Early Growth Response Genes -2 and -3 are essential for optimal immune response

-To study T Cell Receptor Signalling and Autoimmune disease in EGR-2 and EGR-3 deficient mice

A thesis submitted for the degree of Doctor of Philosophy by

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Declaration

I hereby declare that I am the author of this work, except where otherwise specified, and has not been submitted for any other degree.

Emma Ghaffari

The work presented here using the EGR-2cKO, EGR-3KO and EGR-2Tg mice were established as previously described in the thesis. The generation of EGR-2 and EGR-3 double knockout mice were established at Brunel University, Uxbridge by Dr S.Li. I was involved in the genotyping and confirmation of the deletion of EGR-2 and EGR-3 in the mice by RT-PCR and Immunoblotting (Figure. 1-4). The administration of MOG antigen for EAE disease development in the mice was performed with Dr Li at Brunel University, Uxbridge (Figure. 6, B). Furthermore the histological examinations were performed by Dr T. Miao at Queen Mary, University of London (Figure. 5, D and 6, B).

In addition the radioactive labelling of tritium for thymidine incorporation to measure T and B cell proliferation was performed by Dr T. Miao at Queen Marys, University of London (Figure. 7, B and 11). Furthermore the probe sequence and radioactive probe labelling for EMSA analyses throughout this thesis was performed by Dr T. Miao at Queen Marys, University of London, where I was actively involved in the rest of the experimental stages (Figure. 8, 11, D). Also, the EGR-2 and Batf constructs and purchase of cDNA was performed by Dr T.Miao at Queen Marys, University of London (Figure. 9 and 10). Finally the siRNA oligonucleotide sequence was made by Dr T. Miao at Queen Marys, University of London (Figure. 11).

Abstract

Early Growth Response Genes (EGR) is a family of four transcription factors containing a unique zinc finger domain. EGR-2 and EGR-3 are important for hindbrain development and myelination. These transcription factors are also necessary for lymphocyte function however, the mechanisms are still unclear. Previous findings have shown that EGR-2cKO mice develop lupus-like autoimmune disease with high levels of pro-inflammatory cytokines despite showing normal T and B cell proliferation after mitogenic stimulation. Therefore we established the CD2-EGR-2$^{-/-}$EGR-3$^{-/-}$ mouse model to explore the phenotype, susceptibility to autoimmune disease and relevant lymphocyte function. We discovered that CD2-EGR-2$^{-/-}$EGR-3$^{-/-}$ mice developed severe systemic autoimmune disease and expressed high levels of inflammatory cytokines. More importantly we discovered a novel finding that CD2-EGR-2$^{-/-}$EGR-3$^{-/-}$ T and B cells had impaired cell proliferation after mitogenic stimulation.

Further investigations revealed that the molecular mechanism defected in the T cell receptor signalling pathway is due to a dysfunction in Activator Protein-1 (AP-1). AP-1 is a heterodimeric protein composed of AP-1 family members including Jun, Atf and Fos. Our data shows that EGR-2 and EGR-3 directly bind with the Atf family member Batf, which prevents Batf's inhibitory function on AP-1 activation. This research demonstrates that EGR-2 and EGR-3 intrinsically regulate chronic inflammation and also positively regulate antigen receptor activation. In conclusion EGR-2 and EGR-3 are essential for providing optimal immune responses, whilst limiting inflammatory immunopathology. We propose that this new model could be used for studying autoimmune disease.

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Abbreviations

PAMP	Pathogen Associated Molecular patterns
APC	Antigen Presenting Cell
MHC	Major Histocompatibility Complex
DNA	Deoxyribonucleic acid
cDNA	Complementary DNA
RNA	Ribonucleic acid
mRNA	Messenger RNA
TCR	T cell Receptor Signalling
λ	Lambda
α	Alpha
β	Beta
γ	Gamma
ζ	Zeta
δ	Delta
ε	Epsilon
κ	Kappa
θ	Theta
CD	Cluster of differentiation
ITAMs	Immunoreceptor tyrosine based activation motifs
PTK	Protein tyrosine kinase
LAT	Linker for Activated T cells
GEF	Guanine-nucleotide exchange factors
PLCγ	Phospholipase Cγ
PKC	Protein Kinase C
DAG	Diaglycerol
NFAT	Nuclear Factor of Activated T cells
NF-κB	Nuclear Factor κB
AP-1	Activator Protein-1
ERK	Extracellular signal-regulated kinases
FOXO3	Forkhead box O3
MAPK	Mitogen Activated Protein Kinase
CREB	cAMP response element-binding protein
JNK	c-Jun terminal kinase
IL	Interleukin
PKCθ	Protein Kinase Cθ
IKK	IκB
DN	Double Negative
DP	Double Positive
ATF	Activating Transcription Factor
Maf	Masculoaponeurotic fibrosarcoma
bZIP	Basic Leucine Zipper domain
TPA	12-O-tetra decanoylphorbal-13-acetate response
CRE	cAMP-Response Element
CCR	C-C chemokine receptor

BCL-6	B-cell lymphoma 6 protein
NH2	Amidogen
Mef2	Myocyte enhancer factor 2
GR	Glucocorticoid Receptor
ROR	Retinoic acid receptor
GSK3	Glycogen synthase kinase 3
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
TNF-α	Tumor Necrosis Factor alpha
Th	T helper cell
DBD	DNA binding protein
NKT	Natural Killer T cells
WT	Wild Type
EAE	Experimental Autoimmune Encephalomyelitis
NAD	Nicotinamide adenine dinucleotide
TGF-β	Transforming Growth Factor β
EGR	Early Growth Response Gene
K2/3	EGR-2 and EGR-3 deficient mice
UV	Ultra Violet
Cdk	Cyclin Dependent Kinase
PPAR	peroxisome proliferator-activated receptors
GATA-3	Trans-acting T-cell-specific transcription factor
STAT	Signal transducer and activator of transcription
RUNX3	Runt-related transcription factor 3
MMP	Matrix metalloproteinases
IFN	Interferon
ICOS	Inducible T-cell Costimulator
CTLA	Cytotoxic T-Lymphocyte Antigen
PD-1	Programmed Cell death-1
SOCS-1	Cytokine Suppressor of cytokine signalling-1
FoxP3	Fork head box P3
JAK	Janus Kinase
MOG	Myelin basic protein
VEGF	Vascular endothelial growth factor
STIM	Stromal interaction molecule
PCR	Polymerase Chain Reaction
RT-PCR	Real Time PCR
Taq	Thermus aquaticus
TAE	Tris-acetate-EDTA
BSA	Bovine Serum Albumin
PBS	Phosphate buffered saline
MgSO4	Magnesium sulphate
LB	Luria-Bertani
FITC	Fluorescein isothiocynate
PE	Phycoerythrin
BrdU	Bromodeoxyuridine
PMA	Phorbal 12-myristate 13-acetate
DMEM	Dulbecco's Modified Eagle Medium

ME	Mercaptoethanol
FBS	Fetal bovine serum
PMSF	Phenylmethylsulphonyl fluoride
EDTA	Ethylenediaminetetraacetic acid
DTT	Dithiothreitol
KCl	Potassium Chloride
SDS	Sodium dodecyl sulphate
NaCl	Sodium Chloride
PAGE	Polyacrymalide gel Electrophoresis
LDS	Lithium dodecyl sulphate
APS	Ammonium Persulphate
HRP	Horseradish Peroxidase
TBST	Tris Buffered Saline with Tween 20
Ig	Immunoglobulin
TBE	Tris/Borate/EDTA
µl	Microlitre

Publications derived from this work

Li, S., T. Miao., M. Sebastian., P. Bhullar., **E. Ghaffari**., M. Liu., A. L.J. Symonds., and P. Wang. (2012). The Transcription Factors Egr2 and Egr3 Are Essential for the Control of Inflammation and Antigen-Induced Proliferation of B and T Cells. *Immunity* **37**: 685–696.

Miao, T., M. Raymond., P. Bhullar., **E. Ghaffari**., A. L. J. Symonds., U. C. Meier., G. Giovannoni., S. Li., and P. Wang. (2013). Early Growth Response Gene-2 Controls IL-17 Expression and Th17 Differentiation by Negatively Regulating Batf. *Journal of Immunology* **190**: 58-65.

Conference Presentation

The work presented in this thesis was exhibited at the following conference:

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Chapter 1: Introduction

1.1 Function of the Immune System

The Immune System is a complex system that involves many different cellular processes and molecules to primarily protect the body from the invasion of infectious pathogens such as bacteria, viruses, fungi and parasites outside of the body. It is also involved in protection from overt proliferation causing cancer and auto reactivity in autoimmunity and is divided into two main branches; innate and adaptive immunity. The innate immune system is the first to encounter the pathogen and produces a fast and rapid immune response. Adaptive immunity in contrast is an immune specific cell mediated response (Naik*.,* 2003; Marcos *et al.,* 2003).

1.1.1 Innate and Adaptive Immunity

Both the Innate and Adaptive immune systems work together for the effective clearance of a pathogen. Innate immunity is a non-specific type of response and is the first to encounter a pathogen. Innate immune molecules are situated near pathogen entry sites making chemical and physical barriers whilst circulating in the blood. These types of entry sites include enzymes in sweat, tears and hair movements in bronchial epithelium. Innate molecules that circulate in the blood include neutrophils, macrophages, complement molecules and monocytes.

Some of the pathogens have proteins and cell surface markers called pathogen associated molecular patterns (PAMP) that are recognised by macrophages and allow them to be identified and ingested. These types of PAMP include lipopolysaccharides

(LPS) on bacteria, cell wall components on gram negative and positive bacteria, double stranded RNA in viruses and flagellin (Marcos *et al.,* 2003). Uptake of the pathogen by macrophages can release cytokines to recruit other cells from the immune system and provides a link between innate and adaptive immunity (Mackay and Rosen*.,* 2000).

Adaptive immunity is an antigen specific immune response by the recognition of the pathogen on antigen specific receptors on T and B lymphocytes. T cells recognise the foreign antigen from antigen presentation by professional antigen presenting cells (APC) where the pathogen is complexed to the major histocompatibility complex (MHC) (Mackay and Rosen*.,* 2000).

T cells are further divided into two types, cytotoxic T cells and helper T cells. Cytotoxic T cells are defined as CD8+ cells based on this receptor that they bear. Upon encountering a pathogen, they release perforins to perforate the cell membrane of the pathogen and lytic enzymes and granzymes to lyse and kill. Helper T cells bear the CD4+ receptor and they are responsible for helping to recruit and activate other cells of the immune system such as macrophages, B cells and CD8 T cells by releasing cytokines (Mackay and Rosen*.,* 2000; Marcos *et al.,* 2003).

B cells on the other hand produce a humoral immune response, as they recognise the pathogen by the B cell receptor, they release antibodies to neutralise the antigen preventing it from adhering to other cells or recruiting other cells like macrophages to phagocytise (Mackay and Rosen*.,* 2000; Marcos *et al.,* 2003).

1.2 Adaptive Immune Response

1.2.1 How B and T cells recognise antigens

The adaptive immune response in contrast to the innate immune response as already mentioned, generates an antigen specific immune response to a wide variety of different pathogens. For B cells, their antigen receptor contains immunoglobulins (Ig) which are specific to a wide variety of pathogens; each B cell has a different membrane bound immunoglobulin. Once an interaction is made between the pathogen and B cell, the immunoglobulin generates antibodies to eliminate the pathogen and recruit other immune cells to the infected area. The antigen receptor of B cells contains a variable region and constant region. The variable region is different for each B cell and the constant region is important for eliciting B cell effector function.

For T cells they contain a T cell receptor that contains variable and constant regions, however they do not directly bind to the pathogen, they require the protein to be processed and presented with the association of MHC on APC. There are many different varieties of MHC across species and populations; MHC class I molecules are present on most cells whereas MHC class 2 is restricted to antigen presenting cells and B lymphocytes (Surh and Sprent*.,* 2000; Williams and Bevan*.,* 2007; Murphy., 2008; Arens and Scoenberger*.,* 2010).

1.2.1.1 B cell receptor structure

As already described the B cell receptor contains immunoglobulin which is secreted as antibodies once activated. Antibodies are y-shaped molecules that contain

two heavy chains and two light chains. The heavy chains link to the light chains with disulphide bonds to maintain the structure. The heavy chain distinguishes the classes of immunoglobulin. There are 5 different classes of immunoglobulin, IgM, IgD, IgA, IgE and IgG, which is the most abundant. The classes of immunoglobulin are distinguished from each other by the carboxy terminal in the heavy chain.

The light chains vary amongst the immunoglobulins and can contain either the lambda (λ) or kappa (κ) chains. The y shaped structure of the antibody is assembled with a light chain and the heavy chain, whilst both the variable and constant regions pair up separately joining the two light and heavy chains together. The antibody structure also contains Fab (fragment antigen binding) and Fc (fragment crystallisable) fragments. The Fab fragment is involved in antigen binding whereas the Fc fragment is important for binding to effector molecules (Murphy., 2008).

Below is a figure showing the immunoglobulin structure (Figure. 1)

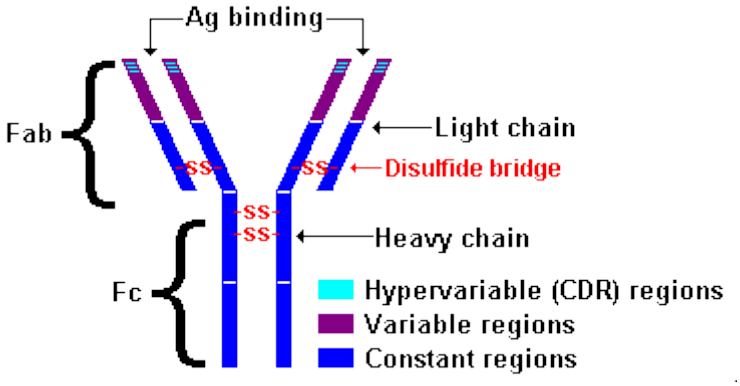


Figure 1: Immunoglobulin structure. (Fix, D.F.,1997-2013)

1.2.1.2 T cell receptor structure

The T cell receptor (TCR) contains T cell receptor α (TCRα) and β (TCRβ) chains. These chains intercalate with each other through disulphide bonding and are important

for antigen binding. The TCR is very similar to the B cell receptor in that it contains both variable and constant regions and held together by disulphide bonds. Each TCR is different on each T cell within the variable region (Murphy., 2008).

Displayed below is a figure showing the TCR structure (Figure. 2). More detail about the TCR will be described in the TCR signalling section.

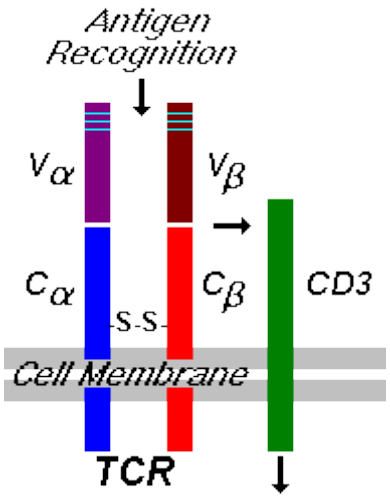


Figure 2: T cell receptor structure. (Fix, D.F., 1997-2013)

1.2.2 CD4 and CD8 cell surface markers distinguish T cells

As already explained, T cells require APC to process the pathogen with the associated MHC before T cells can be activated. T cells themselves are classified into two major categories with different effector functions. They are differentiated from each other by the expression of the cell surface proteins CD4 or CD8 as already described. These two different subsets are activated based on which MHC molecule is associated with the pathogen. CD4 T cells recognise MHC class II, whereas CD8 T cells recognise MHC class I. The CD4 cell surface antigen is composed of four domains, D1/D3, and D2/D4. MHC class II binds to the CD4 receptor through the D1 domain. The CD8 co-receptor is

different to CD4, in that it does not contain domains, rather it has α and β chains linked together by disulphide bonds (Murphy., 2008).

1.2.3 Gene composition in the immunoglobulin and T cell receptor

The immunoglobulin is composed of the variable (V), joining (J) and diversity (D) gene segments which are important for generating the heavy and light chains. Rearrangement of the V, J and D segments give rise to varying receptors amongst B and T cells. This gene rearrangement is carried out by recombinase enzymes called RAG (Recombination activating genes)-1 and RAG-2 and are expressed during lymphocyte development in the bone marrow. B cells can further undergo gene rearrangement known as somatic hypermutation which allows even more diversity in the B cell receptor. The TCR gene rearrangement is similar to immunoglobulins, except T cell development occurs in the thymus (Murphy., 2008).

1.2.4 T cell Receptor (TCR) signalling

T cell responses to self-antigen and foreign antigen, are networked by different signalling pathways. It is the ability of the TCR to have a low activation to self MHC whilst maintaining homeostasis and a high activation to foreign antigens. Once TCR and APC engagement has occurred, this will set up the TCR signalling pathway which will allow the T cell to become activated.

The TCR signalling pathway depends upon responses by chemokines, cytokines, transcription factors and other molecules which will manipulate the TCR complex.

TCR signalling involves three major stages, CD3 phosphorylation, calcium mediated signalling and Ras-mitogen activated protein kinase signalling, which are outlined below in figure 4 (Quintana *et al.,* 2005; Morris and Allen*.,* 2012).

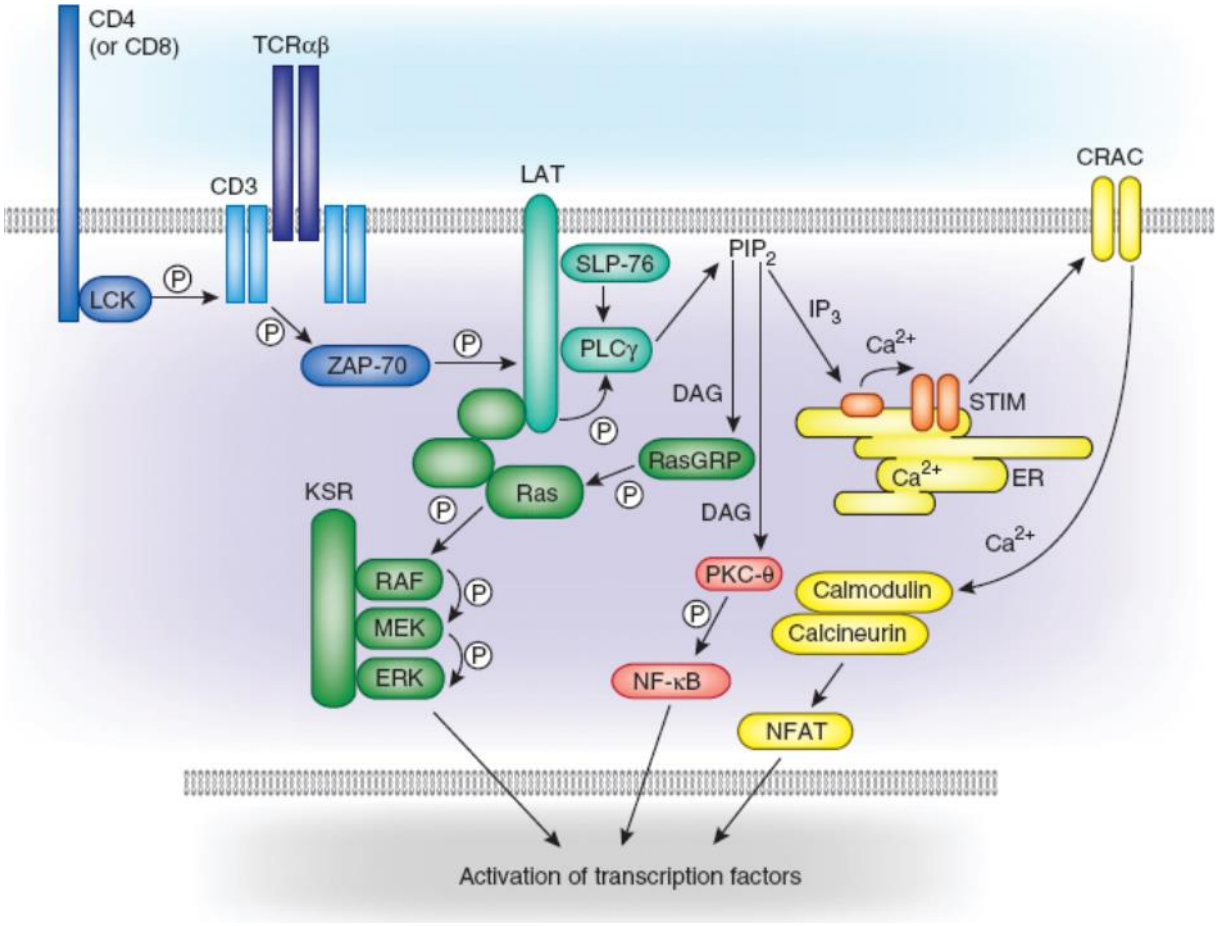


Figure 3: TCR signalling events. (Morris and Allen*.,* 2012)

1.2.4.1 CD3 phosphorylation

CD3 phosphorylation occurs in the TCR where there is a conformational change in the CD3 chains. The TCR contains α and β transmembrane chains that recognises antigens. The αβ chains form a heterodimer with one of the homodimers containing ζ-chains and CD3 (one γ, one δ and three ε-chains). CD3 phosphorylation involves conformational changes in CD3ε and CD3ζ chains to expose immunoreceptor tyrosine based activation motifs (ITAMs) on the plasma membrane. The γ,δ, ζ and ε chains in the TCR make the ITAMs to initiate signal induction into the cell. This initialises Src kinases, Fyn and Lck to phosphorylate the tyrosine residues in the ITAM. CD3 phosphorylation initialises phosphorylation of other adaptor molecules.

Once CD3 is phosphorylated, this recruits the protein tyrosine kinases (PTK) like ζ-chain associated protein of 70kDa (ZAP-70), p59fyn and p56lck. ZAP-70 is involved in phosphorylating the adaptor proteins LAT and SLP76 and subsequently activates Ras through guanine-nucleotide exchange factors (GEF) and phospholipase Cγ (PLCγ) by Src-like tyrosine kinase Tec. Signalling through protein kinase C (PKC) to activate PLCγ involves the splicing of phosphatidylinositol 4, 5 bisphosphate (PIP2) which generates intermediate molcecules diacyglycerol (DAG) and Inositol 1, 4, 5 trisphosphate (InsP3). InsP3 then binds to its receptor on the endoplasmic reticulum to release stored calcium ($Ca2^{+}$).

The mechanism of how CD3 phosphorylation is different in optimal immune responses and anergy is still unclear. However it is believed that CD3 phosphorylation to foreign antigens is higher whereas TCR signalling to self pMHC involves incomplete CD3 phosphorylation which generates little or no T cell activation. The binding strength of the

TCR-pMHC complex to develop into T cell activation or not could also be important (Rellahan *et al.,* 1997; Quintana *et al.,* 2005; Zehn *et al.,* 2012).

1.2.4.2 Calcium mediated signalling

Once InsP3 associates with its InsP3 receptor (InsP3R) on the endoplasmic reticulum, this allows the channelling of $Ca2^{+}$ from the stored compartment to the cytosol. The $Ca2^{+}$ channelling system is activated when there is a low $Ca2^{+}$ in the plasma membrane causing the influx into the cytosol. Low stored levels of $Ca2^{+}$ initialise calcium release activated $Ca2^{+}$ (CRAC) channels which elevate $Ca2^{+}$ levels in the plasma membrane that in turn activates phosphatase calcineurin. The flow of Ca2+ is modulated by the membrane polarity, where depolarisation decreases Ca2+ entry and hyperpolarisation increases Ca2+ entry. Calcineurin then dephosphorylates nuclear factor of activated T cells (NFAT) by revealing its nuclear localisation signals and allowing its shipment from the cytosol to the nucleus. The influx of $Ca2^{+}$ can act as a stimulus for activating NFAT, nuclear factor-κB (NF- κB), PKC and activator protein-1 (AP-1). NFAT also forms complexes with AP-1 for activation.

Further downstream of $Ca2^{+}$ signalling, Extracellular signal-regulated kinases (ERK) signalling is important allowing T cells to survive and proliferate whilst initiating a T cell response. ERK phosphorylates FOX03 and causes its degradation, which leads to the inhibition of Bim and allows T cells to survive. ERK also plays a role in regulating AP-1 activation. Upstream of ERK, PKC-θ, DAG and RasGRP1 further activate the T cells and enhance IL-2 production. (Rellahan *et al.,* 1997; Quintana *et al.,* 2005; Zehn *et al.,* 2012)

Recent data has also shown that Ca2+ induction requires the upregulation of EGR-1 that will increase c-Jun and CREB phosphorylation in glucose stimulated insulinoma cells for ERK signalling (Quintana *et al.,* 2005; Muller *et al.,* 2012).

1.2.4.3 Ras-mitogen-activated protein kinase (MAPK) signalling

Ras then downstream activates the mitogen-activated protein kinase (MAP kinase) signalling pathway. The MAPK pathway, involves a signalling cascade with the eventual activation of AP-1 and IL-2 production, via phosphorylation of ERK-1, ERK-2 and the c-Jun amino-terminal kinases (JNK-1), JNK is for phosphorylating c-Jun at Ser63 and Ser73. JNK activation is believed to be initiated by MAPK kinase kinase MEKK1 and involves the TCR/CD3 or CD28 pathway. These molecules are phosphorylated by active p21Ras (GTP-21Ras) from the GDP form. p21Ras also activates a transcription factor, ELK-1 that is involved in initiating the transcription of Fos genes, vital for forming heterodimers with Jun in AP-1 activation. This mediates the activation of the transcription factors NFAT, NF-κB and AP-1 and IL-2 transcription (Huang *et al.,* 2012). Furthermore it is the activation of NF-κB and AP-1 signalling that is crucial for full T cell activation otherwise, causing anergy. NF-κB and AP-1 are regulated upstream by Protein kinase C θ (PKCθ) (Quintana *et al.,* 2005; Schwarz., 2007; Lupino *et al.,* 2012; Morris and Allen*.,* 2012).

