Egr2/Egr3 are Essential Tumour Suppressor Genes for Lymphomagenesis

A thesis submitted for the degree of Doctor of Philosophy by

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Declaration

I hereby declare that the research presented in this thesis is my own work, except where otherwise specified, and has not been submitted for any other degree.

Punamdip Kaur Bhullar

Abstract

Non-Hodgkin's lymphoma is the fifth most common cancer in the UK, accounting for 4% of all new cases. The control of lymphomagenesis still remains a challenge. Early growth response gene (Egr) 2 and 3 are zinc finger transcription factors. Egr2 plays an important role in the development of both central nervous system and lymphocytes. However the mechanism of action in lymphocytes is still unknown.

In order to fully understand the function of Egr2, in lymphocytes, we developed Egr2 and 3 double knockout mice ($Egr2^{-/-}Egr3^{-/-}$) by crossbreeding lymphocyte specific Egr2 knockout mice (CD2-$Egr2^{-/-}$) with Egr3 knockout mice ($Egr3^{-/-}$), as previous reports suggested that Egr3 compensates for the role of Egr2. In the absence of Egr2 and 3, the homeostasis of T cells is dysregulated with hyper-homeostatic proliferation of effector like phenotype cells. More importantly the development of spontaneous B and T cell lymphoma was found in more than 70% of $Egr2^{-/-}Egr3^{-/-}$ mice. The lymphoma cells from $Egr2^{-/-}Egr3^{-/-}$ mice were highly proliferative and metastatically spread into other non-lymphoid organs, such as lung, liver and kidney. In additional to this lymphoma development the $Egr2^{-/-}Egr3^{-/-}$ mice showed signs of chronic inflammatory disorder. This inflammatory disorder was characterised by glomerulonephritis and an increase in serum cytokines, which may provide the microenvironment for the lymphoma development.

To explore the molecular mechanism of tumour development in $Egr2^{-/-}Egr3^{-/-}$ mice, the transcriptional profile of Egr2 was studied by microarray and ChIP-on-chip. We found firstly that Egr2 directly binds to the promoter regions of

Ikaros and FOXO3. The deletion of Egr2 and 3 in lymphocytes led to the downregulation of Ikaros, Aiolos and FOXO3 expression. The impaired expression was found to be associated with proliferative disorder and the development of T and B cell lymphoma. Secondly Egr2 strongly inhibits STAT3 transcriptional activity by regulating SOCS3, which is a known inhibitor of STAT3. The breakdown of this regulation could be an important mechanism in lymphomagenesis.

A model is proposed which defines Egr2 and Egr3 as the backbone of important tumour suppressor genes that control cell fate decision and regulates homeostasis in the lymphoid system. Thus, our results suggest that Egr2 and 3 are important regulators of lymphocyte function by their involvement in multiple cell signalling pathways, which could potentially be key genes for future cancer therapy.

Acknowledgments

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It would not have been possible to write this doctoral thesis without the help and support of the kind people around me, to only some of whom it is possible to give particular mention here.

I cannot find words to express my gratitude to Sheba. This journey we have travelled together, just like a roller-coaster ride. Every high and low we have experienced together and without your support, motivation and true friendship, I cannot image what it would have been like. “Thank you Sheba”, for always being there when needed, especially during the write up of my thesis. I would also specially like to thank Dr Sahar Al-Mahdawi and Dr Chiranjeevi Sandi for their

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If I have missed anybody out, please forgive me and know that it was not intentional.

Dedication

This thesis is dedicated to my husband, Jagdeep and children, Har-Simran and Rehmath. I give my deepest expression of love and appreciation for the encouragement that you gave me and the sacrifices you made during this PhD. I hope this work can be an inspiration for my children to achieve high in their lives.

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List of Abbreviations

β	Beta
µl	microliter
APAF-1	Apoptotic protease activating factor-1
BAFF	B-cell-activating factor
BAFFR	BAFF receptor
BCR	B cell receptor
CDK	Cyclin-dependent kinases
ChIP	Chromatin immune-precipitation
CKI	CDK Inhibitors
CLP	Common lymphoid progenitor
CMP	Common Myeloid progenitor
CSA	Cyclosporine A
dH2O	Distilled Water
DMEM	Dulbecco's Modified Eagle Medium
DN	Double negative
DNA	Deoxyribonucleic acid
DP	Double positive
DSB	Double strand breaks
Egr2	Early growth response gene
ELISA	Enzyme-linked immunosorbent assay
F(ab')$_2$	Fragments of goat anti-mouse IgM
FACS	Fluorescence activate cell sorting

FADD	Fas-associated death domain
FASL	Fas ligand
FBS	Foetal Bovine Serum
FLT3	Tyrosine kinase
FO	Mature follicular B cells
G1	Gap phase 1
G2	Gap phase 2
HC	Heavy chain
HEL	Hen-egg lysozyme
HL	Hodgkin's Lymphoma
HSC	Hematopoietic stem cells
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
INO	Ionomycin
LC	Light chain
LHβ	Lutenizing hormone
LIP	Lymphopenia induced proliferation
LN	Lymph node
LPS	Lipopolysaccharide-stimulation
M	Mitotic phase
MACS	Magnetic Activated Cell Sorter
MOMP	Mitochondrial outer membrane permeabilisation
MPP	Multipotent progenitors

MZ	Marginal zone B cells
NFAT	Nuclear factors of activated T cells
NHEJ	Non-homologous end joining
NHL	Non-Hodgkin's Lymphoma
NK	Natural killer cells
PBS	Phosphate-buffered saline
PCR	polymerase chain reaction
PMA	Phorbol myristate acetate
PMSF	Phenylmethylsulfonyl fluoride
RAG	Recombination activating genes
RNA	ribonucleic acid
RPMI1640	Roswell Park Memorial Institute 1640
RSS	Recombination signal sequence
S	Synthesis phase
SLE	Systemic lupus erythematosus
TCR	T cell receptor
TGF-β	Transforming growth factor- β
T_H	T Helper Cell
TNF	Tumour necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
WHO	World Health Organisation

Chapter 1 - Introduction

Cancer is one of the most common diseases in the world. In the UK around 424,128 new cases of cancer were diagnosed in year 2010, and more than 1 in 3 people will develop some form of cancer during their lifetime (CancerResearch 2011). The incidence rate for cancer in Great Britain, alone, has increased by 26% between 1979 and 2008. There are many types of cancers, around 200 different types, but five of them – breast, lung, colorectal, prostate and non-Hodgkin's lymphoma account for over half (58%) of all new cases (CancerResearch 2011).

1.1 Hallmarks of Cancer

In the past 20 years an enormous progress has been seen in our understanding of cancer at molecular level; resulting in a large number of exciting new targets for the development of efficient therapies for treatment. Every cancer has different features, which depend on the origin of the cancer cell. However the end result is the same. In 2000, Hanahan and Weinberg defined six hallmarks of most if not all cancers, which established the logical framework for understanding the astonishing diversity of cancer development (Hanahan and Weinberg 2000). A decade later, Hanahan and Weinberg proposed further four emerging hallmarks (Hanahan and Weinberg 2011), that further explained other factors contributing towards cancer growth (Figure 1.1).

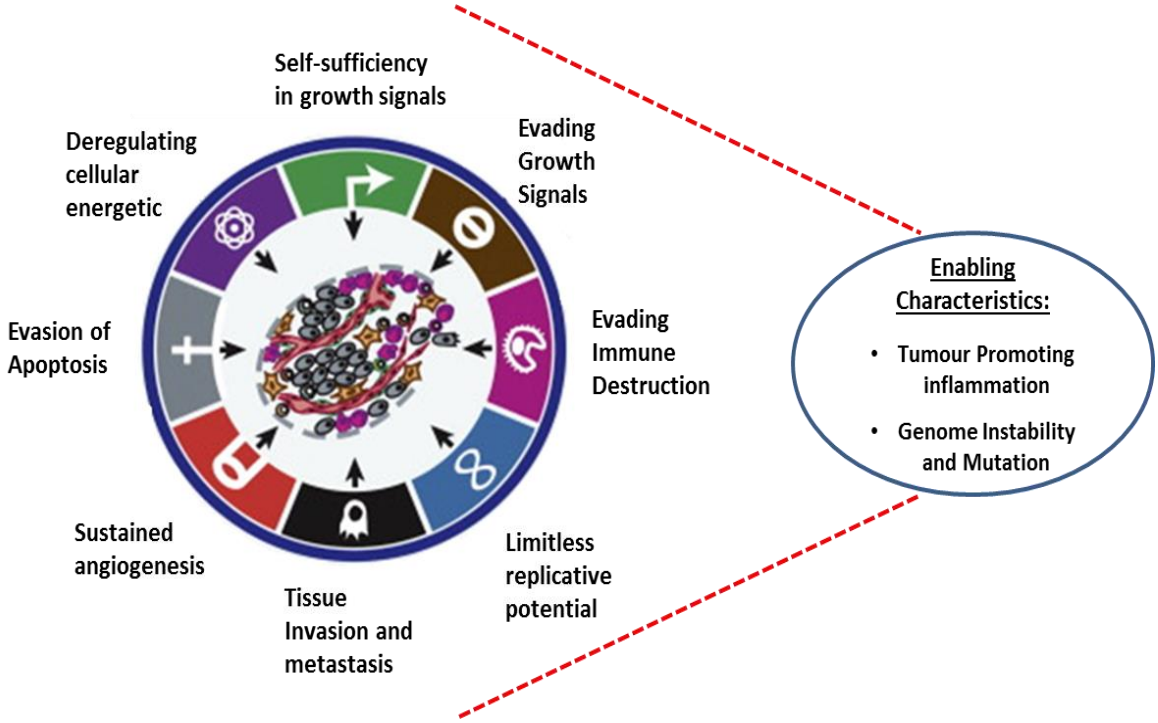

Figure 1.1: Hallmarks of Cancer, adapted from (Hanahan and Weinberg 2000).
The six hallmarks of cancer are illustrated here in conjunction with two enabling characteristics. Activation of oncogene, suppression of tumour suppressor genes, destruction of apoptosis, limitless replication, metastasis and angiogenesis were proposed by Hanahan and Weignberg in 2000. In 2010 they added a further two hallmarks, destruction of cell metabolism and the evading of the immune destruction. The two enabling characteristics are indicated as tumour promoting inflammation and genome instability.

Cancer has long been known as a genetic disease. In 1951, Muller suggested that a cell must comprise more than one mutation in order to transform into a neoplasm (Muller 1951). Since then a number of findings have described that cancer is a step-wise process, where the cell acquires different qualities at each step, initiating the neoplasm. This has also supported the clonal expansion theory of tumour development by Nowell in 1970s, stating that a normal cell which acquires a somatic mutation will achieve a growth advantage over adjacent normal

cells which eventually leads to clonal expansion (Nowell 1976; Nowell 2002; Sarasin 2003).

The normal process by which normal cells become progressively transformed to malignancy is known to require the sequential mutations, which arise as a consequence of damage to the genome (Bertram 2000). Mutations in the context of carcinogenesis can occur in a number of different ways, but using the extensive classification these include:

- Point mutations, resulting in an amino acid substitutions; frame-shift mutations or mutations to stop codons which either truncate the protein product or ascent its sequence.
- Chromosomal instability or imbalance, resulting in amplification, over-expression or inappropriate expression of a particular gene.
- Loss of a gene or its fusion with another gene as a result of chromosomal breakage and rearrangement resulting in a chimeric protein with altered functions.
- Epigenetic modifications to DNA of which the most important is the methylation of cytosine in CpG islands leading to gene silencing.

Mutations within developing cancer cells have two basic functions: mutations which increase the activity of the protein that encourage proliferation, these are called oncogenes; or mutations which inactivate genes that suppress cell proliferation and growth, hence referred to as tumour suppressor genes.

1.2 The Cell Cycle and Cancer development

1.2.1 Cell Cycle Regulation

During the life of a cell, it will undergo cell proliferation, which involves the reproduction of a cell to form two identical daughter cells. The daughter cells will further reproduce themselves into two more daughter cells, and so on. This cyclic process is a sequence of stages through which a cell passes between one cell division and the next is called the cell cycle (Figure 1.2). The phases of the cell growth are the same whether in a tumour cell or in a normal cell.

