TCR signalling strength directly affects proliferation levels in naïve CD4 T cells from Wild type and Egr2-/Egr3-/- mice

Naïve CD4 T cells from wild type mice were stimulated with different doses of anti-CD3 (1µg/ml, 0.5µg/ml, 0.25µg/ml) or combined stimulation of anti-CD3 (1µg/ml) and anti-CD28 (2µg/ml) for 24 hours. Cells were washed and re-challenged for a further 72 hours and proliferation assessed by addition of 3H-Trd for 8 hours. Error bars calculated from standard error values. Data is representative of three experiments.

3.3.3.2 The levels of cytokine expression in naïve T cells is regulated by the doses of TCR signalling

T cell receptor signalling plays a crucial role in the T cell activation; in order for in vitro T cell anergy to take place a TCR signal is essential while the costimulatory as from CD28 is usually absent (Shwartz, 2003). As TCR plays such a crucial role in the mechanism of induction of tolerance, we looked at the effects of antigenic strength in vitro using cytokine production on stimulation. Using a sample treated with anti-CD3 and anti-CD28 as a control which showed increased IL-2 expression, we assessed the effect of anti CD3 concentration, specifically the strength of antigenic stimulation on IL-2 production within CD2-Egr2-/-Egr3-/- CD4 T cells.

On treating cells with increasing doses of anti-CD3 and a stable CD28 at 5μg/ml, IL-2 expression increased with increasing anti-CD3 concentration (Figure 41). However we did not find a similar correlation with varying anti-CD28 stimulation with anti-CD3 at 1μg/ml (Figure 42), whereas varying anti-CD3 stimulation gave a clear relationship with IL-2, anti-CD28 concentration was unrelated to IL-2 production highlighting the role of TCR stimulation in T cell proliferation.
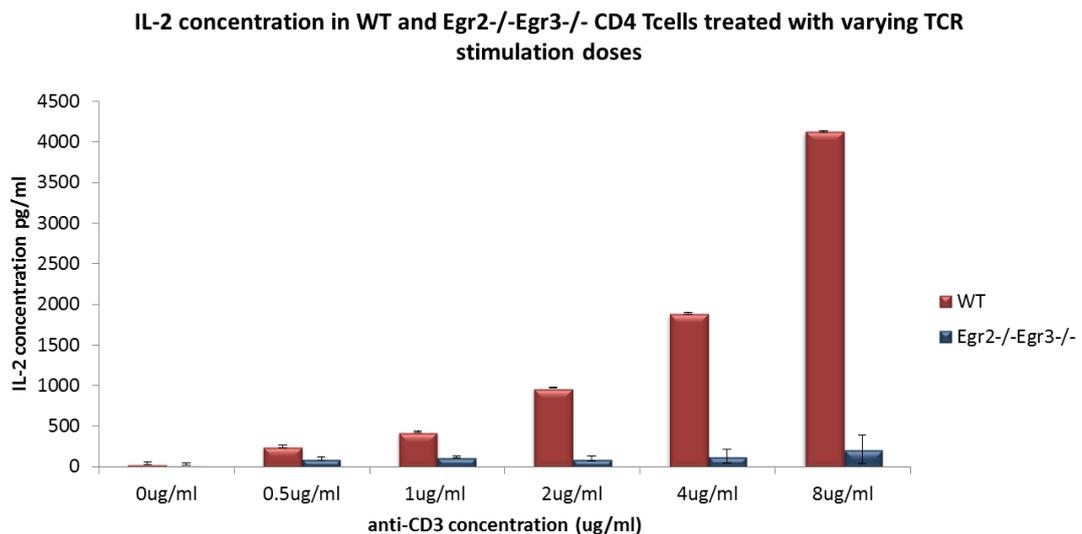
Figure 41: IL-2 expression increased according to increasing anti-CD3 concentration within WT and Egr2-/-Egr3 -/- CD4+ T cells

Naïve CD4 T cells from wild type mice were stimulated with 5μg/ml anti-CD28 and varying concentrations of anti-CD3 (0, 0.5ug, 1ug, 2ug, 4ug and 8ug) for 48 hours before medium collection. IL-2 concentration was measured using ELISA. Data is representative of three individual experiments
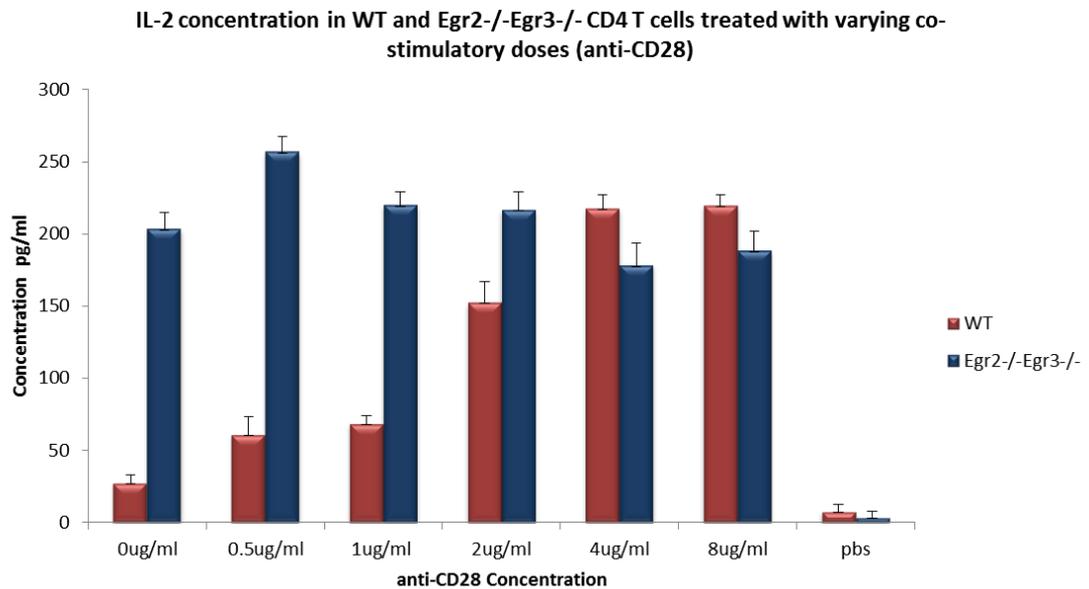
Figure 42: IL-2 expression unaffected by co-stimulatory signalling in Egr2-/-Egr3-/- CD4 T cells

Naïve CD4 T cells from wild type mice were stimulated with 1μg/ml anti-CD3 and varying concentrations of anti-CD28 (0, 0.5ug, 1ug, 2ug, 4ug and 8ug) for 48 hours. Medium was collected and IL-2 measured by ELISA. Error bars calculated from standard error values. Data shown is representative of three individual experiments.

With CD2-Egr2-/-Egr3-/- CD4 T cells producing increased amounts of IFN-γ, we were curious to see if the correlation applies in regard to inflammatory cytokine production. Since within Egr2 and 3 null mice the T cells are skewed toward the production of Th1 predominant cytokines (Li *et al.*, 2012), we hypothesised that, like the production of IL-2, the production of IFN-γ would correlate with TCR stimulation. We found the highest production of IFN-γ was seen after a full stimulation of Egr2-/-Egr3-/- CD4 T cells (Figure 43A). On treatment using only anti-CD3 at different concentrations we found a decrease at 1μg/ml in IFN-γ production compared to treatment with anti-CD3 and anti-CD28. This production then gradually decreased with decreasing concentration of anti-CD3 in a linear manner B). These results correlate with (Yamamoto, Hattori and Yoshida, 2007) where they monitored the expression of IFN-γ with varying concentrations of TCR stimulation within TH1 cells. They found decreasing concentrations of IFN-γ with decreasing antigenic stimulation. We showed a clear correlation between IFN-γ production and antigenic strength by generating a linear graph from the flow cytometry results obtained previously shown in Figure 44.
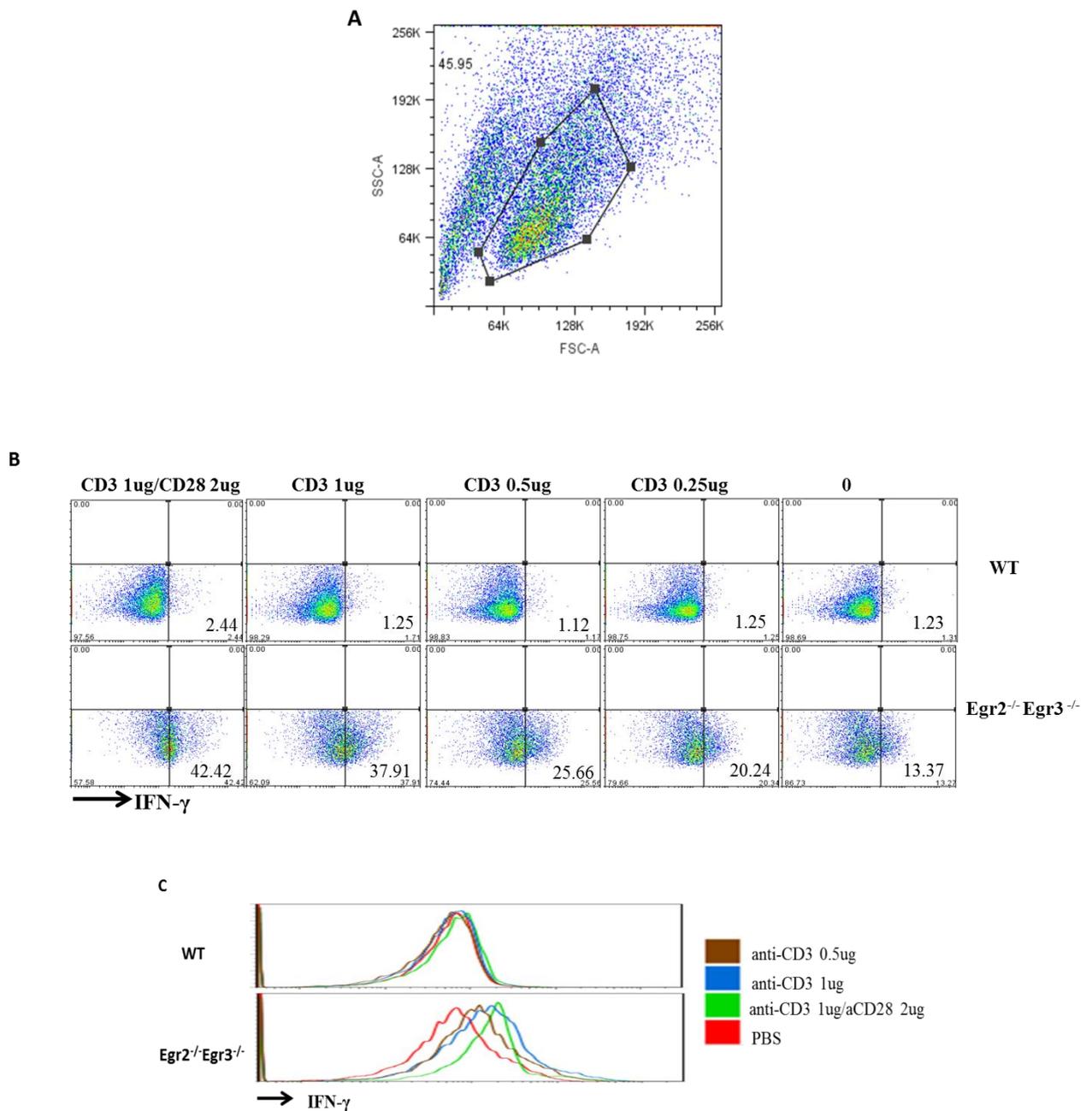
Figure 43: Induction of IFN-γ producing Egr2 and 3 deficient CD4 T cells in response to both active and tolerant stimulation

CD4 T cells from wild type and Egr2-/-Egr3-/- mice were stimulated with different doses of anti-CD3 (1µg/ml, 0.5µg/ml, and 0.25µg/ml) or combined stimulation of anti-CD3 (1µg/ml) and anti-CD28 (2µg/ml) for 24 hours. CD4 T cells were stained for IFN-γ and expression monitored using Flow cytometry. (A) CD4 cells after gating as side scatter (SSC) and forward scatter (FSC) (B) IFN-γ producing CD4 T cells under indicated stimulation conditions displayed as SSC and IFN-γ. (C) MFI of IFN-γ in wild type and Egr2 and 3 deficient CD4 T cells under indicated stimulation conditions. Data are from pooled cells of four mice and representative of three independent experiments.
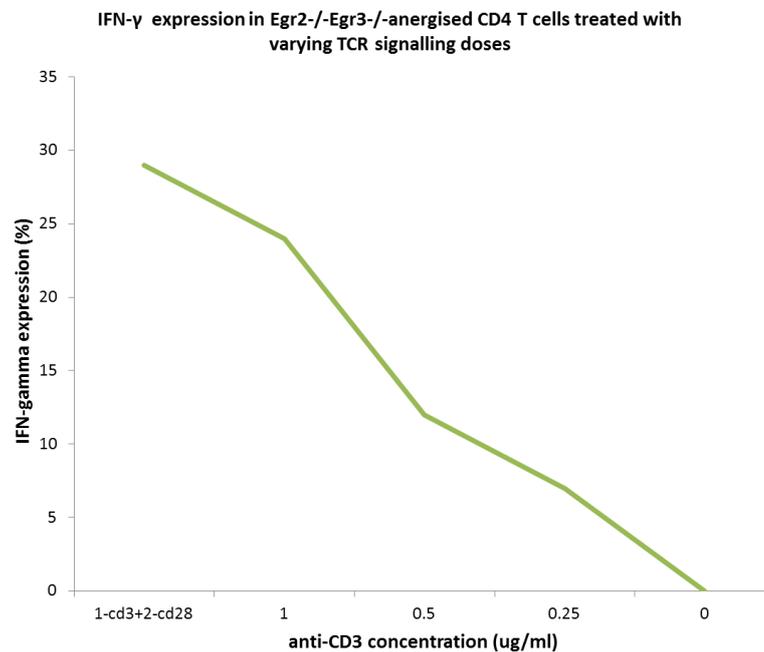
Figure 44: Linear reduction of IFN-γ producing cells in anergic Egr2 and 3 deficient CD4+ T cells challenged by different doses of anti-CD3

Linear graph adapted from FACS values obtained in Figure 43 ; where Naïve CD4 T cells from wild type and Egr2-/-Egr3-/- mice were stimulated with different doses of anti-CD3 (1µg/ml, 0.5µg/ml, and 0.25µg/ml) or combined stimulation of anti-CD3 (1µg/ml) and anti-CD28 (2µg/ml) for 24 hours.

Our results show Egr2 expression, IL-2 production and IFN-γ expression is all dependant on TCR stimulation in a dose dependant manner. These results in fact show that different antigenic stimulation results in altered effects and thus altered functionality of CD4 T cells, including proliferation and inflammation, emphasising the importance of TCR signalling strength in the production of effector cytokines.

3.3.3.3   Downstream effects of TCR signalling in tolerant Egr2-/-Egr3-/- CD4 T cells

Having shown the importance of TCR signalling in T cell activation, we concentrated on factors downstream of immediate TCR activation. For the activation of NFAT (nuclear factor of activated T cells) and the production of IL-2, a calcium flux must take place after the TCR meets the antigen. The importance of the calcium flux has been highlighted by multiple groups with the use of calcium ionophore ionomycin to induce anergy (Macian *et al.*, 2002)(Shwartz, 2003). Ionomycin is able to bypass the TCR and cause a calcium flux, thus ionomycin treatment alone is often used for CD4 T cell anergy induction in TH1 cells (Macian, 2005). This still results

in reduced IL-2 and proliferation, although studies with Th1 cells showed induction with ionomycin requires a longer time to induce compared to achieve than with anti-CD3 (Macian *et al.*, 2002). Our attempted anergy induction using ionomycin in Egr2-/-Egr3-/- CD4 T cells resulted in decreased IL-2 and although proliferation decreased it was not significantly viable in ionomycin induction. It is not as reliable or robust as the most commonly used plate bound anti-CD3 induction and is often very easily reversible over time (Fathman and Lineberry, 2007). Li *et al.* earlier reported a defect in AP-1 expression within Egr2-/-Egr3-/- CD4 T cells, resulting in decreased IL-2 production (Li *et al.*, 2012a). Similarly, we showed this using and EMSA (Figure 45A) and confirmed deceased expression by measuring the density of the bands (Figure 45B). EMSA or electronic mobility shift assay is a technique used to represent the binding of a transcription factor with the promoter region of a particular gene. Once identification of the gene was achieved, an AP-1 probe was designed. CD4 T cells were then stimulated with 1µg/ml anti-CD3 and 2µg/ml anti-CD28 overnight and protein extracted. A binding mix was then prepared for each sample with fluorescence markers and probes. The samples were run on a polyacrylamide gel as viewed in Figure 45 A and B, and measurements were then taken to confirm intensity.
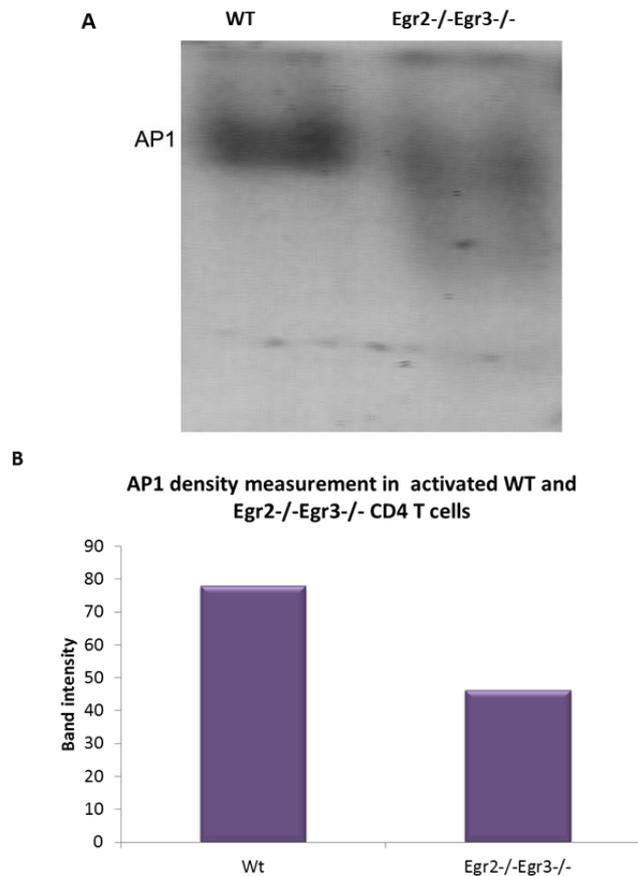
Figure 45: Decreased AP-1 expression within Egr2-/-Egr3-/- CD4 T cells

CD4 T cells from WT and CD2-Egr2-/-Egr3-/- were stimulated with anti-CD3 and anti-CD28 overnight and following protein extraction the expression of AP-1 assessed using EMSA (A). Results showed decreased expression in Egr2-/-Egr3-/- CD4 T cells. AP-1 density measure was carried out on bands from the EMSA experiment (B). The data are representative of 3 individual experiments using four mice per setting.

Following the calcium flux, activation of NFAT must take place to allow the dephosphorylation and mobilisation of NFAT into the nucleus (Harris *et al.*, 2004). We wanted to examine the expression of nuclear factor NFAT within active and tolerant wild type and Egr2 and 3 knockout CD4 T cells. To achieve this we once again used an EMSA carried out by Dr. Tizong Miao at QMUL to visualise NFAT expression. First, cells were treated with 1μg/ml anti-CD3 and 2μg/ml anti-CD28 and compared this to CD4 T cells stimulated with anti-CD3. NFAT probes were designed and once protein extraction was achieved, a reaction mix was prepared. An acrylamide gel was run to visualise each sample as shown in Figure 46. This showed NFAT signalling is similar within Egr2 and 3 knockout CD4 T cells and WT in both the active and tolerant states and confirmed by measuring the density of both the NFAT bands (Figure 47).

This result emphasises the importance of calcium signalling and NFAT activation within in vitro tolerance induction and proves it is not affected by the absence of Egr2 or 3.
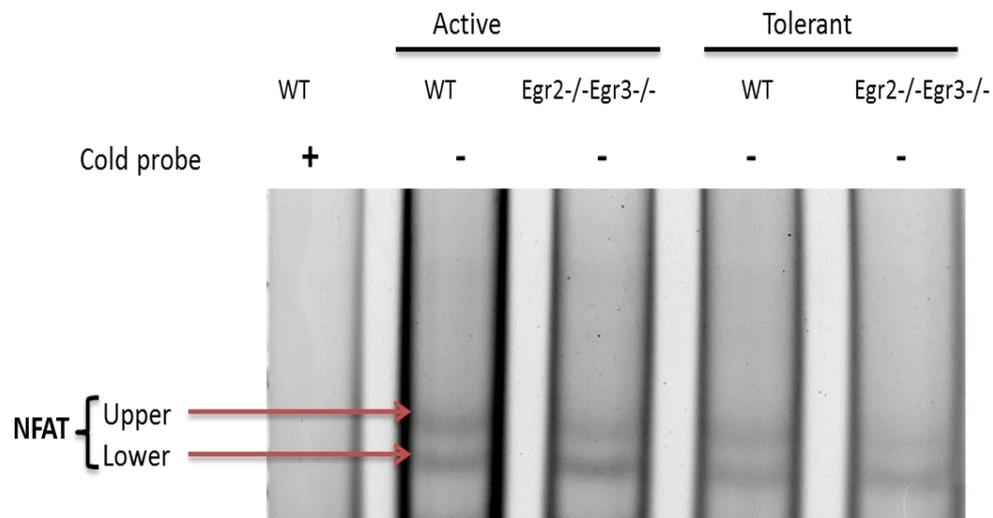
Figure 46: NFAT is activated in both active and anergic CD4 T cells

Naïve CD4 T cells form WT and Egr2-/-Egr3-/- mice were stimulated overnight with anti-CD3 overnight and re-stimulated with anti-CD3 and anti-CD28 for 3 hours to induce anergy. Activated samples were stimulated with anti-CD3 and anti-CD28 for 3 hours before harvesting.