NFAT is a transcription factor that contains 3 members, NFAT1, NFAT2 and NFAT4. NFAT1 is the main protein in naïve T cells. As already shown, NFAT is activated by the influx of $Ca2^{+}$ into the cytosol and subsequent calcineurin dephosphorylation of NFAT.

NF-κB is a transcription factor composed of dimers that are normally associated with the inhibitory molecule, IκB. Once a T cell has been activated, phosphorylation occurs and causes the breakdown of IκB and release of NF-κB into the nucleus (Lupino *et al.,* 2012).

AP-1 is a dimeric complex composed of Jun and Fos families, a more detailed description is described in section 1.2.4.5 and it's activation is mediated by co-stimulation in the CD28 pathway.

IL-2 production via AP-1/NFAT signalling is essential for T cell activation and proliferation to elicit an immune response after TCR activation. AP-1 has two promoter sites in the IL-2 gene. Whereas NFAT has five binding sites in the IL-2 promoter and one of those allows both AP-1 and NFAT to bind together (Folleta *et al.,* 1998).

1.2.4.4 Pre-TCR signalling

TCR signalling is also important in thymocyte development. In the early stages of thymocyte development, there is no TCRαβ and pMHC influence, in fact, signalling and cytokine secretion from IL-7 and Notch1 are sufficient. Latter stages of thymocyte development require TCR rearrangement and expression of CD4 and CD8 coreceptors.

The commitment to form fully functional TCRαβ heterodimers from double negative (DN) thymocytes is complex. The DN thymocytes $CD4^-CD8^-$ rearrange TCRβ with invariant pre-TCRα chain to form the pre-TCR complex by dimerization. The dimerization ensures correct development into the $CD4^+CD8^+$ double positive (DP) stage. The pre-TCR complex will associate with CD3 and proceed for further signalling.

After the formation of the TCR complex, β-selection follows. β-selection initiation is formed by the clustering of pre-TCRα molecules followed by CD3 phosphorylation, Zap-70, Lat and molecule SLP-76 (sets up TCR signals). DN thymocyte signalling requires low TCR interaction compared with the latter stages of thymocyte development to provide correct TCR signalling. High TCR-pMHC signalling is eliminated at this stage to ensure there are no self-reactive TCR's.

After DN transition, the thymocytes mature into DP thymocytes. This involves appropriate TCRα rearrangement that can bind with TCRβ to be able to recognise self MHC. Selection of TCR's that are able to recognise self-MHC molecules are allowed to survive.

At the DP stage, TCR's that have a functional TCRα and TCRβ chains will be selected by positive selection for their affinity to interact with self-MHC. In this instance, TCR's with the correct orientation will be selected for its ability to recognise self-MHC on cortical thymic epithelial cells. TCR's that do not recognise self-MHC are eliminated.

Negative selection then eradicates TCR's that bind strongly to self-MHC, so that TCR's with low affinity for self-MHC can differentiate into CD4 or CD8 single positive (SP) T cells. It is believed that negative selection is caused by increased recruitment of Zap70 and Lat to the TCR complex, with high ERK signalling (Schwarz., 2007; Kreslavasky *et al.,* 2010; Blanco *et al.,* 2012; Huang *et al.,* 2012; Morris and Allen*.,* 2012; Zehn *et al.,* 2012).

1.2.4.5 AP-1 signalling

1.2.4.5.1 AP-1 family and function

AP-1 is a transcription factor that comprises over twenty proteins from dimeric complexes of Fos (c-Fos, FosB, Fra-1, Fra-2 and smaller FosB splice variants FosB2 and Deltas FosB2), Jun (c-Jun, JunB, JunD), ATF (activating transcription factor) (Atf2, Atf3/Lrf1, Batf, Jdp1 and Jdp2) and Maf (musculoaponeurotic fibrosarcoma) (c-Maf, MafB, MafA, MafG/F/K and Nrl) proteins. AP-1 was first discovered as a transcription factor involved in the association with the cis-element of the human metallothioneiniIIa promoter. Dimerisation in this family involves heterodimeric and homodimeric associations between proteins and other genes making AP-1 to have many functions including, metastasis, proliferation, survival, angiogenesis, apoptosis and invasion (Zhou *et al.,* 2005; Karamouzis *et al.,* 2007; Meng and Xia*.,* 2011).

Fos and Jun are the main members of the AP-1 family. They were first discovered as viral oncoproteins, v-Fos and v-Jun from an avian sarcoma virus and Finkel-Biskis-Jinkins osteosarcoma in 1987. They were discovered to be involved in cell transformation as their over-expression gave a positive effect (Ivorra *et al.,* 2005; Karamouzis *et al.,* 2007; Shaulian*.,* 2010).

The AP-1 family of proteins contain the basic leucine-zipper domain (bZIP), basic domain and some members have a transactivation domain. Figure 4 shows the domain structures of Jun; Fos and Batf family members. The bZIP is involved in dimerisation with other protein members. Where the bZIP contains a leucine zipper motif which is involved in creating the dimer associations; however, it also contains a DNA binding domain that can interact with DNA consensus sequences in target genes. The bZIP domain consists of

α-helices, where every seventh amino acid is a leucine. Once they have bound to DNA they can go and activate or repress target genes. Fos and Jun recognise the 12-O-tetra decanoylphorbal-13-acetate response (TPA) element found in promoter regions of target genes. Other elements they recognise include cAMP-Response Element (CRE), the MAF recognition elements (MARES) and the antioxidant response elements (ARES). Normally, Jun-Fos heterodimers bind to the TPA sequence (5'-TGA (C/G) TCA-3') and Jun-Atf binds to CRE (5'-TGACGTCA-3') (Ivorra *et al.,* 2005; Karamouzis *et al.,* 2007; Shaulian*.,* 2010).

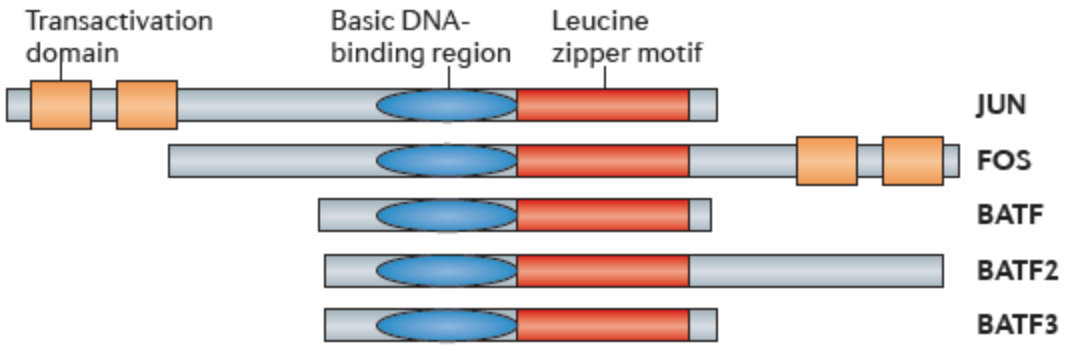


Figure 4: Domain Structures of Jun, Fos and Batf. (Murphy *et al.,* 2013)

As already described the homo and heterodimers are important for AP-1 function and the combinations between AP-1 family members is important for stabilisation and transcription of other genes. Jun family members can form homo or heterodimers with Fos or Atf members. Jun and Fos heterodimers are more stable than Jun/Jun homodimers. Figure 5 shows the Fos and Jun heterodimer formation that bind to AP-1 sites in promoter regions of genes. c-Maf and Nrl can form heterodimers with c-Fos or c-Jun but MafB/G/F/K form dimers with only Fos not Jun. For example c-Jun-Atf2 binding triggers growth factor independence, whilst c-Jun-c-Fos binding activates anchorage

independent growth. These different dimer forms can be important for tumorgenesis. Also, Fra-2 and JunD heterodimers are important for CCR4 and cell proliferation in Adult T cell leukemia. Fra-1 and JunD or Fra-2 and JunB or Fra-2 and JunD heterodimers bind to proto-oncognenes like c-Myb and BCL-6 which are important proliferation. Typically, AP-1 can bind with other elements or genes, not just AP-1 members that contain core transcriptional sequences like TATA box in target genes. AP-1 binding to target genes is also influenced by growth factors, cytokines and oncoproteins that can have profound effects on proliferation, survival, differentiation, transformation, apoptosis and TCR signalling (Foletta *et al.,* 1998; Karamouzis *et al.,* 2007; Nakayama *et al.,* 2008; Shaulian*.,* 2010).

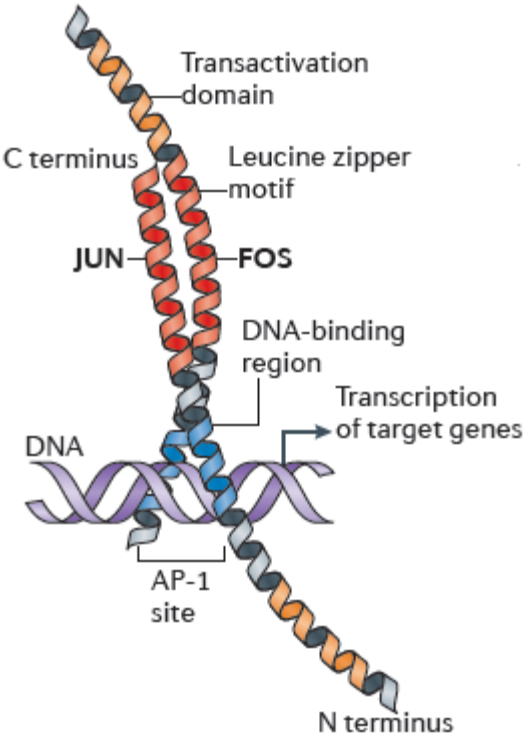


Figure 5: Jun and Fos heterodimer binding which initiates AP-1 activation and binds to AP-1 binding sites in target gene promoter sites. (Murphy *et al.,* 2013)

1.2.4.5.2 Regulation of AP-1 activity

Protein kinases are important for the transmission of extracellular signals through the cytoplasm and phosphorylating other transcription factors including AP-1 family members Jun. Both JNK and ERK are members of MAPK. They are activated through phosphorylation on tyrosine and threonine residues in the motif. Phosphorylation is achieved by MAP kinase kinases (MAPKKs). The MAPKKs MEK4 and MEK7 activate the JNKS; whilst, MEK1 and MEK2 activate ERK and MEK3 and MEK6 activate p38. MAPKKs are further phosphorylated by MAPKK kinases (MAPKKK).

The MAPKKK family is made up of more than 20 protein kinases including Rafs, MEK kinases, germinal centre kinases (GCK), mixed lineage kinases (MLK), apoptosis-stimulated kinase 1 (ASK-1) and TGF-beta activated adaptors (TAK) (Karamouzis *et al.,* 2007; Higai *et al.,* 2009; Shaulian*.,* 2010).

Once MAPK activates JNK, JNK gets translocated into the nucleus and phosphorylates Jun. JNK can also phosphorylate and activate JunD and Atf-2. JNK expression is affected by hormones, cytokines, growth factors, deregulated oncogene expression and cellular stresses including UV.

There are three different genes in JNK; JNK1, JNK2 and JNK3. JNK1 and JNK2 are ubiquitously expressed in cells, whilst JNK3 is expressed only in the brain. JNK phosphorylates c-Jun at serine 63 and 73 and threonine 91 or 93 for activation and prevents ubiquitin dependent degradation of c-Jun. The ability of JNK to phosphorylate JunB has been controversial; however, it is believed to be phosphorylated at threonine 102 and 104. Commonly JNK binding to Jun proteins enhances their ability to engage in gene transcription. Data has shown that JNK1 deficiency does reduce expression of c-Jun

and cyclin D, thus reducing cell proliferation (Karin., 1995; Ukarami *et al.,* 1997; Jochum *et al.,* 2001; Eferl and Wagner*.,* 2003; Karamouzis *et al.,* 2007; Shaulian*.,* 2010).

1.2.4.5.3 Atf-like (B-atf) function

Batf is a component of the AP-1 family of bZIP transcription factors that forms heterodimers to Jun proteins and inhibits AP-1 transactivation, as shown in figure 6. Batf contains three family members Batf, Batf2 and Batf3. Batf and Batf3 contain two domains, the leucine zipper and DNA binding domain; whereas Batf2 also contains an unknown function carboxy-terminal domain. The Batf gene is found on human chromosome 14q. Batf is expressed in Th1, Th2, Th17 and T_{FH} T cells. Batf3 is expressed in Th1 T cells and nominal in Th17 and Th2 T cells (Murphy *et al.,* 2013). Batf can form dimers with Jun which are less transcriptionally active (Logan *et al.,* 2012). This is supported by data showing over expression of Batf blocks AP-1 associated signalling and reducing cell growth. Phosphorylation of Batf occurs at serine 43 by DBD (DNA binding domain) which oxidises the serine residues and inhibits AP-1 mediated gene expression and DNA binding. DBD is also found in other bZIP family members like c-Jun and c-Fos (Deppmann *et al.,* 2003).

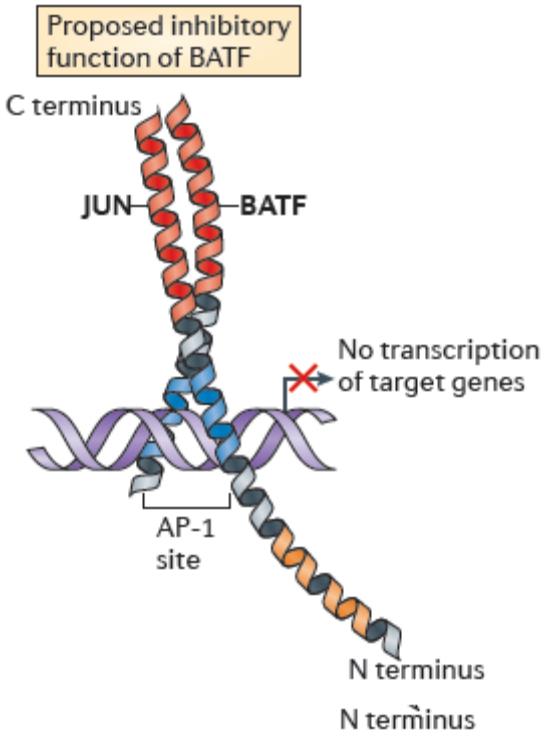


Figure 6: Batf heterodimers with Jun, inhibit AP-1 signalling and prevent binding to AP-1 binding sites in target gene promoter sequences. (Murphy *et al.,* 2013)

Batf was suggested to produce inactive transcriptional activity when it is part of a complex as forced expression of Batf slows down the growth and transformation of fibroblast cells (Echlin *et al.,* 2000).

This was confirmed by Echlin *et al.,* 2000 group who isolated portions of the Batf protein and fused with the DNA binding domain of the yeast GAL4 promoter. The fusion protein was transfected into C3H10T1/2 cells along with the chloramphenicol actyl transferase (CAT) reporter plasmid. Measurement of the CAT reporter gene showed that none of the Batf fusion proteins were transcriptionally active. However, Batf is also important for thymocyte maturation. Batf mRNA expression in thymocytes from WT mice is detected in DN and SP thymocytes, however, Batf expression is not detected in DP

thymocytes. These results indicate the differential expression of Batf as thymocytes gain the CD4 and CD8 T cell markers, they lose Batf expression, however, maturation into single positive thymocytes requires Batf expression (Williams *et al.,* 2001). Transgenic mouse cell lines that over-express Batf in thymocytes or splenocytes were crossed with C57BL/6 mice that carry the AP-1 luciferase reporter gene to define how Batf regulated AP-1 activity. Protein extracts from these mice and AP-1 only reporter gene cells were stimulated with concanavalin A and AP-1 luciferase activity was measured. The data showed repression in AP-1 trans-activation from AP-1/Batf luciferase thymocytes. This was the first report to show that Batf inhibits AP-1 activation following stimulation in T cells and was further supported from the AP-1 luciferase reporter assay from cultured mouse fibroblast cells (Williams *et al.,* 2001).

However, further research has identified Batf to be important for gene expression. Batf has shown to be essential for thymic NKT cell development and is expressed by both T and B cells. Evidence has shown that Batf transgenic T cells have less caspase-3 (apoptotic marker) compared with WT to show that Batf is involved in regulating apoptotic signalling. Data has found that over expression of Batf in mice leads to the development of lymphoid tumours and this is not due to hyperproliferation, this is due to an inadequacy in apoptosis. However, Batf can also be a positive regulator of gene expression. This has been shown by in-vivo results indicating that Batf knockout mice show a defect in CD4 Th17 and T_{FH} helper cells. Batf deficient mice have also been shown to be resistant to the induction of EAE disease (Logan *et al.,* 2012).

Batf has been further shown to be important for Th17 differentiation. Batf knockout mice have a normal thymus, spleen and lymph nodes; they have normal Th1 and Th2 differentiation, whilst Th17 was impaired as ROR-γt is low, thus IL-17 cytokine

production was also reduced. The Th17 cytokines include IL-17A, IL-17F, IL-21, IL-22 and IL-26 (Schramal *et al.,* 2009; Montelione *et al.,* 2011). In contrast Batf transgenic mice had increased expression of Th17 cytokines. In this study Batf transgenic mice developed EAE disease, whereas Batf knockout mice were resistant, displayed by low IL-17. Batf directly binds to the IL-17 promoter. Batf controls Th17 differentiation by regulating IL-6, TGF-β and subsequently STAT3 (Schraml *et al.,* 2009).

Batf is expressed after T cell activation and is shown to be involved in T cell differentiation at the point of signal three which requires IL-12 for full T cell activation. Batf and c-Jun inhibit Sirt1 (histone deacetylase that promotes gene silencing). By inhibiting this enzyme, there will be an increase in histone acetylation of other target genes including T-bet, a gene involved in effector cell differentiation, which increases NAD^+ for glycolysis, TCA and oxidative phosphorylation resulting in production of ATP levels to initiate T cell differentiation and survival (Kuroda *et al.,* 2011; Liao *et al.,* 2011).

1.2.5 T cell mediated immune activation

As already described the adaptive immune response generates a specific response orchestrated by T and B cells. Predominantly the immune response is classified into three major phases. The first phase is termed the effector phase where naïve T cells are primed with antigen on the APC cells. In the effector phase, T cell activation requires three signals before differentiation and expansion. Naïve T cells will differentiate into either CD8 cytotoxic T cells or the CD4 helper T cells that contain further subtypes Th1, Th2 or Th17. The second phase is termed the contraction phase where the effector T cells carry out their effector function for pathogen clearance and up to 90% of those cells once their

effector function has been carried out, will undergo programmed cell death. The final phase is called the memory phase where the remaining 5-10% of the effector T cells will become long lived memory T cells that will circulate in the periphery. Lymphocyte numbers in the secondary peripheral lymphoid numbers are maintained at constant numbers prior to an infection and when the infection is cleared. This process is regulated by cytokines. When there is no invasion of any pathogen, there will be 1 in 100,000 naïve T cells that will be specific to a pathogen. However, once there is an invasion of a foreign pathogen, the antigen specific T cells for those will be activated and expand typically for fourteen days in humans to 50,000 cells (Boymen *et al.,* 2007; Williams and Bevan*.,* 2007; Zehn *et al.,* 2012).

1.2.5.1 Naïve T cell presentation of antigen by antigen presenting cells

A sustained interaction between the T cell and APC is important for initiating sufficient T cell activation. APC's are a subset of cells that are involved in innate immunity because they do not specifically bind to any particular pathogen, however they have receptors that recognise common pathogen cell surface molecules such as LPS, peptidoglycan, dsRNA, ssRNA etc. There are three professional APC's in the immune system; these are macrophages, dendritic cells and B cells. Macrophages and dendritic cells are able to recognise foreign pathogens such as bacteria, fungi and viruses through interactions with their cell surface receptors like, Toll-like receptor, DC-SIGN, mannose receptor, scavenger receptor etc. Once a pathogen enters the body and the common pathogen cell surface markers are recognised, the pathogen is then processed to become

peptides and associated with MHC for naïve T cell activation. For B cells, they can directly elicit a humoral response.

Constantly APC's not only present foreign antigens, they also present self-antigens to T cells; this evokes the tolerance mechanism, where there is no activation of the immune response to self (Surh and Sprent., 2000; Williams and Bevan., 2007; Murphy., 2008; Arens and Scoenberger., 2010).

Naive T cell activation requires the pathogen to be loaded and processed into a peptide associated with MHC on APC's. In the absence of MHC, naïve T cell numbers will deplete. Depending on which MHC molecule the antigen presenting cell has, this will activate the appropriate T cell. Dendritic cells have the unique ability to evoke a much stronger T cell immune response when stimulated with inflammatory cytokines like IFN that will drive the differentiation of resting dendritic cells into an activated state. Only in the activated state the dendritic cell will mediate a T cell response. The dendritic cells also express cytokines and chemokines to drive the T cell immune response. These PAMPS will induce activation of the APC to present to T cells by releasing cytokines, costimulatory molecules and migration into secondary lymphoid organs (Surh and Sprent., 2000; Williams and Bevan., 2007; Arens and Scoenberger., 2010).

1.2.5.2 Naïve T cell activation and expansion by antigen presentation priming

There is a wide diversity of the TCR to be able to interact with a variety of different pathogens. T cell activation through appropriate TCR-peptide-MHC complex requires activation signals. Without these signals, T cells cannot be appropriately

activated and will become unresponsive. Figure 7 outlines all the required T cell signals for activation.

Signal 1 is generated by the engagement of the TCR on the T cell and the peptide-MHC complex on the APC. This then causes the conformational change known as CD3 phosphorylation as already described to initiate T cell activation. Engagement of signal 1 alone induces anergy (Surh and Sprent., 2000; Williams and Bevan., 2007; Arens and Scoenberger., 2010).

The second signal is generated through the expression of costimulatory molecules on the APC. The costimulatory receptors on the T cell drive T cell activation, whereas insufficient costimulation will evoke the T cell into anergy. One of the main costimulatory receptors on T cells is the immunoglobulin member CD28. CD28 is expressed on all naïve T cells. CD28 will interact with the B7 receptor on the APC. Other common interactions are between DC-SIGN (on APC) and ICAM-1 (T cell) or between CD40 (APC) and CD40L (T cell). There are also other co-receptors like CTLA-4, PD-1, ICOS and 4-1BB which can evoke immunosuppressive signals. The interactions in signal 2 evoke survival and differentiation into effector cells by upregulation of IL-2, IL-7 and the down regulation of TGF-β (Oehen and Brduscha-Riem., 1998; Surh and Sprent., 2000; Williams and Bevan., 2007; Blache *et al.,* 2009; Arens and Scoenberger., 2010).

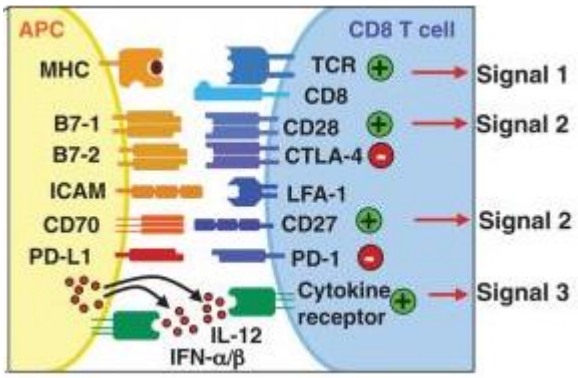


Figure 7: Antigen Presenting cell and CD8 T cell activation. (Arens and Scoenberger*.,* 2010)

A third signal has also been identified to be important along with signal 1 and 2. The third signal is generated through the up regulation of cytokines involved in expansion and effector functions seen in T cells, these cytokines include IL-12, IFN-γ and Type 1 IFN produced by APC's. Research has shown that in the absence of IL-12, CD8 T cells have an impaired ability to differentiate into effector T cells. When CD8 T cells were supplemented with IL-12, they produced a better protection against an infection outlining the importance of generating the third signal for an immune response (Oehen and Brduscha-Riem*.,* 1998; Surh and Sprent*.,* 2005; Williams and Bevan*.,* 2007; Arens and Scoenberger*.,* 2010).

1.2.5.3 Role of cytokines in T cell activation and expansion

When all three activation signals have been generated, the naïve T cells will undergo massive expansion and proliferation into the contraction phase resulting in an infiltration of effector T cells to carry out their function (Surh and Sprent*.,* 2005). To enable naïve T cell numbers to undergo expansion, certain cytokines and genes are up-regulated for the survival and homeostatic pathways. The main players involved include, IL-2, IL-4, IL-7, IL-18, IL-19, IL-15, IL-21 and IFN-γ (Oehen and Brduscha-Riem*.,* 1998; Tan *et al.,* 2001; Karman *et al.,* 2004; Badovinac *et al.,* 2005; Ishimaru *et al.,* 2006; Snyder *et al.,* 2008; Sener *et al.,* 2009; Keller *et al.,* 2010).

IL-2 is the major cytokine involved in the activation and proliferation of T cells. IL-4, IL-7, IL-15 and IL-21 are pivotal for the survival of these effector T cells (Schluns and Lefrancois*.,* 2003; Ishimaru *et al.,* 2006).

The cytokine suppressor of cytokine signalling-1 (SOCS-1) has been shown to be essential for regulating the activation of naïve T cells to self-antigens with IL-15. It has been reported that SOCS-1 deficient naïve CD8 T cells that were adoptively transferred into normal mice showed high T cell pool numbers when there is no IL-15 (Boymen *et al.,* 2007).

There are also cytokines that are involved in suppressing the immune system. Transforming growth factor-β (TGF-β) has been shown to be important for suppressing the immune system. Also IL-10 has been shown to have suppressive properties by reducing the expression of cytokines IL-2 and IFN-γ (Boymen *et al.,* 2007; Sprent *et al.,* 2008; Cardone *et al.,* 2010).