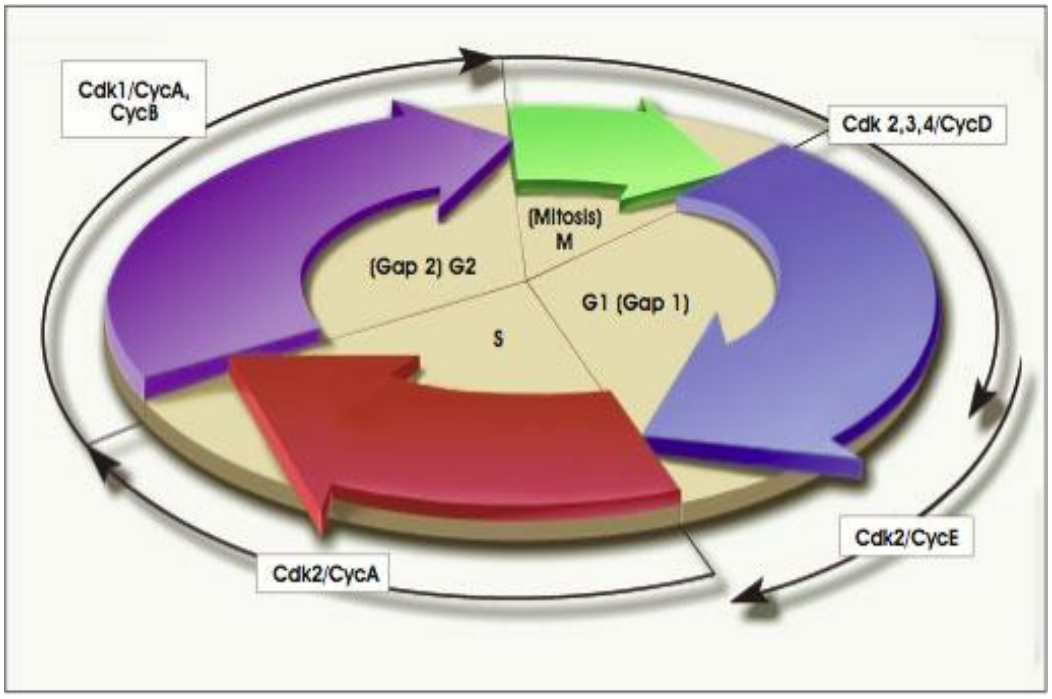

Figure 1.2: Cell Cycle Control.
There are four stages to the cell cycle; gap phase (G1) before the DNA replication (S phase), followed by another gap phase (G2) and the mitotic phase (M phase). The cell cycle process is regulated by cyclin dependent kinases (CDK) and cyclin genes. (Taken from: National Institute of Health, Appendix A: Early Development. In *Stem Cell Information*)

The cell cycle for the eukaryotic cells is composed of four stages; the gap phase before the DNA replication (G1), the DNA synthesis phase (S Phase), the gap phase after DNA replication (G2), and the mitotic phase, which leads the cell to divide (M phase) (Hartwell and Weinert 1989). In addition to the four phases, there is a G0 phase, which is an inactive phase outside of the cell cycle. During this phase the cells are known to be quiescent, which plays a major part of non-growing, non-proliferating cells (Vermeulen, Van Bockstaele *et al.* 2003). Most of the cells in an adult are not in the process of cell division but can enter the cell cycle at G1 due to mitogens or growth factors. At G2 checkpoint, preceding mitosis, there is also a quality control of the cell material, checking the newly synthesised DNA is identical to the original DNA and that division occurs equally between the daughter cells (Hickman, Moroni *et al.* 2002).

The cell cycle is a complex process, which requires a coordination of a variety of macromolecular synthesis, assemblies and movement. This shift from one cell cycle phase to another is carefully regulated before replication the quality of the DNA is checked (G1 phase) (Sandal 2002).

The cell cycle is mainly regulated by cyclin-dependent kinases (CDK). There are a number of regulation mechanisms for the CDKs which ensures that the correct timing of kinase activity occurs during the cell cycle. CDKs alone cannot regulate the cell cycle but are only active once they have formed a complex with their associates, cyclin (Table 1.1). Cyclins were so named due to their expression levels oscillating throughout the cell cycle. As the CDKs function at different phases of the

cell, they are both negatively and positively regulated during the cell cycle and hence provide the driving force for the cell cycle progressions (Sandal 2002).

CDK	Cyclin	Cell Cycle Phase
CDK1 (cdc2)	Cyclin A	G2/M phase transition
CDK1 (cdc2)	Cyclin B	Mitosis
CDK2	Cyclin E	G1/S phase transition
CDK2	Cyclin A	S phase
CDK4	Cyclin D1, D2, D3	G1 phase
CDK6	Cyclin D1, D2, D3	G1 phase

Table 1.1: CDK/cyclin complexes at different phases of the cell cycle Adapted from (Sandal 2002).

1.2.2 Inhibitors of CDK

The regulation of the CDK/cyclin complexes is highly monitored due to the proteins expressed at different stages of the cell cycle. The CDKs and the cyclins are regulated by many different proteins, such as CDK inhibitors (CKI), some of which are p21CIP/KIP, p27cip/kip and p16INK4a. The CKI mediate cell cycle arrest in response to several anti-proliferative signals (Kaldis, Pitluk *et al.* 1998).

The cell cycle control is also maintained by other genes known as the proto-oncogenes and tumour suppressor genes. The activation of one and the evasion of the other, respectively are key hallmarks of cancer development.

1.2.3 Self-Sufficiency in Growth Signals

The most fundamental trait of cancer cells involves their ability to sustain chronic proliferation. Normal tissues maintain homeostasis of cell number by the careful production and release of growth promoting signals that instruct entry into and progression through the cell growth and division cycle. Proto-oncogenes are involved in the process of regulating proliferation, cell cycle progression and apoptosis. The proto-oncogenes become activated (oncogenes) by one of the means of mutations described earlier, which leads to the deregulation of cell growth. Distinct oncogenes have been specifically associated with the different subtypes of non-Hodgkin's lymphoma (Anderson, Reynolds *et al.* 1992). Table 1.2, summarises the oncogenes activated in some of the non-Hodgkin's lymphoma.

Oncogene/Pathway	NHL subtype.
Bcl-2	Follicle Lymphoma, Diffuse large B-cell lymphoma
Cyclin D1	Mantle cell lymphoma
c-Myc	Burkitt lymphoma
NF-kB	MALT lymphoma, diffuse large B-cell lymphoma
Bcl-6	Diffuse large B cell lymphoma, follicular lymphoma

Table 1.2: Oncogenes which are activated in different types of lymphoma Adapted from Hachem, A and Gartenhaus, R.B 2005 (Hachem and Gartenhaus 2005).

1.2.4 Evading Growth Suppressors

In addition to the cell capability of inducing and sustaining positively acting growth stimulatory signals, cancer cells must also evade powerful programs that negatively regulate cell proliferation, which depend on tumour suppressor genes. Tumour suppressor genes are normal genes that negatively regulate the cell division, repair DNA damage or direct the cells to undergo apoptosis. The inactivation of tumour suppressor genes deregulated the cell cycle control and leads to cancer. The first tumour suppressor gene discovered was the human Retinoblastoma protein (pRb) by Alfred Knudson (Knudson 1971; Knudson, Meadows *et al.* 1976). Over the years many such tumour suppressor genes have been identified. The most famous is the p53 gene, known as the "Guardian of the Genome" (Lane 1992), where the inactivation of the gene is an almost common step in the development of over 50% of human cancers (Hollstein, Sidransky *et al.* 1991). The tumour suppressor genes are usually defected when the gene is inactivated on both alleles, "two-hit" theory (Knudson 1971). Table 1.3 shows a number of tumour suppressor genes affected in lymphoma.

Tumour Suppressor Gene	NHL subtype.
BRCA1	Diffuse large B cell lymphoma
BRCA2	T-cell Non-Hodgkin's lymphoma
TP53	Burkitt's lymphoma, Chronic myelogenous leukemia, B cell lymphoma
IKZF1	T Cell lymphoma, Acute lymphoblastic leukemia, T Cell leukemia
IKZF3	B Cell lymphoma, Chronic lymphocytic leukemia

Table 1.3: Tumour Suppressor Gene which are deactivated in different types of lymphoma Adapted from Skibola, C.F., *et al* 2007 (Skibola, Curry *et al.* 2007).

1.2.5 Evasion of Apoptosis

Tissue homeostasis is characterised by the balance between proliferation and cell growth versus the cell death. In response to stressful stimuli, the cell usually supports a cellular stress response to ensure survival (Kultz 2005; Lockshin and Zakeri 2007). Apoptosis, also known as programmed cell death is the cell's intrinsic death program that regulates various physiological as well as pathological processes. Apoptosis was initially described by its morphological characteristics, including cell shrinkage, membrane blebbing, chromosome condensation and nuclear fragmentation (Wyllie, Kerr *et al.* 1980; Kerr, Winterford *et al.* 1994; Lowe and Lin 2000). There are two major apoptosis signalling pathways, that is, the death receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway (Figure 1.3) (Hengartner 2000).

The extrinsic pathway is initiated by the binding of an apoptotic-inducing to the cell surface death receptors associated with Fas-associated death domain (FADD) or tumour necrosis factor (TNF) receptor, respectively (Debatin and Krammer 2004). The well-studied death ligands are Fas ligands and TNF ligands, which binds to the transmembrane death receptors such as Fas receptor or TNF receptor, respectively. Upon ligand binding the FADD become activated and interact with initiator enzymes of the caspase cascade, mostly caspase 8 and 10, resulting in downstream activation of caspase 3 and 7 (effector caspases) and ultimately programmed cellular destruction through proteolytic cleavage of caspase substrates (McKenzie and Kyprianou 2006).

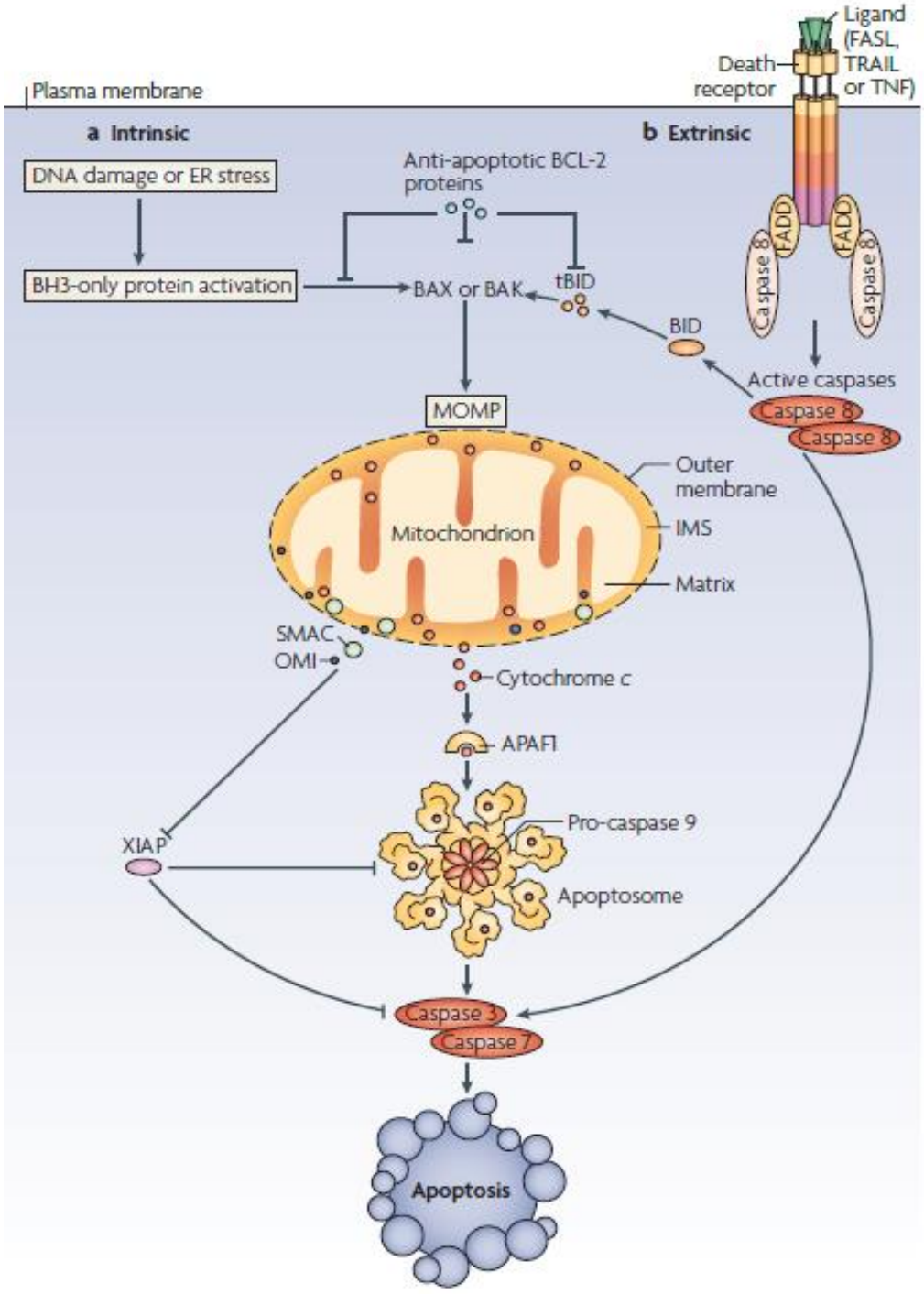

Figure 1.3: Apoptotic extrinsic and intrinsic pathways (Tait and Green 2010).
There are two apoptotic pathways; extrinsic pathway, which is initiated by the activation of death receptors. The intrinsic pathway is mitochondrial mediated. The permeabilisation of the mitochondria releases the cytochrome C into the cytoplasm, activating the caspases.