Nuclear extracts were analysed for NFAT transcription factor expression by EMSA. Anergic samples in WT and Egr2-/-Egr3-/- CD4 T cells show decreased expression compared to their respective active samples. Representative of a single experiment.

A

NFAT upper band density measurement in tolernant CD4 T cells

B

NFAT lower band density measurement in tolerant CD4 T cells
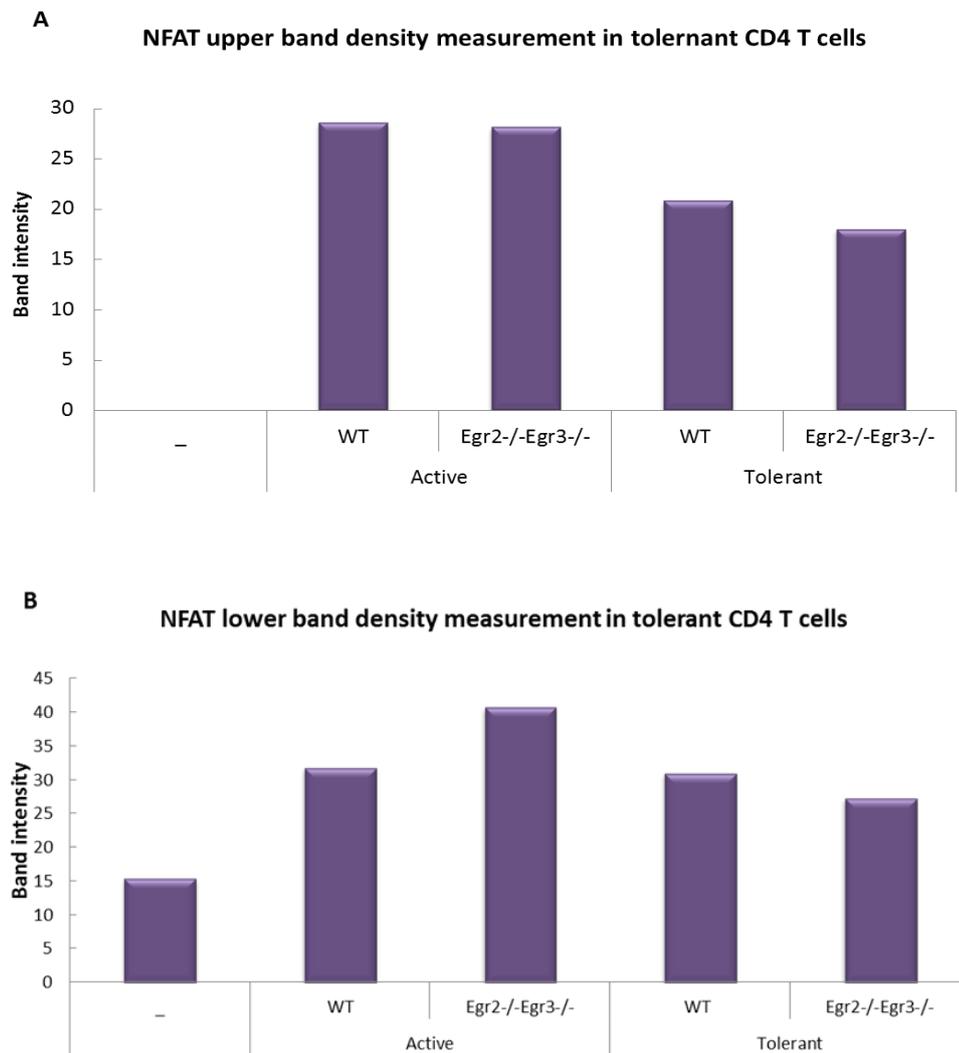
Figure 47: NFAT band binding reduced in tolerant CD4 T cells

Density measurement of the NFAT upper band was taken from NFAT EMSA from Figure 46. Results showed reduced levels of NFAT expression in the WT and Egr2-/-Egr3-/- tolerant cells. (A) NFAT upper band binding reduced in tolerant CD4 T cells. (B) NFAT lower band binding reduced in tolerant CD4 T cells.

3.3.4 Gene expression during in vitro tolerance induction

3.3.4.1 Tolerogenic gene expression in T cells

Egr2 and 3 have been shown to regulate a number of anergy genes that aid in the maintenance of this hyporesponsive state (Safford *et al.*, 2005). On TCR stimulation and calcium flux during anergy, NFAT dephosphorylation allows for the expression of such genes, including Egr2 and 3 (Shwartz, 2003); whereas the lack of MAPK activation by lack of co-stimulatory signalling allows for the expression of CBLB, GRAIL and ITCH (Fathman and Lineberry, 2007). These genes are upregulated during tolerance and include a multitude of functions: dyglycerol kinase alpha or DGK-alpha generates phosphatic acid, reducing the availability of dyglycerol after anti-CD28 stimulation and the two forms of DGK-α have been found to be upregulated during anergy. Zha *et al.* (2006) found the expression of DGK-α to be five times more in anergic TH1 clones as compared to cells treated with anti-CD3 and anti-CD28(Zha *et al.*, 2006)

. DGK-α expression was found to increase within wild type CD4 T cells treated in the anergic conditions compared to fully activated samples (Figure 48). Intriguing was the increased expression of DGK alpha within the anergic Egr2-/-Egr3-/- CD4 T cells compared to the cells treated with anti-CD3 and anti-CD28. According to our results, there was increased expression of DGK-alpha within the Egr2 and Egr3 knockout anergic CD4 T cells similar to that of the wild type.
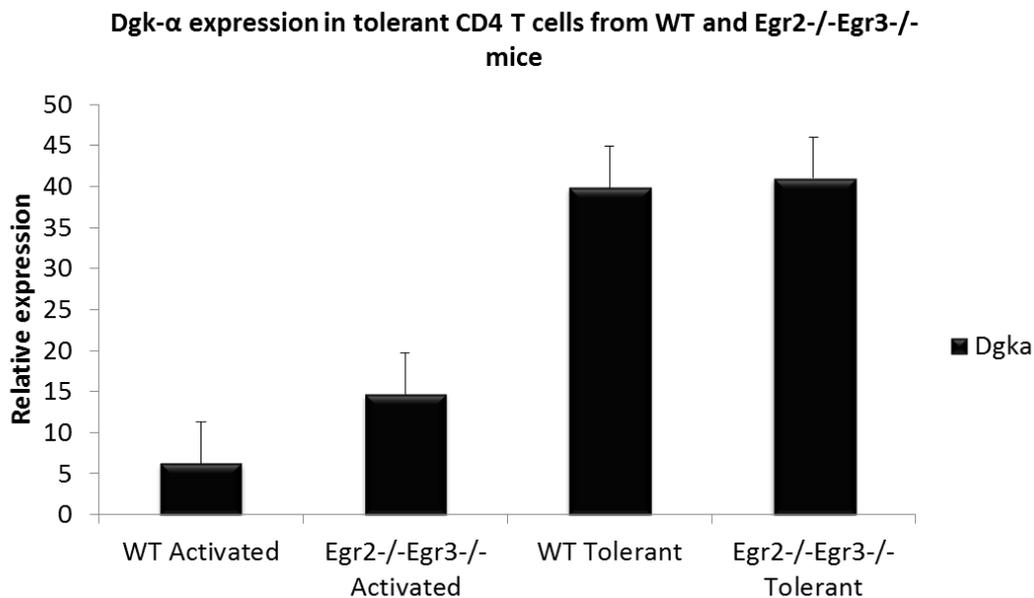
Figure 48: Dgk-α expression upregulated in anergic CD4 T cells from WT and Egr2-/-Egr3-/- mice

CD4 T cells from WT and Egr2-/-Egr3-/- mice were stimulated with anti-CD3 (1μg/ml) in the absence of anti-CD28 for 24 hours for anergy induction, washed and re-challenged for a further 24 hours with anti-CD3 (1μg/ml) and anti-CD28 (2μg/ml) prior to harvesting. Following RNA extraction RT-PCR was carried out to measure expression of DGK-alpha and data normalised against gapdh. Error bars calculated from standard error values. The data presented is a mean of four replicates and represent three independent experiments with similar results.

3.3.4.2   Ubiquitin ligase gene expression within anergic CD4 T cells

Along with DGK-α, a large number of genes upregulated downstream of TCR activation during anergy are ubiquitin ligase genes. During T cell activation ubiquitin serves as a key molecule controlling the process of ubiquitination. The attachment of these genes targets proteins for degradation among other targets anergic T cells have been associated with increased expression of ubiquitin genes Cblb, Itch and Grail (Fathman and Lineberry, 2007). These genes act to degrade key signalling molecules such as PLC-gamma and PKC-theta. Cblb functions as an E3 ubiquitin ligase via its RING domain. Multiple studies have been carried out on cblb-/- mice, with mice exhibiting spontaneous autoimmune diseases. More importantly cblb-/- mice are completely resistant to T cell anergy (Chiang *et al.*, 2000).

CD4 T cells were treated in anergic settings over 24 hours revealed increased levels of Cblb expression in the wild type and in the Egr2-/-Egr3-/- CD4 T cells compared with their

respective fully activated counterparts treated with anti-CD3 and anti-CD28 (Figure 49). Of note, the Cblb expression within the Egr2-/- Egr3-/- CD4 tolerant T cells was much higher than that in the WT, exhibiting a 2-fold increase. On the analysis of ITCH, we found similarly increased expression within anergic CD4 T cells from both the wild type and the Egr2 and 3 knockouts compared to their counterparts treated with anti-CD3 and anti-CD28 (Figure 50). Itch-/- mice have also been found to develop autoimmune disease associated with scratching, thus mice are referred to as 'itchy'. The role of Egr2 or 3 expression has not been directly studied in regard to ITCH expression within the anergy setting.
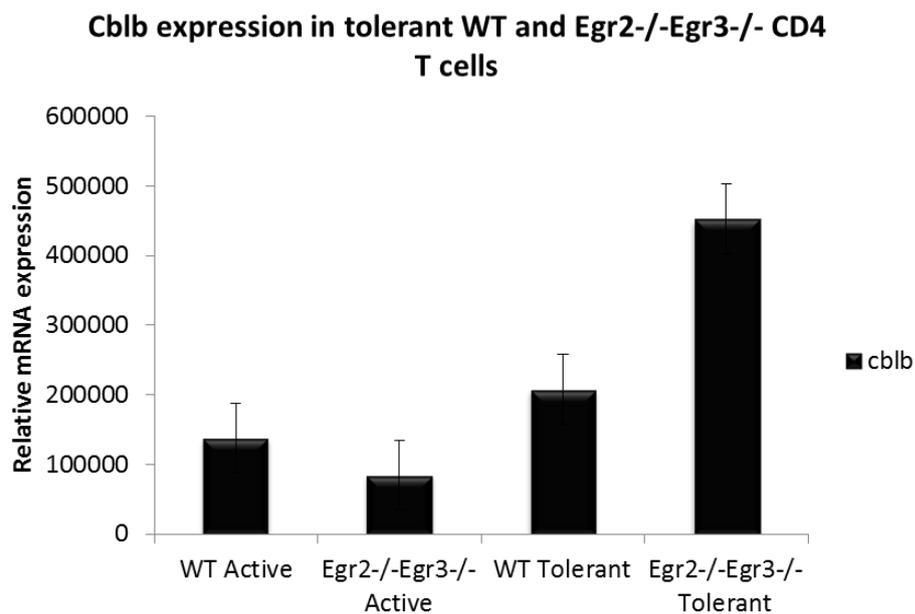
Figure 49: Cblb expression upregulated in tolerant CD4 T cells from WT and Egr2-/-Egr3-/- mice

CD4 T cells from WT and Egr2-/-Egr3-/- mice were stimulated with anti-CD3 (1µg/ml) in the absence of anti-CD28 for 24 hours for anergy induction, washed and re-challenged for a further 24 hours with anti-CD3 (1µg/ml) and anti-CD28 (2µg/ml) prior to harvesting. Following RNA extraction, RT-PCR was carried out to measure expression of Cbl-b. Error bars calculated from standard error values. The data is relative expression against housekeeping gene gapdh and presented is a mean of three replicates and represent three independent experiments with similar results.
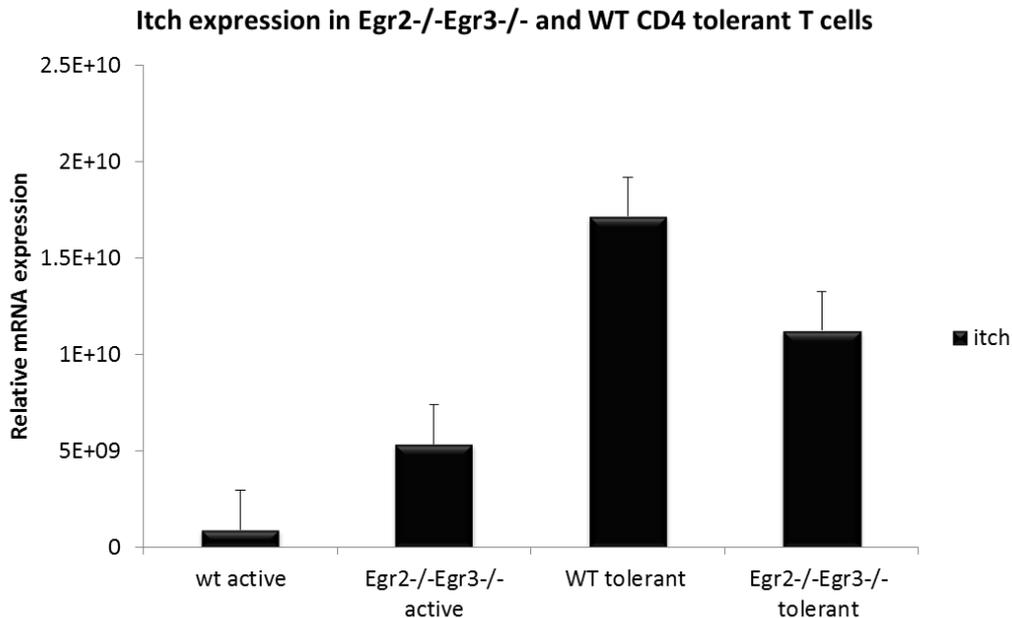
Figure 50: Itch expression is upregulated in tolerant CD4 T cells from WT and Egr2-/-Egr3-/- mice

CD4 T cells from WT and Egr2-/-Egr3-/- mice were stimulated with anti-CD3 (1µg/ml) in the absence of anti-CD28 for 24 hours for anergy induction, washed and re-challenged for a further 24 hours with anti-CD3 (1µg/ml) and anti-CD28 (2µg/ml) prior to harvesting. Following RNA extraction RT-PCR was carried out to measure expression of Itch. Error bars calculated from standard error values. The data is relative expression against housekeeping gene gapdh and presented is a mean of three replicates from four mice and represent three independent experiments with similar results.

3.3.4.3 Egr2 and 3 tolerance induction has no effect on T regulatory cell production

There are a number of peripheral mechanisms that allow for the evasion of autoimmunity. One T cell subset that contributes to this is T reg cells (T regulatory) cells. It is known T reg cell development is dependent on the expression of the transcription factor FOXP3 (Hsieh, Lee and Lio, 2012; O'Garra and Vieira, 2004). Another transcription factor has recently been identified that aids in the governing of T reg cells, Bach2 (Roychoudhuri *et al.*, 2013). Recently Bach2 has been found not only to be expressed within B cells but also in T regulatory cells where it is been found to be essential for the development of both naturally occurring T reg cells and induced T reg cells produced within the periphery (Roychoudhuri *et al.*, 2013). Egr2 and 3 have been found to have no effect on the production of T reg cells, and studies carried out on Egr2-

/-Egr3-/- found the function of CD4+CD25+ regulatory T cells (T reg) were not changed (Li *et al.*, 2012a).

We then sought to find out if the induction of tolerance would affect the production of T reg cells using Bach2 as a marker. To do this we treated WT and Egr2-/-Egr3-/- with anti-CD3 and anti-CD28 as an activated sample and with anti-CD3 alone as a tolerant sample. We then re-stimulated the samples with anti-CD3 and anti-CD28 before assessing the expression of Bach2. Our results showed WT samples had unchanged expression between the activated and the tolerant. The Egr2-/-Egr3-/- tolerant samples had an insignificant change between the activated and tolerant samples similar to that of the WT (Figure 51). This result confirms earlier studies that the function of T reg cells is not affected by the lack of Egr2 and Egr3. It also highlights this within the expression of peripheral T reg cells, that iTreg cells are not affected, even in the tolerant state. The induction of T reg cells remains unchanged within the tolerant state in CD2-Egr2-/-Egr3-/- mice.
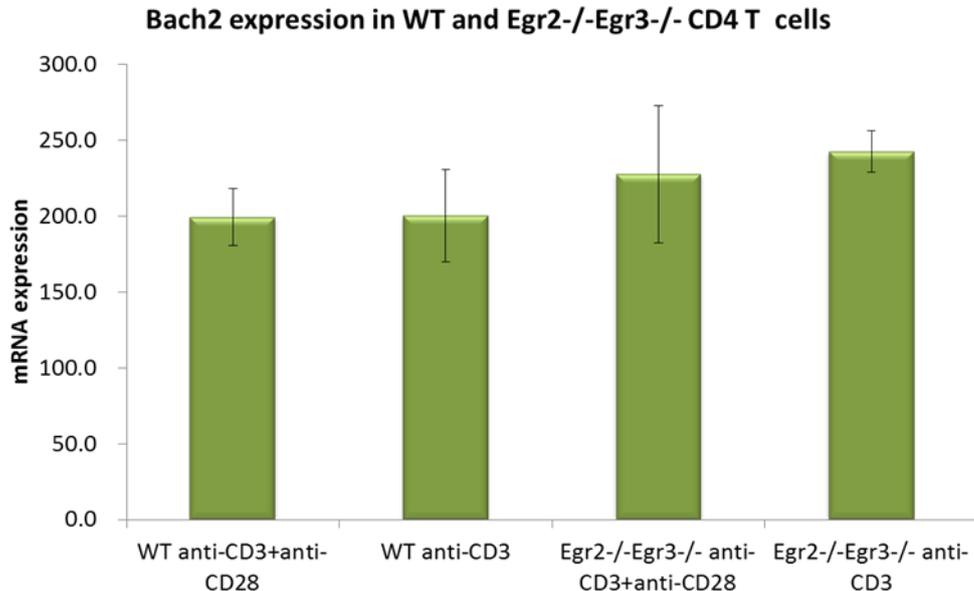
Figure 51: Bach 2 expression in active or tolerant CD4 T cells

Naïve CD4 T cells from wild type and Egr2 and 3 deficient mice were induced to anergy with 1μg/ml anti-CD3 for 24 hours. This was followed by re-stimulation with 1μg/ml anti-CD3 and 2μg/ml anti-CD28. Expression was assessed using RT-PCR where gapdh was used as the housekeeping gene. Error bars calculated from standard error values. The data is representative of three individual experiments.

3.3.4.4 TCF-1 is downregulated within tolerant Egr2-/-Egr3-/- CD4 T cells

T cell differentiation is one of the factors that influence tolerance and so T cell fate lays an essential role. Egr2 has been reported to regulate the development of T cells (Li, 2011). During T cell development, T cells can be influenced toward specific fiats. One transcription factor that has been found to play a role in differentiation regulation is TCF-1 (T cell receptor factor 1) (Choi, 2013). TCF-1 has been found to play an essential role in the regulation of the commitment to the CD4 T cell linage (Steinke, 2014). To investigate the role of Egr2 and 3 within differentiation and during tolerance, we used RT-PCR to measure the expression of TCF-1 within tolerant CD4 T cells.