Transcription factors are important in carrying out an immune response, including, NFAT and NF-κB (Oehen and Brduscha-Riem*.,* 1998; Surh and Sprent*.,* 2000; Ganusov *et al.,* 2007; Blache *et al.,* 2009; Sener *et al.,* 2009).

1.2.5.4 Naïve T cell markers

Naïve T cells and all the other T cell subsets, like effector or memory, have phenotypic cell surface markers that are expressed to make them distinct from other cell types. For naive CD8 T cells they bear IL-7^{+}, TGF-β^{-}, $CD122^{int}$, IL-$7R\alpha^{hi}$, $CD5^{hi}$, $CD195^{hi}$, $CD197^{hi}$, $CD134^{hi}$, $CD152^{hi}$ and $CD25^{hi}$. For naive CD4 T cells they have IL-7^{+}, TGF-β^{-},

CD122$^{lo/int}$, IL-7Rα^{hi}, CD5^{hi}, CD195^{hi}, CD197^{hi}, CD134^{hi}, CD152^{hi} and CD25^{hi} (Boymen *et al.,* 2007; Williams and Bevan*.,* 2007; Wells*.,* 2009; Zhang *et al.,* 2009).

1.2.5.5 T cells differentiate into subclasses of effector T cells

1.2.5.5.1 CD4 effector T cells

1.2.5.5.1.1 CD4 effector T cell subclasses

Helper T cells are further differentiated into distinct subtypes capable of carrying out different helper T cell functions. Activation of transcription factors and differential cytokine expression will give rise to a different CD4 subtype as shown in Figure 8.

The Th1 helper T cells are type 1 effector cells which mediate expression of IFN-γ and IL-2 to help clear the pathogen through helping the activation of CD8 T cells and macrophages. CD4 T cells have been shown to secrete signals that are vital for CD8 activation independently or dependently. It has been shown that sustained and effective CD8 T cell response requires CD4 help to be able to maintain efficient IFN-γ production.

The Th1 subtype is mediated through IL-12, Type 1 IFN, IL-1β, IL-21, TNF-α secretion and the transcription factor T-bet expression to enable an effector function. Th1 T cell development is governed by STAT1 activation induced by the expression of IFN-γ and IL-27 cytokine production by APCs and NK cells. In turn this induces the master regulator of Th1 differentiation, the transcription factor T-bet which allows the expression of the signature cytokine IFN-γ (Haynes *et al.,* 2003; Anderson *et al.,* 2006; Williams and Bevan*.,* 2007; Okamura *et al.,* 2009; Sallusto and Lanzavecchia*.,* 2009; Putheti *et al.,* 2010).

Another CD4 helper T cell subtype is Th2. These cells are involved in activating B cells to release antibodies and protection against helminth infections. They require the transcription factor GATA-3 and c-Maf to be able to carry out the effector function and to mediate the expression of cytokines, IL-4, IL-5, IL-13 and TGF-β. Th2 development requires STAT6 and leads to the induction of the transcription factor GATA-3 and MAF for Th2 differentiation. The activation of these transcription factors induces the expression of the signature cytokine of Th2, IL-4 (Haynes *et al.,* 2003; Anderson *et al.,* 2006; Wang *et al.,* 2006; Williams and Bevan*.,* 2007; Okamura *et al.,* 2009; Sallusto and Lanzavecchia*.,* 2009; Putheti *et al.,* 2010).

Another CD4 helper T cell subtype is Th17, which are involved in initiating an inflammatory response. Th17 T cells are also involved in protecting against bacteria and fungi and circulate in mucosal tissues. Th17 effector function is carried out by the expression of the transcription factor Retinoic-acid-receptor-related orphan receptor- γt (RORγt) and the release of the cytokines, IL-17A, IL-17F, IL-22, IL-6, TGF-β, IL-1β, IL-23, IL-12 and IL-21. Th17 cells also require STAT3 for this lineage type, as STAT3 suppresses IL-10 inhibitory function. Th17 differentiation has been shown to be driven by TGF-β and IL-6 secretion. Th17 T cells have been implicated in the development of various autoimmune diseases including psoriasis, arthritis and multiple sclerosis (Rangachari and Kuchroo*.,* 2013).

Mostly over reactivity of Th2 T cells causes allergic immune responses, whereas abnormal Th1 and Th17 responses causes autoimmune diseases (Haynes *et al.,* 2003; Anderson *et al.,* 2006; Wang *et al.,* 2006; Sallusto and Lanzavecchia*.,* 2009; Wilson *et al.,* 2009; Putheti *et al.,* 2010; Zhu *et al.,* 2010).

The final main CD4 helper T cell subset is the T regulatory cells (Treg). They express $CD4^+$ $CD25^+$ on their cell surface. These T cells play an important role in causing anergy when there is insufficient co-stimulation for T cell activation. They are able to cause anergy through up regulation of E3 ligase, cbl-b, GRAIL, Itch and Nedd4. Treg T cells are characterised by the expression of the Forkhead transcription factor (FoxP3) and are essential for preventing autoimmune disease and maintaining homeostasis. FoxP3 deficiency in mice causes lymphoproliferation and autoimmune disease (Walzer *et al.,* 2002; Fontenot *et al.,* 2003; Kmieciak *et al.,* 2009; Putheti *et al.,* 2010).

It is believed that there are two types of Treg T cells, one that causes anergy and the other controls tissue inflammation. One is believed to circulate in the thymus and the other in the periphery. The anergenic Treg is characterised by cell surface expression $CD4^+$, $CD25^+$ and $FoxP3^+$. The Treg that controls tissue inflammation is thought to secrete IL-10, $FoxP3^-$and $CD4^+$ (Okamura *et al.,* 2009; Sallusto and Lanzavecchia*.,* 2009; Cooles *et al.,* 2013).

The above four Helper T cell effector T cells are the most established subtypes. However, it is now emerging that there could be even more subtypes all with different functions. There is believed to be a Th3 subtype that is thought to be involved in inducing IgA antibodies and suppress autoimmune diseases, like encephalomyelitis (Anderson *et al.,* 2006). There is also the Follicular helper T cells (T_{FH}) subtype that is involved in helping B cells. They express the chemokine receptor CXCR5 that stimulates ICOS-1 expression. T_{FH} T cells are able to produce IL-21 and expresses ICOS, PD-1 and CXCR5, which are thought to be the homing receptor for residence in the B cell follicle. T_{FH} cells aid the expansion of germinal centres (Sallusto and Lanzavecchia*.,* 2009; Pepper and

Jenkins*.,* 2011). T_{FH} T cells are also important for class switching in the germinal centre (Zhu *et al.,* 2010).

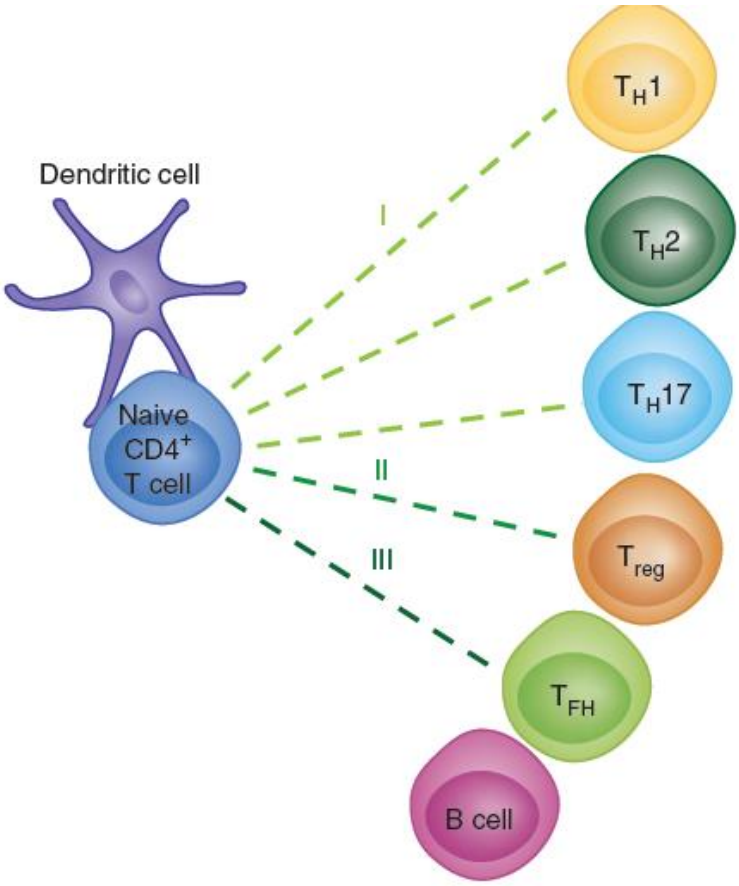


Figure 8: Differentiation of CD4 T cells into subtypes. (Pepper and Jenkins*.,* 2011)

1.2.5.5.2 CD8 effector T cells

CD8 T cells are predominately termed cytotoxic T cells due to the fact they target infected cells. CD8 T cells are vital for the protection against viral and bacterial infections. These cytolytic T cells express a different set of phenotypic markers compared to other lineages, including, IL-12^{+}, IL-2^{+}, IFN-1^{+}, IFN-γ^{+}, T-bet^{+}, BCL-3↑, CD122^{int}, IL-7Rαlo, IL-7^{+} and IL-15^{+} (Boymen *et al.,* 2007; Williams and Bevan*.,* 2007).

1.2.5.5.2.1 Cytotoxic T cells direct killing of pathogen to trigger apoptosis

The CD8 effector T cells main function is to directly kill the pathogen by exocytosis. CD8 T cells release perforin and promote the release of lytic enzymes and molecules, like granzymes, perforin and cathepsin to adhere to target cells. This triggers programmed cell death of the pathogen by exocytosis when perforin lysis the pathogen cell membrane. This allows the entry of granzymes into the cytoplasm of the pathogen and ligation to the Fas death receptor triggering apoptosis.

IFN-γ is one of the key cytokines that are produced by CD8 T cells into driving cytolytic activity. They also produce IL-2, IL-4, IL-7, IL-15 and IL-21 needed for survival and proliferation. IL-2 is needed for T cell proliferation, survival and effective IFN-γ expression. IL-4, IL-7 and IL-15 play the role in cell survival of the effector T cells to enable them to carry out their functions (Schluns and Lefrancois*.,* 2003; Liu *et al.,* 2004; Badovinac *et al.,* 2005; Boymen *et al.,* 2007; Williams and Bevan*.,* 2007).

For cytotoxic T cell activation, a number of transcription factors and genes including, STAT4, RBP-J, CREB-1, T-bet, Eomes and cKrox are required for the release of toxic molecules like granzymes, to kill the pathogen after endocytosis (Kaech *et al.,* 2002; Haynes *et al.,* 2003; Williams and Bevan*.,* 2007).

1.2.5.5.2.2 CD8 effector T cell function requires CD4 helper T cells

To mediate cytotoxic T cell activation, CD8 T cells need CD4 T cell help for effector function. The effector CD4 T cell subset, Th1 CD4+ T cells are involved in priming CD8 T

cell activation by secreting IFN-γ and IL-12 production which further enhance CD8 T cells cytotoxic function (Williams and Bevan*.,* 2007).

1.2.5.5.3 Memory T cells and homeostasis

Memory T cells represent 5-10% of the T cell population and have a TCR that is specific to a particular foreign antigen that has been encountered in a previous infection. The difference between naïve T cells and memory T cells is that memory T cells can mount an immune response when there are lower levels of antigen. They will be able to mount a much faster response in a second encounter with the same antigen.

The mechanisms of the generation of memory T cells and how they are maintained is largely unknown. Their ability to undergo proliferation when naïve T cells are adoptively transferred into irradiated or immunodeficient mice shows that memory T cell numbers are maintained in a process of homeostatic proliferation. Some of the homeostatic proliferation mechanisms involve competition in basal levels of IL-7. It is believed that when there are low levels of IL-7 this triggers homeostatic proliferation. T cells compete for IL-7 for survival and the consumption of IL-7 would be higher under normal conditions. In turn this increases the expression of JAK1, STAT5, BCL-2 and Akt, all involved in maintaining cell survival. However, when there are low T cell numbers, there would be low IL-7 consumption and this would trigger signalling pathways involved in homeostasis including expression of Eomes and triggering the Wnt pathway (Kaech *et al.,* 2002; Tanchot *et al.,* 2002; Schluns and Lefrancois*.,* 2003; Liu *et al.,* 2004; Jameson*.,* 2005; Surh and Sprent*.,* 2005; Williams and Bevan*.,* 2007; Surh and Sprent*.,* 2008; Sener *et al.,* 2009).

Memory T cells have a distinct set of phenotypic markers that differentiate them from other cell lineages: $CD44^{hi}$, $Ly6C^{hi}$, $CD122^{hi}$, $CD62L^{hi}$, $CD127^{hi}$, L-$selectin^{lo}$ and $CD44RB^{lo}$ (Boursalian and Bottomly*.,* 1999; Goldrath *et al.,* 2000; Kaech *et al.,* 2002; Badovinac *et al.,* 2005).

Memory T cells are divided into two different subsets, central or effector memory T cells. They have different cell surface homing receptors and cytolytic function. Central memory T cells have the lymphoid homing receptor $CCR7^{+}$, $CD45RA^{-}$ and $CD62L^{hi}$. The central memory T cells are found in the secondary lymphoid organs and they have a high proliferative capacity but a low effector function. It is believed that the central memory T cells are the first to encounter an antigen, proliferate and then differentiate into effector memory T cells to carry out their cytolytic functions. Central memory T cells have the phenotypic markers, $CD44^{int}$, $CD62^{hi}$, $CD122^{-}$, $CCR7^{+}$ and $CD127^{+}$ (Walzer *et al.,* 2002; Badovinac *et al.,* 2005; Williams and Bevan*.,* 2007; Surh and Sprent*.,* 2008).

Effector memory T cells on the other hand, stay in non-lymphoid organs and they possess the ability to have cytolytic function through up regulating the expression of IFN-γ, however, they have a low proliferative potential. They are involved in carrying out the removal of the pathogen and are fully differentiated from central memory T cells. They have the phenotypic markers, $CD44^{hi}$, $CD62^{low}$, $CD122^{+}$, $CCR7^{-}$, $CD45RA^{-}$ and $CD127^{-}$ (Kaech *et al.,* 2002; Schluns and Lefrancois*.,* 2003; Badovinac *et al.,* 2005; Surh and Sprent*.,* 2005; Williams and Bevan*.,* 2007; Surh and Sprent*.,* 2008).

Effector memory T cells will be involved in the initial drive to clearing the pathogen whilst the central memory T cells may be involved in the latter stages to boost the immune response (Walzer *et al.,* 2002).

A number of cytokines are produced by effector and naïve T cells to sustain memory T cell numbers. These cytokines include IL-7, IL-15, IL-12, IL-18, IL-21, INF-α/β and IL-2. IL-7 is an important cytokine that is needed for survival and is expressed by naïve and effector T cells. IL-15 and IL-21 also share the same function as IL-7 and these cytokines promote the expression of other transcription factors involved in sustaining survival (Curtsinger *et al.,* 1998; Boursalian and Bottomly*.,* 1999; Walzer *et al.,* 2002; Williams and Bevan*.,* 2007; Boymen *et al.,* 2007; Surh and Sprent*.,* 2008).

In terms for long term survival of both CD4 and CD8 memory T cells, it is believed that mechanisms are independent for both subsets. CD8 memory T cells survive through signalling by IL-15 through regulation of CD122 (IL-2/IL-5Rβ) expression dependent on T-bet and eomesoderm. CD4 memory T cells however, are believed to deplete over time because their survival is not dependent on IL-15 signalling. This could be significant for understanding memory T cell survival and proliferation in both CD4 and CD8 subsets (Kaech *et al.,* 2002; Boymen *et al.,* 2007).

1.3 Failure in host immune defence

1.3.1 Autoimmune disease

Autoimmune disease is caused by a breakdown in the immune system or loss of tolerance (inactive to self) where the immune system generates immune cells against self-cells and tissues which can illicit autoantibodies and inflammation. Autoimmunity is a major cause of mortality across the world and currently there is no cure for the disease. Exploring the genetic mutations and polymorphisms in DNA that leads to the development of autoimmune disease is being studied to identify key therapeutic

strategies. Whilst genomic and proteomic studies are being used to identify genes and mechanisms involved, autoimmunity is believed to arise from genetic, environmental and regulatory defects. Predispose to other infections can also influence the development of autoimmune disease (Fatham *et al.,* 2005; Rioux and Abbas*.,* 2005; Murphy., 2008).

1.3.1.1 Self-tolerance and Autoimmunity

A breakdown in self-tolerance is believed to be essential in the development of autoimmunity. Self-tolerance is a mechanism for which the immune cells recognise self-antigens and do not amount an immune response. Self-tolerance is an important checkpoint carried out during the development of B and T cells where those cells that are autoreactive to self-antigen will be eliminated. Self-tolerance is further divided into two stages, central and periphery (Rioux and Abbas*.,* 2005).

1.3.1.2 Discriminating self and non-self

The ability of the immune system to distinguish between self and non-self is essential for balance in the immune system. A defect in this can cause severe tissue damage and autoimmune disease. There are several checkpoints during lymphocyte development (central tolerance) and in the periphery (peripheral tolerance). Another mechanism of how self and non-self are discriminated is by the levels of antigenic presentation. Self-antigens are expressed on every cell and every tissue, so therefore presentation of these antigens will be low and constant to lymphocytes. Therefore the lymphocytes in this case will become tolerant (inactive) once ligated to the antigen

receptor on the lymphocytes. On the other hand, foreign antigens are not commonly on cells in the body and when they enter the host, they rapidly divide and release high levels of antigen, which is a signal to lymphocytes to generate an immune response (Murphy., 2008).

1.3.1.3 Central tolerance

Central tolerance occurs in the lymphoid organs, T cells are in the thymus whilst B cells are in the bone marrow. The process of central tolerance involves these immature lymphocytes to be presented with self-antigens, if those immature lymphocytes mount an immune response, they will undergo apoptosis or inactivation (Rioux and Abbas., 2005).

1.3.1.4 Peripheral tolerance

Peripheral tolerance is where mature lymphocytes that have exited from the lymphoid organs into tissues are still presented with self-antigens on APC's and if they amount an immune response they are either killed or silenced. Peripheral tolerance mechanisms are characterised into three main types, anergy, deletion and suppression (Rioux and Abbas., 2005).

T cell anergy is defined as causing the silencing or unresponsiveness of the T cell when they encounter self-antigens. Cytotoxic T lymphocyte antigen-4 (CTLA-4) is a receptor that is present on T cells which has an inhibitory function when ligated to the

costimulatory molecules CD80 and CD86 on APC's, thus causing the T cell to become unresponsive. CTLA-4 out competes CD28 and binds to the ligands CD80 and CD86. CTLA-4 knockout mice show systemic autoimmune disease which illustrates the importance of anergy in preventing autoimmune disease. CTLA-4 has been linked to the development of Graves' disease and type 1 diabetes.

The deletion of autoreactive T cells is mediated through apoptosis in the Fas ligand pathway. The fas death receptor contributes to the initiation of apoptosis and a polymorphism in Fas causes a breakdown in tolerance.

Suppression of autoreactive T cells is through Treg T cells. Regulatory T cells that are $CD4^{+}CD25^{+}$ express FoxP3 which has been shown to be important for autoimmunity. FoxP3 deficient mice develop systemic autoimmunity disease caused by a decline in regulatory T cells. They suppress IL-2 secretion and induce the expression of immunosuppressive cytokines IL-10 and TGF-β. The mechanism of how Treg cells suppress immune cells, NK cells and antigen presenting cells are still poorly understood (Kronenberg and Rudensky*.,* 2005; Rioux and Abbas*.,* 2005; Cooles *et al.,* 2013; Rangachari and Kuchroo*.,* 2013).

1.3.2 Autoimmune diseases

1.3.2.1 Multiple Sclerosis (MS)

MS is a chronic demyelinating disease caused by a T cell mediated autoimmune attack on myelin and contributing inflammatory disease in the white matter of the central nervous system (CNS). This causes demyelination lesions and neurone defects. MS is described as displaying massive T cell infiltration, loss of conductivity in the axons and

dysfunctional neurone activity. MS is a common neurodegenerative disease that becomes apparent between the ages of 20-40 years of age and most common in women compared with men (Bar-Or *et al.,* 1999; Hemmer *et al.,* 2002; Rangachari and Kuchroo*.,* 2013).

1.3.2.2 EAE Model

Experimental Autoimmune Encephalomyelitis (EAE) is a mouse model of MS. The EAE mouse model is used to study the manifestation of this degenerative disease in mice. EAE can be introduced into mice by injection of peptides derived from myelin proteins like myelin basic protein (MBP), Myelin oligodendrocyte glycoprotein (MOG) and proteolipid protein (PLP). The disease progression course is observed by clinical scoring (Bar-Or *et al.,* 1999; Hemmer *et al.,* 2002; Rangachari and Kuchroo*.,* 2013).

1.3.2.3 Myasthenia Gravis

Myasthenia Gravis is an autoimmune disease that generates autoantibodies to the nicotinic acetylcholine receptor that is important at neuromuscular junctions and leads to muscle weakness. The mouse model used to explore this autoimmune disease is Experimental autoimmune MG (EAMG) (Sedimbi *et al.,* 2013).

1.3.2.4 Rheumatoid Arthritis

Rheumatoid Arthritis is caused by a systemic inflammatory disease that is Th1 driven in synovial joints. It is characterised by chronic inflammation and cartilage damage

which further amplifies this disease and causes severe pain (Sedimbi *et al.,* 2013). Patients with the disease develop severe joint pain and become less motile. Rheumatoid arthritis has been discovered to involve high levels of IgG antibody and autoreactive T cells. This is due to the presentation of the autoantigen by the APC and activation from pro-inflammatory cytokines (Murphy., 2008).

1.3.2.5 Systemic Lupus Erythramatosus (SLE)

SLE is a systemic autoimmune disease which is caused by the loss of immunological tolerance and allowing the survival of autoreactive T and B cells. Depending on the organ type and immune response generated, this can have profound effects on the severity of the disease caused. As yet there is no cure for this disease and the underlying mechanisms are still unresolved. However, we do know that genetics plays an important part in the susceptibility to develop this disease. So far more than 40 genes have been recognised to be important for various signalling pathways that are involved. The IFN gene has been identified to be closely linked with the severity of SLE patients. The IFN genes have been shown to deregulate DNA repair genes, apoptosis, inflammation, cell proliferation and cell motility.

Indeed genetic factors are important for the susceptibility in developing this disease; however environmental factors could also be important. DNA or RNA methylation and histone modifications have been shown to be important in causing defects or abnormalities in key genes associated with the development of the disease (Frangou *et al.,* 2013).

1.3.3 Autoimmune disease and chronic inflammation

Autoimmune disease is generated from a loss of tolerance and the development of autoantigens. In general autoimmune disease progresses into chronic autoimmune disease due to high levels of autoantigens and chronic inflammation. The chronic inflammation leads onto tissue damage and pro-inflammatory cytokines to further cause tissue destruction and damage (Murphy., 2008).

1.3.4 Genetic basis of autoimmune disease

Some genetic defects have been shown to increase the susceptibility to develop autoimmune disease, in particular Type 1 diabetes. However multiple gene defects are more likely to be the causes for inheritable autoimmune disease. Some single gene defects have been identified to cause X-linked recessive autoimmune diseases. For example, immune dysregulation, polyendocrinopathy, enteropathy X-linked syndrome (IPEX) are caused by a single mutation in FoxP3. IPEX patients have severe allergic inflammation, diarrhea and early death. In some cases, other genes involved in various signalling pathways have been indicated to increase the susceptibility to develop autoimmune disease. In this case CTLA-4 for example, if it is mutated it can cause hyperproliferation (Murphy., 2008).

1.4 Early Growth Response Gene family

The Early Growth response (EGR) gene family consists of four members EGR-1 (Zif268), EGR-2 (Krox20), EGR-3 (Pilot) and EGR-4 (NGF1-2) which are transcription factors. The EGR family contain three zinc fingers in the DNA binding domain, CyS2-HyS2 which interacts with target genes rich in GC consensus DNA motifs. The zinc finger domain across the four family members display 90% homology so there is a possible overlap in binding to target genes. Apart from the DNA binding domain, the activation domains and basic domains do not share homology allowing these EGR molecules to regulate other signalling pathways and have different functions (Joseph *et al.,* 1988; Poirier *et al.,* 2008; Myouzen *et al.,* 2010).

EGR-2 was first discovered to be activated early in the G_0/G_1 stage of the cell cycle after serum stimulation in quiescent fibroblast cells; they found accumulating Krox-20 mRNA (Chavrier *et al.,* 1988). The EGR-2 gene is 2719 nucleotides long and the amino acid composition shows it has residues rich in proline (15%), serine (11%), alanine (8%), threonine (7%) with a Mr of 43,307. The EGR-2 gene is located on chromosome 10q21. Mutations on chromosome 10 are associated with tumors (Joseph *et al.,* 1988; Oehen and Brduscha-Riem*.,* 1998; Walzer *et al.,* 2002; Poirier *et al.,* 2008; Myouzen *et al.,* 2010).

Early Growth Response genes were first recognised to be critical in hindbrain development and long term memory maintenance. EGR-1 in particular has been shown to be critical in neural plasticity and demonstrated in EGR-1 deficient mice, showing a severe impairment in the hippocampus and long term memory signalling (O'Donovan *et al.,* 1999; Poirier *et al.,* 2008). Similarly, EGR-3 knockout mice display deficits in learning,

memory formation and motor neuron abnormalities. EGR-3 knockout mice also display ataxia (Sumitomo *et al.,* 2013).