The intrinsic pathway is initiated by internal stress, such as DNA damage, lack of growth factors (Okada and Mak 2004). The internal stress signals subsequently targets the mitochondrial membrane, leading to mitochondrial outer membrane permeabilisation (MOMP). This causes the release of cytochrome c (Newmeyer and Ferguson-Miller 2003). The release of cytochrome c into the cytosol results in the activation of apoptotic protease activating factor-1 (APAF-1) and caspase 9, which form a functional apoptosome that activates the effector caspase cascade, resulting in programmed cellular destruction (McKenzie and Kyprianou 2006) (Figure 1.3).

As Hananah and Weinberg defined the characteristics of cancer development, they proposed the evasion of the apoptosis as one of the key hallmarks and this has been observed in many human cancers. In principle, the apoptosis pathways can be blocked at different levels of the signalling cascade by upregulation of anti-apoptotic proteins and/or downregulation or dysfunction of pro-apoptotic proteins (Fulda 2009). Examples of altered apoptosis signalling pathways that contribute to stress resistance in human cancers are:

Impairment of death receptors is via downregulation of receptor surface expression as part of the adaptive stress response. For example, in chemotherapy resistance leukaemia, the CD95 expression was downregulated (Friesen, Fulda *et al.* 1997). TRAIL is a death receptor which binds to CD95 ligand to induce apoptosis. Deletions or mutations, resulting in loss of both copies of TRAIL-R1 or TRAIL-R2 were detected in small percentage of NHL (Fulda 2009).

Caspase 8 has been shown to be transcriptionally regulated by splicing in leukaemia and neuroblastoma. The alternative splicing of intron 8 of the caspase 8 gene results in the generation of caspase-8L, which lacks the catalytic site (Miller, Karacay *et al.* 2006).

An increase in the ratio of anti- to pro-apoptotic Bcl-2 proteins has been detected in a number of cancers and has been related to tumour cell survival and apoptosis resistance. For example, overexpression of BCL-2 is a key feature of human follicular lymphoma and is caused by the chromosomal transclocation of the BCL-2 oncogene into the immunoglobulin heavy chain gene locus (Tsujimoto, Finger *et al.* 1984).

1.2.6 Telomere Length – Limitless Replication

Telomeres are long stretches of noncoding DNA located at the end of all eukaryotic chromosomes (Figure 1.4). In vertebrates the telomeres are composed of simple, repetitive non-coding DNA sequences. In human and mouse the telomeres contain a six base pair sequence TTAGGG, repeated many thousands of times. However, the length of the telomere varies from one chromosome to another. The telomere length also varies greatly between species, from approximately 300 to 600 bp in yeast (Shampay, Szostak *et al.* 1984) to kilo bases in humans.

The discovery of chromosomal integrity was proposed by Barbara McClintock in 1941. She studies the telomeres in chromosomes from the maize plants. She proposed that if the chromosomes ends were broken by various means,

then it resulted in adhesion and fusion of their ends, with the consequent formation of dicentric chromosomes (McClintock 1941).

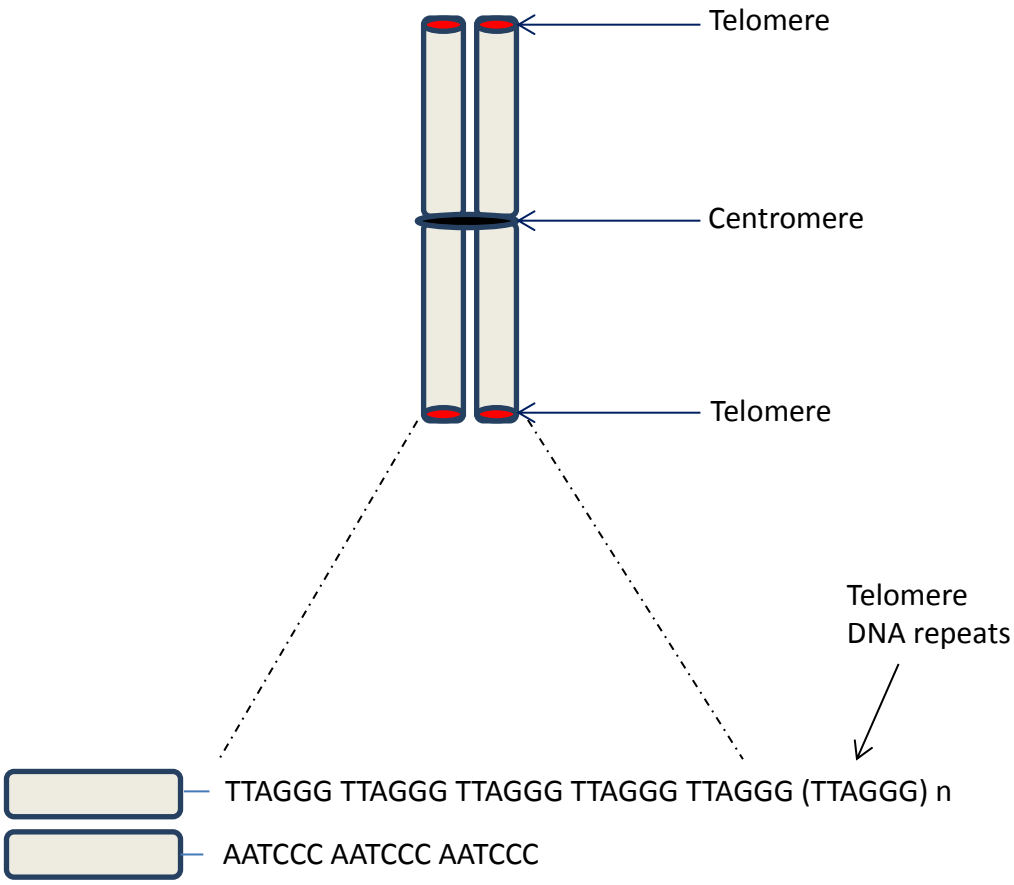

Figure 1.4: Telomeres located at the ends of all eukaryotic chromosomes.
The telomere repeats (TTAGGG)n are present at the ends of all eukaryotic chromosomes. These telomeres shorten after each cell division.

Two years previous to this finding a young American geneticist Hermann J. Muller noticed that the ends of the irradiated chromosomes were different to other genome and could not recover terminal deletions from the Drosophila chromosome ends. He suggested that the chromosome ends are specialised structures and

named them Telomeres from a Greek term “telos” (End) “meros” (Part)(Greider 1996).

A number of scientists have used molecular techniques to confirm the initial finding by McClintock, that telomeres are essential for chromosome stability. In addition to this finding it has also been shown that telomeres have many other critical functions such as:

- Telomeres also participate in nuclear processes such as chromosome positioning in the nucleus (Ferguson and Fangman 1992; Brewer and Fangman 1993; Luderus, van Steensel *et al.* 1996).
- Regulation of gene expression and transcriptional repression (Sandell and Zakian 1993).
- Cellular senescence (Holt, Wright *et al.* 1996; Shay and Wright 2005).

The inability of the conventional DNA polymerase to replicate to the end of the chromosome during lagging strand synthesis results in 50-200 base pairs to be lost from the end of the linear chromosome, during each cell division (Hastie, Dempster *et al.* 1990; Lindsey, McGill *et al.* 1991). This telomere shortening occurs because during DNA replication, the DNA polymerase synthesis DNA in the 5’ to 3’ direction with the aid of RNA primers. The leading strand is synthesised continuously and the lagging strand to be synthesised discontinuously. The RNA primers are degraded by RNase H and the Okazaki fragments are ligated with DNA ligase. However this process leaves the 3’ end of the chromosome as a single stranded DNA, called “end replication problem” (Olovnikov 1973). If the 3’ end is incompletely replicated, the

single-strand DNA is unstable, and this leads to the loss of telomere repeats after each replication (Weng, Palmer *et al.* 1997).

Therefore telomeres have an important role to play to protect the chromosomes from erosion. A study by Olovnikov in 1972 showed that erosion of the chromosome ends lead to the loss of essential genes and an exit from the cell cycle (Olovnikov 1973). In 1990 it was demonstrated by Harley, C.B, *et al* , that telomere shortening accounts for the process of a 'cellular ageing' (Harley, Futcher *et al.* 1990).

The telomere shortening happens with age or with cell division. It also occurs in other types of somatic cells including hematopoietic stem cells, leukocytes, endothelial cells (Harley, Futcher *et al*. 1990; Hastie, Dempster *et al*. 1990; Lindsey, McGill *et al*. 1991; Chang and Harley 1995). However in contrast, the germ line and malignant cells do not appear to undergo telomere shortening with cell division (Allsopp, Vaziri *et al.* 1992). Earlier studies also demonstrated that a significantly shorter telomere length in most cancers compared with noncancerous tissue from the same patient (Hastie, Dempster *et al*. 1990). This suggests that human cancers and germ line cells have developed a mechanism to overcome the telomere loss during cell division.

1.2.6.1 Telomerase

The telomere stabilisation is achieved by reactivation or upregulation of the ribonucleoprotein enzyme telomerase (Greider and Blackburn 1989). Telomerase is an RNA-dependent DNA polymerase, which utilises its RNA as a template for the

addition of TTAGGG repeats to the 3' ends of the chromosomes, therefore compensating for losses due to the end-replication problems. It was first discovered in *Tetrahymena* by Elizabeth Blackburn and Carol Greider (Greider and Blackburn 1985).

Initially it has been proposed that telomerase is expressed in germ-line and malignant cells but not in most somatic cells (Wright, Piatyszek *et al.* 1996), based on the immortality of the germ-line and of malignant cells (de Lange 1994). However a number of studies then indicated that telomerase activity is also detected in normal somatic cells that have the potential to self-renew themselves, such as hematopoietic stem cells (Morrison, Prowse *et al.* 1996), lymphocytes (Weng, Levine *et al.* 1996; Weng, Granger *et al.* 1997; Hathcock, Weng *et al.* 1998; Weng 2002) and skin epithelial cells (Harle-Bachor and Boukamp 1996).

One of the hallmarks proposed by Hanahan and Weinberg in 2000 was the limitless replication potential of a cancer cells. Studies have found that cancer cells have shorter telomere length due to their capability of intense cell proliferation, however this short telomere lengths is maintained with the activation of telomerase enzyme. This was supported by Shay and Bacchetti, who observed an increase in telomerase activity in majority of human cancers (Shay and Bacchetti 1997).

1.2.7 Inflammation and Cancer

A decade after the proposal of hallmarks of cancer by Hanahan and Weinberg, they proposed to include two more hallmarks of cancer, with enabling

characteristics of tumour promoting inflammation and genome instability and mutation (Hanahan and Weinberg 2011).

Inflammation is a physiological process in response to tissue damage resulting from microbial pathogen infection, chemical irritation, and/or wounding (Philip, Rowley *et al.* 2004). In the initial stages of inflammation the neutrophils are the first to migrate to the inflammatory site under the regulation of the molecules produced rapidly responding macrophages and mast cells in tissues (Nathan 2002). As inflammation progresses, various types of leukocytes, lymphocytes and other inflammatory cells are activated and attracted to the inflamed site by a signalling network involving a great number of growth factors, cytokines, and chemokines (Nathan 2002).

There are also control mechanism in place that prevent inflammation, a number of molecules in the body play dual roles of both promoting and suppressing inflammation, such as transforming growth factor-β (Lu, Ouyang *et al.* 2006). The balance between promoting and suppressing inflammation is strictly regulated, if however the inflammation process is dysregulated and becomes favoured towards promoting inflammation, then the cellular response changes to the pattern of chronic inflammation.

However the link between inflammation and cancer was observed as early as 1863 by Virchow, who indicated that cancers tended to occur at sites of chronic inflammation (Balkwill and Mantovani 2001). Epidemiologic studies have also supported that chronic inflammatory diseases are frequently associated with increased risk of cancers (Lu, Ouyang *et al*. 2006). The development of cancers from

inflammation might be a process driven by inflammatory cells as well as a variety of mediators, including cytokines, chemokines, and enzymes, which altogether established an inflammatory microenvironment (Coussens and Werb 2002).

Cytokines, including interleukins (IL), TNF-α, growth factors, and differentiation factors (co-stimulating factors), are secreted molecules or membrane-bound molecules that play a regulatory role in the growth, differentiation, and activation of immune cells (Dranoff 2004). One of the major tumour promoting mechanism is the production of tumour-promoting cytokines by immune/inflammatory cells that activate transcription factors, such as NF-κB, STAT3, and AP-1, in premalignant cells to induce genes that stimulate cell proliferation and survival (Figure 1.5) (Grivennikov, Greten *et al.* 2010).