CD4 T cells from Egr2-/-Egr3-/- mice were treated with anti-CD3 or anti-CD3 plus anti-CD28 before re-stimulation (Figure 52). Levels of TCF-1 decreased between the WT activated and the WT tolerant. Interestingly the Egr2-/-Egr3-/- activated sample had high levels of TCF-1, but the levels decreased by 14 fold within the anergic Egr2-/-Egr3-/- cells. This result indicates Egr2 and 3 may play a role in the control of TCF-1 expression and TCF-1 seems to be downregulated within anergic CD4 T cells. Therefore, Egr2 and 3 may play a key role in the control of T cell differentiation within the tolerant state.
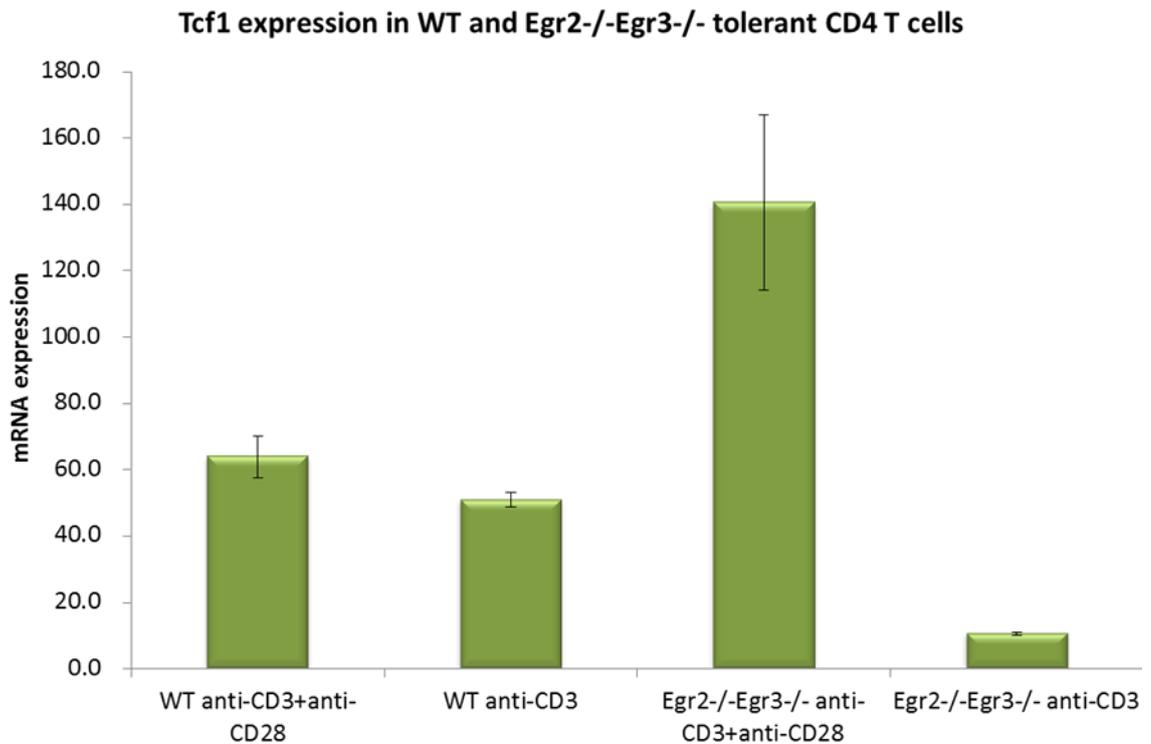
Figure 52: Defective Tcf1 expression in tolerant CD4 T from CD2-Egr2-/Egr3- mice

Naïve CD4 T cells from wild type and Egr2 and 3 deficient mice were induced to anergy with 1µg/ml anti-CD3 for 24 hours. This was followed by re-stimulation with 1µg/ml anti-CD3 and 2µg/ml anti-CD28. Expression was assessed using RT-PCR where Egr2-/-Egr3-/- CD4 T cells treated with anti-CD3+anti-CD28 increased tcf-1 expression compared to WT samples. With tcf expression dropping in the anergic samples both in the WT and Egr2-/-Egr3-/- compared to their activated counterparts. Error bars calculated from standard error values. The data is representative of three individual experiments.

3.3.4.5 Bhlhe40 is upregulated in activated Egr2-/-Egr3-/- T cells

Egr2 and 3 knockout mice have been found to have increased proliferation and differentiation toward the TH1 CD4 T cell subset causing increasing production of inflammatory cytokine IFN-γ (Li *et al.*, 2012a). We have found a similar situation within tolerant Egr2-/-Egr3-/- CD4 T cells. Bhlhe40 is a transcriptional regulator expressed on T cells on TCR stimulation and is known to regulate a number of processes such as apoptosis and differentiation (Miyazaki *et al.*, 2010), and has also been found to control cytokine production in T cells (Lin *et al.*, 2014). As Egr2 and 3 have also been found to control inflammatory response and they may play a role in differentiation, we sought to find any possible link between Egr2 and 3 and bhlhe40. On induction of in vitro anergy, we quantified expression of bhlhe40 by fully activating CD4 T cells

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with anti-CD3 and anti-CD28 or treating cells with anti-CD3 alone followed by re-stimulation with anti-CD3 and anti-CD28 for anergy induction. As with TCF-1 we found increased levels of bhlhe40 expression within activated samples in Egr2-/-Egr3-/- CD4 T cells (Figure 53). This reduced by 4 fold in the anergic sample. However, Blhe40 expression within tolerant Egr2-/-Egr3-/- cells decreased. This result shows that Egr2 and 3 possibly play a double role in the influence of bhlhe40 expression in the regulation of T cell differentiation and that of inflammatory cytokine production.
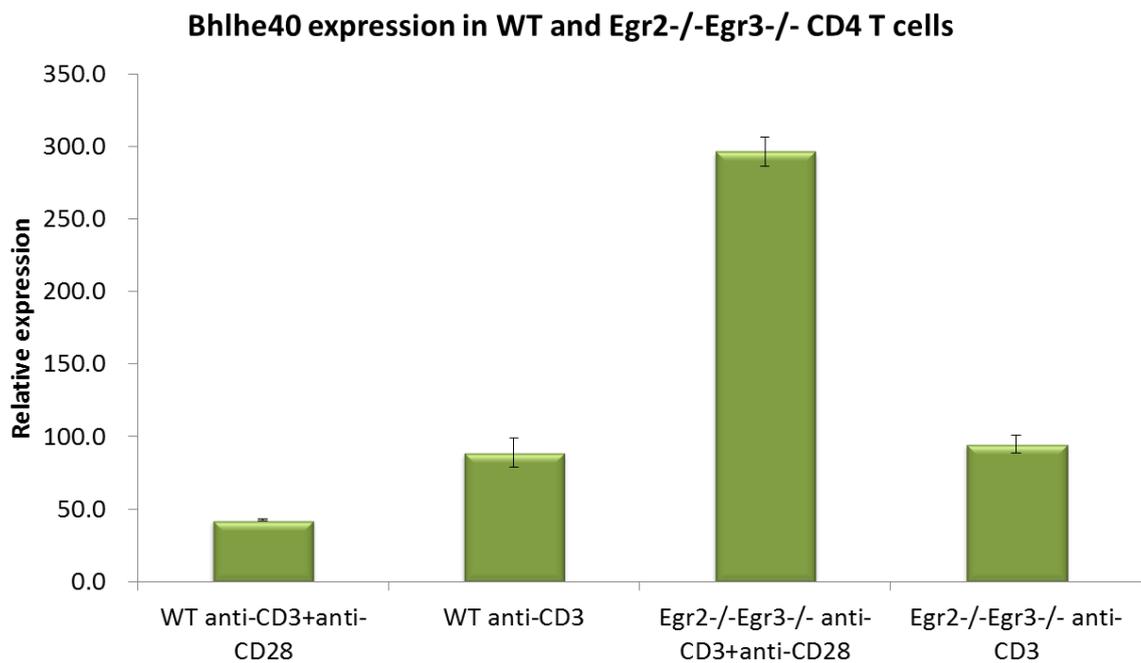
Figure 53: Bhlehe40 expression is increased in active, but not tolerant Egr2 and 3 deficient CD4 T cells

Naïve CD4 T cells from wild type and Egr2 and 3 deficient mice were induced to anergy with 1µg/ml anti-CD3 for 24 hours. This was followed by re-stimulation with 1µg/ml anti-CD3 and 2µg/ml anti-CD28. Expression was assessed using RT-PCR where gapdh was used as the housekeeping gene. Error bars calculated from standard error values. The data is representative of three individual experiments.

3.4 Discussion

This chapter explored the role of Egr2 and Egr3 within mitogenic and tolerogenic conditions in vitro. Both Egr2 and 3 are induced under mitogenic conditions to allow for proliferation and effector function of T cells (Du *et al.*, 2014). We found Egr2 and Egr3 knockout T cells are able to undergo tolerance induction, even though they have been implicated in tolerance induction (Safford *et al.*, 2005). The mechanisms for the induction of T cell anergy involve T cell stimulation through the TCR in the absence of co-stimulation, essentially this is the activation of NFAT in the absence of NFkb and AP-1 (Fathman and Lineberry, 2007)(Shwartz, 2003). The major hallmarks of T cell tolerance being reduced IL-2 production coupled with decreased proliferation (Shwartz, 2003), which is what we viewed on In vitro induction of tolerance in Egr2 and 3 knockout CD4 T cells using anti-CD3. However, it has been reported that Egr2 and 3 are upregulated on anergy induction (Harris *et al.*, 2004). Although most studies carried out have been performed on cell lines (Harris *et al.*, 2004) or in single knockout mice (Zheng *et al.*, 2012) which in one way or another affect the outcome. We on the other hand adopted the use of primary CD4 T cells isolated for CD2-Egr2-/-Egr3-/- mice allowing for a more physiological state of T cell response Figure 28and Figure 29.

On TCR activation there is a calcium influx within CD4 T cells allowing for the activation of NFAT and its transcription into the nucleus (Feske, 2007; Crabtree and Olson, 2002). NFAT activation lies downstream of a calcium flux initiated on TCR signalling. This is the reason in vitro tolerance can also be induced using the calcium ionophore ionomycin (Heissmeyer *et al.*, 2004). As reported by Safford *et al.* (2005), we found the addition of cyclosporine A to Egr2 and 3 knockout T cells with anti-CD3 alone prevented tolerance induction resulting in increased IL-2 and proliferation within Egr2-/-Egr3-/- CD4 T cells (Figure 31 and Figure 32). This has been shown to be one of the trademarks of anergic T cells (Chai and Lechler, 1997). Reports added that reversal could be achieved with the addition of IL-2 to the culture (Shwartz, 2003). Soto-Nieves *et al.* (2009) found NFAT to be key in the induction of T cell anergy, as we have seen within tolerant Egr2-/-Egr3-/- CD4 T cells. In addition, it has recently been reported that Deltex-1(DTX-1) which is upregulated during anergy, is a transcription target of NFAT with dtx-/- mice being more resistant to anergy induction and being more susceptible to autoimmune disease development (Hsiao *et al.*, 2009) proving the importance of NFAT within in vitro anergy induction.

TCR signalling plays an essential role in T cell proliferation and differentiation (Abraham and Weiss, 2004). We have shown a novel relationship between Egr2 and TCR stimulation, wherein Egr2 expression decreases and increases in relation to antigenic strength (Figure 38). Egr2 and 3 deficient T cells are impaired in proliferation response and IL-2 production in response to antigen stimulation. This is due to a defect in AP-1 activity (Li *et al.*, 2012a). On analysis of Egr2-/-Egr3-/- T cells with varying TCR stimulation and Egr2 expression, we also found proliferation to alter in relation to Egr2 expression. This implies over-expression or under-expression of Egr2 or Egr3 has a direct effect on T cell proliferation and IL-2 production. It has been shown that T cells from Egr2 transgenic mice increased proliferation while the lack of Egr2 and 3 exhibited the opposite (Li *et al.*, 2012a). In addition, Harris *et al.* (2004) showed over-expression of Egr2 and Egr3 within transfected cell lines are able to inhibit T cell function and result in decreases IL-2 and proliferation resembling those from in vitro tolerant T cells, where we found a similar result after tolerance induction within Egr2 and 3 knockout CD4 T cells. Therefore, the expression of Egr2 that is governed by TCR signalling can influence proliferation of T cells and in doing so alter effector cytokine production.

On establishing anergy induction in our model, the defect in AP-1 within the Egr2-/-Egr3-/- mice evidently has no effect on tolerance induction. Unlike NFAT, which we have shown to be expressed within anergic T cells, AP-1 formation within anergic T cells is diminished (Sundstedt *et al.*, 1996). It was found that Ras activation which lies upstream of the MAP-K pathway that is activated after co-stimulatory signalling via CD28 is incomplete within tolerant T cells in vitro (Zha *et al.*, 2006). AP-1 is not activated under tolerogenic conditions and we have shown proliferation and cytokine production to be primarily dependent on TCR signalling. Therefore, Egr2 and 3 expression, which lie downstream of AP-1 activation, remain unchanged within the tolerant setting thus allowing NFAT mediated T cell tolerance induction to take place in the absence of Egr2 and Egr3. Significantly, we thus show T cell proliferation is defective in tolerance settings and by extension Egr2 and 3 mediated T cell proliferation is also defective based on the fact that formation of AP-1 is inhibited, proving Egr2 and Egr3 are not essential for the induction of proliferative tolerance.

Activation of NFAT without that of MAPK and NFkb allows for the activation of E3 ubiquitin ligases which are able to attach to molecules and flag them for degradation. These ubiquitin ligase genes have been studied recently within anergic T cells (Fathman and Lineberry, 2007).

On tolerance induction we found increased expression of Cbl-b and ITCH reported by (Heissmeyer *et al.*, 2004) and (Fathman and Lineberry, 2007) to be increased during anergy. The expression of cbl-b has been linked to that of Egr2, with reports indicating expression of Cbl-b may be directly influenced by Egr2 expression (Safford *et al.*, 2005). However, we found the highest levels of Cblb were viewed within Egr2/3 knockout tolerant CD4 T cells. The expression of cbl-b is clearly an important factor within in vitro tolerance induction in CD4 T cells with cblb-/- mice being resistant to tolerance induction in vitro (Chiang *et al.*, 2000). As cbl-b and Itch expression is increased in tolerant T cells within our model, it would indicate an alternative role for Egr2 and 3 in the control of ubiquitin ligase gene expression (Tamiya *et al.*, 2011). The role of Egr2 and Egr3 within the control of ubiquitin ligase expression still has some way to go. With recent reports of DGK-alpha upregulation within anergic T cells (Zha *et al.*, 2006), a link between Dgk and Egr2 was discussed by Zheng *et al.* linking egr2 expression to DGK expression during tolerance (Zheng *et al.*, 2012), once again bringing into question the role of Egr2 within the control of 'anergy associated genes'. A summary of studies within anergy induction and Egr2 is given in Table 5.

In conclusion, we report that Egr2 and Egr3 do not play a major role in the induction of T cell anergy in vitro, with the successful induction in their absence and NFAT upregulation within the CD4 T cells. The expression of Egr2 is governed by TCR signalling strength thus influencing effector cytokine production.

Reference	Mouse model/ cell line	Factor involved in tolerance control	Factor upregulated during tolerance	Summary of findings
Zha, 2006	TCR-Rag-/- mice and IL-2 luc mice TH1 clones	Ras,dgk-alpha, cblb	Dgk-alpha	Ras reverses anergy in Th1. DGK-alpha upregulated in anergic th1 cells
Harris, 2004	AE7	Egr2	-	Anergy cannot be induced in T cells without Egr2
Safford, 2005	A.E7 6.5 TCR transgenic C3-HA	Egr2,Egr3,Cblb	Egr3 and Cblb	Overexpression of Egr2 and Egr3 inhibit T cell function. Egr2 and Egr3 upregulated in anergy in vivo and in vitro. Egr3-/- not capable of anergy induction. CsA can hinder anergy induction
Hsiao, 2009	CD4+CD25- cells primed into Th1 clone from Deltex-1 -/- mice	dtx,cblb,Egr2	DTX-1 and cbl-b	Overexpression of dtx1 suppresses T cell activation. Dtx1 is a transcription target for NFAT. Dtx1 deficiency does not affect t cell development. Egr2 unchanged in dtx1 overexpression but cblb decreased. Deltex 1 addition adds increases Egr2-Cblb expression. Cblb regulated by deltex1 via Egr2
Zheng, 2012	Egr2-/- - th1 clones, and mice	dgk, Egr2	dgk	Egr2 is needed for anergy induction in vitro and in vivo. Egr2 regulates Dgk expression on anergy induction
Oh, 2015	A.E7 and Ndrg1-/- mice CD4 T cells	Ndrg1, Egr2	Ndrg1	Ndrg1 upregulated during anergy,Ndrg1 overexpression mimics anergic state. Ndrg1 is degraded by CD28 signalling. Ndrg1 is a downstream target of transcription factor Egr2. Ndrg1-/- mice resistant to anergy induction
Egr2-/-Egr3-/- knockout model	Egr2-/-Egr3-/- CD4 T cells	Egr2, Cbl-b, Itch, Dgk-alpha, Egr3	Cbl-b, Itch, Itch, Dgk alpha	Egr2 and 3 do not play a role in tolerance induction. Egr2 and 3 play a role in inflammatory cytokine production within the tolerant setting.

Table 5 In-Vitro anergy induction with anti-CD3 studies

Chapter 4.   In-vivo tolerogenic induction in Egr2 and Egr3 knockout CD4 T cells

4.1 Introduction

Self-reactive T cells are still able to bypass central tolerance mechanisms creating a need for peripheral tolerance. This may be due to self-antigens being poorly presented by the MHC molecule, self-antigens being presented at too low an avidity, or some foreign antigens cross reacting to self-antigens resulting in the development of autoimmune disease (Wasterberg, Klein and Snapper, 2008)(Romagnani, 2006; Zehn and Bevan, 2006).

Anergy, the state of functional unresponsiveness, results when the T cells are stimulated in the absence of co-stimulation. As anergy has been studied widely in vitro, there have been a number of proposed mechanisms in vivo which is induced in a different manner (Shwartz, 2003). These mechanisms include repeated antigenic stimulation, adoptive transfer, oral induction through proteins, and the use of superantigen toxins. In the in vivo or adoptive tolerance model, antigen challenge results in a large amount of T cell death with the remaining cells being classified as anergic (Sundstedt *et al.*, 1996). Like in vitro anergic cells, these cells have decreased levels of proliferation and decreased production of IL-2. Superantigens have been reported to stimulate T cells outside of the peptide binding groove, allowing for a much larger T cell number to be activated (20%). Superantigens are primarily toxins that are able to cause diseases such as toxic shock syndrome in humans, but they do not have the same effect in mice. They include staphylococcal enterotoxin A and B (Sundstedt *et al.*, 1994). Reports have shown repeated administration of superantigen to mice will result in a state of in vivo tolerance or adoptive tolerance. However, it has been reported that the mice must be constantly treated to maintain the tolerant state, and if treatment is stopped the mice regain their ability to produce high levels of inflammatory cytokines such as IFN-γ and IL-2. Proliferative function would also be returned. Therefore the use of superantigens is a reversible mode of tolerance induction (Anderson *et al.*, 2006; Sundstedt *et al.*, 1996).

Egr2 has recently been studied in the tolerance condition with some groups also looking at it within the in vivo mechanisms (Zheng *et al.*, 2012), with Egr2-/-Egr3-/- mice exhibiting high levels of inflammatory expression cytokines such as IFN-γ and IL-17. In order to assess the

function of Egr2 and 3 in tolerance, we sought to induce adoptive tolerance within our CD2-Egr2-/-Egr3-/- mouse model to investigate the role of Egr2 and Egr3 in vivo using superantigen staphylococcal enterotoxin A (SEA) to induce tolerance.

4.2 Methods

4.2.1 In-vivo mouse treatment

Mice were housed in the animal unit in Brunel University according to established institutional guidelines under the authority of the UK Home Office project licence. All WT and Egr2-/-Egr3-/- mice were age and sex matched before each experiment and WT mice used as controls.

In order to induce tolerance WT and Egr2-/-Egr3-/- mice were injected interperionatally (IP) 6-8 times with 20μg/ml SEA diluted in 100ml PBS per injection per mouse (16 mice were used on average per experiment). This was done twice a week or 3 days apart. For an activated sample, mice were injected just once 24 hours before experiment end. Control samples went either uninjected or were injected with 100μl 1XPBS. All mice were culled at the same time and samples collected.