EGR-2 on the other hand has been shown to be important for peripheral nerve myelination and hindbrain development. Complete knockout of EGR-2 causes perinatal lethality due to severe defects in hindbrain formation. Therefore, EGR-2 conditional knockout mice were developed. For this, EGR-2 was conditionally knocked out of forebrain neurons. This group found that the other EGR family members do not play a compensatory role when one family member is absent. This group found that EGR family members may play opposing roles in brain development (O'Donovan *et al.,* 1999; Poirier *et al.,* 2008).

EGR-1, EGR-2 and EGR-3 are not only expressed in the central nervous system, they are also expressed in many other tissues. However, the fourth member of the EGR family, EGR-4, is strictly conserved in the central nervous system. Research has found that EGR-4 has a totally different function compared with the other EGR members. Research on EGR-4 has found it to be involved in male fertility and regulating leutinising hormone. EGR-4 is found to be essential for germ cell maturation. EGR-4 deficient mice show male infertility due to apoptosis during early-mid pachytene germ cell development stage (Tourtellote *et al.,* 1999; Tourtellote et al., 2000).

EGR-1, EGR-2 and EGR-3 are also important for T cell development, tumorgenesis, apoptosis, proliferation and anergy (O'Donovan *et al.,* 1999; Poirier *et al.,* 2008).

1.4.1 Role of EGR in T and B cell development

EGR-1, 2 and 3 are all induced upon activation of the pre-TCR, when they are over expressed, this can cause deregulation and forced expression into β-selection. EGR-1 is the earliest transcription factor expressed after TCR stimulation on DP thymocytes. EGR-1 and EGR-3 proteins are involved in the progression from the DN3 to the DN4 stage in thymocyte development. EGR-1 and EGR-3 promote the survival at the β selection point. EGR-3 is known to be involved in the post β- selection proliferation and possibly the most critical at this step (Joseph *et al.,* 1988; Shao *et al.,* 1997; Basson *et al.,* 2000; Unoki and Nakamura*.,* 2003; Wang *et al.,* 2007; Lauritsen *et al.,* 2008; Fukuyama *et al.,* 2009; Lazarevic *et al.,* 2009; Hu *et al.,* 2011).

Positive selection in the thymus is governed by the EGR family of proteins and is dependent on the Ras/MAPK signalling pathway. EGR-1 over expression forces positive selection for low affinity TCR. In contrast EGR-1 deficient mice have impaired positive selection with low CD4 or CD8 single positive cells. Similarly when there is a deficiency in EGR-1 and EGR-3 they show a significant decrease in CD8 single positive cells as well as low CD4 single positive cells.

EGR-2 is also important for DP selection as shown in EGR-2 deficient mice where they have impairments in CD4 and CD8 lineages. Data has shown that when EGR-2 is overexpressed there is an increase in the generation of single positive CD8 thymocytes due to low SOCS-1 expression, whereas a deficiency in EGR-2 leads to reduced single positive thymocyte numbers. This is due to fluctuations in the expression of survival factors and BCL-2 expression in DP cells. Directly EGR-2 has been shown to affect the IL-7 survival pathway shown when there is a deficiency in EGR-2, there is a block in the IL-7

pathway in thymocyte development. Loss of EGR-2 fails to upregulate BCL-2 and increases SOCS-1 expression causing a block in IL-7 signalling. Critically levels of EGR-2 will permit either positive selection or death by apoptosis (Oehen and Brduscha-Riem*.,* 1998; Lauritsen *et al.,* 2008; Lawson *et al.,* 2010).

EGR-1 and EGR-2 are important for invariant natural killer T (iNKT) cells which play a role in protection against foreign pathogens, tolerance and tumor surveillance. iNKT cells develop in the thymus and are a subset made alongside T and B cells. These cells derive from the DP T cells. iNKT cells undergo selection through the invariant TCR on the iNKT cell with $CD1d^{+}$ on DP cells. iNKT cells that express functional TCR are positively selected and undergo proliferation and development and display NK1.1 on their surface. The involvement of calcineurin dephosphorylating NFAT drives the expression of target genes critical for T cell development and activation pathway. Mice deficient in a subunit of calcineurin have a defect in NFAT dephosphorylation and T cell development. Research has found that actually EGR-1 is not critical for iNKT development as EGR-1 knockout mice displayed normal NKT development possibly due to compensation from EGR-2. On the other hand, EGR-2 deficient mice have severely reduced iNKT cell numbers and were unable to mature into fully functional iNKT cells expressing NK1.1, there was a block in this transition showing EGR-2 is important. When both EGR-1 and EGR-2 were knocked out, they displayed a severe defect in cell numbers of iNKT cells (Bassiri and Nichols*.,* 2009; Hu *et al.,* 2011). Also EGR-2 has been shown to bind to Zbtb16 and PLZF adaptors which are important for the NKT lineage commitment (Lazarevic *et al.,* 2009; Hu *et al.,* 2011; Seiler *et al.,* 2012).

EGR-2 and EGR-3 are also targets of the CREB binding protein, which are histone or protein acetyltransferases important for activating target genes in T cell activation and

thymocyte maturation. CREB knockout cells display reduced EGR-2 and EGR-3 expression and showed defects in T cell activation and thymocyte development (Fukuyama *et al.,* 2009).

Our group previously have shown that EGR-2-transgenic (EGR-2Tg) mice showed a defect in thymocyte development. We found that the size of the spleen was one third smaller with increased DN3 and DN4 cells whilst reduced DP cells. On the other hand we also examined B cell development and found a defect in EGR-2Tg mice where there was an increase in pre-pro B cells ($NK1.1^{-}B220^{+}CD19^{-}CD43^{+}$) whilst a decrease in pro-B cells ($NK1.1^{-}B220^{+}CD19^{+}CD43^{+}$), pre-B cells ($NK1.1^{-}B220^{+}CD43^{-}$) and immature B cells ($NK1.1^{-}B220^{+}IgM^{+}$). Mature B cell numbers were normal, similar to T cells. Interestingly the expression of Notch1 and Nur77 genes was severely reduced in ISP cells in EGR-2Tg mice. In pro-B cells Pax5 expression was reduced, this gene is important for lineage commitment. This indicated that EGR-2 is strictly controlled during T and B cell development where it is expressed early on in development after β-selection in DN cells and EGR-2 expression is withdrawn between the ISP and DP thymocyte stage (Li *et al.,* 2011).

1.4.2 Role of EGR in anergy

EGR-2 was first discovered to be involved in the immune system after stimulation with PMA in human lymphocytes. RNA was extracted and an RNA blot was performed which showed high EGR-2 mRNA expression in time points up to 3 hours (Joseph *et al.,* 1988). EGR-1, EGR-2 and EGR-3 are expressed only upon B and T cell activation whilst EGR-4 is continuously expressed in T cells.

Known target genes of EGR include growth factors like vascular endothelial growth factor (VEGF). EGR are modulated through direct interaction from NGFI-A binding proteins (NAB) NAB1 and NAB2. More importantly NAB2 has been shown to be more important for EGR expression. NAB2 can either repress or activate the expression of the EGR family members depending on cell activity. For example upon T cell activation NAB2 and EGR-1 transactivate the IL-2 promoter vital for full T cell activation and proliferation. In other cases, low NAB2 leads to induced expression EGR-2 and EGR-3 which leads to anergy. Indeed further evidence has shown that EGR-1, EGR-2 and EGR-3 can also positively regulate NAB2 gene expression which plays a role in the negative feedback loop to inhibit NAB2 transcription and prevent the prolonged expression of EGR displayed in melanoma cell lines. They showed EGR-1, EGR-2 and EGR-3 upregulate NAB2 expression and EGR-1 can positively regulate EGR-2 and EGR-3 transcription. EGR-3 can further enhance EGR-2 expression showing that these two transcription factors interplay with each other (Shao *et al.,* 1997; Poirier *et al.,* 2008; Kumbrink *et al.,* 2010; Myouzen *et al.,* 2010).

Anergy induction is believed to involve NFAT as EGR-2 is the target gene. De-phosphorylated NFAT can enter into the nucleus to promote the transcription of genes mediating T cell activation and anergy. NFAT also can transactivate the other members of the EGR family including EGR-3. EGR targets include E3 ligases GRAIL, cbl-b and Itch, which are important in anergy (Rengarajan *et al.,* 2000; Safford *et al.,* 2005; Bassiri and Nichols*.,* 2009; Arias et al*.,* 2010; Kumbrink *et al.,* 2010; Myouzen *et al.,* 2010; Okamura *et al.,* 2010).

1.4.3 EGR-2 and EGR-3 role in Autoimmunity

Autoimmunity is largely caused by a breakdown in tolerance. The Treg T cells are important for preventing autoimmune disease. Treg cells express FoxP3 and mice that have a deficiency in this transcription factor develop lymphoproliferative characteristics and inflammatory immunopathology. Data has shown that the IL-10 regulatory T cells, $CD4^{+}CD25^{-}LAG3^{+}$ express EGR-2 and shows that EGR-2 is important for the development of this subset. Overexpression of EGR-2 increases the susceptibility for systemic lupus disease caused by a breakdown in tolerance and loss of T regulatory T cells (Okamura *et al.,* 2009; Myouzen *et al.,* 2010; Gao *et al.,* 2012).

Previously our group have shown that in EGR-2cKO mice where the EGR-2 gene has been specifically knocked out in the CD2+ lymphocytes, they displayed lupus-like autoimmune disease in older mice (Zhu *et al.,* 2008). The mice displayed huge lymphocyte infiltration in the liver, kidney and spleen along with IgG deposits in the glomerulus (Zhu *et al.,* 2008). The EGR-2 deficient T cells were hyper-proliferative when given sustained antigen stimulation. They displayed high levels of the activation markers IFN-γ, CD69 and infiltrating levels of pro inflammatory cytokines IL-17A and IL-17F (Zhu *et al.,* 2008). EGR-2 deficient T cells also displayed an accumulation of memory phenotype CD44 T cells compared to young mice. In fact the hyper-proliferation displayed in our EGR-2 knockout mice was due to defective expression of p21cip1, as EGR-2 directly activates the p21cip1 promoter (Zhu *et al.,* 2008). Tolerant cells display p21cip1 and loss of p21cip1 causes hyper-activation and proliferation in T cells. This demonstrates that EGR-2 is important for the intrinsic control of proliferation where the loss of EGR-2 leads to a huge accumulation of T cells to self-antigens (Zhu *et al.,* 2008).

1.5 Aims of Study

The role of the EGR family has been well established in the CNS system with these transcription factors to be critical for myelination. Furthermore our group have previously established a CD2 specific EGR-2 conditional knockout mouse model to study the role of this transcription factor in lymphocytes. EGR-2cKO mice develop lupus-like autoimmune disease and high secretion of pro-inflammatory cytokines (Zhu *et al.,* 2008). Despite lupus-like autoimmune disease, lymphocyte proliferation was normal after mitogenic stimulation. To study further the function of EGR-2 in lymphocytes, we generated a CD2-EGR-2$^{-/-}$EGR-3$^{-/-}$mouse model, therefore, the aims of this study were:

1. Establish CD2-EGR-2$^{-/-}$EGR-3$^{-/-}$mice model.
2. To investigate the phenotype of CD2-EGR-2$^{-/-}$EGR-3$^{-/-}$mouse model.
3. To study the susceptibility of developing autoimmune disease pathology by inducing EAE disease in CD2-EGR-2$^{-/-}$ mice.
4. To measure cell proliferation in CD2-EGR-2$^{-/-}$, EGR-3KO and CD2-EGR-2$^{-/-}$EGR-3$^{-/-}$ mice.
5. To identify the molecular mechanism of the T cell receptor signalling pathway using molecular biology techniques.

In conclusion, the hypothesis of my research is that EGR-2 and EGR-3 are critical for controlling inflammatory mediated autoimmune disease and intrinsically regulates lymphocyte activation in response to mitogenic stimulation.

Chapter 2: Materials and Methods

2.1 Mouse Models

2.1.1 EGR-2 Conditional Knockout mice

EGR-2 $^{LoxP/LoxP}$ and hCD2 Cre transgenic mice were gifts from Dr P. Charnay, Institute National de la Santé et de la Recherche Médicale, Paris, France; they were crossed together to create the EGR-2 conditional knockout (EGR-2cKO) mice. The Cre insertion and recombination at the two *Lox*P sites for the EGR-2-transgene were detected in Cre expressing lymphocytes only. Mice were crossed at least three times on C57BL/6 background. The EGR-2cKO mice were maintained in the Biological Services Unit, Brunel University and used in accordance with the institution guidelines under the U.K Home Office project license. Verification of Egr-2cKO mice was carried out by genotyping on tail DNA, for identification of the EGR-2 *lox*P and iCre loci by Polymerase Chain Reaction (PCR) analysis (See Table 1 for primer list). Cre and LoxP specific primers were used in PCR analysis on genomic tail DNA to detect the presence of both the knockout alleles. The PCR products of knockout were Cre 150 bp and Loxp 210 bp or the Wild Type (WT) allele of 195 bp. The selection of the knockout alleles with Cre and LoxP were used to generate the EGR-2cKO mice.

2.1.2 EGR-3 Knockout mice

We obtained EGR-3 KO under C57BL background mice from St Louis, Missouri (Tourtellotte and Milbrandt*,* 1998). To confirm genomic deletion of EGR-3, we carried out genotyping with two primer pairs specifically either to the knockout or WT alleles as shown in Table 1 primer list. The PCR products of knockout allele were 735bp, while WT

was 350bp. The mice were maintained in the Biological Services Unit, Brunel University and used in accordance with the institution guidelines under the U.K Home Office project license.

2.1.3 EGR-2 Transgenic mice

CD2-specific EGR-2 transgenic (EGR-2Tg) mice were established as previously described by our group (Li *et al.,* 2011). Briefly primers were designed for the EGR-2 coding regions and EcoRI flanking regions from the EGR-2 expression construct. PCR was performed to amplify those products and then cloned into the vector containing the human CD2 promoter and injected into fertilised C57/BL/6 oocytes (Li *et al.,* 2011). The detection of the CD2-specific EGR-2Tg mice was performed by genotyping of tail DNA by PCR. The primers listed in table 1, were used for PCR analysis and the correct product size was 350bp.

2.1.4 Genotyping mouse tail DNA by PCR

Mouse tails were collected and prepared with REDExtract-N-Amp Tissue PCR Kit (Sigma) in accordance with the manufacturer's protocol. Mice were identified using PCR to selectively amplify exponential DNA sequences. This technique uses the thermostable DNA polymerase from *Thermus aquaticus* (Taq) that works at exceedingly high temperatures. As this process is specific, sense and antisense primers for EGR-2 knockout alleles, EGR-3 knockout alleles and EGR-2Tg alleles at a final concentration of 0.5µM were used as shown in Table 1. The PCR series of sequences denaturation, annealing and

extension were carried out under specific cyclic conditions as shown in Table 1. The PCR products were observed by Agarose Gel Electrophoresis at 100V in TAE Buffer (Sigma). The gel was visualized under ultraviolet (UV) light by the Alpha Imager.

huCD2-EGR-2 Transgenic	Sense Sequence	CCA CCA GTC TCA CTT CAG TTC C
	Antisense Sequence	CAG CTG CTG CAG AAA ACC ACT G
	PCR cycle	95˚C for 5mins 94˚C for 40 sec 55˚C for 40sec 72˚C for 40sec } 30cyc 72˚C for 5mins Keep in 4˚C
hCD2 EGR-2 KO LoxP	Sense Sequence	AGT TGA CAG CCC GAG TCC AGT GG
	Antisense Sequence	GGG AGC GAA GCT ACT CGG ATA CGG
	PCR cycle	95˚C for 5mins 94˚C for 40sec 65˚C for 40sec 72˚C for 40sec } 30cyc 72˚C for 5mins Keep in 4˚C
hCD2 EGR-2 KO Cre	Sense Sequence	CCA ACA ACT ACC TGT TCT GCC G
	Antisense Sequence	TCA TCC TTG GCA CCA TAG ATC AGG
	PCR cycle	95˚C for 5min 94˚C for 40sec 56˚C for 40sec 72˚C for 40sec } 30cyc 72˚C for 5min Keep in 4˚C
EGR-3KO	Neo Sequence	GAT TGT CTG TTG TGC CCA GTC
	Sense Sequence	CTA TTC CCC CCA GGA TTA CC
	Antisense Sequence	TCT GAG CGC TGA AAC G
	PCR cycle	95°C for 2mins 95°C for 30sec 57°C for 30sec 72°C for 30mins } 35cyc 72°C for 30mins 4°C for 10sec

Table 1: Showing Primers and PCR cycle information for Genotyping

2.2 Cell Line

The Human Embryonic Kidney 293 (HEK293) cell line (sourced by Barts London School of Dentistry and Medicine) was generated in the 1970's by transformation of HEK293 cells with adenovirus 5 DNA. The Human Embryonic Kidney cells were derived from a healthy fetus. This cell line has been extensively used in cell culture as they grow quickly and can be easily transfected. The cell line was maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS (Fetal Bovine Serum), 50 µM 2-mercaptoethanol (2-ME), and 100 U/ml penicillin and 100 µg/ml streptomycin (all purchased from Invitrogen). Culturing cells were kept in a humidified incubator with 5% (v/v) carbon dioxide at 37 °C at a density of 1 x 10^6 per ml.

2.3 Antibodies and Flow Cytometry

2.3.1 Antibodies

Fluorescein isothiocyanate (FITC) conjugated antibody used was IFN-γ. The phycoerythrin (PE) conjugated antibodies used were BrdU, CD3, CD4, CD8, IL-17A and IgM. The above antibodies and CD3 (clone 145-2C11) and CD28 (clone 37.51) stimulation antibodies with 7AAD were obtained from BD Biosciences.

Also we used antibodies specifically for western blot and immunoprecipitation. The anti-Batf antibody was obtained from Santa Cruz Biotechnology. EGR-2 and EGR-3 were purchased from Covance and Santa Cruz Biotechnology. The IgM stimulation antibody containing the F(ab')2 fragments of goat anti–mouse was from Jackson ImmunoResearch Laboratories, Inc. The FITC-conjugated donkey anti-mouse IgG and

Alexa Fluor 594-conjugated donkey anti-rabbit IgG secondary antibodies were purchased from Jackson Immunoresearch. The pErk42/44, myc-tag, flag-tag and histone-3 were from Cell signalling Technology. The anti-Erk, Rabbit anti-c-Fos, Mouse anti-Jun B, c-Jun, and anti-Batf were obtained from Santa Cruz.

2.3.2 Flow Cytometry

Flow Cytometry was used to identify cell surface and intracellular markers on T and B cells from a population of cells using the LSRII (BD Immunocytometry Systems). The data was analysed using the FlowJo software. Also cell sorting for successfully transfected positive cells was done on the FACSAria sorter with the DIVA option (BD Immunocytometry Systems).

2.3.3 Intracellular Cytokine staining

Splenocytes were stimulated with PMA (20 ng/ml) and ionomycin (0.5 µg/ml) with the addition of Brefeldin A for 5 hours at the end of stimulation. The cell surface markers were stained next followed by the intracellular staining using the Fixation and Permeabilization Kit for IFN-γ and IL-17A according to the manufacturers protocol (Ebioscience).

2.4 Preparation of plasmid construct into Lentiviral vector

2.4.1 Lentiviral vectors

Lentiviruses are a type of retrovirus that is used to deliver large quantities of viral RNA into the DNA of host cells for stable expression of a desired gene. Therefore these retroviruses are useful for facilitating the production of endogenous proteins in the host cell. Primarily retroviruses contain three main types of viral genes, *Gag*, *Pol* and *Env* that are used for the effective transfer of the gene vector whilst not producing pathogenic particles. The gene of interest to be integrated into the host genome is sequenced to be inserted in-between the two Long Terminal Repeat (LTR) regions of the virus. The LTR is a sequence of DNA that facilitates the integration of the viral DNA into the host genome.

2.4.2 Amplification of mouse cDNA for cloning

Mouse cDNA for EGR-2 and Batf were tagged with Myc and Flag (Plasmid construct and cDNA sequence obtained by Dr T.Miao, Barts and the London School of Dentistry and Medicine) and amplified using PCR to be able to produce enough cDNA required for ligation and transformation. The following reagents used for PCR were purchased from Invitrogen, 10xPfx Amplification Buffer, 10x Enhancer Buffer, 50mM $MgSO_4$, 10mM dNTP, primers for the cDNA and Pfx DNA Polymerase according to the manufacturer's protocol. The PCR cycle was carried out following denaturation, annealing and extension for 35 cycles specified in the manufacturer's protocol.

2.4.3 Restriction Digestion, Ligation and Transformation

The cDNA was first digested with the appropriate digestion enzyme for correct incorporation into the bacteria at 37°C for 80 minutes. Once this was done, the mouse cDNA and competent bacteria were incubated together for the process of ligation. The bacteria construct used was, NEB 10-beta Competent E. coli (C3019) cells, this type of bacteria construct was used as it is highly efficient for transformation and can uptake large plasmid vectors. The process of ligation covalently links two ends of DNA together. The reagents used for ligation were vector backbone, 10x ligase buffer and T4 ligase enzyme (New England BioLabs) and incubated for 15 minutes at 70°C. Transformation was then performed on the ligation products with the competent bacterial cells by the freeze-thaw method. Transformation is a process involving bacterial uptake of exogenous DNA from its surroundings.

The freeze-thaw method creates a tension on the cell membrane and perforates it to allow the DNA to be taken up. The process involved the ligation products and competent cells to be placed on ice for 5 minutes, followed by heat pulsation at 42°C for 30 seconds and then placed on ice again for a further 5 minutes. The cells were allowed to recover by incubating them with Super Optimal broth with Catabolite repression (SOCS) medium (Sigma) in 37°C for 1 hour at 225rpm. SOCS medium is a nutrient rich growth medium with added glucose that improves the transformation efficiency. Following this, the cells were placed on a Luria-Bertani (LB) solid Agar plate (10g/L NaCl, 10g/L Tryptone, 5g/L Yeast Extract, 2g/L Agar) supplemented with ampicillin antibiotic at 100µg/µl for antibiotic growth selection.

2.4.4 Mini-Prep culture and identification of correct plasmid by separation on a 0.7% Agarose Gel

Single colonies from the agar plate were grown overnight in a small volume of LB broth (10g/L NaCl, 10g/L Tryptone, 5g/L Yeast Extract) supplemented with ampicillin antibiotic at 100ng/µl and incubated at 37°C at 225rpm. Selective colonies were grown and mini-prep was done using the mini-prep kit from Qiagen according to the manufacturer's protocol, for identifying the correct incorporation of the plasmid. Briefly, the addition of P1, P2 and N3 buffers precipitate the DNA and protein into a clear lysate. The lysate was then passed through a silica-gel membrane, coated with PB buffer containing high salt. When the DNA passes through the column it attaches to the membrane. The column was washed with a PE buffer to discard protein, RNA etc. from the lysate. To elude the DNA a low salt buffer, EB was used. Purified DNA was then eluded and plasmid DNA concentration quantified using the Nano-drop.

2.4.5 PCR amplification and separation by Electrophoresis on an Agarose Gel

This method specifically amplifies the desired gene from a high proportion of undesired plasmid DNA by PCR. The reagents required were, forward and reverse primers, the template DNA, 10mM dNTP's polymerase buffer and polymerase enzyme. The PCR products were run on a 0.7% Agarose gel to check the PCR product size. The correct product was then excised and gel purified using the Qiagen kit to remove unwanted PCR debris and undesired plasmid DNA.

2.4.6 Restriction Digestion and separation by Electrophoresis on an Agarose Gel

Restriction digestion was used to identify that the plasmid had been successfully incorporated and that the orientation and position of the insertion was correct. Restriction enzymes were selected from the restriction sites surrounding the point of plasmid insertion into the vector. A restriction digestion was performed from the manufacturer's guidelines (New England Biolabs). The products were heated at 65°C for 1 hour and then separated by electrophoresis on a 0.7% Agarose Gel.

2.4.7 Midi-Preparation

Plasmids that contained the correct orientation were selected and grown in a larger volume LB broth supplemented with Ampicillin and grown overnight in 37°C at 225rpm on a larger scale. Midi-preparation was performed according to the manufacturer's protocol from Qiagen. The protocol used in mini-prep is similar to the midi-prep protocol. However, in this case, large quantities of plasmid can be purified. Briefly, the buffers P1, P2 and P3 precipitated the DNA into a clear lysate. The lysate was then passed through the qiagen tip which further precipitates protein out, leaving the lysate containing DNA. The lysate was then passed through the silica membrane where the DNA binds to the column. The wash steps using QC buffer removes any contaminants. The DNA was eluded using the QF buffer containing low salt. The DNA was quantified using the Nano-drop.

2.4.8 Production of viral particles and Transduction into packaging cell line

The viral gene products, *gag*, *pol*, *env* and pcDNA3 expression vector were transfected into the packaging cell line HEK293 using Lipofectamine plus reagent (Gibco) catalogue number 15338100 for 4 hours at 37°C. Once the vector is inside the cell and transcription begins, the *gag*, *pol*, and *env* genes along with ψ present in the viral RNA, is used to compose the virion particles which bud off from the cell. The viral particles are therefore in the supernatant, so after 48 hours the supernatant was collected and viral particles harvested by ultracentrifugation. The virus titre was quantified to 10^9 transducing units per ml. The viral particles were then used to infect the target cell line HEK293. Batf and EGR-2 expression levels were confirmed by immunohistochemistry and immunoblotting with the antibodies to Myc and Flag.