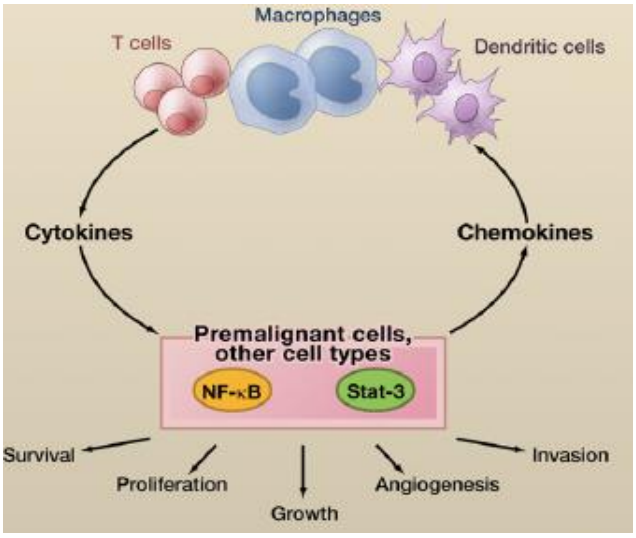

Figure 1.5: Role of inflammation in tumour promotion (Grivennikov, Greten *et al*. 2010).

This schematic representation shows the production of tumour-promoting cytokines by immune/inflammatory cells that activate transcription factors, such as NF-κB, STAT3, and AP-1, in premalignant cells. These factors induce genes that stimulate cell proliferation and survival.

Among these transcription factors, NF-κB and STAT3 are activated in the majority of cancers and act as oncogenes, whose activation in malignant cells is rarely the result of direct mutations and instead depend on signals produced by neighbouring cells, such as IL-6, IL-10 and IL-23 (Yu, Pardoll *et al.* 2009).

Proteins from the STAT family can both inhibit and promote cancer. Anti-tumour responses are increased by the activation of STAT1, which induces IL-12 production leading to the activation of STAT4 by IL-12 receptor engagement, promoting T_h1 responses to produce IFN-γ. IFN-γ stimulate macrophages, natural killer cells and $CD8^+$ T cells, mediating cytolytic activity (Darnell 1998; Takeda and Akira 2000; Chang, Han *et al.* 2009) (Figure 1.6).

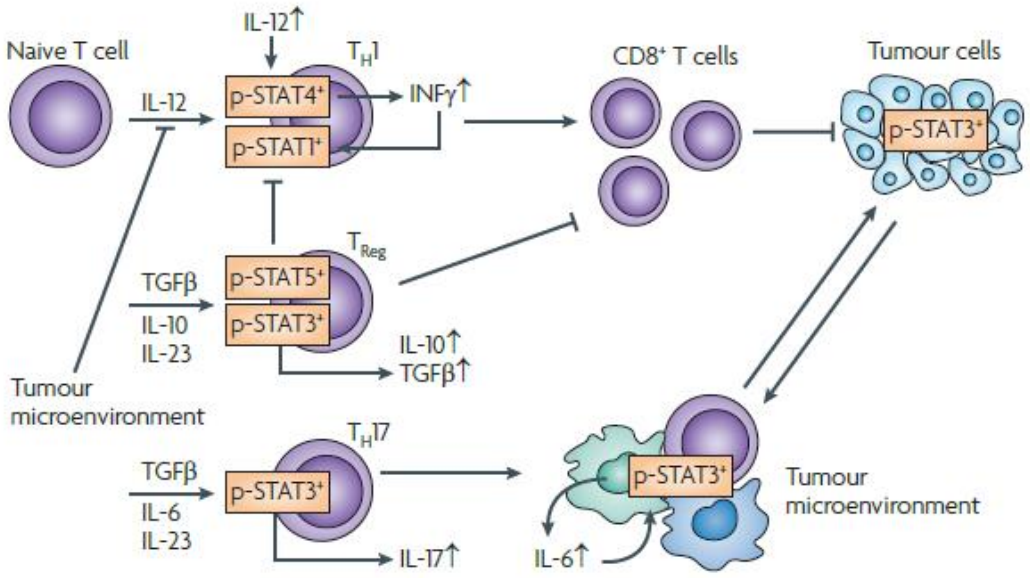

Figure 1.6: The role of STAT proteins in regulation of cancer adaptive immunity (Yu, Pardoll *et al*. 2009)

The STAT family can both inhibit and promote cancer. The activation of STAT1 in T cells produces IFN-γ, which stimulate macrophages, natural killer cells and $CD8^+$ T cells, mediating cytolytic activity. However, STAT3 has the ability to inhibit STAT1 and promote tumour progression.

However, STAT3 has the ability to antagonise STAT1 (Kortylewski, Kujawski *et al.* 2005). Both STAT3 and STAT5 are involved in the expansion of T regulatory cells, which promote tumour progression by inhibiting anti-tumour immune responses that are mediated by T_h1 type $CD4^+$ T cells and $CD8^+$ T cells (Figure 1.6) (Kubo, Hanada *et al.* 2003).

A number of literature publications have associated chronic inflammatory conditions with lymphoid malignancies, one example is provided by mucosa-associated lymphoid tissue (MALT) lymphomas. MALT lymphoma occurs in the context of chronic inflammation caused by infectious agents such as *Helicobacter pylori*, *Borrelia burgdorferi* (Ferreri, Ernberg *et al.* 2009). Other studies have also shown an increase risk of cancer development in patients with systemic lupus erythematosus, a systemic autoimmune disease with chronic inflammation and tissue damage (Pettersson, Pukkala *et al.* 1992). Association is also been recorded in other autoimmune disease and Non-Hodgkin's lymphoma, such as Burkit lymphoma occurs in only X-linked lymphproliferation, T-cell tumours occur in ataxia-telangiectasia and Diffuse large B-cell lymphomas occur in rheumatoid arthritis (Baecklund, Iliadou *et al.* 2006; Grulich, Vajdic *et al.* 2007).

1.3 Lymphoma

Lymphoma is defined as a cancer in the lymphatic system. Lymphoma is a solid tumour of the lymphoid cells. It represents about 4% of the new cases of malignant disorders diagnosed in the UK and US each year, making it the fifth most common cancer diagnosed and is the cause of malignant disorder death (Jemal, Tiwari *et al.* 2004; Illes, Varoczy *et al.* 2009; CancerResearch 2011). The incidence of most malignant disorders is decreasing, whereas the lymphomas are increasing in frequency; the cause of this is unknown as the classification of lymphoma is difficult due to its diverse group of malignant diseases that vary in immunophenotype, genotype, clinical presentation, treatment and prognosis (Illes, Varoczy *et al*. 2009).

The World Health Organisation (WHO) have classified lymphoma on the basis of morphology, immunophenotype, genetic features, clinical features, and a normal counterpart cell is hypothesised for each cancer (Jaffe 2009; Ishida and Ueda 2011)

There are two types of lymphoma; Hodgkin's lymphoma (HL) and non-Hodgkin's lymphoma (NHL). There are more than 35 types of lymphoma, of which, 30 are classified as non-Hodgkin's lymphoma.

1.3.1 Non-Hodgkin's Lymphoma (NHL)

Over the years the understanding of genetic abnormalities has improved. The classification of NHL has steadily evolved as the understanding of the biology of the immune system has advanced (Harris, Jaffe *et al.* 1994; Armitage 2005; Vose

2008; Jaffe 2009). This has led to the development of the WHO classification of lymphoma. The WHO classification is presented in Table 1.4. It subdivides tumours into B cells and T/NK- cell origin and those with an immature or blastic appearance compared to those that are developing from more mature stages of lymphoid development (Armitage 2005).

B-cell neoplasms
Precursor B-cell neoplasm
Precursor B-lymphoblastic leukemia/lymphoma (precursor B-cell acute lymphoblastic leukemia)
Mature (peripheral) B-cell neoplasms
B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma
B-cell prolymphocytic leukemia
Lymphoplasmacytic lymphoma
Splenic marginal zone B-cell lymphoma (with or without villous lymphocytes)
Hairy cell leukemia
Plasma cell myeloma/plasmacytoma
Extranodal marginal zone B-cell lymphoma (with or without monocytoid B cells)
Nodal marginal zone B-cell lymphoma (with or without monocytoid B cells)
Follicular lymphoma
Mantle cell lymphoma
Diffuse large B-cell lymphoma
Burkitt lymphoma/Burkitt cell leukemia
T-cell and NK-cell neoplasms
Precursor T-cell neoplasm
Precursor T-lymphoblastic lymphoma/leukemia (precursor T-cell acute lymphoblastic leukemia)
Mature (peripheral) T/NK-cell neoplasma
T-cell prolymphocytic leukemia
T-cell granular lymphocytic leukemia
Aggressive NK-cell leukemia
Adult T-cell lymphoma/leukemia (HTLV1+)
Extranodal NK/T-cell lymphoma, nasal type
Enteropathy-type T-cell lymphoma
Hepatosplenic gamma delta T-cell lymphoma
Subcutaneous panniculitis-like T-cell lymphoma
Mycosis fungoides/Sezary syndrome
Anaplastic large cell lymphoma, T/null cell, primary cutaneous type
Peripheral T-cell lymphoma, not otherwise characterized
Angioimmunoblastic T-cell lymphoma
Anaplastic large cell lymphoma, T/null cell, primary systemic type

Table 1.4: World Health Organisation Classification of Lymphoid Neoplasms (Armitage 2005)

1.4 Immune System

The immune system is the body's defence system against infections caused by pathogenic microorganisms and viruses. The immune system is a critical system within the body and this is demonstrated by the severity of the immunodeficiencies, hence the complex array of protective mechanisms to control and eliminate the foreign bodies. The immune system defence is provided in two ways; innate and adaptive system. The innate immune system provides a generic and an immediate defence against a toxin or a pathogen, whereas the adaptive immune system manifests exquisite specificity for its target antigens. Even though the innate and adaptive immune systems are described as contrasting separate arms, they generally act together (Chaplin 2010).

The adaptive immunity also plays an important role in immunologic memory and the capacity for the rapid response in the event of re-exposure. The vital cells of the adaptive immune system include the effectors of cellular immune responses, the T lymphocytes and B lymphocytes (will be referred to as T cell or B cell), which are highly mobile. The characteristic of the adaptive immunity is the use of antigen specific receptors on T and B cell to initiate targeted effector responses in two stages. Firstly the antigen is presented to and recognised by the antigen specific T or B lymphocyte leading to cell priming, activation, and differentiation, all of which is carried out within the lymphoid tissues. This is followed by the second effector response, which takes place either due to the activated T cell or due to the release of antibody from activated B cell (Figure 1.7) (Parkin and Cohen 2001).

The ability of both B and T cells to generate antigen receptor diversity provides the immune system the potential to eliminate a vast variety of foreign pathogens.

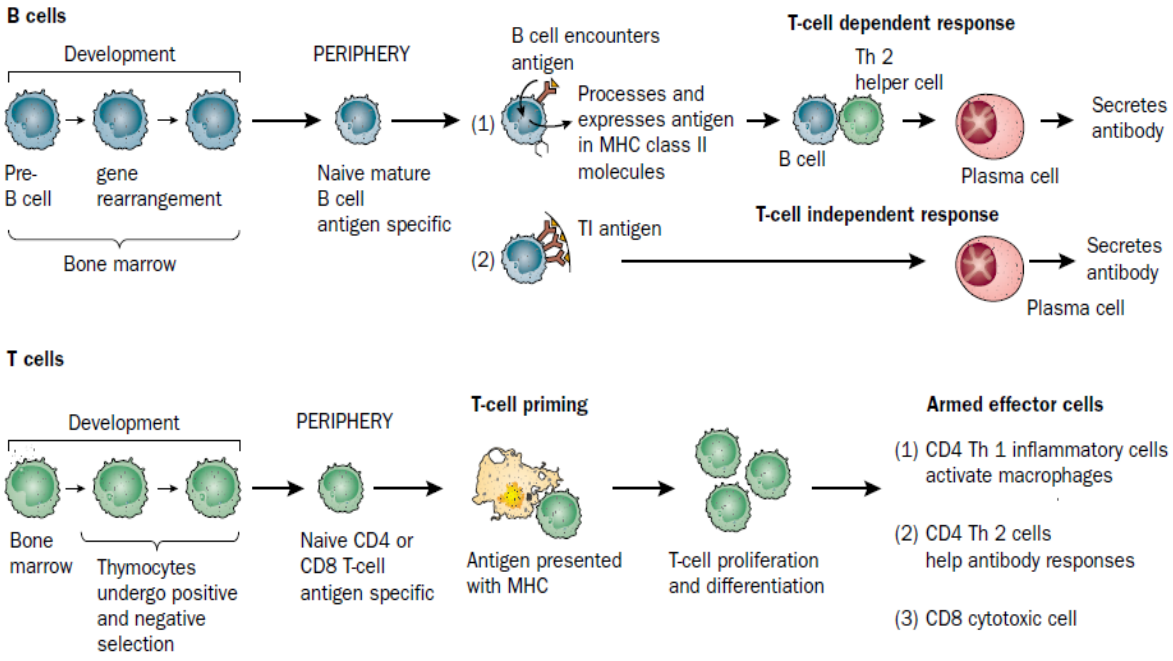

Figure 1.7: The role of T and B lymphocytes in Adaptive Immunity, adapted from (Parkin and Cohen 2001).
T and B cell initiate targeted effector responses in two stages. Firstly the antigen is presented to and recognised by the antigen specific T or B lymphocyte leading to cell priming, activation, and differentiation, all of which is carried out within the lymphoid tissues. This is followed by the second effector response, which takes place either due to the activated T cell or due to the release of antibody from activated B cell.