4.2.2 Ex-vivo cell culture

Spleens were collected the day after the activation injection and red blood cells removed by the addition of 0.8% ammonium chloride. Cells were then treated with CD4 beads for 20 minutes and using a positive selection kit, CD4 positive cells were isolated by Magnetic Activated Cell Sorting (MACS). CD4- T cells and APCs were then treated with mitomiycin to stop intracellular function for 30 minutes at 37°C. APCs were loaded with SEA at 10μg/ml SEA for 3 hours, washed and cultured with CD4+ T cells at a 50:50 ratio. Some cells were collected here for flow cytometry and RT-PCR (see Chapter 2). Cells were stimulated for 48 hours before supernatant collection for ELISA and 72 hours later were treated with 3[H]-TdR for 16 hours.
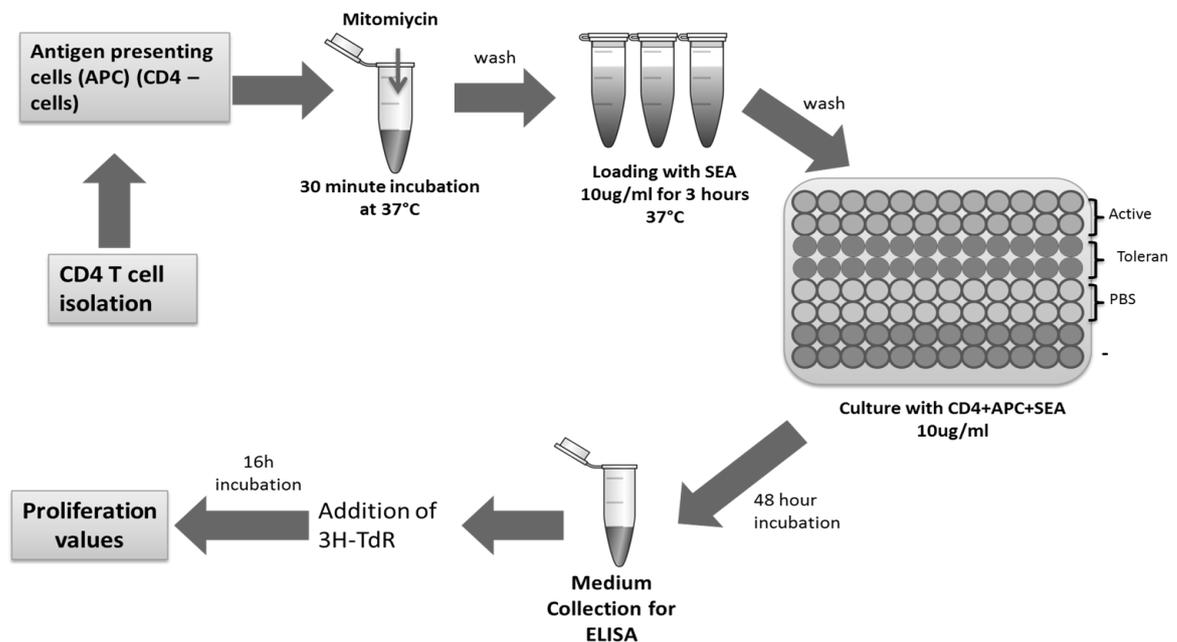
Figure 54: Ex-vivo cell culture

WT and Ehr2-/-Egr3-/- mice spleens were collected and CD4 T cell isolation carried out. CD4- T cells were used as antigen presenting cells (APCs). They were firstly treated with mitomiycin and then loaded with superantigen staphylococcal enterotoxin A (SEA) at a concentration of 10µg/ml. APCs were then cultured with CD4+ T cells at a 50-50 ratio for 48 hours at 37°C before collection of supernatant for ELISA. After 72 hours 3H-TdR (tritium) was added for 16 hours and proliferation values assessed.

4.3 Results

Once we determined in vitro anergy within CD2-Egr2-/-Egr3-/- mice, we looked into in vivo anergy induction. In vivo anergy induction is often referred to as adoptive tolerance and anergy has been shown to differ in a number of ways compared to the in vitro process. One similarity though is that Egr2 and 3 have also been found to play a critical role in the in vivo process, with previous publications showing increased levels of Egr2 and Egr3 in tolerant T cells (Zheng *et al.*, 2012).

4.3.1.1   Egr2 and Egr3 have no effect on the CD4+Vβ3 T cell subset

Adoptive tolerance or in vivo anergy is a state that causes the functional underactivity within T cells in vivo (Schwartz, 2004; Shwartz, 2003). This is due to the continuous stimulation of T cells with superantigens.

CD2-Egr2-/-Egr3-/- mice were developed in the C57BL/6 background (Li *et al.*, 2012a). This allowed for the use of superantigens staphylococcal enterotoxin A or B to stimulate T cells within mice. Superantigens are able to affect the V beta regions within T cells, thus the superantigen used is dependent on this crucial factor. It is known that superantigens activate T cells expressing certain TCR variable region beta chains (Vβ). Binding of the superantigens to MHC class II does not require processing and the sequences on the MHC ii and TCR vβ involved are distinct from the parts engaged in interaction with conventional peptide antigens (Dellabona *et al.*, 1990).

To confirm normality of the CD4+ Vβ3 region within Egr2-/-Egr3-/- CD4 T cells, the cells were stimulated with SEA and Vβ3 expression assessed via flow cytometry. A similar level of Vβ3 expression within the WT and Egr2-/-Egr3-/- CD4 T cells was shown (see Figure 55). Thus Egr2 and 3 deficiency does not alter the TCR variable region 3 allowing for optimal stimulation using superantigen staphylococcal enterotoxin A (SEA).
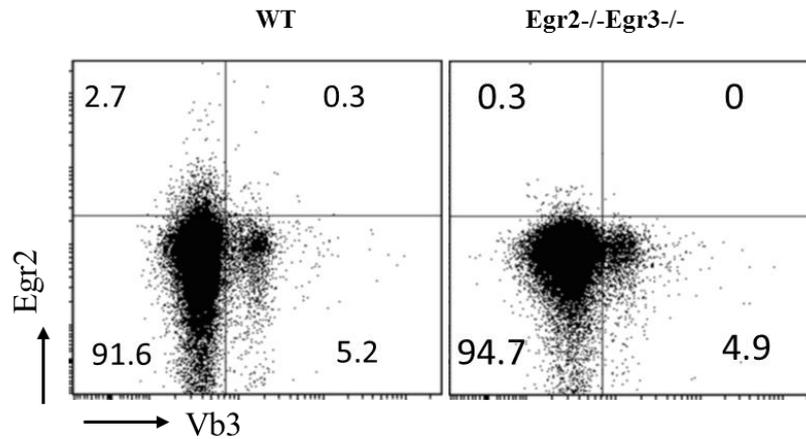
Figure 55 Vβ3 population in naïve Egr2-/-Egr3-/- mice and WT mice

CD4 T cells were isolated from WT and Egr2-/-Egr3-/- mice were collected and stained with Egr2 and Vb3 before FACS analysis. Figure is representative of gating performed before FACS analysis.

4.3.1.2 Egr2 and Egr3 are not essential in the induction of T cell tolerance in vivo

Adoptive tolerance or in vivo anergy is induced by continuous stimulation of a TCR Vβ region using a superantigen. Egr2 and 3 have been found to play a critical role in the in vivo process with previous publications showing increased levels of Egr2 and Egr3 in tolerant T cells (Safford *et al.*, 2005).

Previously it has been demonstrated the induction of adoptive tolerance is due to repeated injections of superantigens to mice. It has been found that superantigens induce an exaggerated initial T cell reaction, with high production of cytokines such as IFN-γ, TNF and IL-2 (Huang *et al.*, 2007). Similarly, on treating both WT and CD2-Egr2$^{-/-}$Egr3$^{-/-}$ CD4 T cells with SEA for 24 hours, there was a dramatic increase in IL-2 compared to samples with CD3 and CD28 (Figure 56). As we have shown, Egr2 and 3 do not affect the activation of Vβ subsets. We injected WT and Egr2$^{-/-}$Egr3$^{-/-}$ mice with SEA at 20μg/100μl PBS once. We collected serum from the mice on days 0, 1 and 4 in order to establish the optimal tolerance induction setting. In accordance with previous publication, IL-2 concentration was at its highest 24 hours post injection. The initial exaggerated reaction is then followed by decrease in effector cytokines (Huang *et al.*, 2007). Indeed by day 4 we found decreased expression of IL-2 (Figure 56).
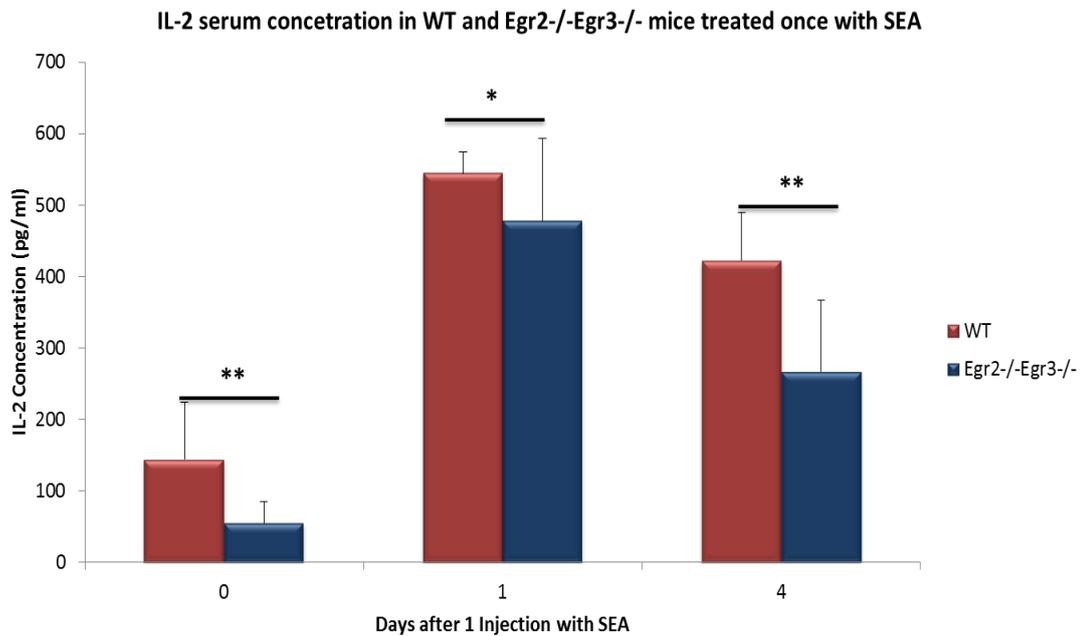
Figure 56 Decreasing IL-2 concentration in serum from wild type and Egr2-/-Egr3-/- mice treated once with SEA

WT and Egr2-/-Egr3-/- mice were treated once with 20µg SEA and serum collected on days 0, 1, 4. IL-2 levels were measured from serum by ELISA. The data presented is a mean of three replicates from two mice and represent three independent experiments with similar results. Error bars calculated from standard error values. Statistical analysis was calculated using students T-test and p values set as follows –P<0.05 *, P<0.01 **, P<0.001***, NS-not significant

We adopted a well-used protocol whereby sex and age matched mice were treated with superantigen SEA twice a week for 4 weeks at a dose of 20µg/100µl PBS via intraperonatal injection or just once as an activated sample. Mice were culled after multiple injections and mice injected just once were used as a positive control. CD4 T cells isolated were further cultured in 10µg/ml SEA ex vivo before analysis. For an optimal activation protocol with antigen presenting cells, we treated both WT and CD2-Egr2$^{-/-}$Egr3$^{-/-}$ APCs with mitomiycin to stop any DNA replication and cultured the cells at a concentration of 10µg/ml with CD4 + T cells. Testing of Egr2$^{-/-}$Egr3$^{-/-}$ APCs and WT APCs gave a similar result in terms of IL-2 production, thus we carried on with WT APCs as our antigen presenting cells. Antigen presenting cells were then loaded with 10µg/ml SEA for 3 hours before culturing alongside CD4 T cells to allow optimal stimulation and production of IL-2 and proliferation (see Figure 57 and Figure 58).
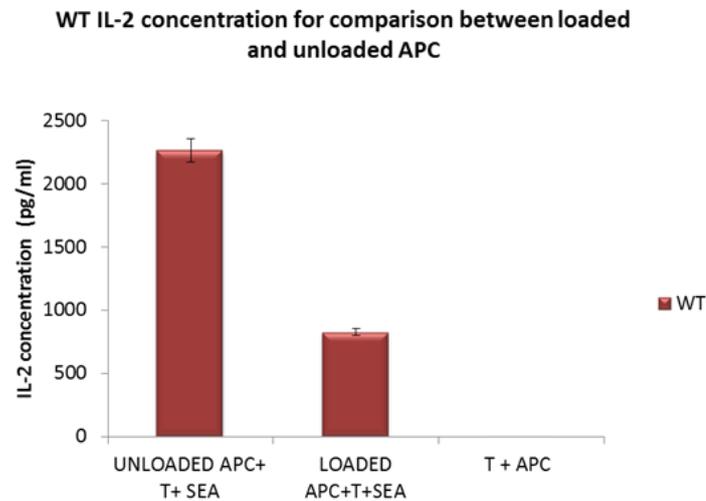
Figure 57 Acceptable IL-2 concentration in CD4 T cells from WT and Egr2-/-Egr3-/- loaded with APCs

Naïve CD4 T cells from WT and Egr2-/-Egr3-/- were cultured for 48 hours with addition of 10µg/ml SEA without loading, loaded first with APCs for 3 hours with 10µg/ml SEA and washed before culture with CD4 T cells or without SEA. After 48 hours, medium collected was assessed for IL-2 production using ELISA. Error bars calculated from standard error values. The data presented is representation three independent experiments with similar results.
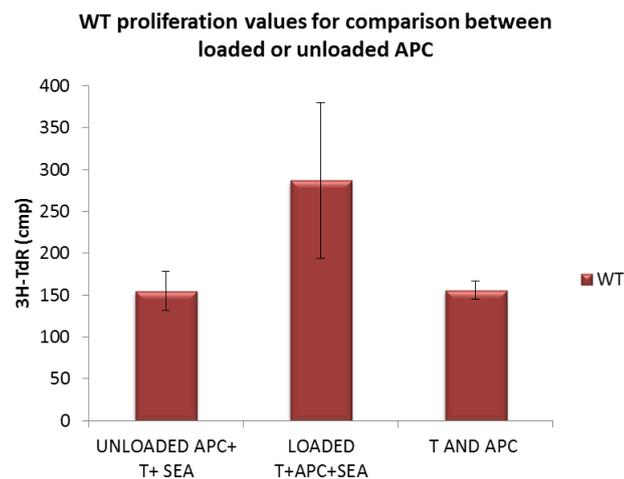
Figure 58 Increased proliferation levels in CD4 T cells from WT loaded with APCs

Naïve CD4 T cells were cultured for 48 hours with addition of 10µg/ml SEA without loading, loaded first with APCs for 3 hours with 10µg/ml SEA and washed before culture with CD4 T cells or without SEA. After 72 hours, proliferation was assessed by addition of 3H over 8 hours. Error bars calculated from standard error values. The data presented is representation three independent experiments with similar results.

Using this protocol wild type and Egr2 and 3 knockout mice were injected 8 times using SEA. CD2-Egr2$^{-/-}$Egr3$^{-/-}$ CD4 T cells have already been shown to have reduced IL-2 levels. In accordance with this previous in vitro observation we found decreased levels of IL-2 in the WT CD4 T cells from mice treated with 8 injections of SEA cells compared to the WT after treatment with one injection of SEA (Figure 59), we also found reduced proliferation levels within samples treated with 8 injections compared to one injection (Figure 59). Importantly we observed a similar view within the Egr2$^{-/-}$Egr3$^{-/-}$CD4 T cells. The reduction of IL-2 and proliferation is significant between the active and tolerant samples, indicating tolerance induction is taking place in the WT and Egr2$^{-/-}$Egr3$^{-/-}$ CD4 T cells.
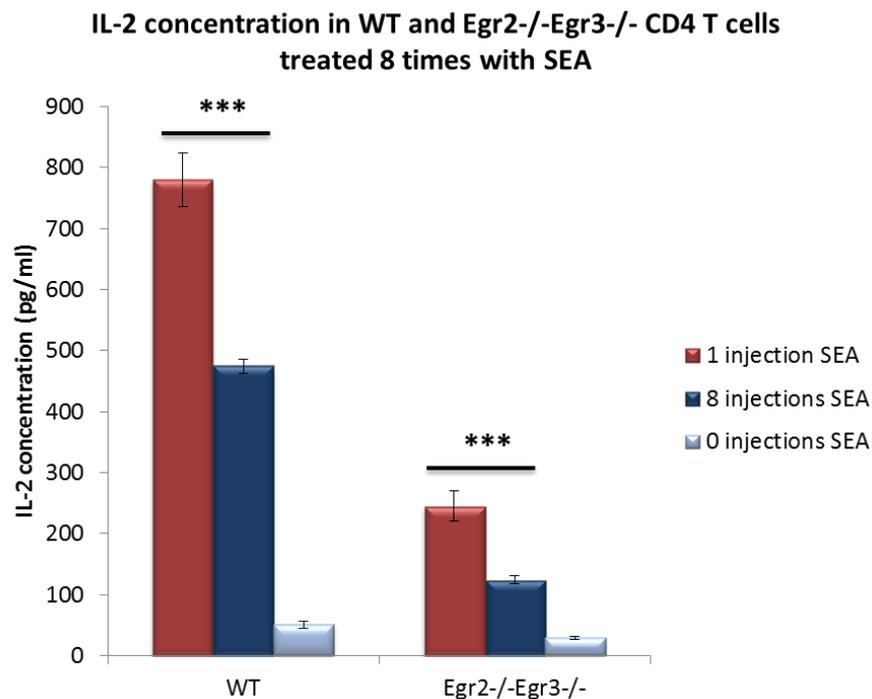
Figure 59 Impaired IL-2 production in WT and Egr2-/-Egr3-/- CD4 T cells in response to tolerogenic stimulation with SEA

WT and Egr2-/-Egr3-/- CD4+ T cells from mice injected ip with SEA once (active) or 8 times (to induce tolerance) or without an injection (unstimulated) and cultured ex vivo in 10µg/ml SEA for 48 hours and supernatant collection. IL-2 production was assessed using ELISA. The data presented is a mean of three replicates from twelve mice per experiment and represent six independent experiments with similar results. Cells without stimuli served as negative controls. Error bars calculated from standard error values. Statistical analysis was calculated using students T-test and p values set as follows –P<0.05 *, P<0.01 **, P<0.001***, NS- not significant.

A
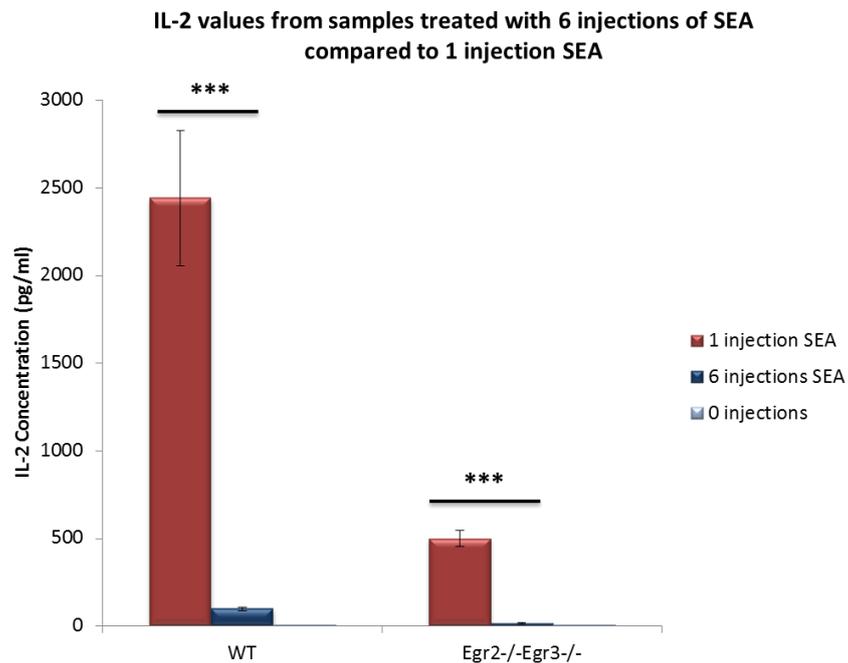
B
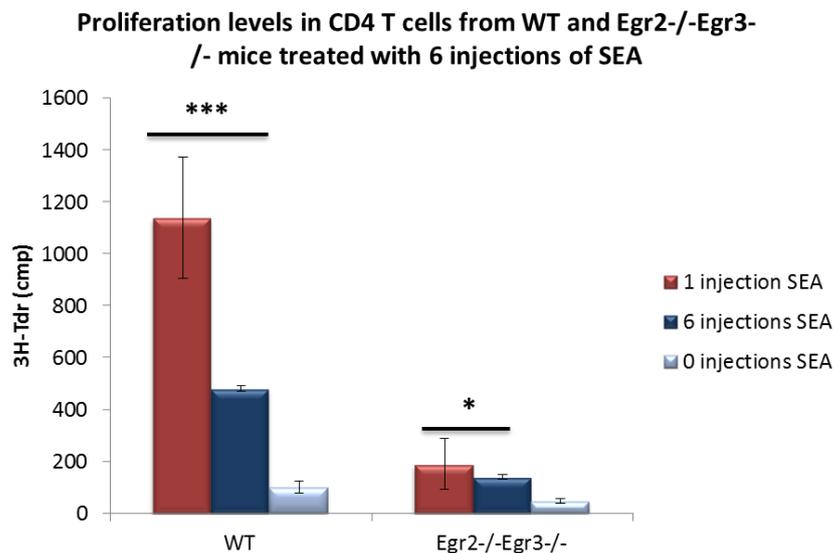
Figure 60 Impaired IL-2 production and proliferation in WT and Egr2-/-Egr3-/- CD4 T cells in response to tolerogenic stimulation using 6 injections of SEA

CD4 T cells from WT and Egr2-/-Egr3-/- mice injected ip with SEA once (active) or 6 times with a three days interval (to induce tolerance) or without an injection (unstimulated) and cultured in ex vivo with SEA-preloaded APCs at ratio of 1:1. After 24 hours, IL-2 production was assessed by ELISA (A). After 72 hours and 3H-Tdr added for a further 16 hours to quantify proliferation (B). The data presented is a mean of three replicates from twelve mice per experiment and represent six independent experiments with similar results. Cells with stimuli served as negative control. Error bars calculated from standard error values. Statistical analysis was calculated using students T-test and p values set as follows –P<0.05 *, P<0.01 **, P<0.001***, NS-not significant.