2.5 Real-Time PCR (RT-PCR)

Total RNA was extracted from $CD4^+$ T cells or B cells were either unstimulated or stimulated, using the RNeasy Kit purchased from Qiagen. The RNeasy kit does not use the phenol/chloroform method and does not cause ethanol precipitation; it allows easy and pure extraction of RNA. This kit separates RNA from proteins and DNA and the method followed was according to the manufacturer's protocol. Briefly, cells were pelleted and lysed using guanidine-isothiocynate which denatures proteins prior to purification in the column. Ethanol was added to coat the RNA structures so that it would bind to the silica membrane spin column. The RNA would bind to the column and other components such

as DNA and protein would be removed in the washing steps. The pure RNA was then eluded using water.

Pure RNA was then reverse transcribed into cDNA using a reverse transcriptase enzyme that makes a complementary cDNA strand from the RNA template. This method uses Oligo (dT) primers from Amersham, Biosciences that bind to the poly-A tails of the mRNA acting like primers and initiates revere transcription.

The resulting cDNA was then subjected to qRT-PCR which is similar to the PCR technique however; this method measures the mRNA transcripts (gene expression) from tissues in real time compared to conventional PCR where products are measured at the end.

The RT-PCR method measures the fluorescence of SYBR green which is emitted after each cycle of PCR and enables you to directly quantify mRNA expression. The melting temperature (Tm) was also determined for each sample to establish where there is 50% denaturing of the DNA to identify non-specific binding. The Tm was calculated using: Tm= 4 x [G + C] + 2 x [A + T]. The reagents used were from the QuantiTect SYBR Green PCR kit (Qiagen) according to the manufacturer's protocol and used at a concentration of 1µl cDNA and 0.2µl sense and anti-sense primers as shown in Table 2. This method was performed on the Rotor-Gene System (Qiagen, Corbett Robotics) and the data interpreted using the Rotor-Gene Software. The program for qRT-PCR consisted of denaturing, annealing and extension steps. The cycle threshold (Ct) was calculated for each sample and measured the number of cycles of the fluorescent signal that is required to reach the threshold level. The Ct value is inversely proportional to the quantity of nucleic acid that is generated; therefore a high Ct value would mean a lower proportion of nucleic acid generated. Each sample was run in duplicate and the relative mRNA

expression levels were normalized against the expression levels of β-actin from the same samples, under the same program. It was worked out by: Relative expression= 2^(CTβ-actin-CT target)*10,000.

Gene	Sense 5'-3'	Antisense 5'-3'
EGR-2	CTTCAGCCGAAGTGACCACC	GCTCTTCCGTTCCTTCTGCC
EGR-3	CGACTCGGTAGCCCATTACAATCAA	GAGATCGCCGCAGTTGGAATAAGGG
β-actin	AATCGTGCGTGACATCAAAG	ATGCCACAGGATTCCATACC
IL-2	GCATGTTCTGGATTTGACTC	CAGTTGCTGACTCATCATCG
IL-17A	AGCGTGTCCAAACACTGAGG	CTATCAGGGTCTTCATTGCG
IFN-γ	CCATCAGCAACAACATAAGC	AGCTCATTGAATGCTTGGCG
IL-21	ATCCTGAACTTCTATCAGCTCCAC	GCATTTAGCTATGTGCTTCTGTTTC
IL-4	CAAACGTCCTCACAGCAACG	CTTGGACTCATTCATGGTGC
GM-CSF	TGGTCTACAGCCTCTCAGCA	CCGTAGACCCTGCTCGAATA
Batf	GAGCTGCGTTCTGTTTCTCC	CCAGAAGAGCCGACAGAGAC

Table 2: Real-Time PCR Primer sequences

2.6 Measuring Proliferation using (^{3}H)TdR incorporation or BrdU

This technique was performed by Dr T. Miao, Barts and the London School of Dentistry and Medicine, London, where I was also actively involved.

WT and K2/3 mice CD4 or B cells were isolated and plated at 5 x 10^4 cells/200µl in 96 well plates in triplicate. To measure proliferation in the cells 1µl Cl (^{3}H)TdR (tritiated thymidine) was added to the last 8 hours of culture before harvesting. TdR is a thymidine nucleotide that has radioactively labelled hydrogen called tritium (^{3}H). The active incorporation of this thymidine occurs when cells are undergoing DNA synthesis, so it is a good marker for proliferation. The cells were then subjected to Scintillation which involves the conversion of radioactive emissions from (^{3}H)TdR, when being incorporated into newly synthesized DNA, into sparks of light. (^{3}H)TdR is measured in a Scintillation counter that contains fluor liquid, which converts the radioactive β-emissions into light and records as counts per minute.

Another way to observe proliferation is using BrdU (Bromodeoxyuridin). Like (^{3}H)TdR, BrdU is incorporated into newly synthesised DNA. BrdU when incorporated into newly synthesised DNA replaces thymidine with bromodeoxyuridine. BrdU incorporation can only be detected with an anti-BrdU antibody. 10µM BrdU was added in the last hour of cell culture before harvesting. The cells were stained with PE-conjugated anti-BrdU antibody and 7AAD using the manufacturers protocol from the BrdU flow kit, BD Biosciences. BrdU incorporation in T cells was measured by Flow Cytometry.

2.7 Nuclear Extraction and Immunoprecipitation

2.7.1 Nuclear protein extraction

Stimulated and unstimulated K2/3 or WT CD4 or CD8 T cells were harvested after three days. Lysis buffer containing Tris (tris(hydroxymethyl)aminomethane) 10mM, KCL 60mM, EDTA (ethylenediaminetetraacetic acid) 1mM, DTT (dithiothreitol) 1mM, 0.1%

Np40 (Tergitol-type NP-40), PMSF (phenylmethylsulphonyl fluoride) 100nM was added to the cell pellet and incubated for 5 minutes on ice to lyse the cytoplasmic membrane. The Lysis Wash Buffer containing Tris 10mM, KCL 60mM, EDTA 1mM, DTT 1mM, PMSF 100nM, was added and spun down at 1200rpm for 5 minutes to remove debris. The cytosolic protein in the supernatant was kept and further washed with Lysis Wash Buffer to remove contaminants. The pellet was then re-suspended in Nuclear Lysis Buffer containing Tris-CL 50mM, EDTA 10mM, 0.8% SDS (Sodium dodecyl sulphate), PMSF 1mM and protease inhibitors (Roche) and incubated for 30 minutes on ice to lyse the nuclear membrane. The cells were centrifuged at 13,000rpm for 10 minutes to pellet the nuclei. The nuclear protein was kept from the supernatant and stored in aliquot at -180°C.

2.7.2 Co- Immunoprecipitation

Co- Immunoprecipitation is a technique used to examine protein-DNA interactions where you can identify transcription factor/DNA binding interactions of interest. Briefly fusion proteins with an affinity tag were used to precipitate the target protein and purified using immobilized protein A beads.

Each precipitation contained 400µl 1x chip buffer (40µl 10x Chip buffer, 360µl water, 2µl Proteinase inhibitor cocktail) and 100µl of each sample (500mg chromatin). 500µl of diluted chromatin, Protein A beads, immunoprecipitation antibodies anti-Myc for Batf, anti-Flag for EGR-2 were incubated overnight.

Tubes were placed on a magnetic rack and supernatant removed. Washes of low salt and high salt were performed to remove non-specific binding to the Protein A beads and chromatin eluded.

2.8 Western Blot

2.8.1 SDS-PAGE Electrophoresis

The samples were prepared for SDS Polyacrymalide gel Electrophoresis (PAGE) which separates proteins through an electric field based on protein size and charge. The proteins travel from the negative to positive terminal and the smaller the protein, the faster it will migrate through the electric field compared with a larger protein.

The samples were prepared prior to denaturing using 4x LDS (lithium dodecyl sulfate) sample buffer, β-mercaptoethanol and the reaction heated in 70°c for 10 minutes at 900rpm. The samples were run along with a Rainbow molecular marker (Novex) on a 6-12% SDS PAGE gel composed of a 10% resolving gel containing 10% acrylamide (0.26% bisacrylamide solution (Protogel), 0.1% SDS, 0.1% APS (Ammonium Persulphate), 0.04% Temed (N,N,N'N'-tetramethylethylene diamine) and 380mM Tris pH8.8), 5% stacking gel containing 5% acrylamide (0.13% bisacrylamide solution (Protogel), 0.1% SDS, 0.1% APS, 0.1% Temed and 187.5mM Tris pH6.8). The gel was placed in SDS running buffer 1L which contained: 50ml SDS and add up to 1000ml with distilled water used for separating the protein bands. Samples were run at 150v for 1hr 30 minutes to resolve proteins that are closer together.

2.8.2 Western Blot Transfer

After gel electrophoresis the gel was prepared for protein transfer onto a nitrocellulose membrane. The transfer was prepared as a stack; four pre-soaked foams in Western Transfer Buffer (10ml methanol, 10ml Transfer buffer (Amersham)) were placed on the bottom. The gel was placed on the top of the nitrocellulose membrane (Amersham), as proteins migrate from the negative to positive terminal and held with filter paper that sandwiched it together. The filter paper was required to assist the buffer flow once a voltage is applied between the gel and the membrane. Finally, the transfer sandwich was stacked with more foam to keep the transfer moist and compact. All of the components were pre-soaked in Western Transfer buffer. The sandwich was then placed in a cassette containing the Western Blot transfer buffer and transfer was allowed to take place at 30v for 1hour 30 minutes. The proteins will transfer onto the nitrocellulose membrane at the same position as on the gel.

2.8.3 Blocking, primary antibody, washing, second antibody and developing

The membrane was placed in 5% blocking buffer containing 5% skimmed milk and TBST (Tris Buffered Saline and 0.1% Tween 20) to prevent any non-specific antibody binding. The primary antibody as described section 2.3.1 was prepared in 5% BSA overnight at 4°C. The membrane was washed to remove unbound primary antibody with 1xTBST (1M Tris, 5M NaCl, 0.5M EDTA pH8 and distilled water) for 15 minutes, then a further three times with TBST for 5 minutes. The membrane was then incubated with the second antibody conjugated with IgG HRP (horseradish peroxidase) corresponding to the

species of primary antibody (Biorad) for 1 hour at room temperature. The membrane was washed 1x TBST for 15 minutes and a further three times in TBST for 5 minutes to remove unbound secondary antibody.

To detect fusion proteins, chemiluminescense from the ECL prime Western Blotting Detection Reagent kit (Amersham) was used according to the manufacturer's protocol. The kit contains detecting reagents that are involved in the oxidation of cyclic diacylhydrazides like luminol which produces light when catalyzed by HRP. The detection reagents are exposed to the nitrocellulose membrane for 5 minutes to detect peak light signals. The luminescence signal was identified by exposure from photographic paper (Amersham) over the nitrocellulose membrane.

2.9 Electrophoretic Mobility Shift Analyses (EMSA)

This technique was performed by Dr T. Miao, Barts and the London School of Dentistry and Medicine, London, where I was also actively involved. The EMSA technique is used to detect protein binding interactions to a DNA sequence labelled by a radioactive isotope. The reaction mixture is then run on a non-denaturing polyacrylamide gel. Successful binding of the proteins to the radioactively labelled DNA sequence will hinder the migration of this complex through the gel. To find specifically whether a protein is involved in a protein complex, the addition of an antibody is used to do a supershift assay. If the desired protein is part of the complex, the antibody that it binds to will further hinder the migration of the complex causing a supershift phenomenon.

2.9.1 Labelling the probe

The following probes were made in Barts and the London School of Dentistry and Medicine, London. The consensus probes for AP-1 (50-AGCTTCGCTTGATGAGTCAGCCG-30),NFkB (50-CAGAGGGGACTTTCCGAGA-30), SP1 (50-ATTCGATCGGGGCGGGGCCAG-30) and NFAT (50- CTGTATCAAACAAATTTTCCTCTTTGG-30) were labelled with [a-32P] dCTP using Ready-to-Go DNA labeling beads (Amersham Biosciences UK Ltd., Pollards Wood, Bucks). 1pmol/µl double stranded primer 2µl,10x Klenow Reaction buffer 2µl,Radiolabelled dCTP 3µl,0.5mM each dGTP, dATP, dTTP.1µl 5U/µl Klenow 1µl,Molecular Biology water 11µl were prepared and incubated at room temperature for 40 minutes. The reaction was stopped by adding 1µl of 0.5M EDTA; this was followed by adding 30µl water. Then 40µl sense and antisense primers (dissolved at 100pmol/µl),10µl 10x annealing buffer (200mM Tris,10mM EDTA, 500mM NaCl pH 8) were added together and heated to 95°C for 5 minutes and then left to cool overnight. The reactions were placed at 4°C for 2 hours and then stored at -20 °C. 2pmol was used for labelling.

The labelled probe was purified using the Qiagen nucleotide removal kit in accordance with the manufacturer's protocol. It contains a silica membrane which single nucleotides bind to the column in the high salt buffer conditions in the first binding step. The second step involves the washing phase where any impurities are flushed through the column. Lastly the elution step involves the collection of the labelled probe under low salt conditions.

2.9.2 Binding Reaction

Nuclear extracts from lymphocytes were stimulated with anti-CD3 and anti-CD28 for T cells or IgM for B cells for 24 hours and restimulated for a further 30 minutes. The cells were incubated with the binding reaction reagents 5x Binding buffer, 20% glycerol, 5mM $MgCl^2$, 2.5mM EDTA, 2.5mM DTT**,** 250mM NaCl**,** 50mM Tris (pH 7.5)**,** 0.25mg/ml poly(dI-dC)**,** labelled probe, nuclear extract and water**.** The reaction tubes were incubated on ice for a further 20-40 minutes. Then 1µl 10xLB was added. For supershift reactions anti-Batf, anti-Myc, anti-cFos and anti-JunB antibodies were added in the first 10 minutes during the labelling time.

2.9.3 Gel Electrophoresis and Developing

The samples were run on a 6% Novex DNA retardation gel (30% Acrylamide 1ml, 10x TBE 300µl, 10% APS 100µl, TEMED 20µl, Water 4.6ml). 0.53xTBE was used in the running buffer and run for 30-60 minutes at 100V. The gel was dried for 40 minutes in a preheated gel dryer. The cassette was placed in x-ray film in dark room and exposed overnight at -80°C and analysed on a Storm 860 PhosphorImager (Molecular Dynamics). The intensity of the AP-1 band was normalized against the activity of the SP1 control.

2.10 Messenger RNA Silencing

This technique was performed by Dr.T.Miao at Barts and the London School of Dentistry and Medicine, where I was actively involved. RNAi also termed post-

transcriptional gene silencing is a technique used to silence the expression of genes to find out their function etc. Double stranded RNA is synthesized in the cell and will contain a sequence complementary to a gene you wish to silence. The long double stranded RNA is cut by the enzyme Dicer into short siRNA segments and made single stranded. One of the strands is termed the passenger strand which gets degraded and the other is the guide strand. The guide strand forms complementary base pair binding with the mRNA and is cleaved so it cannot be translated into protein in the RNA-induced silencing complex (RISC). The siRNA was made against the mRNA sequence of BATF, EGR-2 and EGR-3: siBatf 2, 50-GAACGCAGCUCUCCGCAAA-30; siBatf 4, 50-GGACUCAUCUGAUGAUGUG-30, siEgr2-1, GCUGCUAUCCAGAAGGUAU-30; siEGR2-2, 50-CGACCUCGAAAGUACCCUA-30, and siEGR3-1, 50-GCGACUCGGUAGCCCAUUA-30; and siEGR3-2, 50-GCAGUUUGCUAAAUCAAUU-30, were created. Non-specific short random siRNAs from QIAGEN, CAT: 1027281, were used as negative controls.

Primary naïve CD4+ T and resting B cells isolated from 4 week old mice were transfected with Amaxa Nucleofector according to the manufacturer's protocol using 1 mM siRNA. The Batf siRNA2 sustained specific reductions in Batf expression for at least 72 hrs from RT-PCR results.

2.11 Histology

Histology was performed by Dr T.Miao, Barts and the London School of Dentistry and Medicine. Briefly the tissues were fixed with 10% formalin in PBS to retain the tissue structures and dehydrated prior to paraffin embedding by adding increasing concentrations of ethanol over a period of 5 hours. Then xylene and paraffin wax was

added and tissues mounted on a slide pre-coated with gelatin for adherence, and air-dried overnight in an oven. The Paraffin sections were then deparaffinised to rehydrate the tissue section using xylene, followed by reducing concentrations of ethanol. The slides were then submerged in blocking buffer 3% BSA and primary antibodies were added. Then a second antibody was used and counterstained with DAPI, or sections were stained with haematoxylin and eosin.

The analysis of urine proteins (proteinuria) was done using urinalysis reagent strips (Access Diagnostics Tests, UK) according to the manufacturer's protocol.

To determine the aggregation of Ig deposits 6µm frozen kidney sections were obtained and stained with Texas Red conjugated anti-mouse IgG obtained from Jackson Immunoresearch Research.

2.12 ELISA

Performed by Dr T.Miao, at Barts and the London School of Dentistry and Medicine, London, where I was also actively involved. Enzyme Linked Absorbent Assay (ELISA) is a technique used for the detection of an antigen in a given sample. The process initially begins with immobilization of the sample by adding a capture antibody specific for the antigen, thus causing the antigen to attach on the surface. Non-specific binding to the surface is blocked using a blocking reagent. Then a detection antibody is added specific for the antigen which is detected once the secondary antibody is given. The secondary antibody in this case, is covalently linked to an enzyme like peroxidase which can metabolize colourless substrates into colour. Washing steps were performed in-between to remove non-specific antigen or antibody binding complexes. The detection of

present antigen is performed when the addition of the enzyme substrate is added causing a colour change (optical density) which can be detected by spectrophometry.

Serum concentrations for whole Ig and Ig isotypes were performed by ELISA using the capture antibody goat anti mouse IgG and secondary antibodies HRP conjugated against IgG, IgG1, IgG2a, IgG2b and IgM (Sigma). The optical density readings were normalized against the isotype controls from sigma.

Anti-histone and anti-dsDNA antibodies in serum were measured (as described in Zhu *et al.,* 2008).

2.13 Experimental Autoimmune Encephalomyelitis

Experimental autoimmune encephalomyelitis (EAE) was induced into the mice using a myelin oligodendrocyte glycoprotein $(MOG)_{35–55}$/CFA emulsion kit (EK-0111; Hooke Laboratories) according to the manufacturer's protocol subcutaneously. The mice were induced with 100µl Pertussis Toxin and re-administered 24 hours later. Recordings for the progression of the disease were calculated by clinical scoring relating to the severity of the disease as shown in Table 3. After thirty days from the initial induction, the mice were sacrificed and CNS tissues were taken and immediately fixed with 10% formalin in PBS and embedded in paraffin. Haematoxylin and eosin staining was performed on those paraffin sections.

Stage of disease	Signs
0	Mice appear normal
1	Limp tail/hind limb weakness
2	Limp tail and hind limb weakness
3	Partial hind limb paralysis
4	Complete hind limb paralysis
5	Death, moribund state

Table 3: EAE Clinical Scoring and Severity of Disease

Chapter 3: Results

3.1 Establish K2/3 knockout model

3.1.1 Genotyping of CD2-specific EGR-2 knockout models

The model carries CD2-Cre transgene and floxed EGR-2 at exon 2 under C57/BL background as described previously (Zhu *et al.,* 2008). In order to use this model to create K2/3 in lymphocytes, confirmation of the Cre gene and Egr-2–loxp locus by genomic PCR analysis was performed (Figure. 1). The data showed that the parental mice of CD2-specific Egr-$2^{-}/^{-}$ had both Cre and floxed EGR-2 genes (Figure. 1).

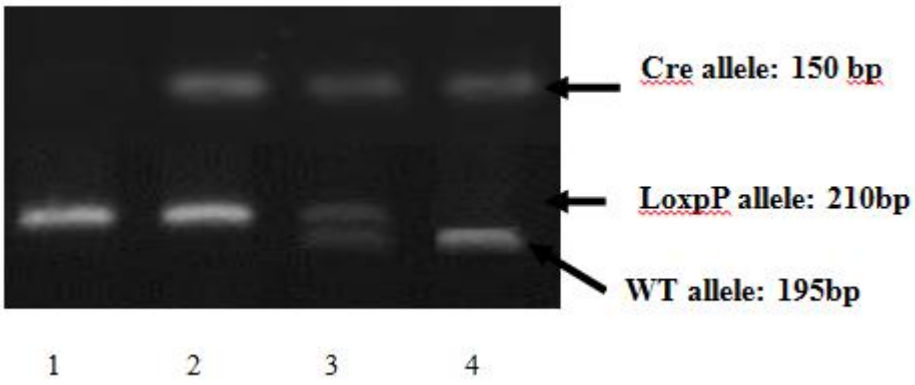


Figure 1: Genotyping of CD2+ cell-specific EGR-$2^{-/-}$ mice. Cre primers were used to detect the presence of the iCre transgene to yield a 150 bp and the LoxP primers to yield 210bp products. The genotype from mouse in lane 1 represents EGR-$2^{f/f}$ as shown by the upper 210bp band and cre transgenic negative. The mouse in lane 2 represents EGR-$2^{f}/^{f}$ CD2-Cre positive. Lane 3 genotype represents EGR-$2^{f/-}$ CD2-Cre. Finally, the genotype of the mouse from lane 4 is CD2-Cre transgenic.

3.1.2 Genotype of EGR-3 KO model

Unlike conventional EGR-2 KO mice, the EGR-3KO is principally normal in phenotypes of major organs with a degree of defect in the central nervous system including ataxia, scoliosis, ptosis and tremors (Tourtellotte and Milbrandt., 1998). Previous studies also found a defect of thymocyte development in EGR-3 KO mice showing thymic atrophy due to a partial block at DN3 which reduces proliferation of subsequent DN4 (Carter *et al.,* 2007) and reduced susceptibility to anergy induction (Safford *et al.,* 2005). However, the function of effector lymphocytes in the periphery is normal. We obtained EGR-3 KO under C57BL background mice from St Louis, Missouri (Tourtellotte and Milbrandt., 1998). Genotyping of EGR-3KO mice was performed by genomic tail DNA PCR analyses. PCR primers were designed to detect the presence of the neo allele that confirmed EGR-3 inactivation of the WT allele. The data showed that the parental EGR-3KO had the neomycin insertion or were WT. Selection of the neo allele was used for breeding for generating the K2/3 model (Figure. 2)

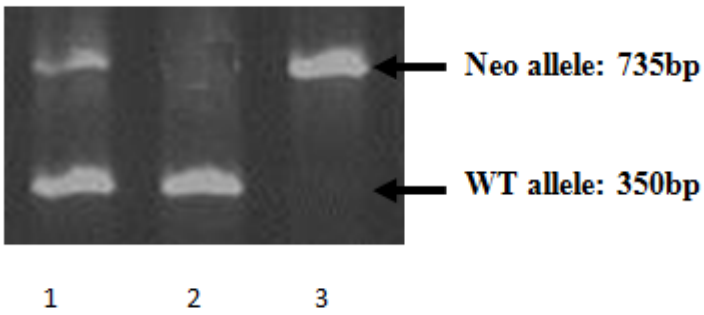


Figure 2: Genotyping EGR-3KO using PCR. Neo specific primers were used to detect the Neo cassette insertion that deleted the total zinc finger domain of EGR-3. This yielded a 735 bp PCR product. (Lane 3) The WT allele yielded a 350 bp product (Lane 2). The presence of one neo allele and one WT allele gave both the 735 bp and 350 bp PCR products (Lane 1). The Neo positive mice were used for subsequent breeding of K2/3 mice (Lane 3).

3.1.3 Creation of the EGR-2 and EGR-3 double knockout mouse model

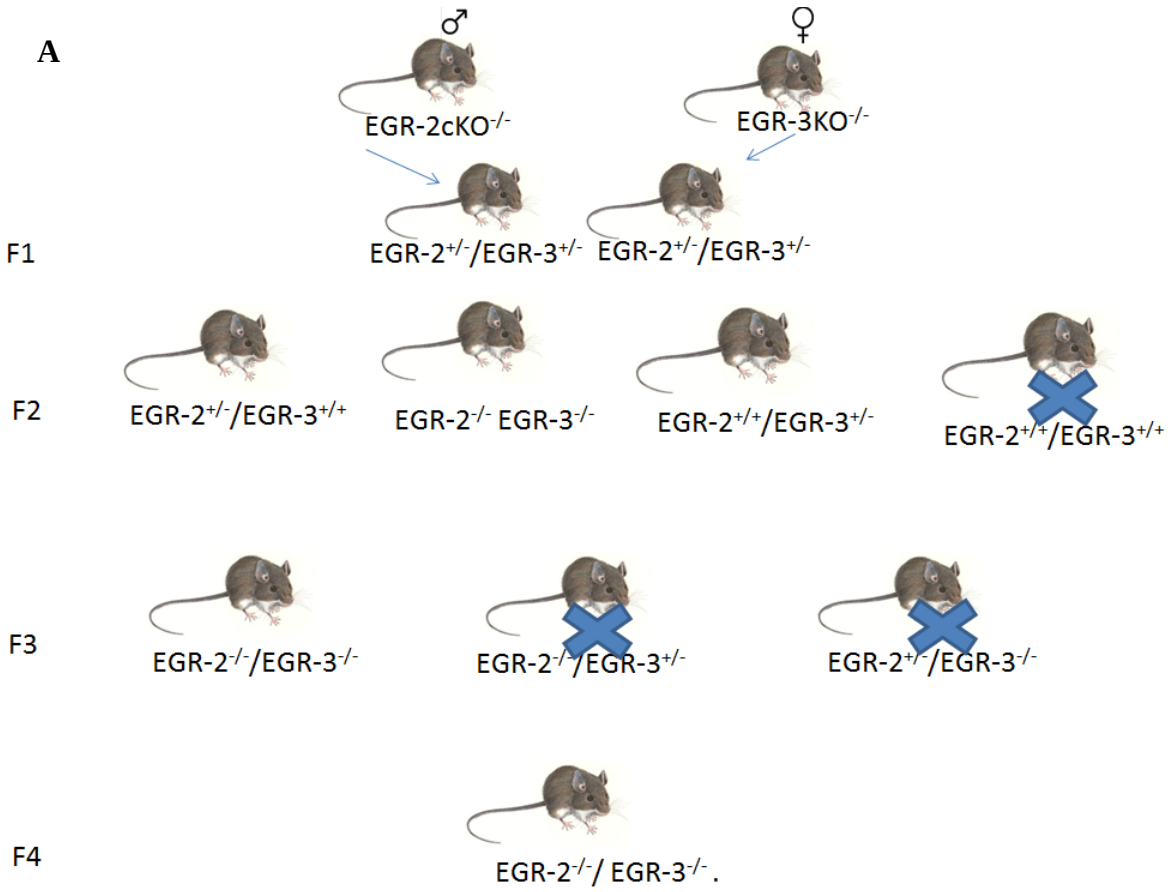
As already described earlier, EGR-2 and EGR-3 family members share similar structure and function. Single EGR-2 and EGR-3 knockout models have shown no significant defect in lymphocyte function. So to explore further EGR-2 and EGR-3's gene function we generated a double knockout mouse model.