1.4.1 Generation of Antigen Receptor Diversity

The lymphoid arm of the immune system has evolved to respond and protect against a diverse set of antigens, encountered by the host. This is achieved

by the limitless diversity of antigen receptors. The generation of unique receptor type for each cell occurs during the development process of both B and T cells. The DNA sequences that present antigen specificity to these B cell receptors (BCRs) and T cell receptors (TCRs) are assembled at seven different loci (three for the BCR; one immunoglobulin heavy chain plus a κ or λ light chain; four for the TCR [α, β, γ and δ]) (Cedar and Bergman 1999). The generation of the antigen-binding diversity is assembled at the variable domain exons of the antigen receptors expressed on B and T cells, which are generated via somatic assembly of component variable (V), diversity (D), and joining (J) gene segments in a process called V(D)J recombination (Lieber 1991).

V(D)J recombination is a site-specific recombination process, which is directed by recombination signal sequences (RSS). Each RSS consists of a conserved heptmer and nonamer sequences, with respective consensus sequences of CACAGTG and ACAAAAACC, separated by intervening spacer sequence that is either 12bp or 23bp in length (van Gent, Ramsden *et al.* 1996). The V(D)J recombination process is initiated by the recombination activating genes 1 and 2 (RAGs) proteins, which induces double-strand breaks (DSB) between the RSS and variable region gene-coding segment (Jung and Alt 2004). The DSB are repaired by ubiquitously expressed nonhomologous end joining (NHEJ) proteins, forming precise signal and joints and imprecise coding end joints (Bassing, Swat *et al.* 2002).

1.4.2 Lymphoid Haematopoiesis

Both T and B cells, which are like all leukocytes, are derived from pluripotent hematopoietic stem cells (HSC), within the bone marrow. HSCs are defined as primitive cells which are capable of both self-renewal and differentiation into any of the hematopoietic cell lineages (Oguro and Iwama 2007). Once the HSCs receive a signal to undergo differentiation, the first biological change in the HSCs is the gradual loss of self-renewal; hence onwards the developmental sequence begins. It is the specialised microenvironment of the bone marrow that provides the signals both for the development of the lymphocytes progenitors from hematopoietic stem cells and for the further differentiation of the B cells. These signals regulate the expression of key genes that direct the control the development program.

The HSCs first differentiate into multipotent progenitors cells (MPPs), which have the ability to produce both myeloid and lymphoid cells. These cells express a cell surface receptor tryrosine kinase (also known as FLT3), which is required for the cells to differentiate to the next stage as either common lymphoid progenitors (CLP) or Common Myeloid progenitors (CMP). The stromal cells provide the FLT3 ligand to the FLT3 receptor to signal the MMP cells to differentiate into CLP. The progenitor cells can differentiation into any leukocyte, which can be separated into two major lineages: the lymphoid lineage, which includes the T, B, and natural killer (NK) cells. The second myeloid lineage is comprised of erythrocytes, megakaryocytes, granulocytes, and monocytes/macrophages (Lai and Kondo 2008; Slavov, Gimenes Teixeira *et al.* 2010).

The process by which lymphocytes are developed is named lymphopoiesis or otherwise formally called lymphoid haematopoiesis.

1.5 B Lymphocyte Development

In mouse, B cells are generated from pluripotent HSCs in the liver during mid-to-late foetal development and in the bone marrow after birth (hence the name B cells) (Hardy and Hayakawa 2001). In the mid- 1960s B cells, were discovered and characterised with the aid of experimental animal models, clinical evaluation of patients with immunodeficiency diseases and the characterization of cell surface molecule (Good and Zak 1956). During lymphocyte development, cells first become committed to the lymphoid, opposed to the myeloid, lineage and then to either B or T cell lineages. In mice the development of B cells initiates with the stromal cells secreting the cytokine IL-7, which is essential for the growth and survival of developing B-lineage cells (Ray, Stoddart *et al.* 1998).

The B cell development rise from the common lymphoid progenitor, and differentiate during a number of different stages, the earliest being pro-B cells, followed by late pro-B cells, large pre-B cell, small pre-B cell, and eventually the mature B cells (Figure 1.8). As mentioned previously the generation of unique and diverse antigen receptors (in B cells known as the immunoglobulin) for each B cell occurs during these developmental stages by DNA recombination. The V_H, D_H, and J_H, rearrangement of the heavy chain (H-chain) together with V_L-J_L rearrangement

of the light chain (L-chain) gene segments generate a B cell repertoire expressing antibodies.

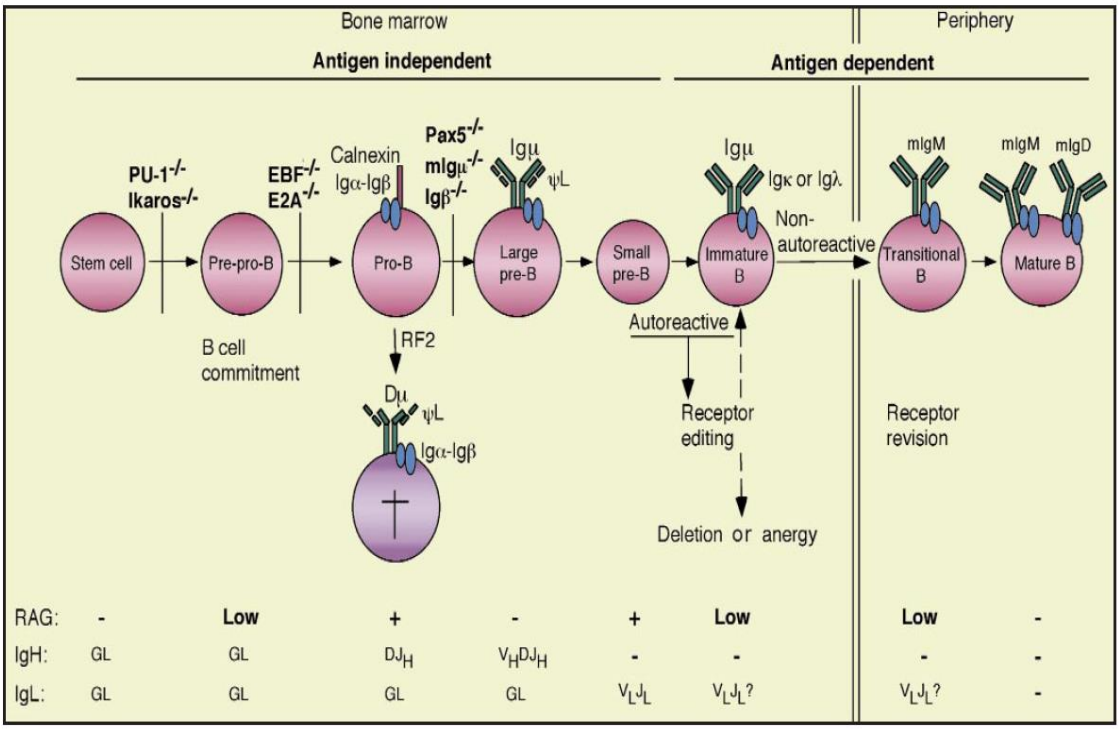

Figure 1.8: B cell development stages (Meffre, Casellas *et al.* 2000).
B cells are developed in the bone marrow from stem cells. During B cell development the V(D)J rearrangement occurs, regulated by a number of genes. The initial immunoglobulin (Ig) gene rearrangement begins at the early pro-B cell stage, with the joining of the D_H to the J_H segments, resulting in the development of late pro-B cells, followed by further rearrangement of V_H to DJ_H. The immature antigen specific B cells are released into the periphery where they are exposed to antigens and matured.

The initial immunoglobulin (Ig) gene rearrangement begins at the early pro-B cell stage, with the joining of the D_H to the J_H segments (Alt, Yancopoulos *et al.* 1984). This leads the cell to become a late pro-B cell, where the second rearrangement of V_H gene segment to the DJ_H. The successful rearrangement of the V_H to DJ_H leads to the production of the intact μ heavy chain, resulting in the

expression of Igμ chain in complex with the surrogate light chain (consisting of λ5 and VpreB chains). The Igμ chain is also associated with two other proteins chains, Ig-α (CD79α) and Ig-β (CD79β). The immunoglobulin-like complex is known as the pre-BCR cell receptor (pre-BCR) (Melchers 2005).

The pre-BCR plays a critical role in B cell development as suggested in a number of studies where deficient mice were created for the components of the pre-BCR, either the μ heavy chain or the λ5 protein (Kitamura, Roes *et al.* 1991; Kitamura, Kudo *et al.* 1992). In both of the studies B cell development was found to be severely impaired at the transition from the pro-B cell stage to the large pre-B cell stage, which resulted in no or poor generation of mature B cells.

The assembly of the pre-BCR mediates a number of signals so that the following process can be performed (Martensson, Almqvist *et al.* 2010).

- The cellular transition from the large pre-B cell stage to the small pre-B cell stage.
- The Igμ heavy chain allelic exclusion and the down-regulation of the recombination by deactivating the recombination-activating genes (RAG).

Allelic exclusion is a process that ensures when one of the two μ heavy chain alleles has achieved a functional rearrangement at the pro-B cell stage and produced the Igμ heavy chain the V(D)J rearrangement at the other allele is inhibited to avoid the generation of B cells with double specificity and a B cell expressing a unique B cell receptor with monospecificity (Karasuyama, Nakamura *et*

al. 1997). It has been demonstrated that the expression of RAG-1 and RAG-2 genes was down-regulated in the large pre-B cells (Grawunder, Leu *et al.* 1995). In contrast, the expression of RAG-1 and RAG-2 were highly expressed in pro-B cells and small pre-B cells, where the rearrangement of the heavy chain and the light chain occurs, respectively.

As cells transit from large pre-B cell stage to small pre-B cell stage, the pre-BCR induces signals for proliferation expansion of large pre-B cells, which undergo two to five rounds of cell division (Rolink, Winkler *et al.* 2000).

As the cells transit into small pre-B cells the expression of RAG1 and RAG2 genes is induced, resulting in the rearrangement of the light chain locus, expanding the population of the cells with a successful IgH chain, ultimately resulting in a more diverse antibody repertoire, which later in development will recombine with and express a unique light chain.

In line with the monospecificity of the heavy chain, the light chains are also enforced with allelic exclusion. The rearrangement at the light chain does not include the D segment, only joining the V to J segment. Like the heavy chain the rearrangement is initiated on only one allele, of which if the VJ rearrangement fails to produce a functional light chain, then the rearrangement is repeated on the other allele. As well as allelic exclusion, light chains also undergo isotypic exclusion, meaning that they express either κ or λ Light chain. However, the expression of κ or λ is not represented equally on the B cells or in the serum Ig (Chen, Trounstine *et al.* 1993).

The successfully rearranged light chain goes on to pair with the Igμ chain, which transforms the cells from small pre-B cells to immature B cells. Immature B cells are the first B lineage cells that express BCRs, they display surface IgM (Meffre, Casellas *et al*. 2000). The immature B cells are positively and negatively selected based on their antigen specificity and migrate from the bone marrow to the secondary lymphoid organ, spleen and lymph nodes.

1.5.1 B cell maturation and subset development

Loder, *et al* (1999) was first to refer Immature B cells as "transitional" (T1 and T2) based on their phenotype and ontogeny and were characterised primarily in the mouse (Chung, Silverman *et al.* 2003) and since then a number of groups have confirmed and further characterised the distinct subsets. The early transitional B cells exit the bone marrow and enter the peripheral circulation. On arriving in the spleen, the cells pass through a series of transitional stages prior to developing into mature follicular(FO), marginal zone (MZ) or B-1 B cells (Allman and Pillai 2008) (Figure 1.9).