4.3.2   Phenotypic identification of in vivo tolerised T cells

The induction of in vivo anergy involves the constant exposure of the antigen to the cell and as a result apoptosis ensues leaving behind an unresponsive group of cells. Egr2 and 3 have been reported to play a significant role in T cell activation and proliferation. There are specific markers to allow for the identification of specific T cell subsets. These markers include CD44 and CD69 for activated memory T cells, and CD62L for identification of naïve T cells (Oehen and Brduscha-Riem, 1998). Anergic T cells can be distinguished from the lack of IL-2 and proliferation but anergic cells can also be identified from non anergic cells using phenotypic expression. To establish this, we used in vivo tolerance induction of WT and CD2-Egr2-/-Egr3-/- mice and identified surface markers using flow cytometry. CD2-Egr2-/-Egr3-/- T lymphocyte subpopulation within tolerant T cells exhibited decreased levels of T lymphocyte activation marker CD69 compared to the activated sample (Figure 61); this result mirrored the situation within WT samples. We found increased levels of CD44-CD62L+ within the tolerant T cells in both WT and Egr2 and 3 knockout (Figure 63A). We found the tolerant Egr2-/-Egr3-/- CD4 T cells had a larger decrease in CD44-CD62L+ expression compared to the WT tolerant (Figure 63B) showing a 2.3 fold increase compared to a 1.5 fold increase in the WT.
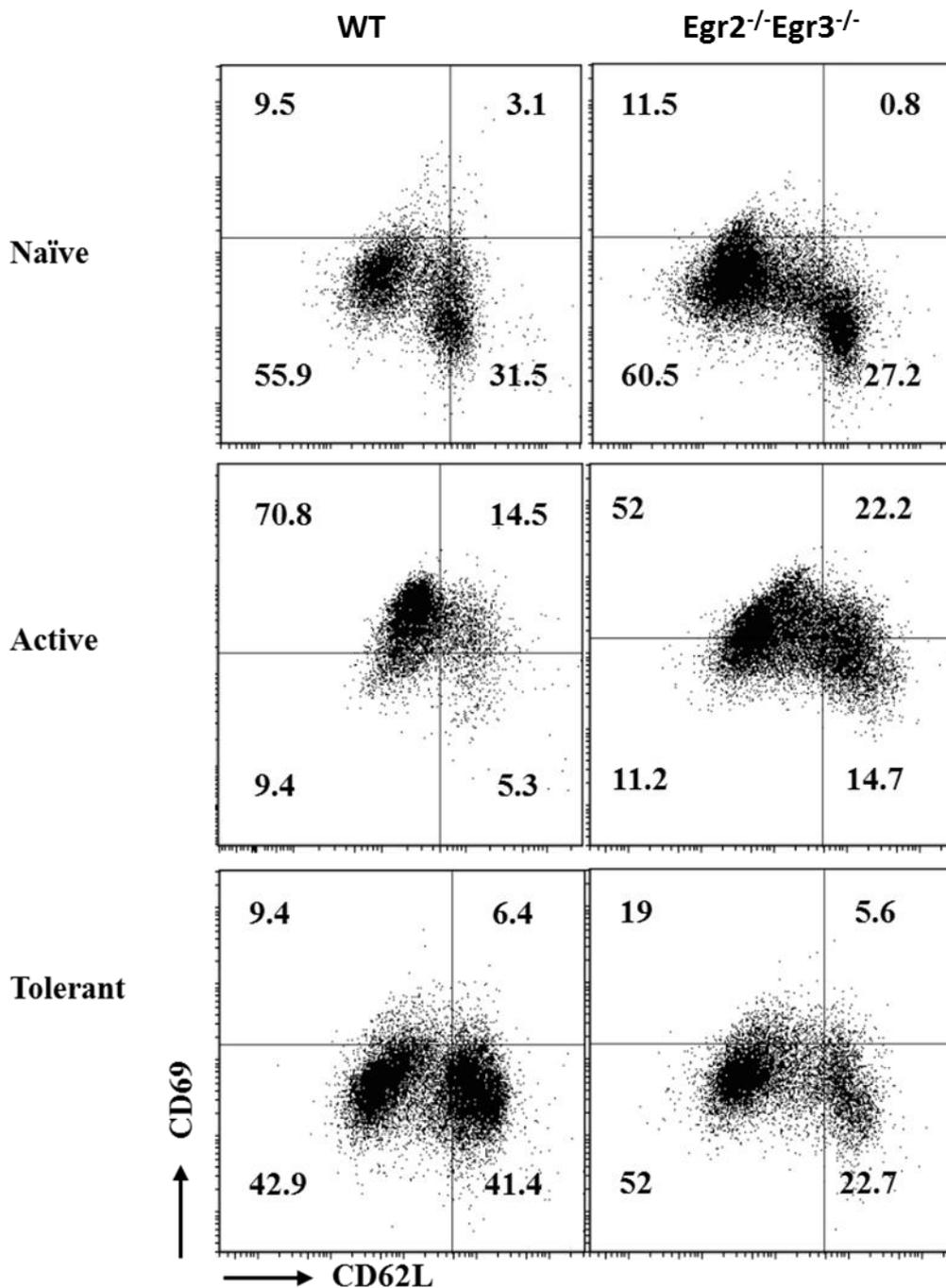
Figure 61 Decreased activation of CD4 T cells from tolerant WT and Egr2-/-Egr3-/- mice

Flow cytometry phenotypic analysis of CD69 and CD62L population in naïve CD4 T cells from WT and CD2-Egr2-/-Egr3-/- mice that were either Naïve (untreated), Active (treated once with SEA at 20μg/injection ip), or Tolerant (treated 8 times with SEA at 20g/injection ip). The data presented is a representation of one of three experiments. Cells were gated on CD4+Vb3+ population.

121

A

B
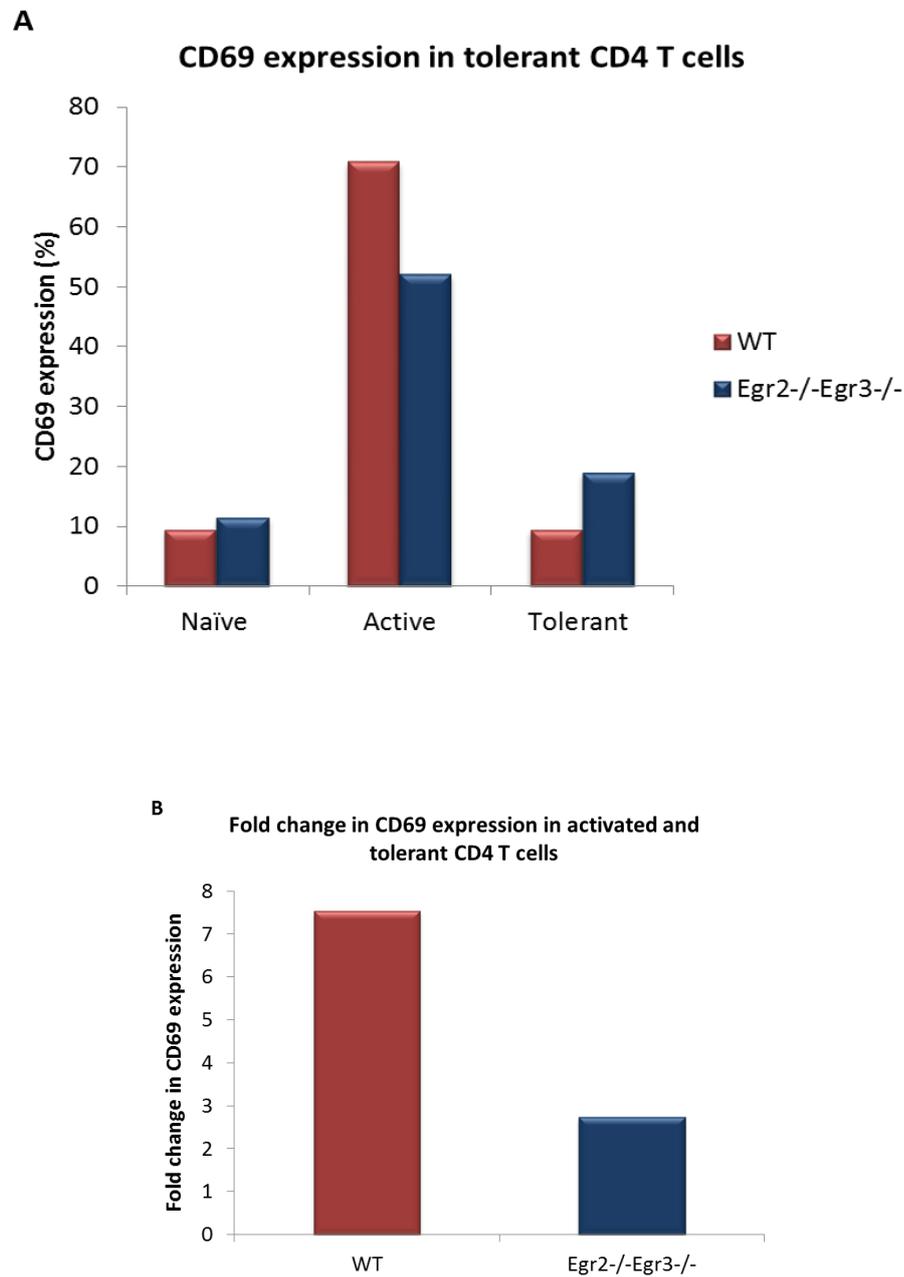
Figure 62 CD69 expression showed tolerant phenotype of CD4 T cells.

(A)Graphical presentation from Figure 61. Flow cytometry phenotypic analysis of CD69 population in naïve CD4 T cells from WT and CD2-Egr2-/-Egr3-/- mice that are: Naïve (untreated), Active (treated once with SEA, or Tolerant (treated 8 times with SEA).(B) Fold change analysis of CD69 expression which decreases by 7.5 fold in the WT and 2.5 fold in the Egr2-/-Egr3-/- tolerant CD4 T cells. The data presented is a representation of one of three experiments. Cells were gated on CD4+Vb3+ population

A CD62L expression in tolerant CD4 T cells

B Fold change in CD62L expression in activated and tolerant CD4 T cells
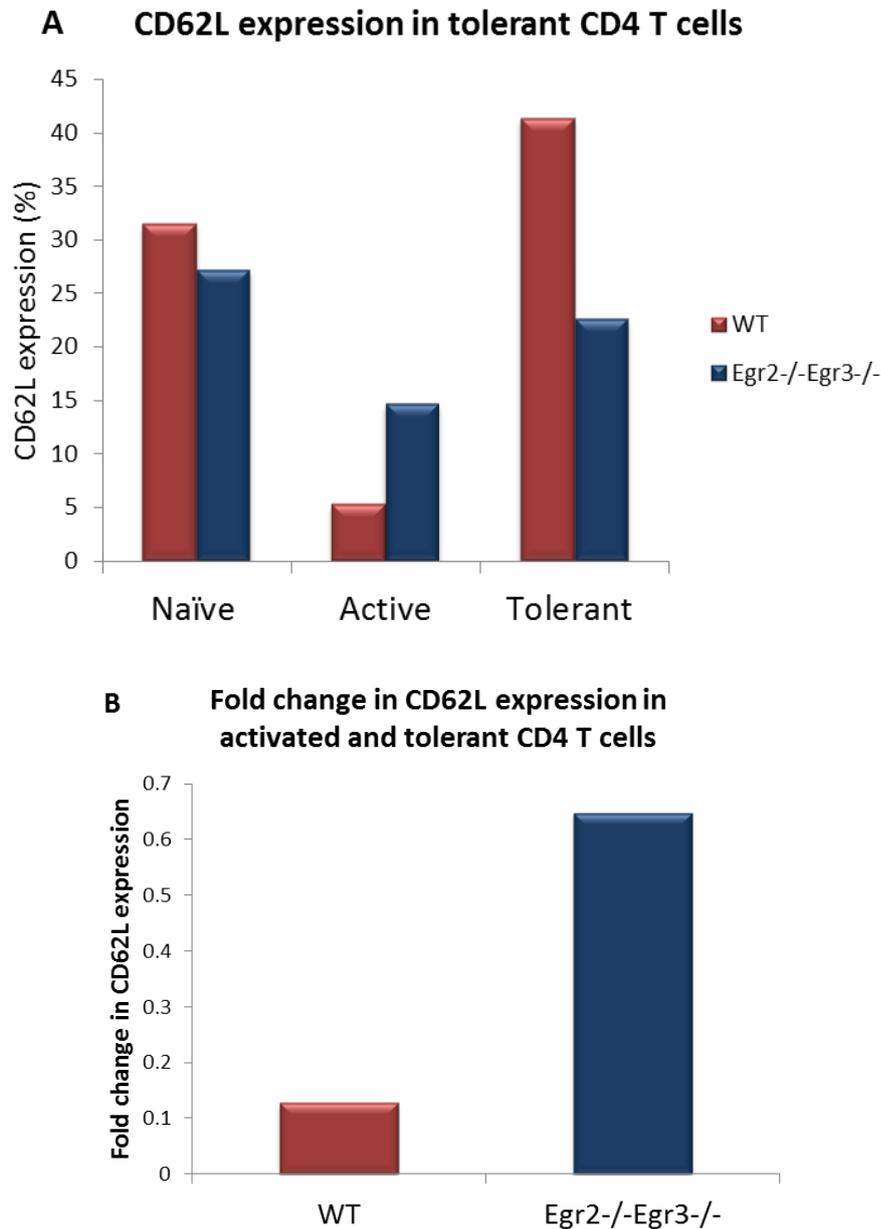
Figure 63 CD62L+ population in CD4 tolerant T cells

(A)Flow cytometry phenotypic analysis, adapted from Figure 61, of CD62L population in naïve CD4 T cells from WT and CD2-Egr2-/-Egr3-/- mice that were either Naïve (untreated), Active (treated once with SEA at 20μg/injection ip), or Tolerant (treated 8 times with SEA at 20g/injection ip). (B) Fold change analysis of CD44-CD62L+ expression, which increases by 1.5 fold in the WT and 2.3 fold in the Egr2-/-Egr3-/- tolerant CD4 T cells. The data presented is representative of three experiments.

We then went on to look at another T lymphocyte activation marker, CD44. On treating CD4 T cells with either 8 injections of SEA (tolerant) or one injection (active). Cells were stained with CD44, CD69, Egr2 and CD62L and flow cytometry analysis carried out on the CD4+Vβ3 population (Figure 64). The CD44 population within tolerant T cells reduced significantly compared to the activated, indicating tolerance induction within our CD2-Egr2-/-Egr3-/- T cells similar to that of the WT (Figure 65). These results therefore highlight in vivo T cell tolerance induction is not fully dependant on Egr2 or Egr3. They also show Egr2-/-Egr3-/- CD4 T cells are able to maintain the tolerant condition of increased naïve T cells and decreased activation markers.
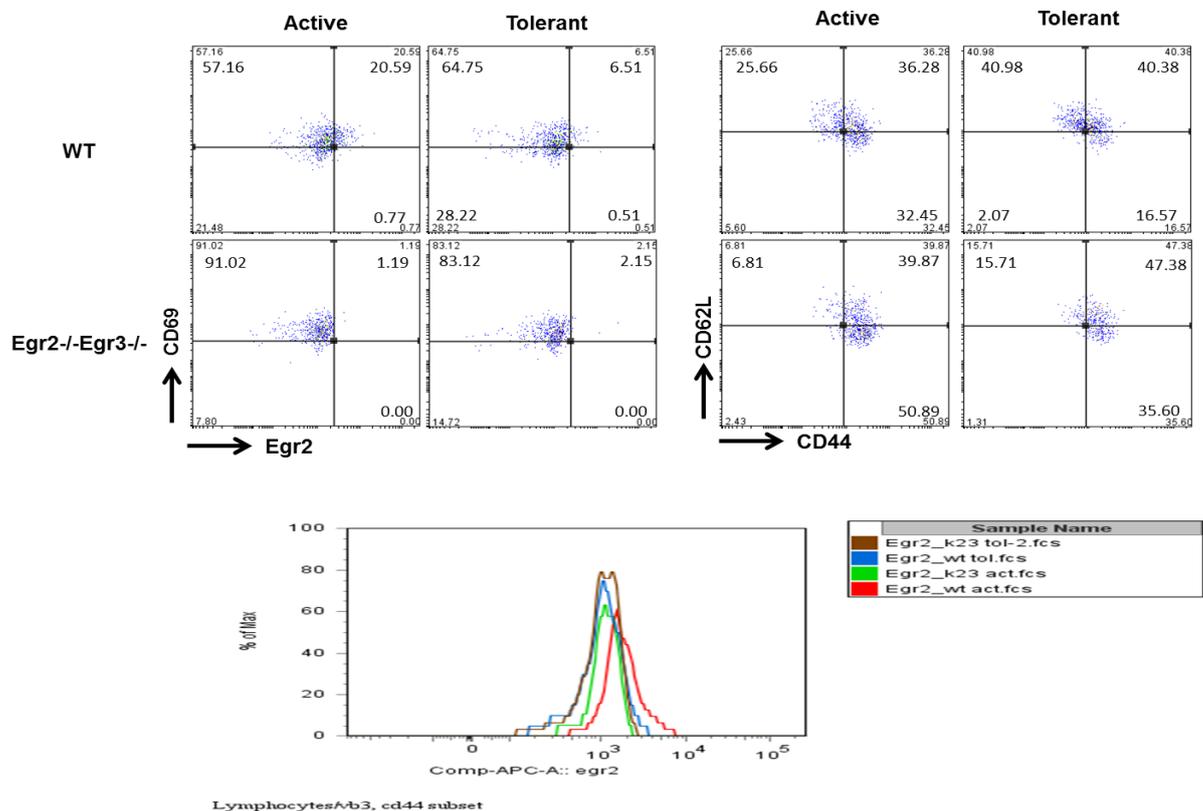
Figure 64 Phenotypes of tolerant CD4 T cells In-vivo

Flow cytometry phenotypic analysis of CD69,Egr2,CD44 and CD62L population in naïve CD4 T cells from WT and CD2-Egr2-/-Egr3-/- mice that were either Naïve (untreated), Active (treated once with SEA at 20µg/injection ip), or Tolerant (treated 8 times with SEA at 20g/injection ip). Cells were gated on the CD4+Vb3+ population. The data presented is a representation of one of three experiments with similar results.

A

CD44 expression in tolerant CD4 T cells

B

Fold change in CD44 expression in activated and tolerant CD4 T cells
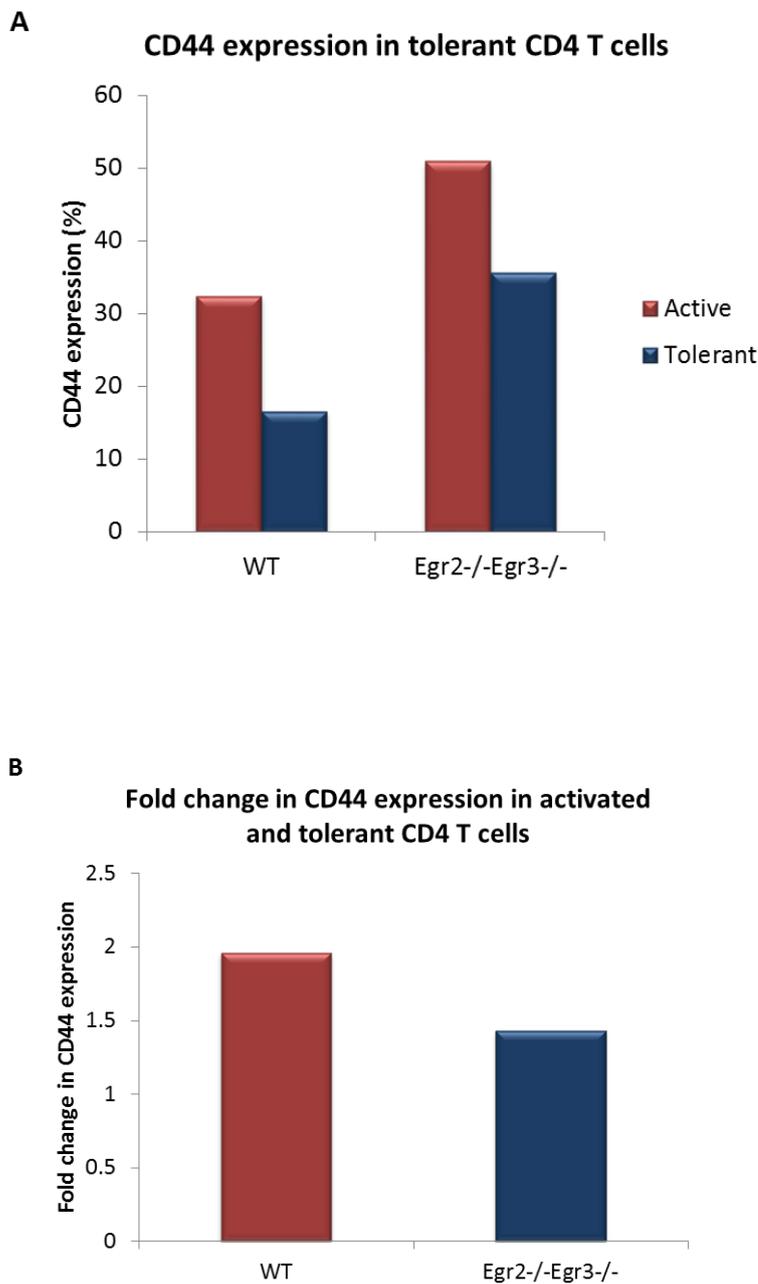
Figure 65 CD44 Expression in CD4 tolerant T cells decreases

(A)Flow cytometry phenotypic analysis, adapted from Figure 64, of CD69 population in naïve CD4 T cells from WT and CD2-Egr2-/-Egr3-/- mice that are: Naïve (untreated), Active(treated once with SEA), or Tolerant (treated 8 times with SEA). Fold change analysis of CD44 expression which decreases by 2 fold in the WT and 1.5 fold in the Egr2-/-Egr3-/- tolerant CD4 T cells. The data presented is a representation of one of three experiments with similar results.