The EGR-2cKO$^{-/-}$ male as previously described was bred together with the female EGR-3KO$^{-/-}$ mouse that produced the F1 generation (Figure. 3, A). Each generation of mice that followed, were tail genotyped by PCR. The F1 generation produced, had the phenotype of EGR-2$^{+/-}$/EGR-3$^{+/-}$ (Figure. 3, A). The F1 littermates were bred with each other and produced the F2 generation that had the phenotype EGR-2$^{+/-}$/EGR-3$^{+/+}$, EGR-2$^{-/-}$ /EGR-3$^{-/-}$, EGR-2$^{+/+}$/EGR-3$^{+/+}$ and EGR-2$^{+/+}$/EGR-3$^{+/-}$ (Figure. 3, A). Twenty five per-cent of the litter were EGR-2 and EGR-3 homozygous or wild type and fifty per-cent of the litter were EGR-2 and EGR-3 heterozygous.

From the F2 generation, EGR-3$^{-/-}$ or EGR-3$^{+/-}$ and EGR-2$^{-/-}$ or EGR-2$^{+/-}$ were selected for breeding pairs from the double knockout mice. The F3 generation of mice had the phenotype, EGR-2$^{-/-}$ and EGR-3$^{-/-}$ homozygous, EGR-2$^{-/-}$ EGR-3$^{+/-}$ and EGR-2$^{+/-}$ EGR-3$^{-/-}$

heterozygote's (Figure. 3, A). One third of the litter were EGR-2 and EGR-3 homozygous and two thirds were EGR-2 or EGR-3 homozygous and heterozygous for either gene.

From the F3 generation, more EGR-2 and EGR-3 homozygotes could be selected as breeding pairs for the double knockouts and were used for breeding for the F4 generation. The final breeding pair to generate the double knockout generation was EGR-$2^{-/-}$ EGR-$3^{-/-}$ (Figure. 3, A). We confirmed the genotype of the EGR-$2^{-/-}$ EGR-$3^{-/-}$ mice by genomic tail PCR analysis. The primers used to test for Cre, LoxP and Neo are described as before in 3.1.1 and 3.1.2 (Figure. 3, B).



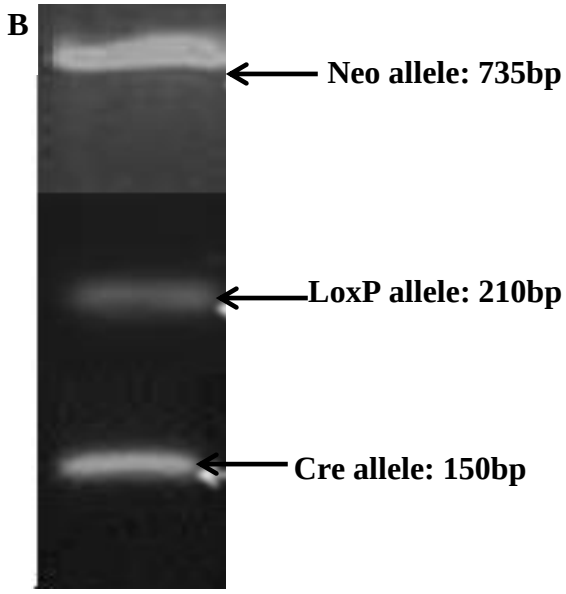


Figure 3: Generating the EGR-2$^{-/-}$ EGR-3$^{-/-}$ double knockout final breed. (A) Schematic overview of how we created the EGR-2$^{-/-}$ EGR-3$^{-/-}$ double knockout breed. Parental EGR-2$^{-/-}$ and EGR-3$^{-/-}$ were used for the generation of the F1 hybrid EGR-2$^{+/-}$ EGR-3$^{+/-}$. These mice were then bred together to produce the F2 hybrid where the EGR-2$^{+/-}$ EGR-3$^{+/+}$, EGR-2$^{-/-}$ EGR-3$^{-/-}$ and EGR-2$^{+/+}$ EGR-3$^{+/-}$ were used as breeding pairs that generated the F3 hybrid. From the F3 hybrid the EGR-2$^{-/-}$ EGR-3$^{-/-}$ were bred to generate the final breeding pair F4. **(B)** Genotyping was performed to check the presence of Cre, LoxP $^{f/f}$ and Neo in the tail DNA.

This thesis focusses on the K2/3 mouse model; however we also generated an EGR-2Tg mouse model to study the overexpression of EGR-2 in CD2 specific cells (Li *et al.,* 2011). Genotyping was performed on tail DNA by PCR analyses to yield a 350bp PCR product (data not shown).

3.1.4 Confirmation of the defect in EGR-2/EGR-3 expression in K2/3 mice at mRNA and protein level

As previously shown, we confirmed genomic deletion of EGR-2 and EGR-3 (Figure. 3, B). We further validated this at mRNA and protein expression levels. Both CD4 and B cells from WT and K2/3 mice were either unstimulated or stimulated for 6 hours with anti-CD3 and anti-CD28 for T cells or IgM for B cells. EGR-2 and EGR-3 have been shown to be expressed after antigenic stimulation (Anderson *et al.,* 2006), for both the mRNA and protein expression levels, unstimulated samples were used as a negative control (Figure. 4, A, B). The WT sample was used as a positive control to show the endogenous EGR-2 and EGR-3 mRNA expression in both the unstimulated and stimulated conditions in T and B cells (Figure. 4, A). For the mRNA expression levels of EGR-2 and EGR-3 , WT and K2/3 CD4 and B cells were stimulated for 6 hours with indicated antigen and RT-PCR was performed relative to β-actin expression (Figure. 4,A). The results showed that from the unstimulated results, WT and K2/3 CD4 and B cells showed no EGR-2 and EGR-3 mRNA expression (Figure. 4, A). From the stimulated results, WT CD4 and B cells showed high EGR-2 and EGR-3 mRNA expression (Figure. 4, A). Whereas K2/3 CD4 and B cells, showed no EGR-2 and EGR-3 mRNA expression in stimulated cells, which confirms that there is complete excision of EGR-2 and EGR-3 (Figure. 4, A).

Complete excision of EGR-2 and EGR-3 in the CD2 expressing cells had been confirmed by RT-PCR (Figure. 4, A), we also wanted to further confirm this at protein level by immunoblotting. Immunoblotting (referred to as Western Blotting) is a technique used to detect protein in a given sample. In this case we used the same cell extracts from WT and K2/3 CD4 and B cells and stimulation conditions as described for RT-PCR. We showed

here that for both WT and K2/3 unstimulated CD4 and B cells, there was no detectable EGR-2 or EGR-3 protein expression consistent in our confirmation of complete excision of EGR-2 and EGR-3 in CD2 expressing cells from K2/3 mice (Figure. 4, B). More importantly we showed that after stimulation, there is no EGR-2 or EGR-3 protein expression in K2/3 mice, whereas our positive control, WT showed high protein expression in both CD4 and B cells (Figure. 4, B). In conclusion, we demonstrated here that the K2/3 mice showed complete excision of EGR-2 and EGR-3 at mRNA and protein expression levels.

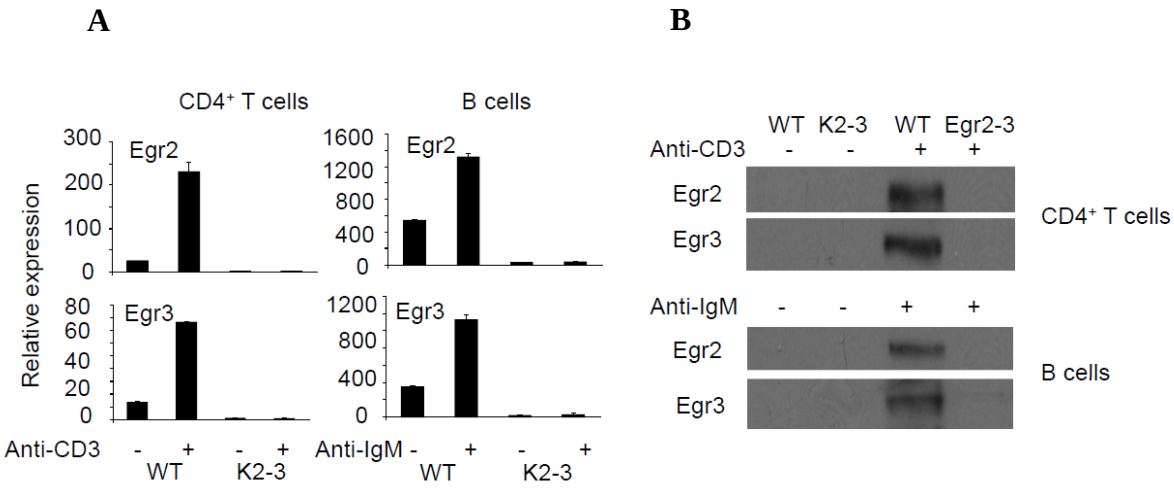


Figure 4: Defective expression of EGR-2 and EGR-3 confirmed by RT-PCR and immunoblotting. Splenic CD4 and B cells from WT and K2/3 mice were either unstimulated or stimulated with anti-CD3 and anti-CD28 for CD4 T cells or anti-IgM for B cells for six hours. **(A)** mRNA expression levels of EGR-2 and EGR-3 were confirmed by RT-PCR and **(B)** protein expression levels of EGR-2 and EGR-3 were confirmed by immunoblotting. The RT-PCR data was represented relative to β-actin expression. The data presented here was representative from two experiments (The data is taken from Li *et al.,* 2012, in which I am one of the co-authors).

3.2 Development of autoimmune disease in K2/3 mouse model

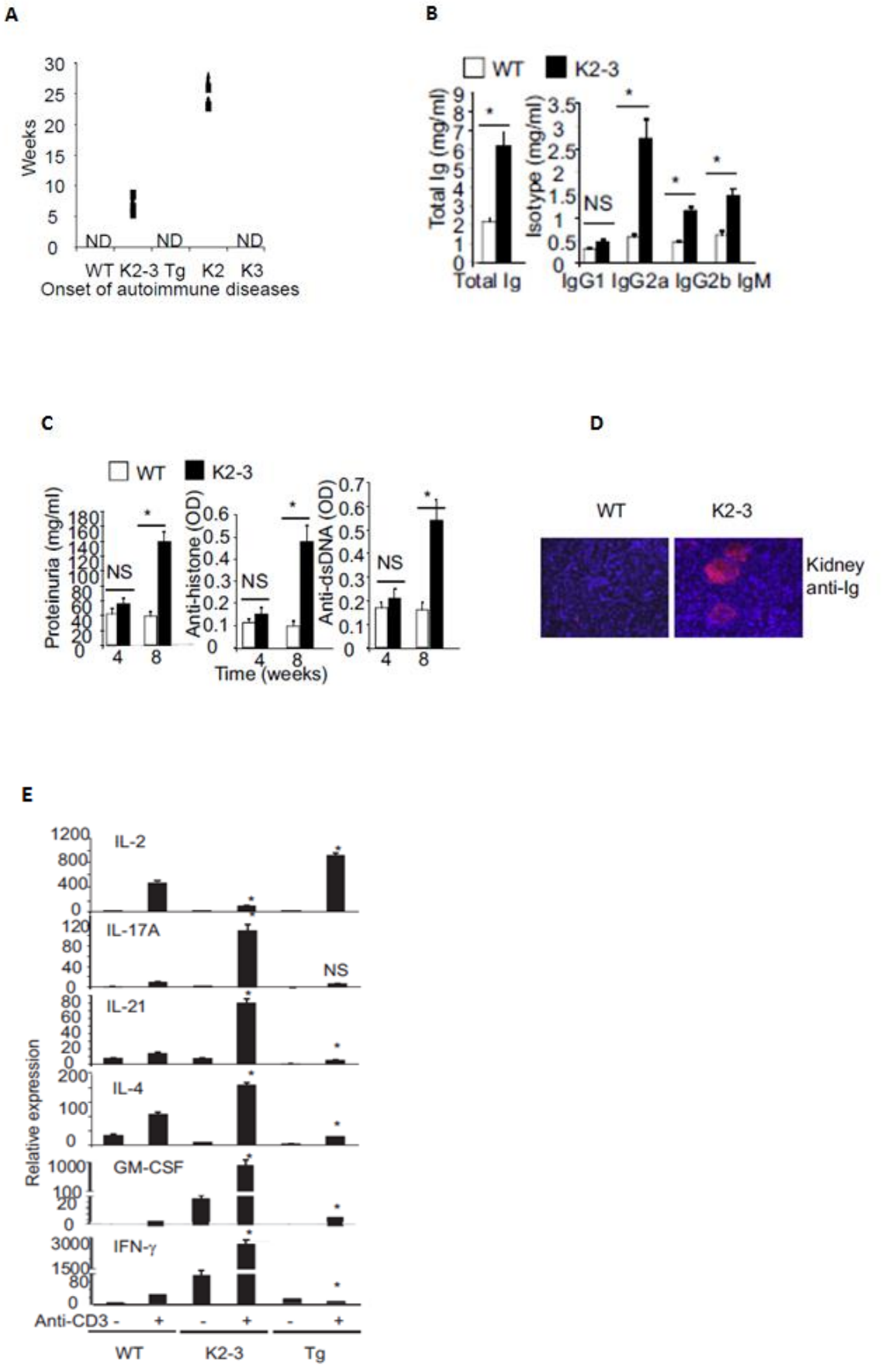
We have already shown that in EGR-2cKO mice they developed lupus-like autoimmune disease with a high infiltration of mononuclear cells in the liver, kidney and spleen (Zhu *et al.,* 2008). The phenotype of the accumulating cells expressed CD25, CD44 and CD69 (Zhu *et al.,* 2008). EGR-2cKO histology sections show high infiltration of cells in the liver and kidney, whilst more white pulp in the spleens of 15 month old mice (Zhu *et al.,* 2008). We therefore wanted to investigate the phenotype in EGR-2 and EGR-3 deficient mice. From observing the mice, when the K2/3 litter reached 2-3 months of age, they began to become less motile and reduced their food intake. The mice after 8 months became increasingly less motile and some were moribund. This suggested that possibly EGR-2 and EGR-3 regulated the development of autoimmune disease. Therefore we measured cytokine expression levels and self-reactive antibodies from WT, K2/3, Tg, K2 (EGR-2cKO) and K3 (EGR-3KO) mice over time for 30 weeks. We observed that K2/3 mice showed increasing levels of IFN-γ and self-reactive antibodies as early as 5 weeks after birth whereas WT, Tg and K3 mice showed undetectable levels (Figure. 5, A). Interestingly we showed that K2 mice displayed IFN-γ expression and self-reactive antibodies between 20-25 weeks of age which confirms our group's previous result that EGR-2cKO mice develop autoimmune disease in later life (Zhu *et al.,* 2008) (Figure. 5, A). When there is a deficiency of both EGR-2 and EGR-3, the onset of autoimmune disease is much earlier in life which we proposed is due to compensatory role that EGR-3 plays in the absence of EGR-2. Our positive control in this experiment was the Tg mice who showed no detectable signs of autoimmune disease explaining why these transcription factors are essential (Figure. 5, A).

We also wanted to investigate the Ig serum levels from our K2/3 mice as our group previously found high serum IgG in EGR-2cKO mice (Zhu *et al.,* 2008). We showed here that there are also high serum IgG concentrations in K2/3 mice from ELISA results, particularly IgG2 which is important for the Th1 mediated inflammatory response (Zhu *et al.,* 2008) (Figure. 5, B). However, IgG1 was low, which is similar to what our group previously published (Zhu *et al.,* 2008) as it mediates a Th2 response, not associated with inflammation (Figure. 5, B).

To study the development of autoimmune disease in K2/3 mice, we measured self-reactive antibodies, a hallmark of autoimmune disease. Urine protein levels and anti-dsDNA in K2/3 and WT mice were also measured. Urine protein levels were measured because the kidney is one of the major organs that show tissue damage and inflammation in SLE patients. We not only measured urine protein levels, anti-dsDNA and histone were also examined at two time points, 4 weeks and 8 weeks; this is because we have already shown from Figure. 5, A that the mice develop autoimmune disease early so we chose those time frames. dsDNA refers to the antigen specific autoantibodies and histone is the DNA and protein interactions that generate the autoantigens, both are believed to be important for detecting autoimmunity. We found urine protein levels increased fourfold between 4-8 weeks in K2/3 mice compared with WT mice (Figure. 5, C). The self-reactive antibody levels were fivefold higher in 4-8 week K2/3 mice compared with WT mice (Figure. 5, C). To further analyse this, we also did immunohistochemistry and took kidney sections and fluorescently labelled them with Texas Red Ig to show the IgG deposits. We showed here that there were large deposits of IgG in the K2/3 kidney section compared to WT (Figure. 5, D). These results confirmed high levels of autoreactive antibodies and

IgG deposits in the glomeruli which indicated that the K2/3 mice had developed systemic autoimmune disease.

We wanted to observe pro-inflammatory cytokines in CD4 T cells isolated from WT, K2/3 and Tg 4 week old mice and compare un-stimulated or stimulated conditions with anti-CD3 and anti-CD28 (Figure. 5, E). mRNA expression levels for IL-2, IL-17A, IL-21, IL-4, GM-CSF and IFN-γ were measured by RT-PCR (Figure. 5, E). We showed that in unstimulated conditions, K2/3 mice had increased production of pro-inflammatory cytokines IL-17A, IL-21, IL-4, GM-CSF and IFN-γ compared with WT and Tg (Figure. 5, E). Furthermore, when the cells were stimulated, K2/3 mice showed a threefold increase in these cytokines, compared with Tg mice (Figure. 5, E). Interestingly the K2/3 mice showed impaired IL-2 mRNA expression once stimulated, compared to Tg which show a fourfold increase in comparison (Figure. 5, E). These results showed that EGR-2 and EGR-3 play an important role in regulating autoimmune disease and inflammation.


A
Weeks
ND
ND
ND
WT K2-3 Tg K2 K3
Onset of autoimmune diseases
B
WT
K2-3
Total Ig (mg/ml)
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NS
Total Ig
IgG1 IgG2a IgG2b IgM
C
WT
K2-3
Proteinuria (mg/ml)
Anti-histone (OD)
Anti-dsDNA (OD)
NS
Time (weeks)
D
WT
K2-3
Kidney
anti-Ig
E
IL-2
IL-17A
IL-21
IL-4
GM-CSF
IFN-γ
NS
Relative expression
Anti-CD3
WT
K2-3
Tg

Figure 5: EGR-2 and EGR-3 deficient mice develop severe systemic autoimmune disease and display increased inflammatory cytokines. **(A)** Measurement of onset of autoimmune disease by serum IFN-γ and self-reactive antibodies comparing WT, K2/3, Tg, K2 and K3.**(B)** ELISA serum levels of Ig from WT and K2/3 3 month old mice. **(C)** Measurement of self-reactive antibodies and protein in urine from 3 month old WT and K2/3 mice **(D)** Texas Red anti-mouse Ig labelled for IgG deposits in the glomeruli from 3 month old K2/3 mice. **(E)** WT, K2/3 and Tg CD4 T cells after stimulation with anti-CD3 and anti-CD28 for 16 hours were measured for cytokine levels in mRNA expression by RT-PCR. The data from **[A]** was representative of 4 mice from two experiments. The data from **[B]**, and **[C]** were the mean standard deviation from four mice. **[D]** was representative of four mice and **[E]** was measured relative to β-actin from three experiments. ND= Not detected (the figures are taken from Li *et al.,* 2012, in which I am one of the co-authors).

3.3 Susceptibility of EAE autoimmune disease in EGR-2cKO mice

EGR-2 and EGR-3 deficient mice developed autoimmune disease where a deficiency in both transcription factors resulted in an earlier onset of autoimmunity compared with EGR-2cKO (Figure. 5). EGR-2cKO mice were used to see if we can induce autoimmune disease. There are many autoimmune disease models including EAE, adjuvant arthritis and systemic lupus erythematosus (Farine., 1997). We decided to use the EAE mouse model because you can observe early stages of the disease and monitor the progression using clinical scoring (Farine., 1997). To study the susceptibility of autoimmune disease using the EAE mouse model in 10-12 week WT and EGR-2cKO mice, immunization with MOG_{35-55} peptides was used. These peptides are autoantigens derived

from CNS myelin and they are involved in inducing a high T and B cell response which manifests into demyelination and plaque formation giving rise to tissue damage and an inflammation immunopathology. The mice were induced with pertussis toxin and monitored up to 30 days and clinical symptoms from clinical scoring were recorded. The early signs of development of the disease were similar from both WT and EGR-2cKO at day 9 (Figure. 6, A). However, the progression of the disease was more severe in the EGR-2cKO mice as shown by a higher clinical score and symptoms compared with WT (Figure. 6, A). The pathology of EAE disease was examined by H and E staining on WT and EGR-2cKO from CNS tissues with clinical scoring of I. The staining results revealed that there was a massive infiltration of mononuclear cells situated around the spinal cord in the EGR-2cKO cells compared with WT (Figure. 6, B). The infiltration of cells found in the EGR-2cKO CNS tissues confirmed the severity of clinical symptoms and scores we obtained (Figure. 6, A, B). To examine the phenotype of the infiltrating cells found in the spinal cord, mononuclear cells were isolated for CD4+ cells and double stained with IL-17 and IFN-γ, cytokines expressed in autoimmune disease. The results showed a fivefold increase in expression of IL-17 from CD4+ cells in EGR-2cKO compared with WT (Figure. 6, C). The results indicated that the mice were more susceptible to the development of autoimmune disease when EGR-2 is deleted.

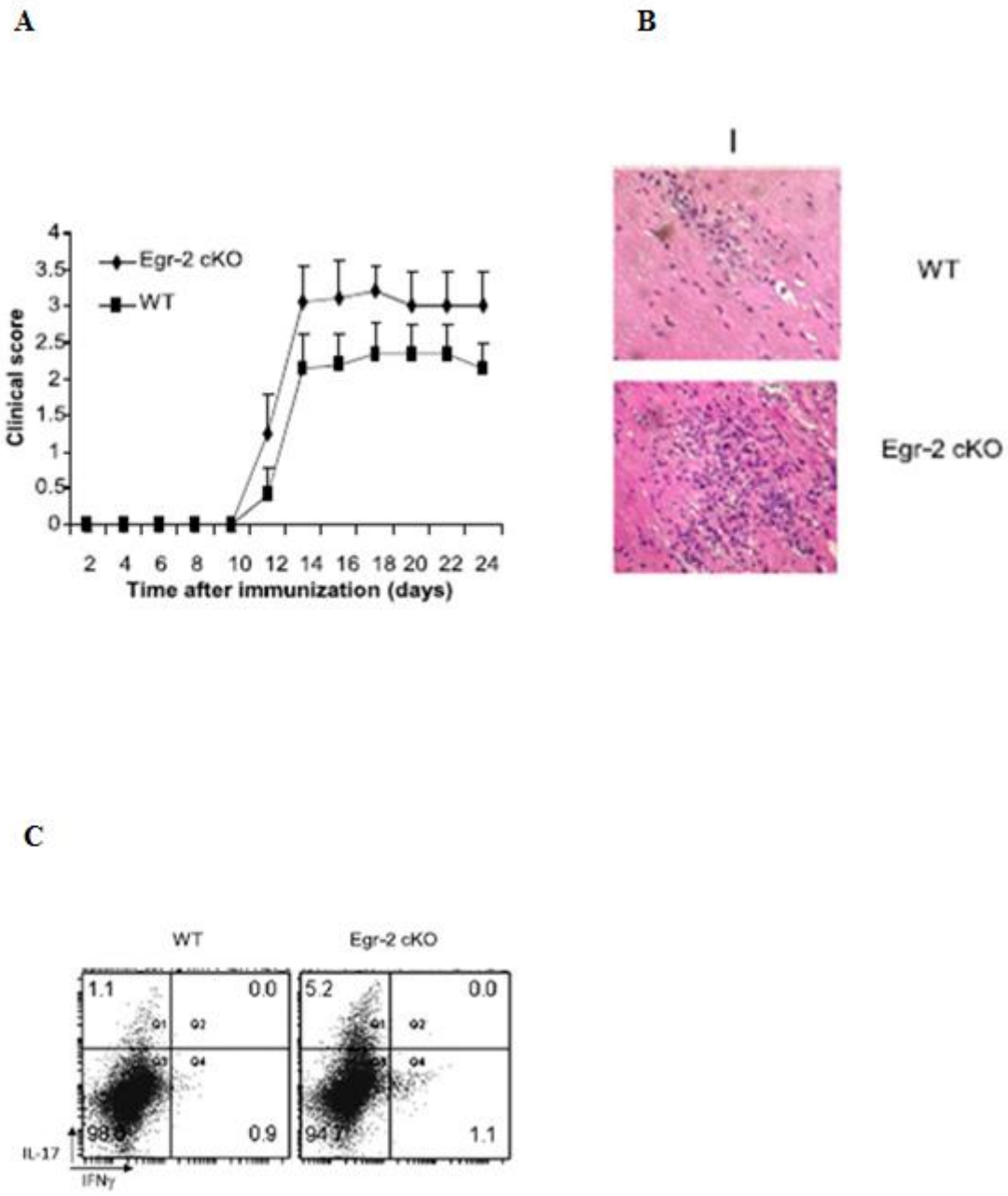

Figure 6: EGR-2cKO has increased susceptibility to develop EAE disease. **(A)** Data showing clinical scoring of stage and symptoms after immunisation with MOG_{35-55} from five WT and EGR-2cKO mice with as shown in Materials and Methods. **(B)** H and E staining from CNS tissues with a clinical scoring of I at 30 days post immunization at x10 modification. **(C)** CNS tissues from five immunized mice were isolated for mononuclear cells and stained with CD4, IFN-γ and IL-17A and gated on CD4+ cells. All the data

presented were representative of three experiments on five mice in each (the figures are taken from Miao *et al.,* 2013, in which I am one of the co-authors).