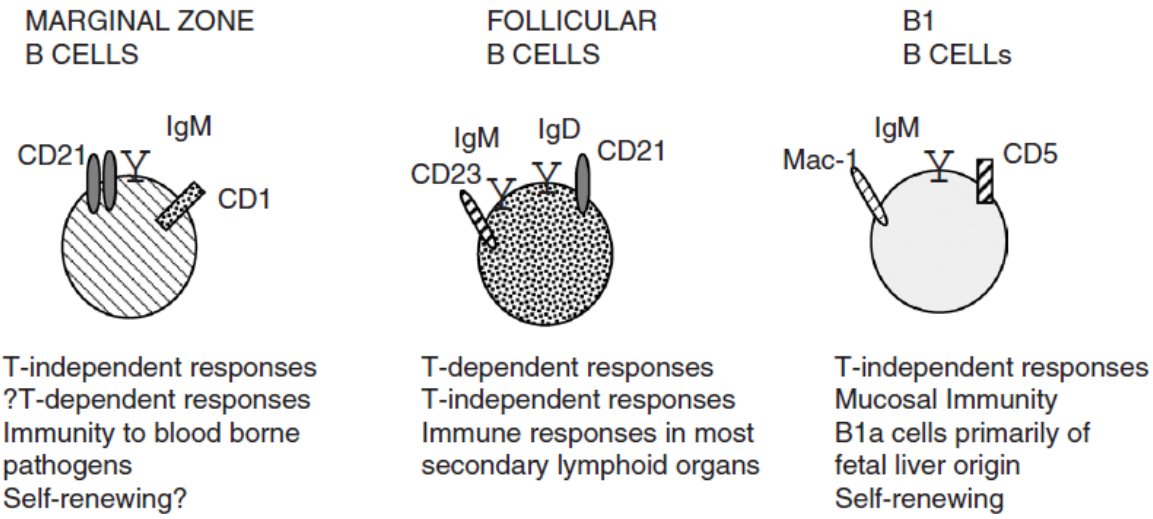

Figure 1.9: Characteristics of the three peripheral B cell subsets (Pillai, Cariappa *et al.* 2004).

Mature FO B cells are always found adjacent to T cell zones. This arrangement allows activated FO B cells and activated T helper cells to migrate towards each other and interact. Therefore FO B cells participate in T cell-dependent germinal centre-based immune responses to protein antigens. FO B cells also occupy a second niche; they also recirculate through the bone marrow and are known to respond to T cell-independent manner to blood borne pathogens (Pillai and Cariappa 2009).

In contrast to FO B cells, MZ and B-1 B cells respond in a T cell-independent manner and can develop into antibody producing plasma cells upon antigen-stimulation (MacLennan, Toellner *et al.* 2003). MZ B cells are considered to be like innate-like cells that can be induced to differentiate into short lived plasma cells in the absence of BCR ligation (Allman and Pillai 2008). B-1 B cells are the majority B cell subpopulation in pleural and peritoneal cavities (Baumgarth 2011).

1.5.2 B cell Tolerance

The development and the maturation process of B cells are tightly regulated by testing the antigen receptor for tolerance to a self-antigen. There are two processes of B cell tolerance; central tolerance, where the antigen receptor of the immature B cell is tested in the bone marrow. The second process is peripheral tolerance, where self-reactive B cells have escaped the central tolerance and become mature B cells; hence the name peripheral tolerance as the process occurs in the secondary lymph node organs.

Understanding the tolerance mechanisms and the identification of the developmental stages of B cell tolerance has been difficult. But the recent availability of transgenic mice in which majority of lymphocytes express an immune receptor against a given antigen has enabled the identification of several mechanisms.

1.5.2.1 Clonal Deletion

During the B cell development, the immune system generates potentially harmful self-reactive B cells that must be distinguished from useful lymphocytes. These self-reactive B cells are eliminated from functional repertoire during the transition from the pre-B to mature B-cell stage in the bone marrow, even low-affinity interactions can lead to central deletion (Nemazee and Burki 1989; Chen, Nagy *et al.* 1995), by the process of BCR ligation.

1.5.2.2 Clonal Anergy

Clonal anergy was first proposed in the 1980s by Pike and Nossal, when they found that B-cell precursors cultures with high levels of antibody against surface IgM impeded any B-cell development (Pike, Boyd *et al.* 1982). Anergic lymphocytes persist in tolerant animals, but are functionally inactivated. Goodnow group demonstrated clonal anergy *in vivo* using transgenic B-cell receptor (BCR) against hen-egg lysozyme (HEL). They found that when these mice were crossed with mice expressing a soluble form of HEL, the transgenic B cells became Anergic (Goodnow, Crosbie *et al.* 1988).

1.5.2.3 Receptor Editing

Although many self-specific B cells undergo clonal deletion, some of them can undergo further somatic recombination to make new V_H and V_L combination that are not specific, which is known as receptor editing. The ability of receptor editing to rescue some self-specific B cells by changing their specificity was demonstrated by the Nemazee and Weigert groups, with the aid of mice carrying Ig transgenes encoding self-MHC-specific BCR. They found that total B cell numbers were greatly reduced in the periphery, but of these few B cells that were produced in the mice were not self-specific because they have been able to make new recombination of both heavy and light chains in the V region (Gay, Saunders *et al.* 1993; Tiegs, Russell *et al.* 1993).

1.5.3 Homeostatic proliferation of B cells

When B cells emerge from bone marrow into the periphery, they are still functionally immature. Due to the high expression levels of surface IgM and low levels of IgD, not many immature B cells survive to develop into mature B cells, however majority of peripheral B cells are long-lived (Gaudin, Rosado *et al.* 2004). The failure of most of the newly formed B cells to survive for more than a few days is due to the access of follicles in the secondary lymphoid organs. The follicles cannot accommodate all the B cells that are generated, hence if the new immature B cells do not enter the follicles, and then they cannot survive due to the competition for entry. Hence the follicles provide signals necessary for B cell survival, one particular signal is from B-cell-activating factor (BAFF, also known as BLyS) belonging to the TNF family (Schneider, MacKay *et al.* 1999).

BAFF and a proliferating-inducing ligand (APRIL) are related members of the TNF family. BAFF binds to the BAFF receptor (BAFFR, also known as BR3) with high affinity. In the absence of BAFF, B cell maturation proceeds normally in the bone marrow, however splenic B cell are reduced, especially transitional T2, mature and MZ B cells (Schiemann, Gommerman *et al.* 2001; Bossen, Tardivel *et al.* 2011). Hence BAFF, upon binding to the receptor BR3 enhances survival of semi-mature and mature B cells and it has been reported that this occurs by promoting expression of BCL-2 family members (Harless, Lentz *et al.* 2001). BAFF also mediates longevity of mature B cells. This was observed when treatment with soluble BAFF receptors resulted in the rapid loss of most of the mature peripheral B cells (Gross, Dillon *et al.* 2001).

Fitness of survival of naïve B cells does not only rely on competitive processes but are also dictated by BCR specificity. Continuous signalling through B cell receptor also has a positive role in the maturation and continued recirculation of peripheral B cells. This is supported by the existence of more restricted immunoglobulin (Ig) variable region gene repertoire in mature B cells than that seen in their immature precursors (Levine, Haberman *et al.* 2000).

1.6 T Cell Development

T cells arise from hematopoietic stem cells that migrate to the thymus (Starr, Jameson *et al.* 2003). The thymus is an organ that supports the differentiation and selection of T cells (Takahama 2006). Figure 1.10 shows the overall scheme of the T cell development within the thymus.

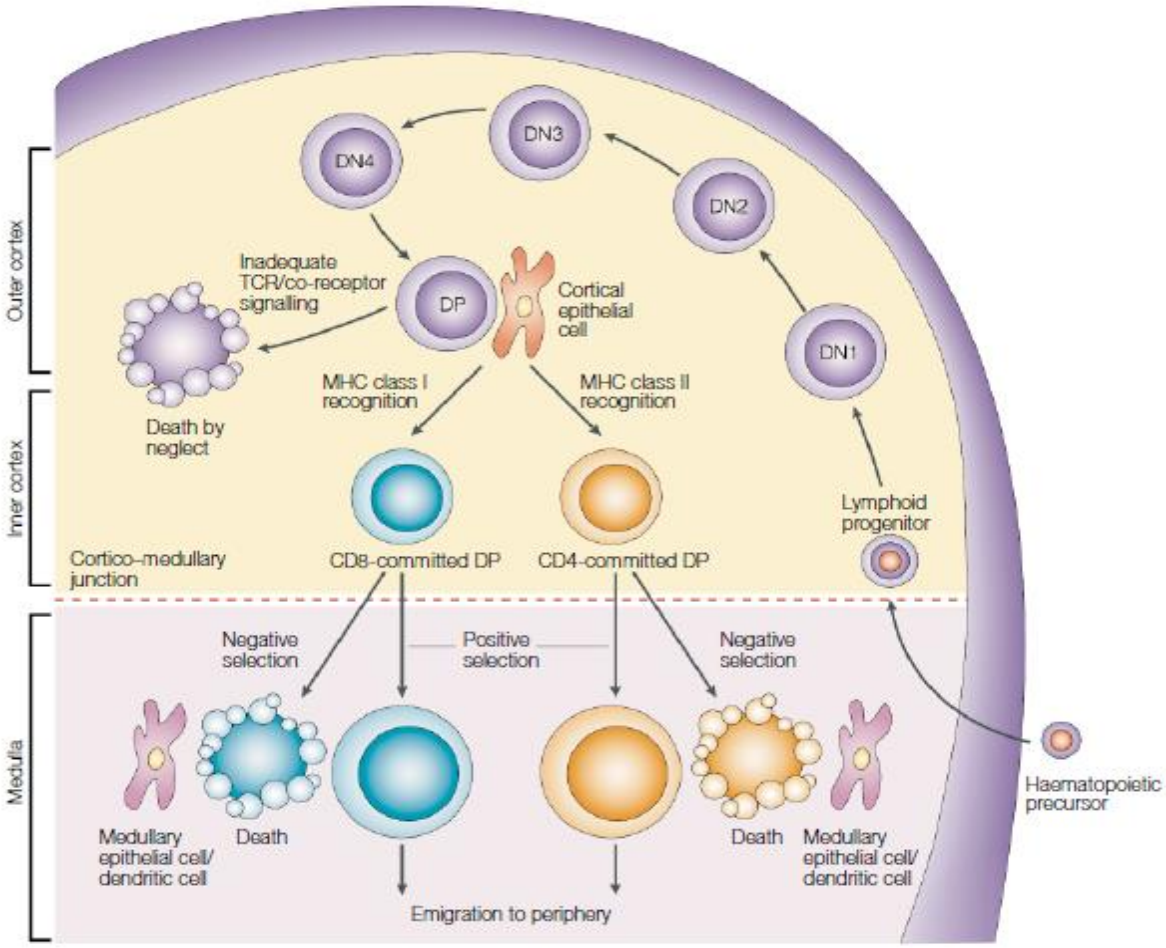

Figure 1.10: Overall summary of T cell development in the Thymus (Germain 2002).

T cells development occurs in the thymus. The hematopoietic stems cells migrate to the thymus were they differentiate and mature into specialised T cell.

Lymphoid Progenitors arise in the bone marrow and migrate to the thymus. In the thymus these cells lose the potential for B cell and natural killer cell development (Michie, Carlyle *et al.* 2000). During T cell development in the thymus the T cell precursors, called thymocytes, lack expression of CD4 and CD8 and are called double negative (DN). DN thymocytes can be subdivided further into four sequential stages of differentiation, which are known by their surface expression of

CD44 and CD25: DN1, $CD44^{+}CD25^{-}$; DN2, $CD44^{+}CD25^{+}$; DN3, $CD44^{-}CD25^{+}$; and DN4;$CD44^{-}CD25^{-}$ (Godfrey, Kennedy *et al.* 1993). As the cells progress from DN2 to DN4 stages, they express the pre-T cell receptors (pre-TCR), which is composed of the non-rearranged surrogate pre-Tα Chain and a rearranged TCR β-chain, forming a pre-TCR complex (von Boehmer and Fehling 1997). Both TCRα and β genes have variable (V) and joining (J) segments, while β genes also have diversity (D) segments. The rearrangement of these genes follows the same mechanism as B cell developments, with the aid of RAG 1 and RAG 2. Only the cells that succeed in a functional in-frame rearrangement of the gene encoding the TCR β-chain are selected for further differentiation beyond this DN3 stage. The selection events are required to select only those cells that are able to weakly recognise self-MHC molecules loaded with self-peptide (Michie and Zuniga-Pflucker 2002). The cells that have a functional β chain and recognise self-peptide/MHC molecules are termed as β-selection.

1.6.1 Positive Selection and Negative Selection

In the thymus cortex, the successful expression of the pre-TCR complex on the cell surface, along with Delta-Notch interaction, initiates the signals to progress the development to double positive (DP) thymocytes that express the TCRαβ antigen receptors (Takahama 2006). During the transition of DN4 to DP the RAG proteins expression is switched off and the pre-TCR expression leads to substantial cell proliferation.

At the DP stage of the thymocyte differentiation, the RAG genes are once again expressed and the cells will undergo the rearrangement of TCR Vα and Jα, producing a new rearranged TCRα chain that can pair with the existing rearranged TCRβ chain, which allows the surface expression of TCRαβ-CD3 complex (Goldrath and Bevan 1999).