4.3.3 Inflammatory cytokine production within in vivo tolerant CD4 T cells

Superantigen stimulation has been shown to induce an exaggerated cytokine response within CD4 T cells. As in vivo anergy induction is dependent on peptides such as superantigens, the increase in cytokines included those of the inflammatory nature (Sundstedt *et al.*, 1994). On treatment of CD4 T cells from WT and Egr2-/-Egr3-/- mice once with SEA, we observed increased levels of IFN-γ within WT and Egr2-/-Egr3-/- CD4 T cells. Egr2/3 knockout mice have already been reported to have increased levels of IFN-γ which is evident in our results (Figure 66). As Egr2 and 3 have been found to play a role within in vivo anergy induction (Safford *et al.*, 2005), there has been no speculation of their roles within the control of cytokine production.

In vivo tolerance is maintained by repeated injections of the antigen, with a decrease in cytokine expression taking place after a number of injections (Pape *et al.*, 1998). Interestingly, after inducing tolerance or activating mice we observed increased levels of IFN-γ in Egr2-/-Egr3-/- CD4 T cells after multiple injections of SEA (Figure 66). This was a completely opposite outcome to the WT where activated samples treated once with SEA showed increased concentrations of IFN-γ within both samples. The WT drastically reduced by 8 fold after 6 injections of SEA whereas the Egr2 and 3 knockout CD4 T cells remained higher. On flow cytometric analysis of CD4 T cells treated either once with SEA or multiple times we further confirmed high levels of IFN-γ production within tolerant Egr2-/-Egr3-/- CD4 T cells compared to WT (see Figure 67 Figure 68).

IFN-γ concentration in CD4 T cells from WT and Egr2-/-Egr3-/- mice treated with multiple injections of SEA
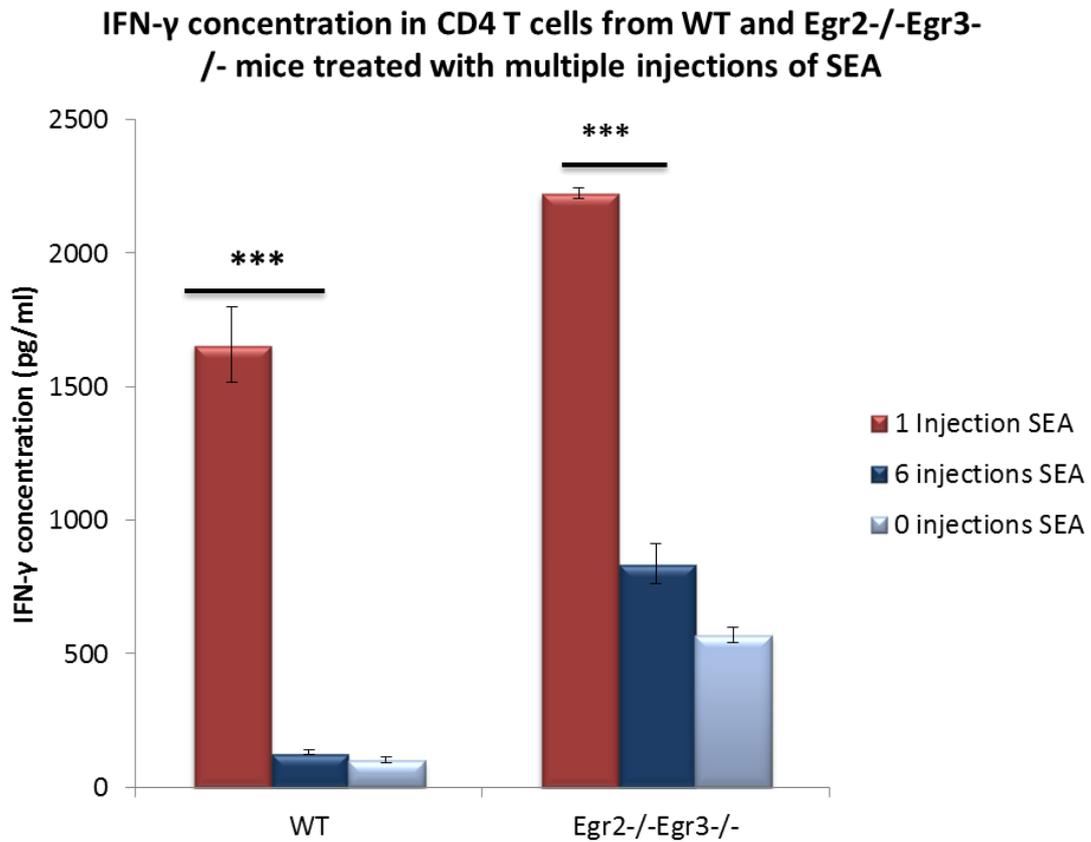
Figure 66 Increased IFN-γ concentration in tolerant naïve CD4 T cells from CD2-Egr2-/Egr3- mice

WT and Egr2-/-Egr3-/- CD4+ T cells from mice treated with once (active) or 6 times with a three-day interval (tolerant) with SEA at 20μg in vivo and cultured with SEA-reloaded APCs in ex vivo for 48 hours before collection of supernatant. IFN-γ quantification was done by ELISA. Cells cultured without stimuli served as negative controls. The data presented is a mean of three replicates from and represent three independent experiments with similar results. Error bars calculated from standard error values. Statistical analysis was calculated using students T-test and p values set as follows –P<0.05 *, P<0.01 **, P<0.001***, NS-not significant
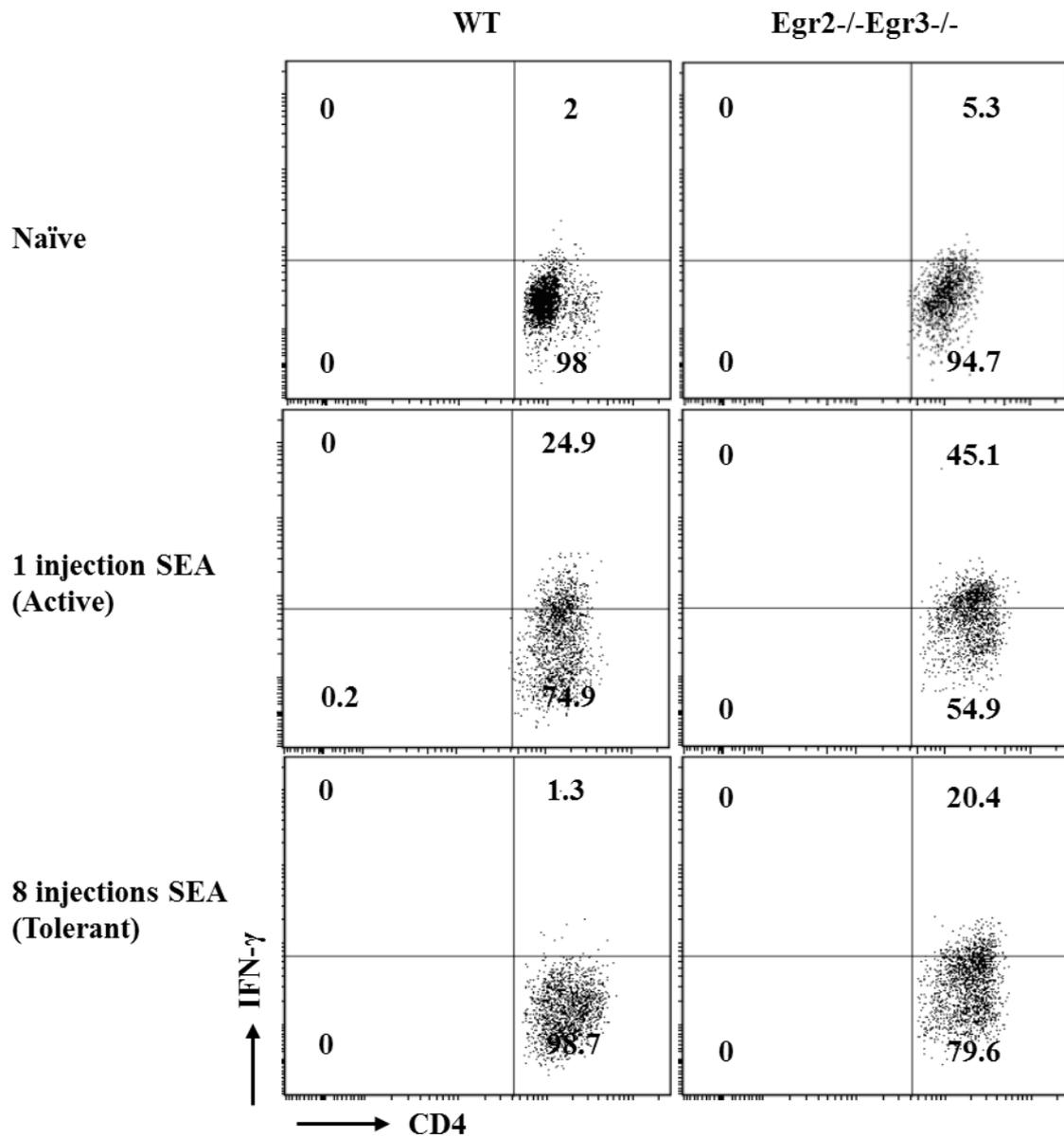
Figure 67 High IFN-γ expression in naïve CD4 T cells from CD2-Egr2-/Egr3- mice treated with SEA 8 times

IFN-γ levels in naïve WT and Egr2-/-Egr3-/- CD4+ T cells from mice treated with once (active) or 8 times (tolerant) with SEA at 20µg/injection and cultured ex vivo with 10µg/ml of SEA for 24 hours before re-stimulation with PMA and INO before flow cytometry analysis. Cells were stained with CD4 and IFN-γ and gated on the Vb3+ population. The data presented represent three independent experiments with similar results.
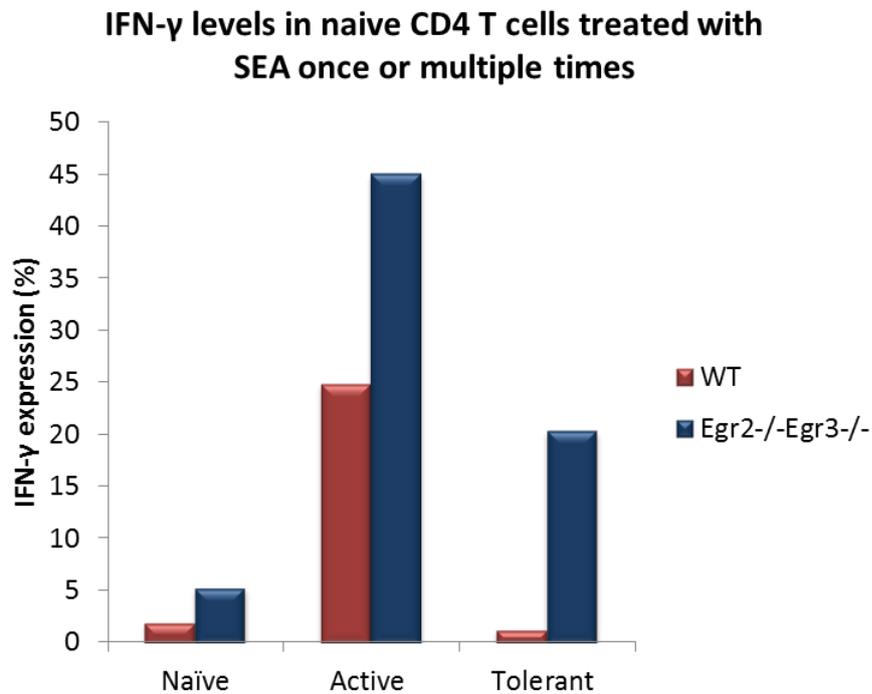
Figure 68 High IFN-γ expression in naïve CD4 T cells treated once or multiple times with SEA.

IFN-γ levels in naïve WT and Egr2-/-Egr3-/- CD4+ T cells from mice treated with once (active) or 8 times (tolerant) with SEA and cultured ex vivo for 24 hours. Adopted from Figure 67. Before flow cytometry analysis. Cells were stained with CD4 and IFN-γ and gated on the Vb3+ population. The data presented represent three independent experiments with similar results.

4.3.4 T-bet induction in anergic T cells

We have shown Egr2-/-Egr3-/- tolerant CD4 T cells to have increased levels of IFN-γ, which has not been reported in other studies. With increased IFN-γ expression within tolerant T cells due to Egr2 deficiency, we investigated the control of the inflammatory cytokine production. T bet or T box expressed in T cells is a transcription factor responsible for the TH1 program of naïve CD4 T cells. T bet is responsible for the activation of IFN-γ after its activation by STAT1 (Jenner *et al.*, 2009). Egr2-/-Egr3-/- mice showed increased activation of STAT1 (Li *et al.*, 2012a). By inducing IFN-γ, T bet is able to control multiple aspects of inflammation (Jenner *et al.*, 2009). As T bet expression has not been investigated within anergic CD4+ T cells, we treated WT and Egr2-/-Egr3-/- CD4 T cells with either one injection of SEA or with 8 injections of SEA. Surprisingly, we found T bet expression in tolerant Egr2-/-Egr3-/- CD4+CD44+ T cells treated 8 times with SEA to be similar to that to WT tolerant CD4 T cells (Figure 69). Activated samples

had increased levels of T bet in the Egr2-/-Egr3-/- CD4 T cells compared to WT. As confirmed in histograms provided via flow cytometry in Figure 70 and Figure 71. The result within the tolerant samples highlights the realisation that T-bet control is essential within the autoimmune disease or inflammatory settings. These results therefore suggest that Egr2 and 3 function to influence the activity of T bet and most importantly the inflammatory cytokine production within tolerant CD4 T cells.
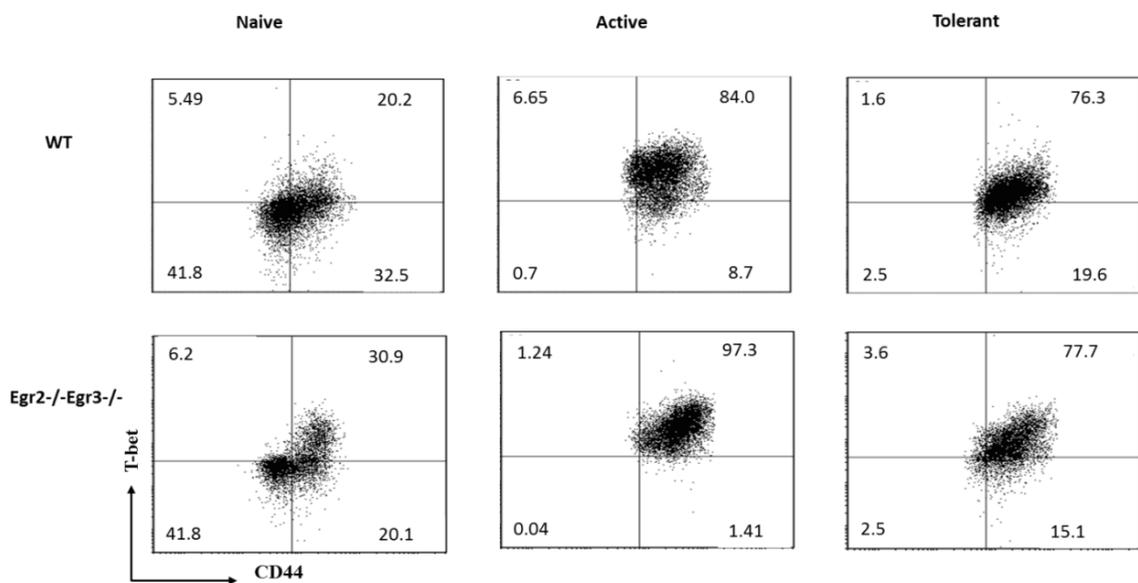
Figure 69 T-bet is co-induced in tolerant CD4+CD44+ T cells from WT and CD2-Egr2-/-Egr3-/- mice

WT and Egr2-/-Egr3-/- deficient mice were treated with 8 injections of SEA ip (20µg per injection) to induce tolerance and compared to samples treated with 1 injection SEA (active) or untreated. CD4 T cells were isolated and stained for T-bet and CD44 and expression checked by flow cytometry. This result is representative of 1 of 3 similar experiments. The data presented is a result of three individual experiments. Cells were gated on the CD4+Vb3+ population.
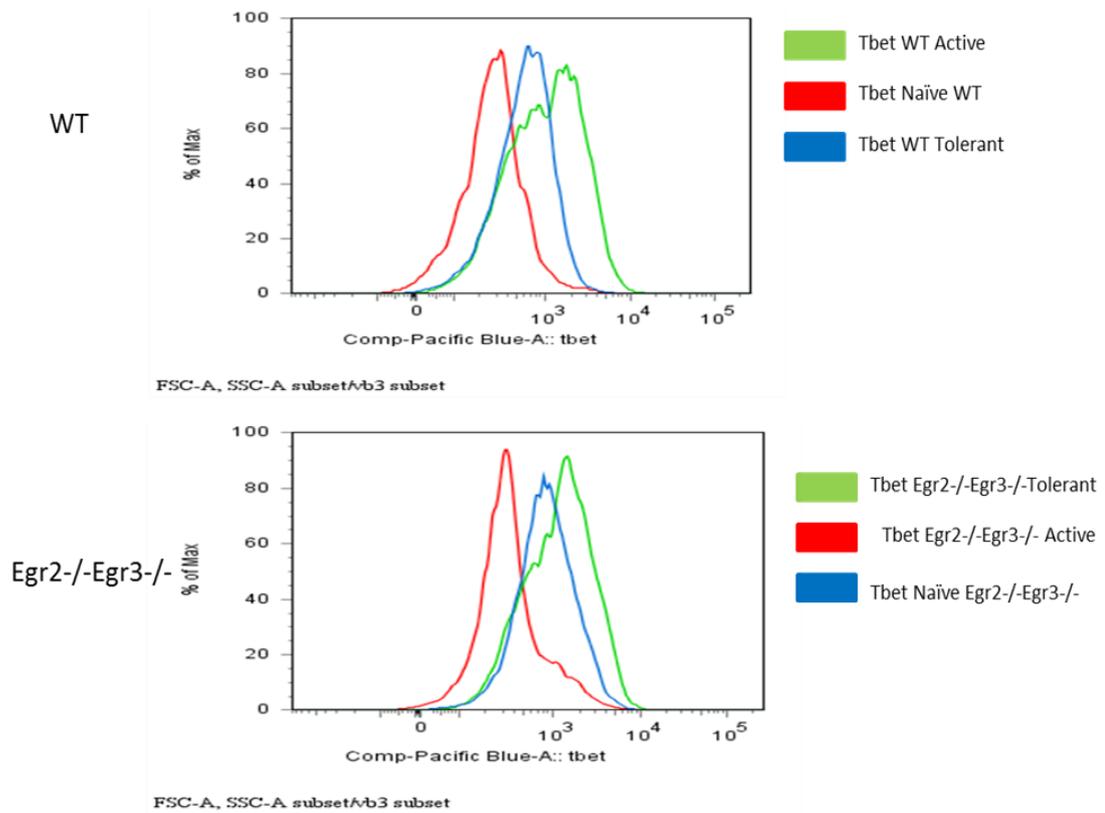
Figure 70 WT and Egr2-/-Egr3-/- T-bet kinetic shift

Flow cytometry histogram, showing the kinetic shift of different samples. WT and Egr2-/-Egr3-/-deficient mice were treated with 8 injections of SEA ip (20µg per injection) to induce tolerance and compared to samples treated with 1 injection SEA (active) or untreated. CD4 T cells were isolated and stained for T-bet and CD44 and expression checked by flow cytometry. Cells were gated on the CD4+ population. Data are representative of three individual experiments.
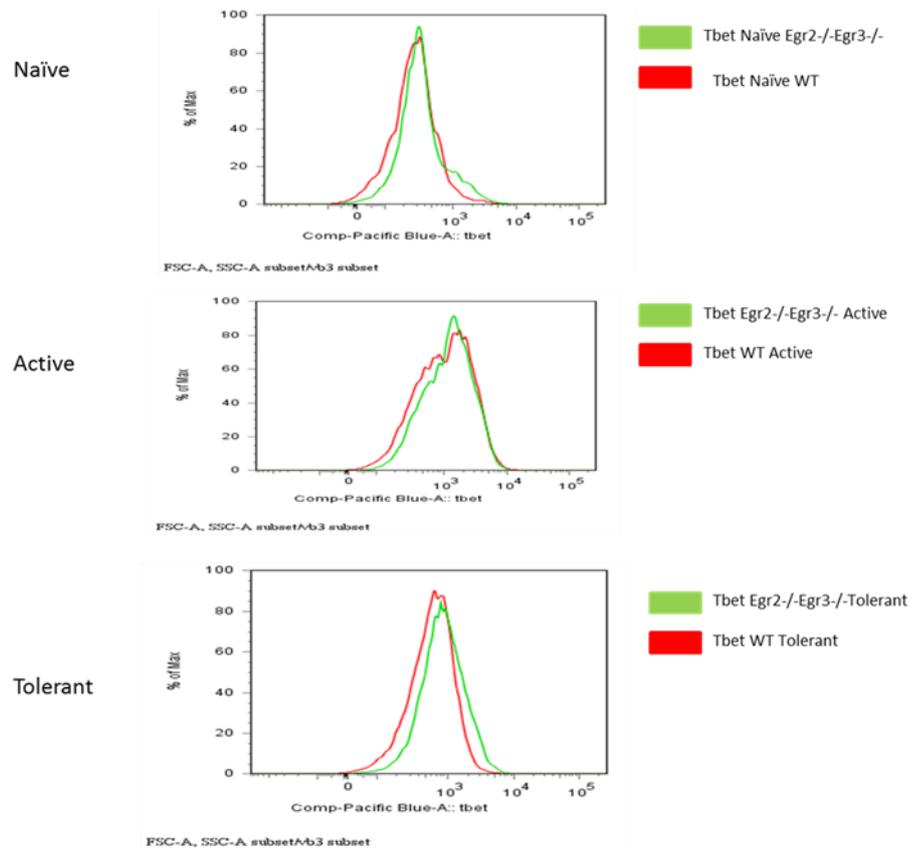
Figure 71 T-bet expression within the tolerance setting

Flow cytometry histogram, showing the kinetic shift of different samples. WT and Egr2-/-Egr3-/- deficient mice were treated with 8 injections of SEA ip (20µg per injection) to induce tolerance and compared to samples treated with 1 injection SEA (active) or untreated. CD4 T cells were isolated and stained for T-bet and CD44 and expression checked by flow cytometry. Cells were gated on the CD4+ population. Data are representative of three individual experiments.