3.4 Role of EGR-2 and -3 in T cell receptor signalling

3.4.1 EGR-2 and -3 double deficiency results in impaired proliferative responses of T cells to antigen receptor stimulation

In Figure. 5, E we showed defective IL-2 mRNA expression in K2/3 mice. Similarly, SLE patients showed impaired in-vitro stimulation after antigenic stimulation (Gottlieb *et al.,* 1979). In rheumatoid arthritis patients, ex-vivo analysis also found a defect in the ability of the T cells to proliferate (Wagner *et al.,* 2004). To study the role that these transcription factors play in regulating T cell proliferation after antigen receptor stimulation, T and B cells isolated from splenocytes of K2, K3, WT, Tg and K2/3 mice were stimulated in-vitro with anti-CD3 and anti-CD28 for T cells and IgM for B cells aiming to mimic antigen stimulation. The proliferation was measured by BrdU or [^{3}H]thymidine incorporation assays. BrdU is a synthetic thymidine that gets actively incorporated into newly synthesised genomic DNA during S phase and it can be detected by BrdU specific antibody conjugated with a fluorescent marker and visualised using Flow Cytometry. The other technique used to measure cellular proliferation was [^{3}H]thymidine. This technique uses radioactive nucleoside [^{3}H]thymidine that gets actively incorporated into newly synthesised DNA during DNA synthesis. The difference between BrdU and [^{3}H]thymidine is that BrdU labelling is specific and can identify what cells are dividing compared to undivided, however, [^{3}H]thymidine cannot distinguish specific cells from a population that are dividing. The mice from K2, K3, K2/3 and Tg CD4 T cells were stimulated for three

days with anti-CD3 and anti-CD28 for T cells. K2 and K3 cells showed similar proliferation compared with WT, indicating that single knockout of these transcription factors did not have an effect on T cell proliferation (Figure. 7, A). Significantly K2/3 cells showed a defect in cell proliferation compared with WT (Figure. 7, A). Similar results were shown by ^{3}H-TdR incorporation (Figure. 7, B). In contrast, EGR-2 expressing CD4 T cells from Tg showed a fourfold increase in cell proliferation following antigenic stimulation in both BrdU and ^{3}H-Thymidine incorporation assays (Figure. 7). This demonstrates that EGR-2 and EGR-3 are important for activating T cell proliferation after mitogenic stimulation. The absence of both EGR-2 and EGR-3 causes a severe defect in the ability of cells to proliferate after mitogenic stimulation.

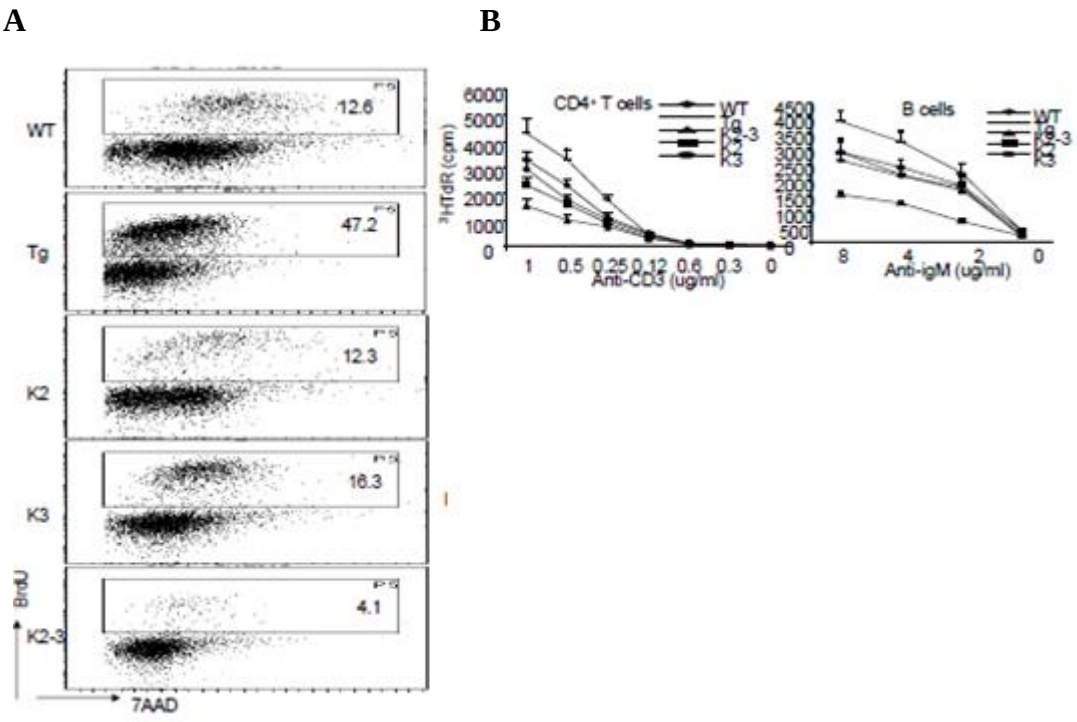

Figure 7: EGR-2 and -3 plays a role in regulating T cell receptor signalling. **(A)** Proliferation results from BrdU incorporation into naïve CD4 T cells from WT, K2, K3, Tg and K2/3 after stimulation with anti-CD3 and anti-CD28 for three days. **(B)** CD4 and B cell

proliferation results measured by ^{3}H-TdR incorporation after anti-CD3 and anti-CD28 stimulation for three days for T cells and IgM and LPS for B cells. Data represented three independent experiments (figure is taken from Li *et al.,* 2012, in which I am a co-author).

3.4.2 The impaired proliferation in EGR-2 and -3 deficiencies is due to defective AP-1 activation in both T and B cells

As previously shown EGR-2 and EGR-3 deficient mice have the impaired ability to proliferate after antigen receptor stimulation (Figure. 7, A) and play important roles in the development of B and T cells (Li *et al.,* 2011). EGR-2 and EGR-3 have been shown to be targets for NFAT which mediates calcineurin pathway in TCR signalling and also induces FasL expression in T cells (Rengarajan *et al.,* 2000). To identify the role that both of these transcription factors play in TCR signalling we explored which of the signalling pathways were affected. TCR signalling is a series of multiple steps starting with T cell and antigen presenting cell engagement, followed by CD3 phosphorylation and further downstream signalling to activate MAP kinase proteins such as ERK, Fos and AP-1 (Rengarajan *et al.,* 2000). Data has shown that AP-1, NFAT and NF-κB are activated once TCR engagement has been initialised which can then go on to induce IL-2; therefore we wanted to explore these signalling proteins (Kim *et al.,* 2009). We observed ERK, NFκB, NFAT and AP-1 signalling components for TCR signalling. Naïve T and resting B cells from WT and K2/3 knockout cells were either stimulated or un-stimulated with anti-CD3 and anti-CD28 or IgM (Figure. 8, A). Cytoplasmic extracts were used to measure ERK phosphorylation and other proteins such as AP-1, NFAT and NFκB are present in the nucleus so nuclear extracts were used for those. To check if there was an association of

EGR-2 and EGR-3 with these proteins, EMSA was performed on stimulated and un-stimulated T and B cells. EMSA is able to detect DNA and protein complexes from nuclear extracts to a consensus probes for a particular binding sequence. The EMSA technique is based on the fact that free DNA and probe will migrate faster through the gel, whereas protein-DNA complexes will migrate slower. The addition of another antibody can cause a supershift which can reveal a further protein involved in the complex that will migrate even slower through causing a supershift.

Erk detection was used as a control to show cellular expression before T cell activation in WT and K2/3 stimulated and un-stimulated T and B cells (Figure. 8, A, row 2). Consensus probes for AP-1, NFκB and NFAT were used in the binding reaction with nuclear extracts from WT or K2/3 T and B cells stimulated or un-stimulated. The presence of proteins pERK, NFκB and NFAT and AP-1 were shown to be only produced after antigenic stimulation, whereas the negative control using un-stimulated WT or K2/3 cells showed no expression of these proteins (Figure. 8, A, lane 1, 2, 5 and 6). The results indicated that MAP Kinases (phosphorylation of Erk), NFκB and NFAT were not defected in B and T cells in K2/3 as compared with WT (Figure. 8, A, row 2, 4 and 5). However, AP-1 activation was impaired in K2/3 samples compared to WT (Figure. 8, A). Interestingly, despite reduced AP1 binding in EMSA, the results showed a lower alternative binding complex in B and T cells from K2/3 mice compared with WT (Figure. 8, A and B). In order to compare the levels of differences, the intensity of AP1 binding complexes detected in EMSA was measured by the phosphorimager. The relative intensity of AP1 binding complexes in CD4 T cells from WT was threefold higher than that of EGR-2 and EGR-3 deficient CD4 T cells after normalization to the internal control SP1 (Figure. 8, B). Thus, the activity of classic AP-1 was impaired in CD4 T cells from K2/3 mice, demonstrating

EGR-2 and EGR-3 are important for the induction and/or maintenance of AP-1 activation in response to antigen receptor stimulation.

To further confirm AP1 binding anti-cFos and anti-JunB antibodies were used to do super-shift. The anti-cFos antibody shifted up in WT CD4 cells while in K2/3 CD4 cells; there was no supershift (Figure. 8, C). Interestingly the EMSA results (Figure. 8, A and C) showed that typical AP-1 activation was impaired in the K2/3 samples however, the addition of anti-JunB antibody caused a supershift in the EMSA gel revealing that this protein was important in the AP-1 binding complex in K2/3 compared with WT that showed no change (Figure. 8, C). AP-1 activation is composed of many different proteins that dimerise with each other to bind to DNA binding sequences that can activate other genes. Here we showed that part of the AP-1 defect in EGR-2 and EGR-3 deficient mice did involve the protein JunB. However, there are lots of other proteins in the AP-1 family. So further analysis using the AP-1 DNA binding probe against anti-cFos in WT or K2/3 revealed that c-Fos appeared to not be important for the deficiency of AP-1 activation seen in K2/3 compared with WT (Figure. 8, C). This indicated a possible link between EGR-2 and EGR-3 binding with JunB for AP-1 activation.

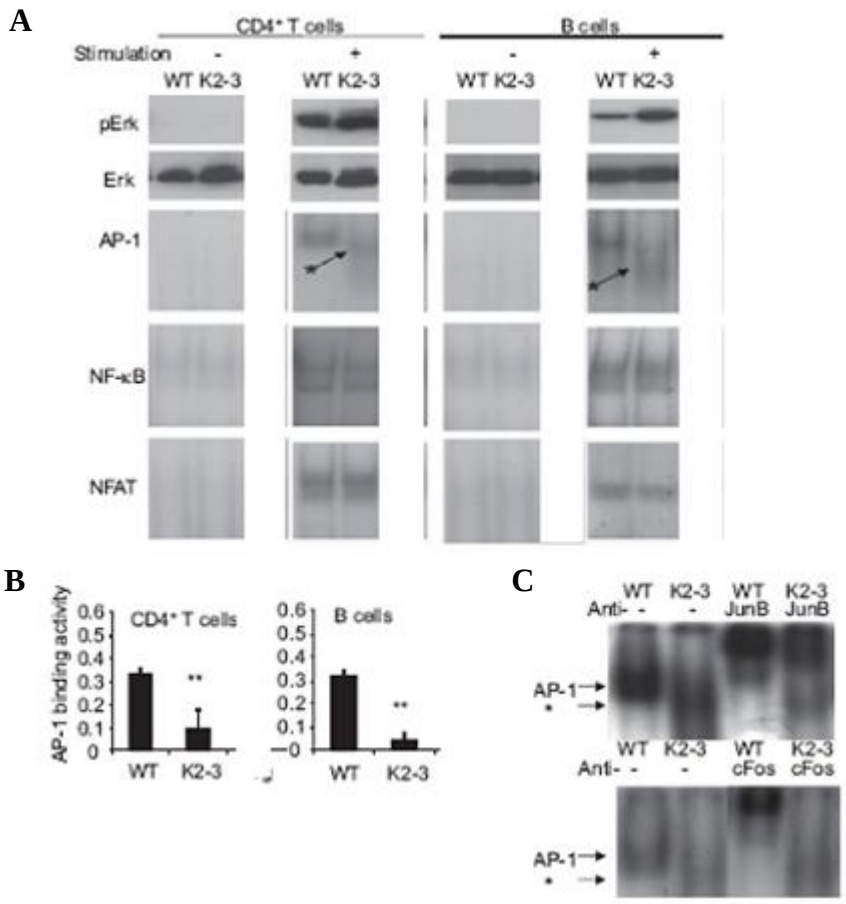

Figure 8: EGR-2 and -3 KO show reduced AP-1 activation in lymphocytes. **(A)** Naive CD4 and resting B cells were stimulated with anti-CD3 and anti-CD28 for T cells or anti-IgM for B cells. Cytoplasmic extracts was measured for Erk phosphorylation. Nuclear extracts were subjected to EMSA for NFκB, NFAT and AP- 1 with the consensus probe and the arrows are indicative of other complexes that are also bound to the AP-1 probe. **(B)** Phosphorimager results to quantify the AP-1 intensity bands from **(A)** and normalised by using SP1 internal control. **(C)** EMSA results from anti-CD3 and anti-CD28 stimulated CD4 T cells, extraction of nuclear content with AP-1 DNA probe against JunB and c-Fos. Data from **[A]** and **[B]** are representative of three independent experiments, whereas **[C]** is representative of two experiments (the data represented is taken from Li *et al.,* 2012, in which I am one of the co-authors).

3.5 The role of Batf in the regulation of AP-1 signalling

3.5.1 Batf is part of the lower AP-1 binding complex

The impaired AP-1 activation in K2/3 deficient B and T cells indicated that EGR-2 and EGR-3 may be involved directly in the regulation of AP-1 activation. The AP-1 complex is composed of many different proteins that form homo or hetero-dimers with each other to bind to AP-1 consensus DNA. Batf is a small nuclear protein that is expressed in hematopoietic organs such as spleen, thymus and lymph nodes. Batf is a 125 amino acid nuclear protein and is a member of the AP1 family (Williams *et al.,* 2001). However, it does not contain a trans-activation domain (Williams *et al.,* 2001). Batf forms heterodimers with Jun proteins which bind to consensus AP-1 DNA. However, the Batf-AP1 complexes do not have AP1 function as they cannot transactivate the target genes. Also forced expression of Batf in fibroblast cells blocks cellular transformation of the cells and increases the longevity of the cells in culture (Williams *et al.,* 2001). This was confirmed by Echlin *et al.,* 2000 group who isolated portions of the Batf protein and fused with the DNA binding domain of the yeast GAL4 promoter. They demonstrated that Batf could not trans-activate the CAT reporter plasmid from the different portions of the Batf protein. Batf can however, regulate Th17 differentiation, by binding with the IL-17 promoter (Schraml *et al.,* 2009). EMSA results from whole Th17 cell extracts subjected to the AP-1 DNA probe, revealed two complexes in the Batf positive Th17 cell extracts (Schraml *et al.,* 2009). Only the upper complex was found in Batf knockout Th17 cell extracts. To show Batf directly interacts with AP-1, supershift analyses was performed on CD2-N-Flag-Batf-transgenic Th17 cell extracts using the anti-flag antibody (against Batf) which supershifted the lower complex, revealing the lower complex that binds to the AP-

1 DNA probe from Th17 cell extracts contains Batf (Schraml *et al.,* 2009). Batf is also essential for thymic NKT cell development and a deficiency in Batf leads to inadequate differentiation of Th17 T cells (Logan *et al.,* 2012).

The EMSA results shown in Figure. 8, A and C revealed a lower complex from AP-1 binding in K2/3 T and B cells. As already shown from Schraml *et al.,* 2009 data, Batf was shown to be in the lower complex from the AP-1 binding probe in Th17 cell extracts. To investigate whether Batf may also be involved in the alternative lower complex seen in the K2/3 T and B cell EMSA results (Figure. 8, A), EGR-2, Batf or double transfected HEK293 cells were used to perform EMSA to examine AP-1 DNA binding activity (Figure. 9). An AP-1 DNA probe was labelled with ^{32}P and used for binding assay. Figure. 9, lane 1 represented un-transfected negative control cells, showing no AP-1 binding when neither EGR-2 nor Batf were present. Figure. 9, lanes 2 and 3 represented positive controls from either EGR-2 transfected or Batf transfected HEK293 cells showing that EGR-2 directly bound with AP-1 (lane 2) or Batf directly bound with AP-1 forming an alternative lower complex (lane 3). Figure. 9, lane 4 showed the HEK293 cells co-transfected with EGR-2 and Batf blocked the binding to the consensus AP-1 probe. To test which complex contained Batf, supershift analysis using an anti-myc antibody (for Batf) in HEK293 cells transfected with Batf was used (Figure. 9, lane 5). The addition of an anti-Batf antibody supershifted the lower AP-1 binding complex. To test whether another molecule, JunB was important in the AP-1 complex from EGR-2 transfected HEK293 cells, anti-JunB antibody was used for supershift analyses (Figure. 9, lane 6). The results showed that the addition of anti-JunB antibody supershifted the upper AP-1 complex (Figure. 9).

The results revealed EGR-2 was part of the AP-1 binding complex, whilst Batf blocked the conventional AP-1 complex; however, it was part of the alternative lower AP-

1 consensus binding complex. Co-transfection of Batf and EGR-2 blocked the conventional AP-1 consensus DNA binding.

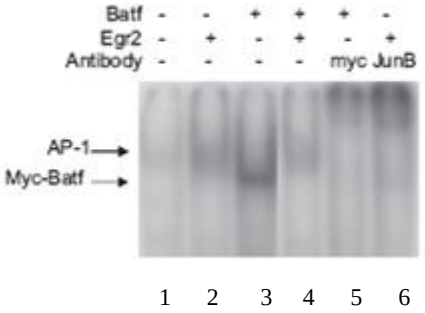

Figure 9: Batf is part of the lower complex in AP-1. HEK293 cells were transfected with Flag-tagged EGR-2 or Flag-tagged EGR-2 plus Myc-tagged Batf. The lysates were immunoprecipitated with anti-Flag-tag or anti-Myc-tagged antibodies. EMSA supershift analysis was performed with the AP-1 DNA probe. The data is representative of three experiments (Li *et al.,* 2012).

3.5.2 Direct interaction between EGR-2 and Batf

To examine whether EGR-2 can interact with Batf, HEK293 cells were co-transfected with flag tagged EGR-2 plus myc tagged Batf (Figure. 10). Flag or myc tagging allows detection of tagged protein with antibodies specific to the tag. The expression of both proteins was confirmed by immunoblotting with antibodies specifically to Flag or Myc, respectively (Figure. 10). To examine the interaction, the total protein lysates from transfected cells were immunoprecipitated with antibody either against Flag (for Flag

EGR-2 cells) or Myc (for Myc Batf cells), this allowed the pulling out of a tagged molecule and the molecules associated with it. The immunoprecipitates were separated and transferred onto a nitrocellulose membrane with antibodies for anti-EGR-2 or anti-Myc for anti-Flag added to the precipitates, this allowed you to see whether EGR-2 and Batf presented in each other's precipitates. Indeed, EGR-2 was detected in the precipitates of myc-tagged Batf while Batf was detected in the EGR-2 precipitates (Figure. 10). To further confirm the interaction, a reciprocal co-immunoprecipitation test was carried out on the same lysate. The results were consistent (Figure. 10). The immunoblotting with EGR-2 on total lysate from immunoprecipitated Flag tagged EGR-2 cells was used as a control to show total EGR-2. The results showed that EGR-2 did directly bind with Batf (Figure. 10).

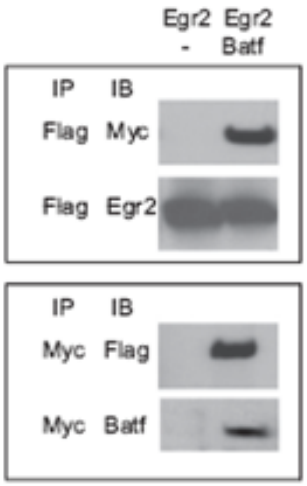

Figure 10: Direct interaction of EGR-2 with Batf. The same HEK293 cells transfected as indicated (figure. 9) were used to perform Western Blot with anti-Myc and anti-EGR-2 for the anti-Flag precipitated samples or anti-Flag anti-Batf for anti-Myc precipitated samples. The data is representative of two experiments (Li *et al.,* 2012).

3.5.3 siRNA knockdown of Batf, restores AP-1 consensus DNA binding in K2/3 T cells

The results so far have shown that when EGR-2 binds with Batf, AP-1 activation is inhibited (Figure. 9). To confirm this, AP-1 activation should be restored if Batf is removed, thus knockdown of Batf was created. Knockdown of Batf was achieved by the addition of short interfering oligonucleotides (siRNA) against Batf, EGR-2 and EGR-3 mRNA. By adding these siRNA's against specific sequences of mRNA, those target genes will have reduced expression. As a negative control, siRNA not specific for any mRNA (con) was also transfected into the cells, to serve as a basal level for comparison with targeted siRNA. To check whether mRNA expression is reduced, RT-PCR was performed. CD4 T and CD19 B cells were transfected with siRNA (control) or siRNA specific for Batf from WT or K2/3 T cells. RT-PCR analysis was performed on Batf expression (Figure. 11, A). The cells were stimulated for 16 hours with anti-CD3 and anti-CD28 for T cells or IgM for B cells and un-stimulated cells served as a control. From the un-stimulated B and T cell RT-PCR results, in both WT and K2/3 cells, Batf expression was low because Batf will only be expressed after T cell receptor activation (Figure. 11, A). From the stimulated results, WT and K2/3 T and B cells transfected with siRNA (con) showed high expression of Batf (Figure. 11, A). The WT and K2/3 cells transfected with siRNA specific for Batf showed efficient knockdown whereby Batf mRNA expression was reduced (Figure. 11, A).

Knockdown of Batf was proved by reduced Batf mRNA expression levels, to further confirm reduced expression levels, Western Blot was performed (Figure. 11, B). Protein expression levels confirmed the RT-PCR results. WT and K2/3 T and B cells transfected with siRNA (con) and blotted with anti-Batf antibody showed normal Batf protein expression. However, cells transfected with siRNA specific for Batf,

immunoblotted with anti-Batf antibody showed very low protein expression (Figure. 11, B). Immunoblotting with β-actin was used as a control to show basal protein expression levels in WT and K2/3 transfected cells (Figure. 11, B). The RT-PCR and Western Blot results showed that siRNA knockdown of Batf in WT and K2/3 cells was efficient (Figure. 11).

Previously Batf has been shown to inhibit AP-1 activation and slow down cell growth (Lio *et al.,* 2011). This group found that M1 cells (Mouse myeloid leukemia cell line) transfected with Batf-RNAi and monitored for cell growth for 7 days in the absence or presence of LIF/IL-6 cytokines grew slower with fewer cells compared with WT cells (Lio *et al.,* 2011). As Batf inhibits AP-1 activation, knockdown of Batf should restore lymphocyte proliferation in K2/3 cells, thus ^{3}H-thymidine incorporation and IL-2 expression was explored in those knockdown cells. WT and K2/3 CD4 and CD19 cells were transfected with siRNA as already described and stimulated with anti-CD3 and anti-CD28 for T cells and IgM for B cells with the addition of ^{3}H-thymidine for 48 hours. The negative control used was un-stimulated cells, where both WT and K2/3 T and B cells showed reduced cell proliferation and low IL-2 expression as expected (Figure. 11, C). In contrast, CD4 and B cells from WT transfected with control siRNA showed high levels of proliferation, however, the same transfected cells from K2/3 showed low proliferation levels and IL-2 expression, due to impaired AP-1 activation (Figure. 11, C). Also CD4 and B cells stimulated from WT cells transfected with siRNA for Batf showed similar proliferation levels compared with control siRNA. Interestingly from K2/3 results, proliferation and IL-2 expression was rescued after knockdown of Batf, proving that EGR-2 binding with Batf does have a functional effect in lymphocytes (Figure. 11, C). As Batf represses AP-1 activation, to observe whether knockdown of Batf can restore AP-1

activation, the same samples as described were used for EMSA. The results from WT CD4 and B cells transfected with control or Batf siRNA, showed normal AP-1 activation (Figure. 11, C). On the other hand, K2/3 T and B cells transfected with control siRNA showed reduced AP-1 activation as Batf inhibits AP-1 activation; however formation of a lower complex was present. K2/3 cells transfected with siRNA for Batf revealed the formation of two complexes, showing that the lower complex contains Batf (Figure. 11, D).