From the large number of DP thymocytes, the ones that are best suited to function in the host environment are permitted to mature and migrate to peripheral lymphoid tissues. This selection is characterised by four processes; death by neglect, Negative selection, positive selection and lineage-specific development (Figure 1.11) (Germain 2002).

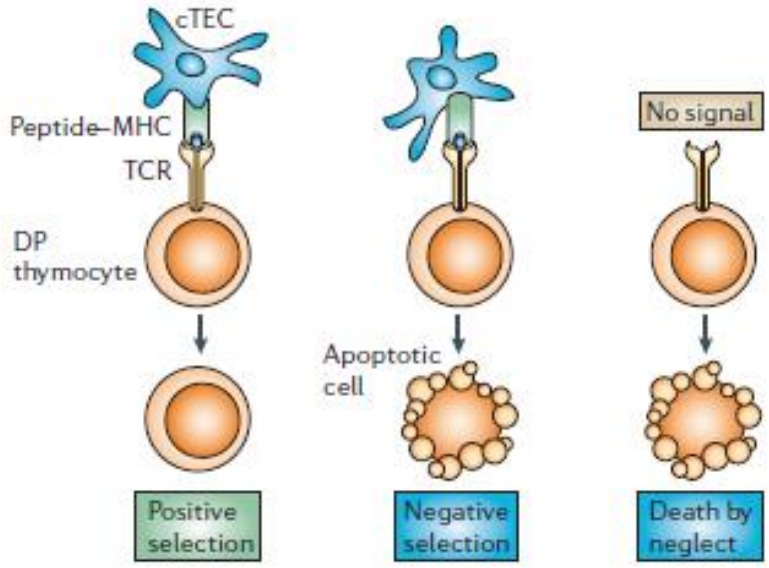

Figure 1.11: Positive selection process, negative selection process and death by neglect (Takahama 2006)

The double positive (DP) thymocytes undergo both positive and negative selection to delete any auto-reactive lymphocytes. Those thymocytes that have receptors that bind strongly to self-antigens are removed by the induction of apoptosis of the auto-reactive cells.

Death by neglect is initiated when there is too little signalling between the TCR and the self-peptide-MHC ligands, which result in delayed apoptosis. Too much signalling can promote acute apoptosis, which is caused in negative selection. Positive selection occurs when the signalling is at an intermediate level, where TCR signalling initiates effective maturation (Germain 2002).

Positively selected DP thymocytes are induced to differentiate into single positive (SP) thymocytes, which are $CD4^{-}CD8^{+}$ (MHC class I reactive) or $CD4^{+}CD8^{-}$ (MHC class II reactive) (Hollander and Peterson 2009). These thymocytes spend approximately 12 days in the medulla before travelling to the thymus. During this period the SP thymocytes go through a maturation process. This is accompanied by further deletion of self-reactive thymocytes that have escaped negative selection in the cortex. This process is particularly important in central tolerance to tissue specific antigens (Kyewski and Derbinski 2004).

As well as the deletion mechanism that ensures self-tolerance, it is also thought that the medulla is the place for the production of regulatory T cells (Treg) (Takahama 2006). Treg cells have emerged to play a critical role in suppressing the response of the immune system to self-antigen (Kuhn, Beissert *et al.* 2009).

1.6.2 T cell Maturation and T Cell Tolerance

After completing their maturation in the thymus, the T cells migrate to the periphery. Although these T cells are mature, they are termed immunologically naïve T cells, as they have not been activated in the periphery. Naïve T cells express molecules such as CD62L (Gallatin, Weissman *et al.* 1983; Resino, Navarro *et al.*

2001) that enables their circulation through the blood and secondary lymphoid organs such as lymph nodes and the white pulp area of the spleen. During the circulation it enables them to come in contact with thousands of antigen presenting cells (APC) in the lymphoid tissues and interact with peptides on MHC-molecule. APC are cells that present antigen peptide associated MHC molecule to inactivate T cells and activate them for the first time, such as macrophages, dendritic cells and B cells.

The level of stimulation that the T cell receives upon contact with the APC presenting peptide-MHC molecules governs the response of that T cell. Only those T cells that bind and receive a strong signal upon interaction with an APCbecome activated and induce an immune response.

The outcome of a T cell response to antigen stimulation is regulated by the interplay and complex interaction of positive (stimulatory) and negative (inhibitory) pathways (Zhu, Symonds *et al.* 2008). It is important that there is some mechanism to prevent auto reactivity of the T cells after they have emigrated from the thymus. This is achieved through the T cell tolerance process (Figure 1.12). Although most of the self-reactive T cells are eliminated in the thymus, negative selection has limitations and additional mechanisms of tolerance are required to limit autoimmunity (Bandyopadhyay, Soto-Nieves *et al.* 2007).

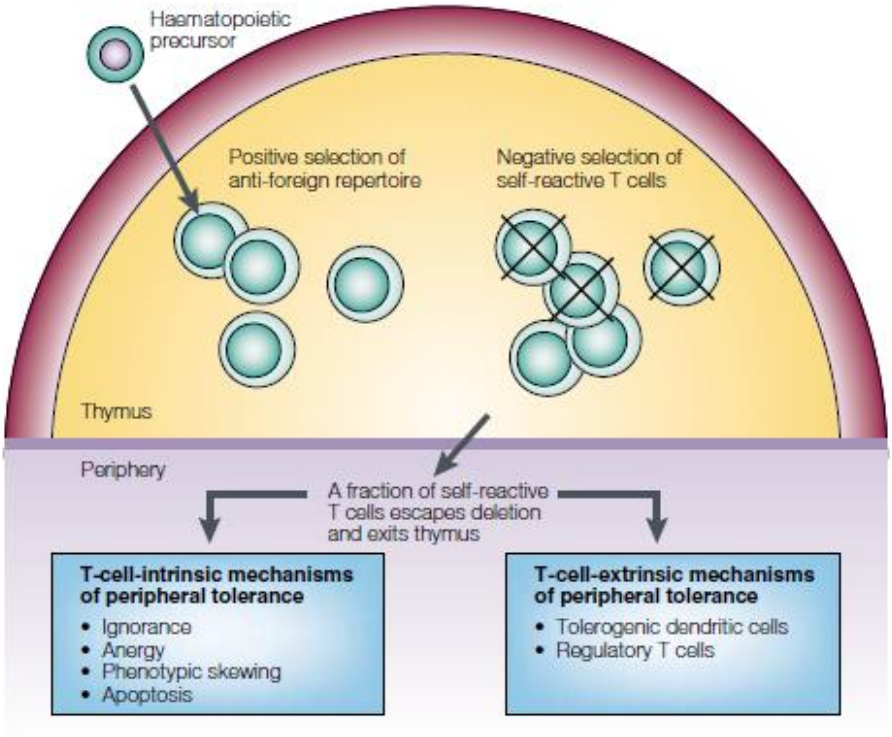

Figure 1.12: Pathways to tolerance (Walker and Abbas 2002).

T cells undergo both central tolerance and periphery tolerance. A few self-reactive T cells escape the central tolerance and leave the thymus, in the periphery they go through further selection and auto-reactive T cells are removed.

1.6.3 T cell activation

T cell activation is initiated by the interaction of the TCR-CD3 complex with an antigenic peptide bound to either MHC class I or class II molecules on the surface of APC. However this interaction alone does not activate the T cells. A further two signal are required for the T cells to be activated once they come in contact with the APC, a co-stimulatory molecule, such as CD28 and cytokines that trigger the rapid growth and proliferation of effector T cells and are thought to increase the cytotoxic effects of the $CD8^+$ T cells (Figure 1.13).

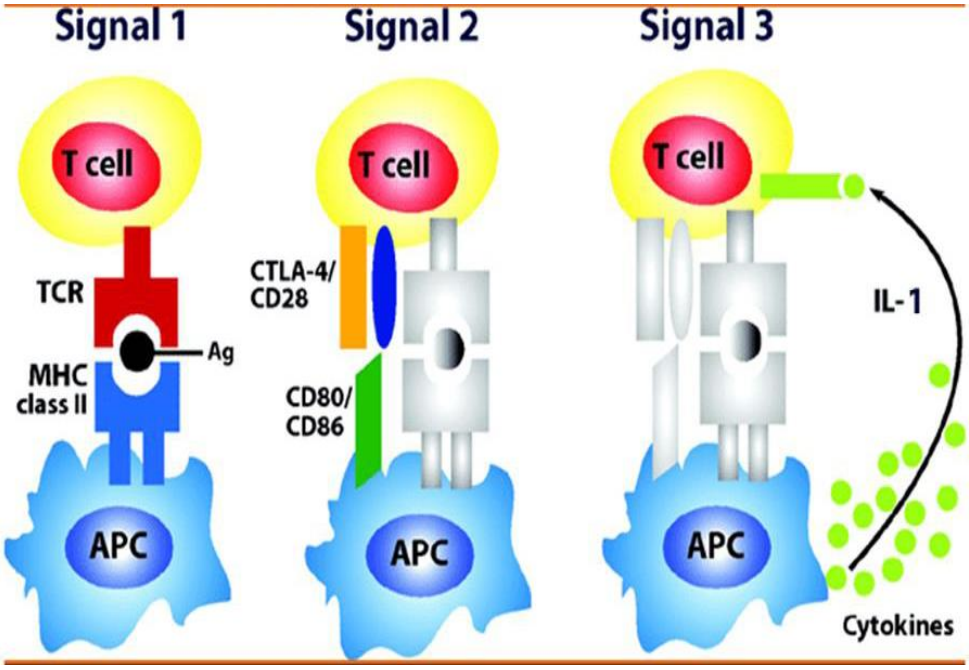

Figure 1.13: T cell Activation (Pietropaolo, Surhigh *et al.* 2008)
Three signals are required to activate the T cell receptor; the TCR-CD3 complex interacts with either MHC class I or class II molecules, co-stimulatory molecules and cytokines, which trigger rapid growth and proliferation of the effector T cell.

A strong signal in the T cell by APC surpass the threshold for T cell activation, which leads to a cascade signalling molecules, resulting in the generation or release of second messengers, such as Ca^{2+}. This signalling cascade eventually results in the induction of transcription factors, such as nuclear factor of activated T cells (NFAT) (Dolmetsch, Lewis *et al.* 1997) and Early Growth Response (Egr) (Safford, Collins *et al.* 2005; Anderson, Manzo *et al.* 2006). The transcription factors initiate the process of proliferation and also induce the expression of cytokines, such as IL-2 or Il-17, leading to the differentiation of the effector T cells.

On antigen recognition, naïve T cells differentiation from naive T cells into effector T cells with enhanced functional (Wan and Flavell 2009). Based on the cytokines produced these effector cells can be classified into different groups with specialised functions (Table 1.5).

Cytokine Produced	T cell Type	Function
IFN-γ and IL-2	T_H1	Macrophages activating effector cell. Cell-mediated immunity
IL-4 and IL-5	T_H2	Activation of B cells.
IL-17 and IL-6	T_H17	Promote acute inflammation by neutrophil recruitment
TGF-β and IL-10	T_{reg}	Produce inhibitory cytokines. Involved in tolerance.

Table 1.5: T cell effector subsets and function adapted from (Zhu and Paul 2008)

After the effector cells have cleared the infection the majority of the responding effector T cells die, but a small cohort of cells remains for long-term protection. These cells, are termed memory cells, and can respond more rapidly than naïve T cells upon re-exposure to the same antigen *in vivo* (Sallusto, Lenig *et al.* 1999; Veiga-Fernandes, Walter *et al.* 2000).

1.6.4 T Cell Homeostasis

T cell production and survival are tightly constrained by competition for cytokines and self-peptide MHC ligands. Such competition allows the size of T cell population to increase gradually during young life and then declines progressively with age (Sprent, Cho *et al.* 2008). Throughout this period the cellularity of the spleen and lymph nodes remains relatively constant (Surh and Sprent 2008), T cell homeostasis is the mechanism that exists to serve the maintenance of T cell number and cell survival. Homeostatic processes are vitally involved in T cell development, survival, and maintenance, as well as following antigen-driven responses (Boyman, Letourneau *et al.* 2009). The homeostasis of naïve and memory T cells is maintained differently. In the case of naïve T cells, prolonged survival of these cells in interphase is dependent on a combination of transform TCR signalling from interaction with self-peptide MHC ligands plus interaction with IL-7 and to a lesser degree with IL-15. In contrast, most of the memory T cells are MHC independent but rely heavily on contact with a mixture of IL-7 for cell survival and IL-15 for cell proliferation (Surh and Sprent 2008).