4.4 Discussion

Within this chapter we investigated the role of Egr2 and Egr3 within the tolerance induction in vivo, where we found Egr2 and Egr3 knockout mice are able to undergo tolerance induction. A number of studies have looked at the induction of the tolerance in vivo in regard to Egr2 and Egr3, but they did not use the Egr2-/-Egr3-/- mouse model. Our model allows for the Egr2 deletion within lymphocytes where the CD2 promoter is present in conjunction with the systemic deletion of Egr3. Other groups reported that the presence of Egr2 and 3 may be required for the induction of tolerance; Zheng *et al.* used Th1 cell lines in vitro from an Egr2 knockout model (Zheng *et al.*, 2012). However, it was earlier published that if Egr2 is absent, Egr3 is able to functionally compensate. Making the Egr2-/-Egr3-/- model an exceptional model to carry out in vivo studies.

Superantigens are able to stimulate the V beta subsets of T cells and achieve T cell activation outside the peptide binding groove and were used to investigate the induction of tolerance in the absence of Egr2 and 3. This method has been used by a number of groups such as (Zheng *et al.*, 2012). It has been reported that in vivo tolerance results in decreased expression of AP-1 (Sundstedt *et al.*, 1996). Before use we assessed Egr2-/-Egr3-/- CD4 T cells and found they showed a reasonable population following SEA stimulation, similar to that in WT and CHIP sequencing data from Egr2-/-Egr3/- CD4 T cells treated with anti-CD3 and anti-CD28 showed no changes in the MHC genes related to the Vb3+ T cell subset thus Egr2 and Egr3 have no effect on the Vb3 subset. We reported repeated stimulation of Egr2-/-Egr3-/- mice with Staphylococcal enterotoxin A (SEA) resulted in decreased production of IL-2 and proliferation (Figure 59 and Figure 59) similar to that within the WT mice. Results showed repeated stimulation with superantigen causes an initial upregulation of effector cytokine production such as IL-2 and IFN-γ and TNF-α (Shwartz, 2003), a sudden decrease in cytokine expression takes place after 3 days coupled with apoptosis with the cells being left behind termed anergic. However, some studies within tolerance induction in regard to Egr2 and 3 used just one injection of superantigen stimulation. As the constant treatment is needed to maintain a state of tolerance (Shwartz, 2003), our experimental setting seemed more fitting. Proving Egr2 and Egr3 are not imperative in the process of tolerance induction.

Reference	Mouse model/ cell line	In-vitro induction using anti-CD3	In-Vivo induction method	Egr2 or Egr3 upregulated during tolerance?	Summary of findings	Similarity to our model
Zha, 2006	TCR-Rag-/- mice and IL-2 luc mice TH1 clones	yes	antigenic administration	no report	Ras reverses anergy in Th1. DGK-alpha upregulated in anergic th1 cells	Yes
Safford, 2005	A.E7 6.5 TCR transgenic C3-HA	yes	Adoptive transfer	yes	Overexpression of Egr2 and Egr3 inhibit T cell function.	Yes
Zheng, 2012	Egr2-/- - th1 clones, and mice	yes	Superantigen	yes	Egr2 is needed for anergy induction in vitro and in vivo. Egr2 regulates Dgk expression on anergy induction	partial
Egr2-/-Egr3-/- knockout model	Egr2-/-Egr3-/- CD4 T cells	yes	Superantigen	no	Egr2 and 3 do not play a role in tolerance induction. Egr2 and 3 play a role in inflammatory cytokine production within the tolerant setting.	-

Table 6 In-vivo tolerance induction in regard to Egr2 and Egr3 expression

Tolerant cells had to be identified successfully. On T cell activation CD44 and CD69 are expressed while the expression of CD62L is a marker of naïve T cells. In vivo, tolerant T cells have been reported to have decreases in activated T cell phenotypes coupled with increases in naïve T cell markers (Shwartz, 2003). On tolerance induction we found a similar result within our Egr2-/-Egr3-/- CD4 T cells (Figure 61). Both the tolerant Egr2-/-Egr3-/- and WT cells had increased expression of CD62L (Figure 63). Thus tolerance can take place within Egr2 and 3 knockout mice and phenotypically match the phenotype of

tolerant T cells. We surprisingly found the upregulation of inflammatory cytokine IFN-γ following tolerance induction within Egr2-/-Egr3-/- mice compared to the WT (Figure 71). This result is in accordance with our in vitro data and has yet to be reported. It was previously reported that Egr2-/-Egr3-/- mice showed increased inflammatory cytokine production (Li et al., 2012b), and we were curious to see how this is controlled molecularly and what factors controlled the high expression of IFN-γ within a tolerant setting, a setting that mimicked that of autoimmune disease. Autoimmune disease is found to be due to a breakdown in tolerance,

with an   inflammatory environment reported to be one of the major contributors to autoimmune disease (Lacotte et al., 2009). The inflammatory setting within our model showed the increase of IFN-γ, thus we investigated the role T-bet plays in this state. T bet is a transcription factor that is able to control the expression of IFN-γ and differentiation into the Th1 subset. On looking at T bet expression within the tolerant setting we found its expression to be similar to the WT and Egr2-/-Egr3-/- Tolerant samples (Figure 72), indicating that Egr2 and 3 play a role in the control of inflammatory cytokine production. In conclusion this chapter shows Egr2 and Egr3 do not play a role in in vivo T cell tolerance induction but instead play a role in the control of inflammatory cytokine production.

Chapter 5.   General Discussion

Self-tolerance is an important function of peripheral T cells. In homeostatic conditions, self-tolerance of T cells is controlled by extrinsic mechanisms such as regulatory T cells (T reg) and also intrinsic mechanisms induced by partially stimulation of T cell receptors (TCR) (Klein *et al.*, 2009; 2014; Vieira, 2004). Although in animal models the induction of tolerance renders T cells resistant to proliferate and produce IL-2, T cells from autoimmune disease patients such as lupus show poor proliferative response and impaired IL-2 production in response to TCR stimulation, but display hyper-active and inflammatory phenotypes (Kammer *et al.*, 2002). The findings from this study demonstrate a novel mechanism for the control of inflammation tolerance mediated by Egr2 and 3 in T cells. We demonstrated that tolerant T cells are not only resistant to TCR mediated proliferation, but also resistant to inflammatory stimulation. Proliferative tolerance refers to the ability of the T cells to proliferate and produce IL-2 on tolerance induction while inflammatory tolerance is the ability of CD4 T cells to produce inflammatory cytokines under tolerogenic conditions. In this study we have reported that Egr2 and Egr3 are not essential for the induction of proliferative tolerance, but of inflammation tolerance by controlling inflammatory cytokine production.

5.1 Tolerance induction in the absence of Egr2 and Egr3

The upregulation of Egr2 and Egr3 has been reported within tolerogenic and mitogenic conditions (Du *et al.*, 2014). Anergy induction is reported to result in hindered IL-2 production and decrease in proliferation within CD4 T cells (Jenkins *et al.*, 1987b), with the most common method of tolerance induction in vitro being the use of stimulation of the TCR with anti-CD3 in the absence of co-stimulation or CD28. The mechanism of anergy in vitro is mimicked in vivo and referred to as adoptive tolerance. Egr2 and 3 have been found to play a role within tolerance with studies showing its upregulation following anergy induction (Safford *et al.*, 2005). Our Egr2 and 3 knockout mouse model acted as a suitable tool to investigate the role of Egr2 and 3 within tolerance as no other group has used a similar double knockout model. We have reported decreased IL-2 and proliferation within Egr2 and 3 knockout CD4 T cells in vitro following treatment with plate bound anti-CD3 as seen within WT mice (Figure 28 and Figure 29). Previous reports have demonstrated the use of single knockout models, transfection of Egr2 into T cells and T-cell cell lines, however the overexpression of Egr2 has been shown to

result in apoptosis which also results in reduced IL-2 and proliferation thus other methods result in altered physiological condition (Safford *et al.*, 2005).

On investigating the hallmarks of anergic cells, reports show anergy induction in vitro involves the translocation of NFAT into the nucleus allowing for its expression as we have shown within our Egr2-/-Egr3-/- CD4 T cells. Reversal of anergy can be achieved in two ways, firstly by the addition of IL-2 into culture and secondly by the addition of cyclosporine A. We found CsA addition to result in increased IL-2 and proliferation within Egr2-/-Egr3-/- CD4 T cells. Reports have shown that single knockout Egr3 mice cannot undergo anergy induction (Safford *et al.*, 2005), similarly on knocking out only Egr3 from CD4 T cells we found a similar result, meaning Egr2 and 3 play another role within the tolerance setting. Importantly we have shown the large role TCR signalling plays on the expression of Egr2. As Egr2 has been found to play multiple roles including that of T cell development, further studies are looking into its role within T cell differentiation. We reported Egr2 expression decreased with decreasing TCR signalling strength which provides a key result to understating the significance of Egr2 within promotion of proliferation and in doing so T cell differentiation.

In vivo tolerance can be induced by the persistence of antigen stimulation causing unresponsiveness to further antigenic stimulation (Shwartz, 2003). Similarly, it adopts the MAPK and NFKβ pathways with the addition of the mammalian target of rapamycin (MOTR) pathway (Krakauer, 2013) which is induced directly by the presence of cytokines (McKay, 2001). Harris *et al.* (2004b) reported the upregulation of Egr2 and 3 within the in vivo setting as well, while we found tolerance induction to be possible in the absence of Egr2 and 3 following treatment with Superantigen SEA, similar to that in wild type mice. As Egr2 and 3 have been found to play a role in this, methodology of tolerance induction may play a role in the different outcomes viewed, however with both IL-2 and proliferation levels dropping significantly within Egr2 and 3 knockout cells after treatment with superantigen we can say tolerance was induced within Egr2 and 3 knockout CD4 T cells. Once again proving Egr2 and 3 play an alternative role within this mechanism other than that of tolerance induction.

Within both the in vivo and in vitro tolerance induction we found increased IFN-gamma expression within Egr2-/-Egr3-/- tolerant T cells compared to the WT Figure 36 and Figure 67. As this is yet to be reported, we looked into the control of said cytokine. T bet is the

transcription factor responsible for T cell differentiation into the Th1 subset, allowing for inflammation within the body. On inducting tolerance on WT and Egr2-/-Egr3-/- knockout mice we discovered expression in tolerant WT to mimic that within tolerant Egr2-/-Egr3-/- CD4 T cells, revealing a possible role for Egr2 and Egr3 within the control of inflammatory tolerance.

A number of anergy associated genes have been investigated within the tolerance setting. We have reported that within Egr2-/-Egr3-/- CD4 T cell tolerance induction, there is still high expression of tolerance associated genes. This includes E3 ubiquitin ligase genes which carry out the final step in the ubiquitination cascade, catalysing transfer of ubiquitin from an E2 enzyme to form a covalent bond with a substrate lysine. cbl-b and itch have been found to be upregulated within anergy. In addition, we looked at the expression of diaglycerol kinase alpha (DGK-α) which we found to be upregulated, as did other groups (Zheng *et al.*, 2012). Recently the gene Ndrg1 has been investigated with regard to Egr2, and we found similarities between the upregulation of cbl-b, itch and dgk alpha. Ndrg1 has been reported to be upregulated within the anergy induction. Its overexpression has been shown to result in the induction of a state similar to that of anergy. Its expression has been found to fall downstream of Egr2 (Oh *et al.*, 2015).

We looked at the expression of Bach2 which we found to be unchanged within the anergic setting in WT and Egr2-/-Egr3-/- CD4 T cells. BACH2 is expressed with T regulatory (Treg) cells (CD4+CD25+) and has been reported to be promote tumour immunosuppression (Roychoudhuri *et al.*, 2013). It has been reported to be highly expressed within T cells with a high affinity for an antigen. Our results confer with previous results from Li *et al.* in that Treg expression remained unchanged within WT and Egr2-/-Egr3-/- CD4 T cells (Li *et al.*, 2012b), which correlated with the development of autoimmune disease. It has been reported that CD4+CD25+ cells are able to facilitate anergy induction (Ermann *et al.*, 2001). We also assessed the expression of TCF-1 and Bhlhe40 and found reduced TCF-1 levels with both Egr2-/-Egr3-/- and WT tolerant CD4 T cells. Bhlhe40 has been reported to encourage differentiation toward the Th1 lineage (Lin *et al.*, 2014). In activated Egr2-/-Egr3-/-CD4 T cells we found its expression to be upregulated, however the expression fell within tolerant Egr2-/-Egr3-/- CD4+ T cells. Egr2 and 3 knockout CD4+ T cells have been reported to have high levels of Th1 cells with increased levels of IFN-γ (Li *et al.*, 2012b). We also found decreased levels of IFN-γ between Egr2-/-Egr3-

/- activated T cells and Egr2-/-Egr3-/- tolerant T cells that directly correspond to Bhlhe40 expression.

5.2 Egr2, Egr3 and inflammation in the tolerant setting

Inflammation is one of the major mechanisms that lead to tolerance breakdown and thus to autoimmune disease (Theofilopoulos and . Bona, 2002). Egr2 and Egr3 have been implicated in their roles in inflammation. Li *et al.* (2012a) found Egr2 and 3 knockout CD4 T cells exhibited increased production of inflammatory cytokines such as 1L-17A, 1L-17F and IFN-γ, but tolerance within the inflammatory setting had yet to be studied. In doing so we found induction of tolerance within Egr2-/-Egr3-/- T cells sustained elevated expression of IFN-γ, implying the induction of tolerance has an effect on inflammatory cytokine production control.

Inflammation has been found to be a significant factor in the development of autoimmune diseases such as rheumatoid arthritis, which presents in the joint lining and is associated with Th1 expression (Choy *et al.*, 2014). Tolerance induction prevents the activation of self-reactive T cells; however self-reactive T cells that lie latent in the periphery can still be activated by outside stimulus and result in inflammation. This can be seen in patients with RA, where CD4 T cells which lack co-stimulation are activated and skewed toward the Th1 subset resulting in the development of arthrosclerosis (Pasceri and Yeh, 1999). Inflammation can also be triggered by infection by a pathogen, and subsequent activation of dendritic cells in turn leads to the activation of autoreactive T cells eventually leading to autoimmunity (Turley, 2002). This can be seen in patients with thyroid autoimmune disorders such as Graves' disease and Hashimoto's thyroiditis, and allows for autoimmune disease development from an otherwise tolerant state (Millar *et al.*, 2003). Thus inflammation is evidently one of the key factors that control self-tolerance and is a major factor that causes the breakdown leading to autoimmune disease, and multiple studies using mouse models have proven this (see Table 7).

Essentially, the control of inflammatory cytokines is governed by specific transcription factors. The control of IFN-γ is controlled by the expression of T-bet which skews naïve T cells into the Th1 subset. With high IFN-γ levels within tolerant Egr2-/-Egr3-/- T cells we found the expression of T-bet to remain lowered similar to that in WT tolerant cells. T-bet has been implicated in the tolerant setting with studies carried out on T regulatory cells using peptides to induce tolerance. It was reported that Egr2 and T bet worked in this system to maintain

peptide-induced tolerance. However, both Egr2 and T-bet functioned in different ways to maintain this state (Anderson *et al.*, 2006; Anderson *et al.*, 2006; Anderson *et al.*, 2006). With Egr2 and 3 deficient cells exhibiting increased IFN-γ in the tolerant state, even though we showed decreased T bet expression, this would imply a role for Egr2 and 3 in the control of T-bet activation. Thus Egr2 and 3 play a role in inflammatory tolerance via the control of inflammatory cytokine production. We also viewed decreased expression of RORgt, which is the transcription factor that controls Th17 differentiation. These results suggest an alternative mechanism for tolerance induction within the inflammatory setting as expression of transcription factors do not correlate with cytokine production.

Disease	Description	Mouse Model	Pathology	Cytokines/Mechanism	References
Multiple Sclerosis (MS)	Chronic inflammatory autoimmune disease with affects in the central nervous system with cell infiltration and CNS cell demyelination	EAE	Demyelination in CNS due to mononuclear cell infiltration	Involvement of CD4 T cells with Th1 predominant effects including increase in IFN-γ. TH17 cells promote inflammation via IL-17	(Komiyama *et al.*, 2006)(McCarthy, Richards and Miller, 2012)
Rheumatoid arthritis (RA)	Chronic disease associated with systemic effects characterised by swollen joints, autoantibody production to collagen	CIA-collagen induced arthritis using in DBA/1 mice	Inflammatory infiltration, synovial hyperplasia and cartilage and bone erosion	TNF-α, IL-1, IL-17 and IL-6 play biggest roles with IL-6 driving neutrophil activation leading to inflammation	(Smolen *et al.*, 2007)(Asquith *et al.*, 2009)
Systemic Lupus Erythematosus (SLE)	Disease characterised by production of antibodies to one or more self-antigens leading to polysystemic autoimmunity. Associated with renal disease (glomerulonephritis, nephritis), skin rash, hyperactivation of B and T cells, accumulation of inflammatory cells leading to swelling, defective clearance of immune complexes	NZB/W	Gene mutation loss of Fas ligand interaction thus lack of clearance of B and T cells promoting inflammation	Pathology includes inflammation expression increased IFN-γ, IL-6, IL-17A, IL-17F and TNF-α leading to inflammation.	(Abraham and Weiss, 2004; Mok. C.C, 2003) (Perry *et al.*, 2011; Rottmann and Willis, July 2010) (Li *et al.*, 2012a)
		MRL/lpr	high autoantibody titres, development of skin rash and inflammatory arthritis		
		BXSB/Yaa	Gene mutation in the Y chromosome thus disease more susceptible to males unlike others. Genetic defect in TLR-7.		
		CD2-Egr2-/-Egr3-/-	Hyperactive B and T cells increase in inflammatory cytokines IFN-γ, IL-17 A, IL-17F. Development of splenomegaly and glomerulonephritis		

Table 7 Human inflammatory autoimmune diseases and their corresponding mouse model

5.3 Varying Egr2 expression in autoimmune disease

Egr2 and 3 knockout mice have been found to have high numbers of CD4+CD44+ effector phenotypes. These are known to be highly cross-reactive and lead to autoimmune disease development (Li *et al.*, 2012). These mice exhibit lupus-like symptoms within 4 weeks and have lymphocyte infiltration in multiple organs such as the kidney (Li *et al.*, 2012). It is important to differentiate the roles of inflammatory disorders in comparison to breakdowns in inflammation that lead to autoimmune disease. Inflammatory proliferative disorders such as inflammatory bowel disease (IBD) exhibit normal IL-2 expression and proliferation (Fuss *et al.*, 1996), whereas autoimmune disease exhibits decreased IL-2 and proliferation similar to that seen in tolerant Egr2 and 3 knockout mice.