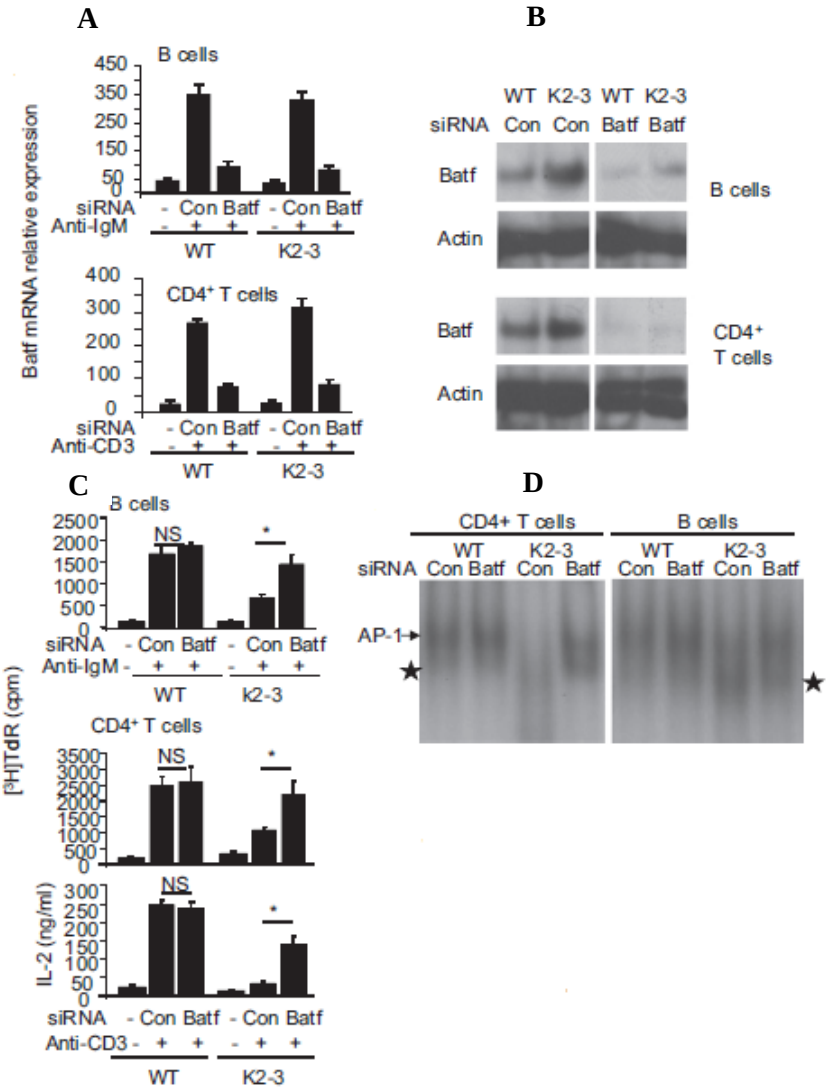

Figure 11: Batf is knocked down in EGR-2 and -3 deficient cells restored AP-1 activation. **(A)** and **(B)** WT and K2/3 CD4 and CD19 cells were transfected with control siRNA (con) or Batf specific siRNA (Batf) to show knockdown of Batf. The cells were stimulated with either anti-CD3 and anti-CD28 or IgM for 16 hours and mRNA expression levels of Batf were measured to confirm knockdown of Batf **(A)** and at protein level **(B)**. **(C)** CD4 and CD19 cells from WT or K2/3 mice were measured for IL-2 and proliferation from transfected cells with control or Batf siRNA after anti-CD3 and anti-CD28 or IgM for 48 hours. **(D)** EMSA was performed on the above transfected cells with the AP-1 DNA probe. The data is representative of three experiments **[A, B and C]** or two experiments **[D]** (the data has been taken from Li *et al.,* 2012, in which I am one of the co-authors).

Chapter 4: General Discussion

4.1 A deficiency in EGR-2 and EGR-3 results in systemic autoimmune disease

The causes of autoimmune disease are still unclear and a lot of research is being done to elucidate the cause and possible treatment strategies. Autoimmune mouse models are extensively used to study autoimmune disease. There are three main types of animal models used to study autoimmune disease, firstly the spontaneous model, secondly, the inducible model and lastly genetically engineered mice (Morel., 2004). The spontaneous model is difficult to manipulate towards human clinical disease. However, non-obese diabetic (NOD) mouse do develop type 1 diabetes spontaneously (Morel., 2004). The lupus mouse model is NZB plus NZW (Farine., 1997). The inducible method is more preferred as autoantigens are administered to the mouse and then stimulated to induce autoimmune disease. The well-established inducible models include EAE for MS and collagen-induced arthritis (CIA) for rheumatoid arthritis (Farine., 1997; Morel., 2004). Another arthritis mouse model is type II collagen-induced arthritis (Farine., 1997). The mouse model for Hashimoto's thyroiditis is experimental autoimmune thyroiditis (Farine., 1997). Lastly, the genetically engineered mice are those that have knockout or over-expression of a particular gene of interest and typically being used for SLE (Morel., 2004). Therefore it will be of interest to unravel more genes and models to study the mechanisms of autoimmune disease.

Previously our group have shown that older EGR-2ckO mice develop lupus-like autoimmune disease and secrete pro-inflammatory cytokines with high levels of IFN-γ and IL-17 at 15 months of age (Zhu *et al.,* 2008), whilst EGR-3KO mice did not develop autoimmune disease symptoms (Li *et al.,* 2012). Our group are the first to develop an EGR-2 and EGR-3 deficient mouse model which showed an earlier onset of autoimmune

disease at 2 months compared to single EGR-2cKO. EGR-2 and EGR-3 deficient mice displayed systemic autoimmune disease with a multiorgan immunopathology and excessive production of autoantibodies in the kidney indicating the possible dose effect of EGR-2 and EGR-3 on other functionally related transcription factors. Previously polymorphisms of EGR-2 show increased susceptibility for the development of systemic lupus disease (Myouzen *et al.,* 2010). Introducing single nucleotide polymorphisms in EGR-2 caused an increased susceptibility to develop SLE shown by a high expression of those genes prone for the disease (Myouzen *et al.,* 2010). Similarly, inducing SLE associated autoimmune disease in mice, displayed defective expression of EGR-2 and EGR-3 in auto reactive T cells (Sela *et al.,* 2008). The defective expression of EGR-2 shown by this group (Sela *et al.,* 2008) is also consistent with what we observed in the expression of EGR-2 in MS patients, where expression levels of EGR-2 were reduced (Miao *et al.,* 2013) indicating the importance of this gene in regulating autoimmune disease.

It has been previously reported that EGR-2 is important for T and B cell development, and forced expression of EGR-2 renders low numbers of DP cells in thymus and immature B cells in the bone marrow, however percentages of mature T and B cells were increased (Li *et al.,* 2011). In our model EGR-2 and EGR-3 deficient mice showed no developmental defects (Li *et al.,* 2012).

To further confirm the role of EGR-2 in controlling autoimmune disease we used EAE which is the mouse model for MS. The data showed that EGR-2cKO mice developed more severe symptoms of the disease from clinical scoring compared with WT. There was an excessive accumulation of mononuclear cells in the spinal cord. The CD4 T cells from these mice displayed increased pro-inflammatory cytokines IL-17, TNF-α, IL-4 and IFN-γ

associated with EAE disease (Bar O' *et al.,* 1999; Stanislaus *et al.,* 2005). In conclusion EGR-2 and EGR-3 intrinsically regulate and protect lymphocytes against the development of autoimmune disease.

4.2 Mechanism for the development of autoimmune disease in EGR-2 and EGR-3 deficient mice

4.2.1 How chronic inflammation plays the role for autoimmune disease development

EGR-2 and EGR-3 deficient mice display lethal autoimmune disease and excessive production of pro-inflammatory cytokines. Despite this, antigen receptor stimulation of T and B cells from EGR-2 and EGR-3 deficient mice displayed defective proliferation and IL-2 production. There was an excessive production of IFN-γ, GM-CSF, IL-17A and IL-17F in the EGR-2 and EGR-3 deficient mice. These cytokines have been shown to be increased in autoimmune disease (Langrish *et al.,* 2005; Harris *et al.,* 2007). GM-CSF has been further shown to be present in synovial joints from autoimmune disease patients (Hamilton., 2002).

Previously another group have shown high expression levels of IL-17 and IFN-γ in EGR-2cKO mice similar to what we have shown (Iwasaki *et al.,* 2013), which confirms EGR-2's important role in cell intrinsic regulation of autoimmune disease.

Pro-inflammatory cytokines have been linked to causing inflammation at the site of tissue damage. Cytokines including TGF-β, IFN-γ, IL-8 and IL-6 help recruit and activate inflammatory type cells like macrophages and endothelial cells. The accumulation of cells and induction of proximal and distal cytokines causes swelling and redness surrounding the local site, to induce an inflammatory cytokine cascade, which further cause's tissue

damage and eventually leads to organ dysfunction (Blackwell and Christman., 1996; Gogos *et al.,*2000; Sultani *et al.,* 2012). This further suggests that the increased expression of pro-inflammatory cytokines is causative of the inflammatory immunopathology observed in EGR-2 and EGR-3 deficient mice.

EGR-2 has been shown to induce Treg (iTreg) that express lymphocyte activation gene-3(LAG-3) (Okamura *et al.,* 2012). FoxP3 deficient mice exhibited splenomegaly and multi-organ autoimmune disease, where FoxP3 is expressed in naturally occurring Tregs (nTreg) and has been associated with the maintenance of tolerance and protecting against autoimmune disease (Fontenot *et al.,* 2003). EGR-2 has also been indicated to be critical for the regulation of IL-10 producing CD4+ Treg cells. EGR-2 transduced cells produce an immunosuppressive function in-vivo suggesting that EGR-2 plays a cell extrinsic mechanism in IL-10 producing CD4+ Treg cells however in our EGR-2 and EGR-3 deficient mice there were no difference for Treg cells compared with WT (Li *et al.,* 2012).

In fact the immunopathology observed in K2/3 mice is not due to excessive proliferation; it is due to uncontrolled production of pro-inflammatory cytokines. EGR-2 and EGR-3 directly regulate SOCS1 and SOCS3 expression (Li *et al.,* 2012). STAT1 and STAT3 are regulated by their suppressor transcription factors SOCS1 and SOCS3 which are important for Th1 and Th17 differentiation (Anderson *et al.,* 2006). We found hyperactivation of STAT1 and STAT3 in EGR-2 and EGR-3 deficient B and T cells after mitogenic stimulation (Li *et al.,* 2012). STAT-3 deficient mice did not develop severe EAE disease compared with WT (Harris *et al.,* 2007). SOCS1 deficient mice contained activated T cells and responded to cytokines without antigen receptor stimulation to produce IFN-γ, they developed multiorgan inflammatory pathology (Marine *et al.,* 1999; Croker *et al.,* 2004) very similar to what we found in EGR-2 and EGR-3 deficient mice. EGR-2 and EGR-3

deficient lymphocytes showed low expression of SOCS1 and SOCS3 and high STAT1 and STAT3 expression and discovered that the promoters of SOCS1 and SOCS3 contain EGR-2 binding sites (Li *et al.,* 2012). Another group have reported that reduced IL-11 cytokine expression leads to hyperactive STAT1 and STAT3 expression resulting in inflammation and gastric tumorgenesis (Ernst *et al.,* 2008). We show that an immunopathology exists once you have hyperactivation of STAT1 and STAT3 in EGR-2 and EGR-3 deficient mice (Li *et al.,* 2012).

The link between chronic inflammation and autoimmune disease shows that elevated levels of inflammatory cytokines are observed in autoimmune diseases (Langrish *et al.,* 2005; Harris *et al.,* 2007). Studying the rheumatoid arthritis model, chronic inflammation plays an important role in the disease and arthrosclerosis in coronary heart disease (Abou-Raya *et al.,* 2007). They show that inflammation and dysregulation in T and B cells causes autoimmune disease and tissue destruction (Abou-Raya *et al.,* 2007). Importantly IL-6, pro-inflammatory cytokine has been implicated in several autoimmune diseases like rheumatoid arthritis and chronic inflammatory disorders to show the link between inflammation and autoimmune disease (Ishihara and Hirano., 2002).

In conclusion, EGR-2 and EGR-3 play an intrinsic role in suppressing inflammatory mediated immunopathology in autoimmune disease.

4.2.2 Th17 mediated inflammatory autoimmune disease

Although EGR-2 and EGR-3 have been shown to mediate the STAT1 and STAT3 inflammatory pathway, STAT3 is also important for Th17 differentiation and pro-inflammatory cytokines (Harris *et al.,* 2007). IL-17 is a pro-inflammatory cytokine that has

been shown to be important for inducing inflammation. IL-17 deficient mice show reduced inflammatory responses in the CIA mouse model for rheumatoid arthritis (Nakae *et al.,* 2003) and inducing autoimmune disease in EAE (Langrish *et al.,* 2005). It has been previously shown that STAT3 is vital for Th17 mediated autoimmune disease, as suppression of STAT-3 can decrease Th17 mediated differentiation and drive a Th1 response. MS patients showed marked increases in mRNA expression levels of IL-17 in mononuclear cells and cerebrospinal fluid showing the importance of IL-17 in inflammatory induced autoimmune disease (Matusevicius *et al.,* 1999). Loss of SOCS3 in T cells resulted in increased Th17 differentiation and IL-10 production (Taleb *et al.,* 2009). Previously, IL-23 a cytokine in CD4 T cells can induce differentiation into Th17 cells that produce IL-17, IL-17A, TNF, and IL-6 production (Langrish *et al.,* 2005). EGR-2cKO mice displayed increased levels of the pro-inflammatory cytokines IFN-γ, IL-17A, IL-17F, IL-6 and IL-21, signature cytokines produced in Th17 CD4 T cells suggesting that EGR-2 is important in regulating the expression of pro-inflammatory cytokines (Miao *et al.,* 2013).

Th17 differentiation has also been shown to be driven by Batf, where Schraml *et al.,* 2009 group found that Batf is important for IL-17 production and they confirm Batf is highly expressed in Th17 cells. The Batf deficient mice showed normal Th1 and Th2 differentiation with normal or reduced expression of IL-4 for Th2 and IL-12 for Th1 CD4 producing cells to illustrate that only the Th17 CD4 producing cells are affected (Schraml *et al.,* 2009). However, this group show that Batf deficient mice have impaired Th17 differentiation and were resistant to developing EAE disease. From EGR-2cKO mice we observed normal Th1 and Th2 differentiation, whilst Th17 differentiation was significantly increased suggesting that only the Th17 lineage was affected (Miao *et al.,* 2013).

CD4 T cell lineage commitment is complex and the ability of Th CD4 T cells to switch between subtypes has been reported. The transcription factor T-bet has been shown to be important for Th1 differentiation whereas GATA-3 has been shown to be important for Th2 differentiation (Zhou *et al.,* 2009). Th1 CD4 T cells produce IFN-γ, IL-12 and Th2 T cells producing IL-4 signature cytokines (Zhou *et al.,* 2009). Expression of these cytokines suppresses IL-17 cytokine expression, thus showing that Th lineages are distinct from one another (Zhou *et al.,* 2009). When we explored the role of EGR-2 in the Th CD4 lineages, we observed that EGR-2 was expressed in the presence of TGF-β and IL-6, important Th17 pro-inflammatory cytokines (Zhou *et al.,* 2009; Miao *et al.,* 2013).

TGF-β has been associated in systemic autoimmune disease similar to what our EGR-2 and EGR-3 deficient mice displayed showing the importance of TGF-β in inflammatory induced autoimmune disease (Leveen *et al.,* 2002; Miao *et al.,* 2013). EGR-2 expression was not induced in the presence of Th1 and Th2 inducers IL-12, IL-4 and IFN-γ which further suggests that EGR-2 regulates Th17 differentiation distinctly (Miao *et al.,* 2013).

Although Th17 CD4 T cells have been established to be important for mediating inflammatory autoimmune disease pathology, increasingly Tregs have also been shown to have a significant importance. The balance of Th17 to Treg is important for the maintenance of homeostasis and anergy (Eisenstein and Williams., 2009) to prevent the development of autoimmune disease. Th17 is important for inducing the release of pro-inflammatory cytokines that in turn increase disease pathology in autoimmune disease causing tissue and organ damage (Waite and Skokos., 2012). The breakdown in tolerance is one contributory factor in autoimmune disease as clinical data has shown that autoimmune disease patients have reduced levels of Treg cells (Dejaco *et al.,* 2005).

However, in our EGR-2 and EGR-3 deficient mice there was no difference in the Treg population or FoxP3 expression (Li *et al.,* 2012).

When EGR-2 was reconstituted in the CD4 T cells, IL-17 production was low and siRNA knockdown of EGR-2 enhanced the expression IL-17 production (Miao *et al.,* 2013). These results confirm Schraml *et al.,* 2009 group showing that EGR-2 interacting with Batf is important for suppressing Th17 pro-inflammatory cytokines and therefore immunopathology.

Previous publications have indicated that other EGR family members are involved in inflammatory disease. EGR-1 has been associated with cholestasis in the liver, which is an inflammatory disease caused by an increase in bile acids in the liver and blood, where EGR-1 deficiency reduced liver inflammation (Allen *et al.,* 2010). A recent group has found that EGR-3 induces the expression of the anti-inflammatory cytokine TGF-β1 in human and mouse CD4 T cells owing to the role that EGR-3 plays in anergy (Sumitomo *et al.,* 2013). However we found that EGR-2 not EGR-1 or EGR-3 suppressed Batf mediated transactivation of the IL-17 promoter (Miao *et al.,* 2013).

A recent study has shown that genetics is not the only risk factor with the environmental factors proving to be increasingly critical (Kleinewietfeld *et al.,* 2013). This study found that increasing salt concentrations can elicit a more profound Th17 mediated CD4 T cell response through activation of the MAPK pathway (Kleinewietfeld *et al.,* 2013). More research needs to be done to discover more about the causes of autoimmunity.

In conclusion, EGR-2 and EGR-3 are critical for controlling autoimmune disease by suppressing pro-inflammatory cytokine production and IL-17 mediated immunopathology.

4.3 The Role of EGR-2 and EGR-3 in positive regulation of T cell receptor signalling

Single EGR-2 or EGR-3 knockout mice display normal proliferation after mitogenic stimulation. However, a deficiency in both EGR-2 and EGR-3, results in defective cell proliferation and IL-2 production. In contrast, EGR-2Tg mice show increased proliferation after mitogenic stimulation. This phenomenon of an autoimmune disease phenotype and defective T cell proliferation in-vitro is similar to autoimmune disease patient's data; SLE patients show impaired T cell proliferation once stimulated in-vitro (Gattlieb *et al.,* 1979), which is consistent with our mouse model. Previously EGR-2 and EGR-3 have been considered to be important for anergy (Collins *et al.,* 2008; Seiler *et al.,* 2012) by increasing E3 ligases leading to the deterioration of the TCR signalling molecules (Harris *et al.,* 2004; Safford *et al.,* 2005; Collins *et al.,* 2008). Safford *et al.,* 2005 group showed that EGR-2 and EGR-3 negatively regulate T cell activation by increasing E3 ubiquitin ligase Cbl-b and evoking immunosuppression in-vitro. IL-2 is an important cytokine for T cell activation and IL-2 knockout mice have shown to develop autoimmune disease and splenomegaly, owing to reduced Treg and other factors (Wolf *et al.,* 2001; Schimpl *et al.,* 2002; Geng *et al.,* 2012).

We propose that EGR-2 and EGR-3 play uncoupled roles in naïve versus anergic T cells. In this case the anergic route is associated with down regulation of MAPK and AP-1 and the increase in NFAT, with deterioration of TCR signalling by inducing E3 ligases (Harris *et al.,* 2004; Safford *et al.,* 2005). We show that EGR-2 and EGR-3 are important for naïve T cell activation in response to mitogenic stimulation where EGR-2 and EGR-3 deficient lymphocytes have a severe defect in proliferation and IL-2 production in response to mitogenic stimulation. The findings represented here are novel.

EGR-2 and EGR-3 have been previously shown to induce apoptosis by directly regulating FasL transcription (Mittelstadt and Ashwell*.,* 1999), however we did not observe any differences in apoptosis in EGR-2 and EGR-3 deficient mice (Li *et al.,* 2012) proving that the defective proliferation is not due to increasing apoptotic activity.

In fact we found that the defect in T cell proliferation after mitogenic stimulation is due to impairment of AP-1 signalling and not MAPK, NFAT or NF-κB. AP-1 activation is vital for initialising T cell activation and IL-2 production (Foletta *et al.,* 1998). We demonstrated that EGR-2 and EGR-3 bind with Batf, whose function it is to suppress AP-1 signalling and is part of the alternative complex seen in EGR-2 and EGR-3 lysates from AP-1 EMSA analyses. Batf does not contain a trans-activation domain and forced expression of Batf in fibroblast cells slows down growth (Echlin *et al.,* 2000; Williams *et al.,* 2001). The mouse F9 teratocarcinoma cells (low AP-1 levels) when transfected with the TRE-Lux luciferase reporter gene and Batf, showed low AP-1 activation (Echlin *et al.,* 2000). We show that EGR-2 and EGR-3 directly interact with Batf and do not regulate Batf expression levels (Li et al., 2012). In these instances antigen-mediated responses in naïve T cells is strictly regulated. At the early stages of antigen-mediated responses, EGR-2 and EGR-3 expression is high and Batf expression is repressed, to allow the expansion of activated T and B cells (Li *et al.,* 2012). Once the effector function of T and B cells has been carried out, EGR-2 and EGR-3 is rapidly arrested and Batf expression levels are restored (Li *et al.,* 2012). This suggests that EGR-2 and EGR-3 are important for the initial expansion of naïve T cells after T cell activation and rapidly degraded once expansion is completed.

Direct interaction between EGR-2 and EGR-3 blocks Batf suppressing AP-1 signalling as shown by EMSA. Conversely we show that silencing of Batf using siRNA in

EGR-2 and EGR-3 deficient CD4 T cells restored AP-1 activation and IL-2 production whilst reducing inflammatory cytokine IL-17A.

Although we show that EGR-2 and EGR-3 directly interact with Batf in the AP-1 signalling pathway, we also identified that EGR-2 and EGR-3 deficient nuclear extracts contained JunB in the complex, suggesting that EGR-2 and EGR-3 interacting with JunB is important for the activation of AP-1. Mice that have a deletion of JunB and c-Jun develop psoriasis-like disease and arthritis in the skin where a huge influx of epidermal cytokines and inflammation was observed, to indicate that AP-1 proteins are important for regulating skin homeostasis and preventing inflammation (Guinea-Viniegra *et al.,* 2009). c-Jun has been shown to promote proliferation and differentiation of keratinocytes in the epithelium (Schonthaler *et al.,* 2011) and its expression is ubiquitous in dividing cells (Johnson *et al.,* 1993). c-Jun knockout primary embryonic fibroblast cells showed reduced growth rates in-vitro (Johnson *et al.,* 1993) showing that c-Jun is important in proliferation during embryonic development. Also c-Jun knockout fibroblast cells from mice showed a severe defect in cell proliferation due to an increase in the tumor suppressor gene p53 (Schreiber *et al.,* 1998). The role of c-Jun and JunB has not been investigated in our EGR-2 and EGR-3 deficient mouse model.

In conclusion we show the essential role of EGR-2 and EGR-3 in positively inducing T cell receptor activation after mitogenic stimulation.

4.4 Future Considerations/Work

The work presented in this thesis show EGR-2 is prerequisite for preventing autoimmune disease and EGR-2cKO mice are more susceptible to inducing autoimmune disease in the EAE model. Future work would be to explore the role of EGR-2 in other autoimmune disease mouse models, for example zymosan induced arthritis (Asquith *et al.,* 2009) as the role of EGR-2 in autoimmune disease is unclear. This could have an impact on the use of EGR-2 as a therapeutic agent for controlling autoimmune disease in future research.

Another important area for future work is the role of EGR-2 and EGR-3 in TCR signalling. Overall our results support that EGR-2 and EGR-3 are important for positive regulation of T cell activation in response to antigenic stimulation through AP-1 signalling. TCR signalling is complex (Morris and Allen*.,* 2012) and we only explored the role of EGR-2 and EGR-3 in MAPK pathway, it would be interesting to examine their role in the other signalling components.

Another area for future research would be to explore the role of AP-1 and inflammation in our model. AP-1 proteins are also associated with inflammation and a report has shown that asthmatic patients show over expression of AP-1 (Jaques *et al.,* 2010). Similarly AP-1 over expression has been shown to increase ICAM-1 in human vascular endothelial cells (Wang *et al.,* 1999). Jun proteins are important in regulating skin inflammation and have been associated in the induction of skin inflammatory diseases where a loss of JunB causes SLE disease and the loss of both c-Jun and JunB results in severe psoriasis (Sconthaler *et al.,* 2011). This could provide a possible link between the autoimmune disease and TCR signalling defect in our EGR-2 and EGR-3 deficient mice. As

EGR-2 and EGR-3 control both these aspects, we would also want to explore how they regulate other transcription factors by using microarray and ChIP sequencing.

4.5 Conclusion

The work presented in this thesis, illustrate the importance of EGR-2 and EGR-3 intrinsically controlling the development of chronic inflammatory autoimmune disease. Therefore the EGR-2 and EGR-3 deficient mouse model could be potentially used as a new model for studying of autoimmune diseases.

More importantly we identified a novel finding to support the role of EGR-2 and EGR-3 in the positive regulation of antigen receptor activation. This therefore shows the profound effect of these transcription factors in the regulation of the immune system. As EGR-2 and EGR-3 play an uncoupled role to suppress the induction of inflammatory cytokines and development of autoimmune disease, whilst being essential in positive induction of T cell activation after mitogenic stimulation. In conclusion the work presented in this thesis supports the essential role of EGR-2 and EGR-3 for desirable immune response whilst restricting immunopathology.

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