Egr2 has been reported to play a role in multiple autoimmune diseases. Dendritic cells DCs play one of the major roles in autoimmune disease development with mDCs and PDCs playing a role in multiple sclerosis development. Egr2 is reported to be expressed in at the later stages of DC development and have decreased expression of SOCS1. Reduced Egr2 expression within DCs has been shown to affect IL-12 generation within MDCs (Miah *et al.*, 2013), with Egr2 knockdown DCs showing increased levels of MHC class I and II molecules and co-stimulatory molecules (Miah *et al.*, 2013). This would suggest that the absence of Egr2 in our model would cause autoimmunity through the increased production of inflammatory cytokines via increased activation of mDC. It has also been reported that Egr2 plays a role in the development of T regulatory cells (Okamura *et al.*, 2012), which are important in the maintenance of the immune system to avoid autoimmune disease. Interestingly, the production of T reg cells within the Egr2/3 knockout mouse model was found to be unchanged (Li *et al.*, 2012b). Our analysis of the BACH2 gene also showed levels of Treg remained similar within the tolerant setting in Egr2/3 knockout mice.

In autoimmune disease, Egr2 has been shown to play roles in systemic Lupus in Japanese populations, where polymorphisms within the Egr2 gene are linked to SLE (Myouzen *et al.*, 2010). In multiple sclerosis which is a chronic inflammatory demyelinating disorder of the central nervous system the levels of Egr2 within patients had decreased and similarly to our model there were increased levels of IL-17 (Miao *et al.*, 2013). Interestingly, this is not the pattern with Egr2 within autoimmune disease, and in studies on systemic sclerosis (SSc) and scleroderma the expression of Egr2 elevated disease severity. It was reported that Egr2 was upregulated in skin biopsies collected from SSc patients and the SSC mouse model (Fang *et al.*, 2011). Thus the balance of Egr2 expression

within autoimmune disease plays a very important role, proving there is still a way to go with its expression in autoimmune disease in relation to therapy.

Egr2 has been shown to play a role during infection. It was found that CD2-Egr2 knockout mice have increased viral load in their lungs compared to WT or Egr1 knockout mice following inflection with influenza (Du *et al.*, 2014). We found CD2-Egr2-/-Egr3-/- mice to have increased viral load coupled with a decrease in neutralising antibody following viral infection, showing Egr2/3 are important in the production of virus specific antibodies that are needed for clearance (Ogbe *et al.*, 2015).

These results therefore show the importance of Egr2 and Egr3 within the control of inflammation, tolerance breakdown and viral clearance. Egr2 can then be applied directly to the treatment of patients with autoimmune disorders in the future as in the case of SSC where the addition of TGF-β stabilises the expression of Egr2 resulting in normal fibroblast production (Fang *et al.*, 2011). Identification of mutations within the Egr2 locus could help not only diagnose the cause of disease but within inflammatory disorders it can be applied to better control the severity of ongoing illness. In this case Egr2 and 3 can be used in the control of autoimmune disease severity.

Chapter 6. Conclusions, limitations and future work

Our previous work has shown a role for Egr2 and 3 within the development of B and T cells coupled with the role of Egr2 in the prevention of autoimmunity. Based on the findings, having already shown inflammation within Egr2 and Egr3 knockout mice, we have now shown that tolerance can be induced in the absence of transcription factors Egr2 and Egr3 in vitro and in vivo. We found tolerance induction in Egr2 and 3 knockout CD4 T cells to be NFAT dependant with Egr2 and 3 knockout T cells upregulating expression of tolerance associated genes. Thus we found that Egr2 and 3 play a more important role in preventing inflammation of tolerant T cells, rather than tolerance induction.

We also looked at the control of Egr2 expression within the mitogenic setting and found this to be dependent on the strength of antigenic stimulation. This suggests that the expression of Egr2 through T cell receptor activation can therefore directly influence T cell proliferation and production of IL-2 within CD4 T cells. As we have previously reported, the induction of tolerance is possible in the absence of Egr2 and 3 as tolerance induction is independent of AP-1 activation. We have also shown a possible role of Egr2 and 3 in the control of inflammation within tolerant T cells, as tolerant Egr2 and 3 negligent mice still exhibit high expression of IFN-γ. A diagrammatic representation of the role of Egr2 and 3 within inflammatory tolerance is shown in Figure 72 .

In summary the presence of Egr2 and Egr3 is not imperative for tolerance induction to take place. We found that TCR signalling governs the expression of Egr2 and thus dictates proliferative function in T cells. Egr2 and 3 thus do not play a role in proliferative tolerance. We found Egr2 and 3 functions to control inflammation within the tolerant setting, and may play a role in the control of activation of T bet. Egr2 and 3 may therefore be able to regulate the effector function of tolerant T cells within the inflammatory setting, possibly influencing the inflammatory severity within autoimmune disease.

As tolerance has yet to be studied within an inflammatory setting, the Egr2 and 3 knockout mouse model is an appropriate mouse model for this. However, this study was not without limitations. The potential role of Egr2 and 3 within inflammatory tolerance has been established, but IFN-γ is only one of the factors that give rise to inflammation. In order to truly look into inflammation and tolerance the consideration of other inflammatory cytokines such as IL-17, IL-6 and TNF-α needs to

be further investigated. Li *et al.* (2012b) reported that Egr2 and 3 knockout mice presented with increased inflammatory cytokines in addition to IFN-γ, but this study did not look into the remaining cytokines which may also contribute to inflammatory tolerance. The molecular mechanisms of Egr2 and 3 direct interactions with that of the transcription factor T bet have also yet to be confirmed. In addition, the Egr2 and 3 knockout model provides only one inflammatory setting on which to work with, where there are multiple models (as seen in Table 5 and **Error! Reference source not found.**) that can be used for similar inflammatory tolerance studies. As Egr2-/-Egr3-/- mice have been found to develop symptoms after 4-6 weeks, autoimmune disease models such as EAE would be more stable to work with as these would have established autoimmune disease at any age.
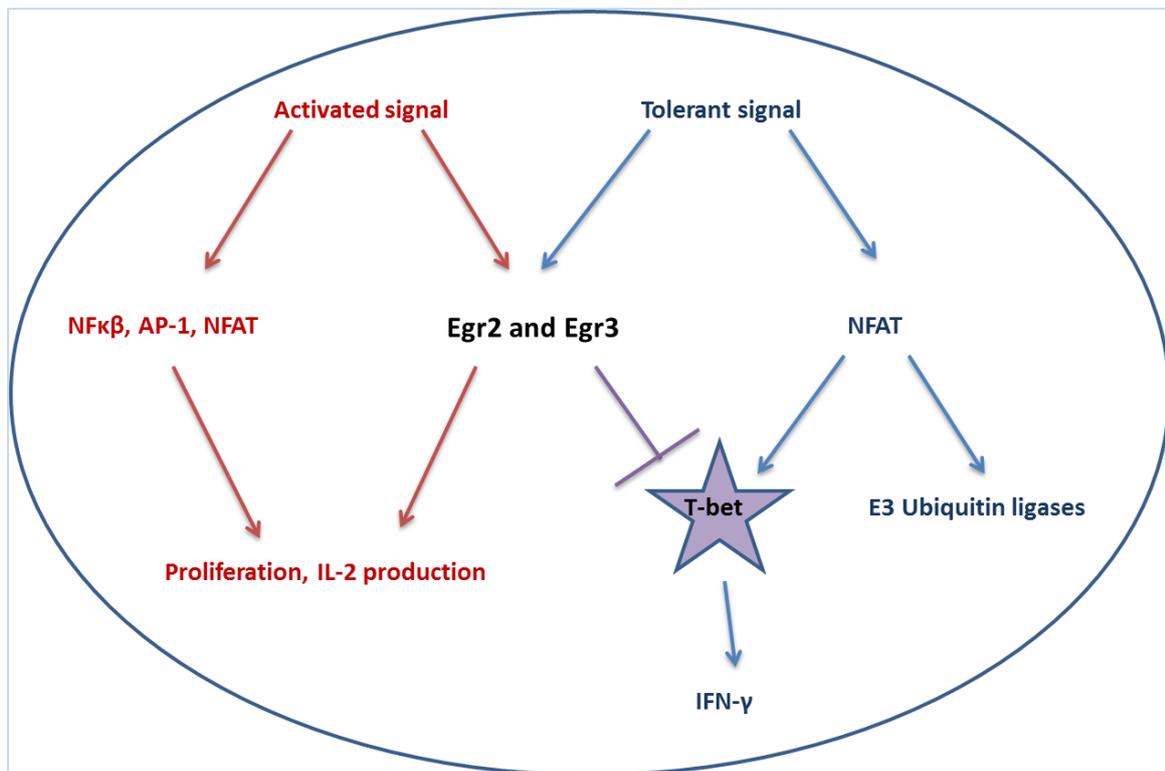
Figure 72 Diagrammatic representation of Egr2 and Egr3 in tolerance inflammation

On tolerance induction TCR signalling leads to NFAT activation which leads to Egr2 and 3 expression. During inflammatory tolerance, the absence of Egr2 and 3 lead to T-bet activation and the production of inflammatory cytokine IFN-γ in the tolerant state. This means that Egr2 and 3 play a crucial role in the control of inflammatory tolerance.

The findings from this study demonstrate a novel tolerogenic mechanism for Egr2 and 3 within the control of inflammation. As we have found the expression of Egr2 to be directly linked to the TCR signalling, it would be interesting to be able to quantify the true expression of Egr2 and 3 during T cell reactions. It would also be interesting to further investigate the expression of other genes within the tolerant Egr2 and 3 negligent CD4 T cells, as we found anergy induction to take place in the absence of Egr2 and 3. Because the role of tolerance has not been investigated within the inflammatory setting, CHIP sequencing or microarray carried out on tolerant Egr2-/-Egr3-/- would allow for a larger array of gene expression to further investigate the mechanisms in the control of inflammation within the tolerant state and identify new genes involved. Egr2 has also been implicated in the expression of 'anergy associated genes'. It would be interesting to investigate this further, especially in terms of the ubiquitin ligase genes and their control within the tolerant setting. As we have already carried out an EMSA for the presence of NFAT, in order to confirm NFAT binding it would be good to look at a super shift analysis where anti-NFAT oligonucleotides would be incorporated to prove the binding of NFAT within the complexes. Egr2-/-Egr3-/- mice have been reported to have a decreased number of thymocytes (Li *et al.*, 2011) and this may be because Egr2 is required for thymocte development in the DN1 and DN2 stages. However the DN4 and DP stages are not affected by Egr2 expression. In order to prove positive selection is completely functional within the model under the tolerance conditions, an MHC class II knockout model could be applied, and mitogens could be used to rule out defects in negative selection as they allow for weak self-antigen monitoring of negative selection.

Additionally, our group has recently come up with a knockin model of Egr2 (Miao et al (2016)-in review). Egr2 knockin has a fluorescence tag to Egr2 expression using GFP. This model opens a whole new array of experimental opportunity with the ability to track Egr2 within homeostatic, mitogenic and tolerogenic conditions. This means the expression of Egr2 within tolerance induction can possibly be shown in vitro as well as in vivo. It would act to confirm what we have already found and would show Egr2+CD44 high to have high proliferation and low IFN-γ production against the Egr2-CD44 high, showing low proliferation and high IFN-γ production. The use of animal models such as cblb-/- mice or itch-/- mice would allow the studies of not just inflammation and autoimmune disease, but also of tolerance. Monitoring the expression of Egr2 and 3 within such models would provide new insight into their role in tolerance induction as well identify novel roles in the control of ubiquitin ligase genes within tolerance.

In a similar manner this study could be expanded and further confirmed using the most commonly used form of pMHC (peptide-major histocompatibility complex) multimurisation called tetramers. Tetramers are used to identify specific T cell populations and are fluorochrome conjugated allowing easy analysis via flow cytometry (Wooldridge *et al.*, 2009). Tetramers have been used in tolerance studies such as by david and group, Where peptide induced tolerance was achieved (David *et al.*, 2014). However our method differed; Bacterial superantigens have been found to provide a strong cross-linking to enable the investigation using pMHCII tetramer use allowing for direct staining of samples ex-vivo. Tetramer use would provide identification of TCRs (Massey *et al.*, 2007). This would allow for a more specific population to be identified within our model following tolerance induction and a much faster analysis. In addition the use of our model backcrossed within the TCR transgenic Rag2-/- background would create an excellent environment for the true nature of Egr2 and Egr3 knockout CD4 T cells. Similarly to safford and group the induction would be monitored within a neutral environment. To add to this, the use o EMSA can be adopted within in-vivo treated CD4 Tcells; by the monitoring of the transcription factors AP-1 and NFκβ.

Toll-like receptors (TLRs) are pattern recognition receptors that are able to recognise distinct pathogen-associated molecular patterns (PAMPS) that are conserved in specific microorganisms. Domininguez-Villar *et al.* (2015) recently showed that TLR7 is able to induce anergy in human CD4+T cells by silencing the expression of TLR7 in CD4+ T cells using the HIV-1 infection, as these induce high levels of CD4+ T cells. They then went on to investigate the calcium flux where NFATc is activated; in doing so they carried out an NFATc knockdown within the CD4 T cells. It would be interesting to see the effects of NFAT knockdowns within the tolerance induction models. Their investigation took place within a viral environment, but it is not clear what would happen in a human autoimmune disease inflammatory environment. Since viruses have been found to be among the factors that influence autoimmune disease development, and so it would be something worth investigating.

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APPENDIX

IL-2 concentration from samples treated with SEA once or 6 times with different dendritic cells.
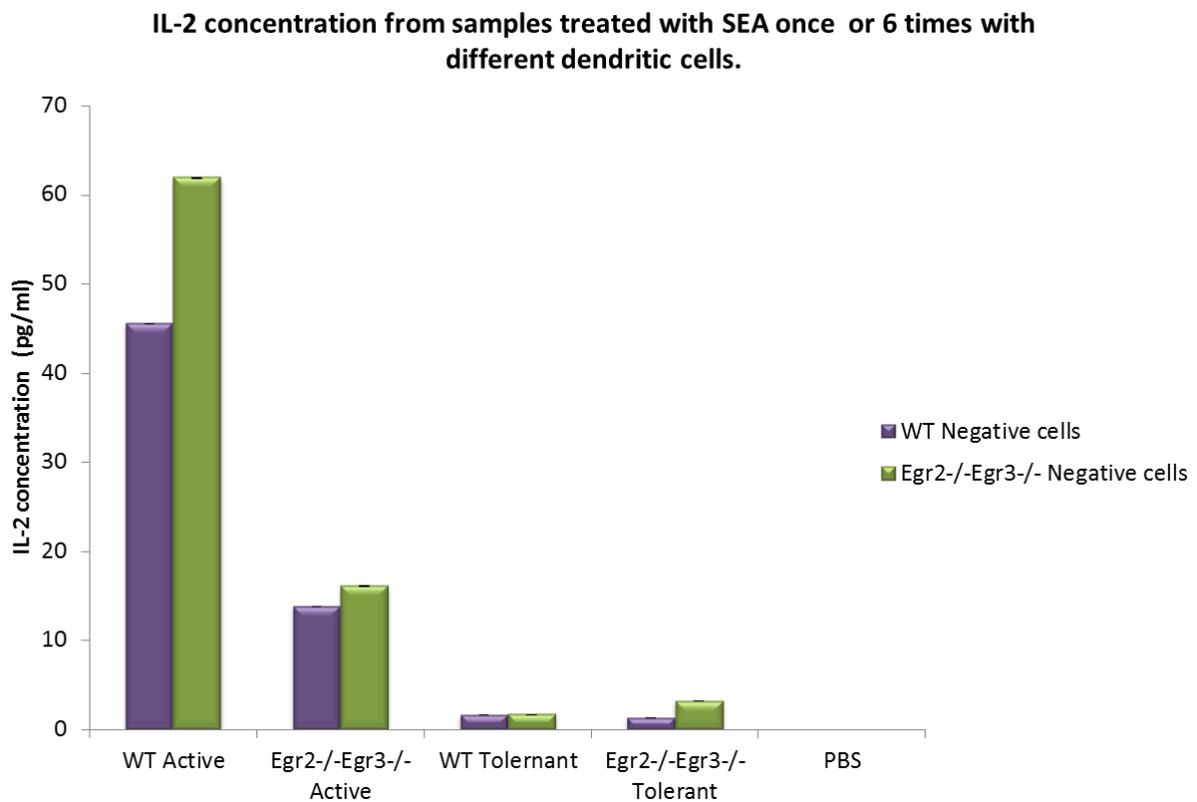
Figure 73 IL-2 concentration in naïve CD4 T cells from wild type and CD2-Egr2-/Egr3- mice with WT or Egr2-/-Egr3-/- negative cells show similar IL-2 production

IL-2 levels in naïve WT and Egr2$^{-/-}$Egr3$^{-/-}$ CD4$^+$ T cells from mice treated with one (active) or 6 injections (tolerant) with SEA and cultured ex vivo in 10µg/ml SEA for 48 hours before IL-2 quantification. WT and Egr2-/-Egr3-/- CD4 T cells from mice treated just once with SEA showed increased IL-2 expression, with expression being increased in the WT. Similarly, samples treated 8 times with SEA from both WT and Egr2-/-Egr3-/- mice showed decreased IL-2 concentrations compared to mice treated once. The difference between WT and WGr2-/-Egr3-/- CD4 T cells is similar as cells show equal significant change.

IL-2 concentration from samples treated with 1 injection of SEA and mice injected 6 times with SEA with or without culturing with SEA
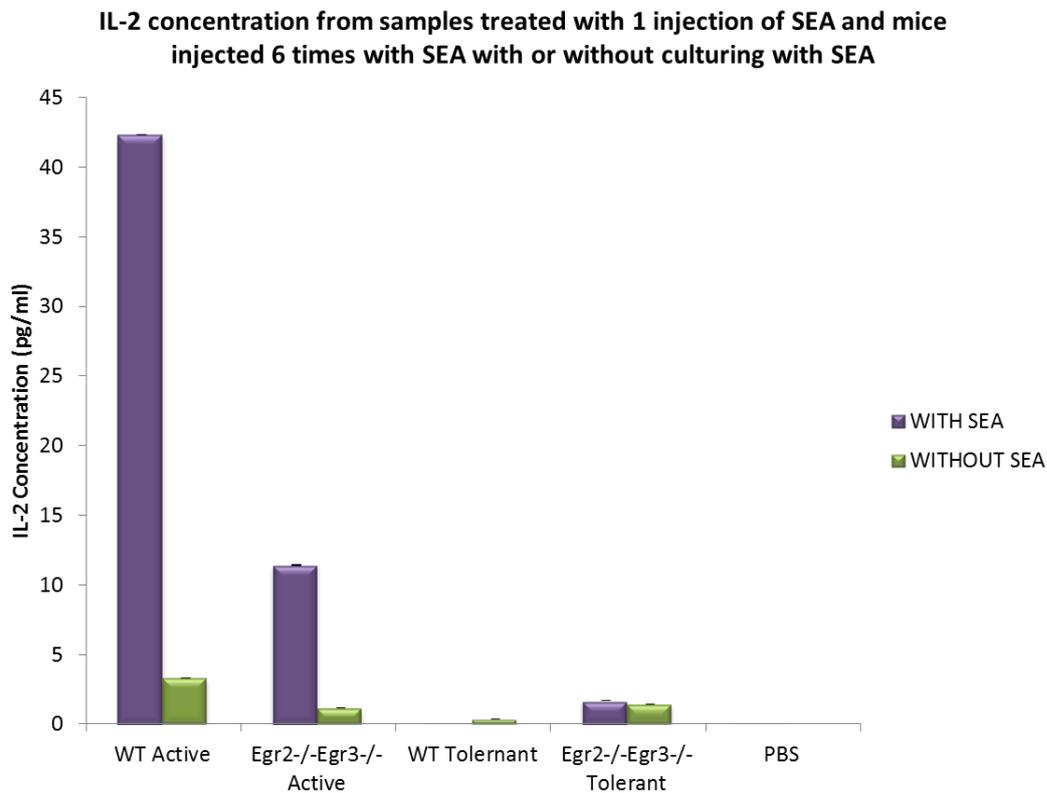
Figure 74 IL-2 concentration in naïve CD4 T cells from wild type and CD2-Egr2-/Egr3- mice with SEA show significant changes compared to those without SEA

Samples with SEA show IL-2 levels in naïve WT and Egr2$^{-/-}$Egr3$^{-/-}$ CD4^{+} T cells from mice treated with one (active) or 6 injections (tolerant) with SEA and cultured ex vivo in 10µg/ml SEA for 48 hours before IL-2 quantification. WT and Egr2-/-Egr3-/- CD4 T cells from mice treated just once with SEA showed increased IL-2 expression, with expression being increased in the WT. Similarly, samples treated 6 times with SEA from both WT and Egr2-/-Egr3-/- mice showed decreased IL-2 concentrations compared to mice treated just once. Samples without SEA show no significant difference.