As mentioned post-thymic naïve T cells depend on contact with IL-7 and self-peptide MHC molecules for their homeostatic survival. Homeostatic proliferation caused by the combination of IL-7 and Self-peptide MHC molecule continues at a slow rate and is readily detected for naïve $CD4^+$ and $CD8^+$ T cells upon adoptive transfer into syngeneic normal hosted that have been made acutely lymphopenic by irradiation, known as lymphopenia-induced proliferation (LIP) (Tan,

Dudl *et al.* 2001). In addition to LIP, naïve T cells can also proliferate in non-lymphopenic animals by increasing the intensity of IL-7 signals, using IL-7/anti-IL7 mAb complexes. These complexes induce slow proliferation of the naïve T cells, particularly $CD8^+$ cells (Boyman, Ramsey *et al.* 2008). Interestingly when naïve T cells are stimulated with other members from γ_c cytokines, such as IL-2 and IL-15, they undergo rapid proliferation. A Group performed adaptive transfer of naïve $CD8^+$ T cells to IL-2Rβ-deficient hosts (they contain elevated levels of IL-2 and possibly IL-15). This led to rapid proliferation and differentiation of the donor T cells into effector T cells followed by the production of memory cells (Cho, Boyman *et al.* 2007). The cytokines and the self-peptide MHC molecules are not the only factors involved in the homeostasis of naïve T cells, as evidence has shown TCR signals are also necessary. The initial prospect used to be that naïve T cells did not recognise self-peptide-MHC ligands in the periphery. However, recently several groups have shown that MHC molecules are important in maintaining peripheral pool of T cells. Individual experiments have demonstrated the importance of MHC class I and class II for the maintenance of naïve $CD8^+$ and $CD4^+$ T cells, respectively. In two individual experiments, TCR transgenic $CD8^+$ T cells were transferred into MHC class I-deficient hosts. In both experiments it was shown that the naïve $CD8^+$ T cells were significantly reduced in the periphery (Tanchot, Lemonnier *et al.* 1997; Murali-Krishna, Lau *et al.* 1999).

Memory T cells often divide in the periphery long after antigen stimulation. The homeostasis of memory T cells is controlled differently. Memory cells are generally recognised by their CD44 marker, which is expressed much lower in naïve

T cells. Studies using antigen specific memory cells establish that cells still continue to proliferate in the absence of MHC molecules (Murali-Krishna, Lau *et al*. 1999). Hence indicating that peptide-MHC ligands are not required for the survival of $CD44^{high}$ cells instead they require the presence of IL-7 and IL-15 cytokines (Boyman, Purton *et al.* 2007; Surh and Sprent 2008).

1.6.5 Peripheral T cell Tolerance

Throughout T cell development T cells maintain self-tolerance, which is divided into central and peripheral tolerance. As discussed previously central tolerance plays a key role in the early life of T cells, it is fostered by apoptosis through elimination of auto reactive lymphocytes in the thymus. Potentially self-reactive T cells do escape central tolerance due to relatively low-avidity for the self-peptide MHC complex molecule (Liu, Fairchild *et al.* 1995). These self-reactive T cells migrate to the periphery where they can lead on to cause autoimmune disease. Therefore the self-reactive T cells that escape the negative selection must be kept under control in the periphery. To simplify the tolerance process the peripheral tolerance can be divided into those that act directly on the responding T cell (T-cell intrinsic) and those that evoke additional subsets of cells, including dendritic cells (DCs) and regulatory cells (T cell extrinsic) (Walker and Abbas 2002). Here, the cell intrinsic, T cell clonal anergy is discussed further.

1.6.5.1 T cell Anergy

The term anergy was first coined by Von Pirquet in 1908 to describe the loss of delayed-type hypersensitivity to tuberculin in individuals infected with measles virus (Schwartz 2003). Following a series of experiments describing antigen-specific B cell tolerance *in vivo*, Nossal and Pike employed the terminology clonal anergy to describe the antigen-specific nature of the unresponsiveness they observed (Nossal 1993). However with respect to T cell tolerance, it is a tolerance mechanism in which the lymphocyte is intrinsically functionally inactivated following an antigen encounter, but remains alive for an extended period of time in the hypo-responsive state (Schwartz 2003). In earlier experiments, it was found that T cell clones that were initially stimulated with formaldehyde fixed APCs failed to proliferate or produce IL-2 upon re-challenge with live APC+peptide (Jenkins and Schwartz 1987). The idea of T cell anergy occurred when T cells were stimulated through the T cell receptor in the absence of co stimulation came when it was found that fixation of APCs inhibited the B7-CD28 interaction (Jenkins and Johnson 1993).

Calcium signalling is critical for the first step of anergy induction (Macian, Garcia-Cozar *et al.* 2002). Calcium- induced anergy is mediated primarily by NFAT, a transcription factor regulated by the protein phosphatase calcineurin, and both NFAT activation and anergy induction are blocked by the calcineurin inhibitors cyclosporine A (CSA) and FK506 (Jenkins, Chen *et al.* 1990; Macian, Im *et al.* 2004; Podojil and Miller 2009). This model was formally proven by Rao's group using cells from NFAT knockout mice and over expressing mutants of NFAT that could not cooperate with AP-1 (Macian, Garcia-Cozar *et al*. 2002).

Activated NFAT travels to the nucleus and up regulates the transcription of several genes, including the genes encoding the transcription factors early growth response 2 (EGR2) and early growth response 3 (EGR3) (Figure 1.14) (Fathman and Lineberry 2007).

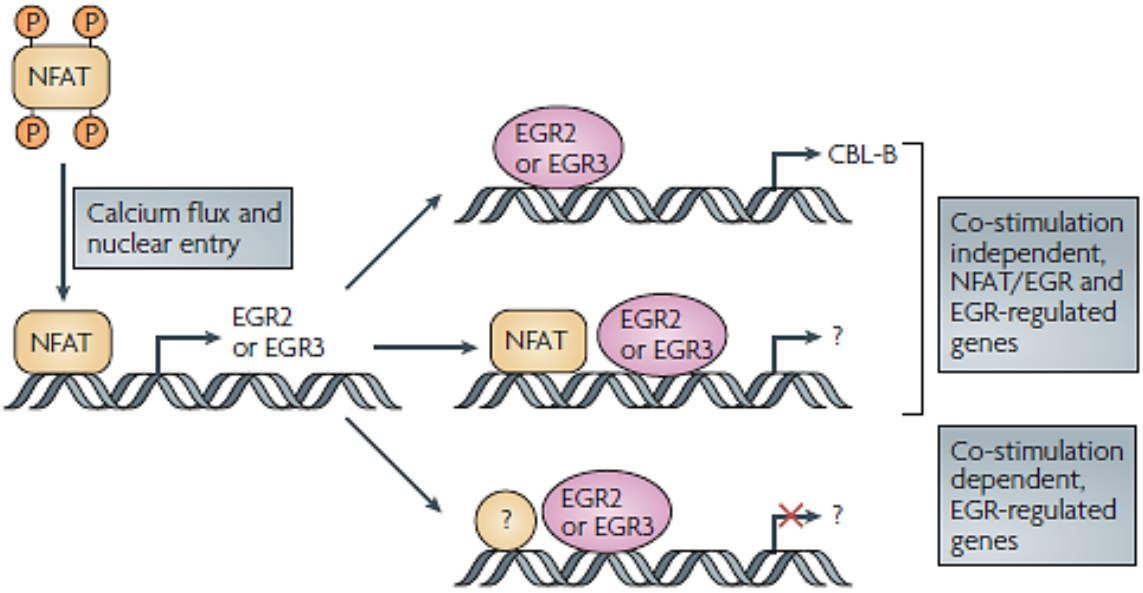

Figure 1.14: Induction of EGR2 or EGR3 by activated NFAT (Fathman and Lineberry 2007).

The initial steps of anergy require calcium signalling, which is mediated primarily by NFAT. Egr2 and Egr3 transcription factor genes are activated by NFAT.

When self-reactive lymphocytes escape from tolerance and become activated, then the immune system will react to itself, leading to autoimmune diseases. It is still not clear how autoimmune diseases occur, however, autoimmunity is thought to result from a combination of genetic variants, acquired environmental triggers such as infections, and stochastic events (Rioux and Abbas

2005). By focusing on how our understanding of cellular and molecular mechanisms of self-tolerance and immunity, it will guide rational solutions to autoimmune disease (Goodnow, Sprent *et al.* 2005).

A number of studies have been carried out to identify the exact process of how self-tolerance is initiated, managed and balanced along with immune response. Each of the mechanisms are essential for maintaining self-tolerance, as loss of one pathway can result in a limited set of autoimmune reactions (Abbas, Lohr *et al.* 2004).

The complexity of the immune system has led to a lot of research in understanding the genetic model for the T cells differentiation into memory cells or the route of achieving self-tolerance. One study by Zhu *et al* has shown that Egr2 controls the self-tolerance of T cells and prevent the development of autoimmune diseases, such as lupus like syndrome (Zhu, Symonds *et al.* 2008).

1.7 Autoimmune Diseases

Autoimmune diseases are still one of the top five diseases that are major causes of morbidity and mortality throughout the world. They are difficult to treat due to the focus on the immune response and self-antigens cannot be eliminated from the immune system. Self-reactive B and T cells are a normal component of the immune system for which many self-tolerance mechanisms are in place. Autoimmune diseases develop when self-reactive lymphocytes escape from tolerance and become activated (Rioux and Abbas 2005). Traditionally autoimmune

diseases were classified as T cell mediated or autoantibody mediated. However with the improved understanding of the immune system , the role of B cells in autoimmune diseases is more understood (Hampe 2012).

The major autoimmune diseases include rheumatoid arthritis, multiple sclerosis, type 1 diabetes mellitus, inflammatory bowel disease and systemic lupus erythematosus (Cho and Gregersen 2011).

Systemic lupus erythematosus (SLE) is an autoimmune disease characterised by the production of antibodies to components of the cell nucleus in association with a diverse clinical manifestations. The key features of SLE patients are inflammation, vacuities, immune complex depositions, and vasculopathy. Most cases of SLE are sporadic but the observation of concordance of SLE in identical twins, and the increase frequency of SLE in first degree relatives, reflect a polygenic inheritance of the disease (Mok and Lau 2003).

The common and basic pathology feature of SLE patients is that of inflammation and blood vessel abnormalities, which includes immune complex depositions. A key diagnoses performed by clinicians in diagnosing SLE is organ pathology, of which the main organ being the kidney (Weening, D'Agati *et al.* 2004). By light and immunofluorescence microscopy, renal biopsy in patients with SLE display mesangial cell proliferation, inflammation, basement membrane abnormalities, and immune complex depositions.(Mok and Lau 2003).

Autoantibody production is the central immunological disturbance in SLE patients. These autoantibodies are directed at several self-molecules found in the

nucleus, cytoplasm, and cell surface, in addition to soluble molecules such as IgG (Arbuckle, McClain *et al.* 2003; Mok and Lau 2003).

In both human patients and mouse models genetic predisposition plays a critical role in the susceptibility of SLE; in human SLE, a number of genes have been identified. Our group identified Egr2 to be a key gene involved in controlling self-tolerance. We produced Egr2 conditional knockout mice, where Egr2 was specifically deleted in cells expressing CD2 promoter, i.e. lymphocytes. These mice exhibited features of SLE disease. The mice displayed hair loss, skin lesions and accumulations of inflammatory monoclear cells in various tissues, of which majority of them were T cells, with a small population of B cells. Kidney histology also revealed deposition of immune complexes in the glomeruli, as SLE patients also manifest glomerulonephritis (Zhu, Symonds *et al*. 2008).

1.8 Early Growth Response Transcription Factors

The Egr family consist of four members; Egr1 (also known NGFI-A, Zif268 or Krox-24), Egr2 (also known as Krox-20), Egr3 (also known as PILOT) and Egr4 (also known as NGFI-C). Three of the Egr genes (Egr1, Egr2 and Egr3) are growth factor inducible, whereas Egr4 is not. (Sukhatme 1990). All four Egr proteins share extensive 90% homology throughout the zinc finger DNA-binding domains and recognise the same consensus DNA-binding motifs (Beckmann and Wilce 1997). All the Egr proteins contain closely related Cys_2-His_2-type of the zinc finger proteins (Sukhatme 1990). However they do have unique functions as revealed by specific

phenotypes in the knockout mice. Egr1$^{-/-}$ mice are characterised by female infertility, Egr2$^{-/-}$ mice have a defective peripheral nerve myelination and hindbrain abnormalities, Egr3$^{-/-}$ mice have motor neuron disorders, such as ataxia, and Egr4$^{-/-}$ mice have male infertility (O'Donovan, Tourtellotte *et al.* 1999). Recent studies have also revealed that the Egr family performing opposing functions. Collins and colleagues demonstrated that Egr family play a critical role in directing the consequences of antigen recognition. Egr1 and NAB2 promote a TCR-induced activation program while Egr2 and Egr3 are responsible negatively regulating T cell function by suppressing Egr1 and NAB2 expression (Collins, Lutz *et al.* 2008)

As well as their DNA binding domains, Egr protein also contain both transactivation and repressor domains, thus they function both as transcriptional activators and repressors (Thiel and Cibelli 2002).

1.8.1 Egr protein DNA binding

The Egr2 gene transcribes early growth response 2 transcription factors and is located at 10q21.3 near the MBS3 critical region (Van Der Zwaag, Verzijl *et al.* 2002; Bult, Eppig *et al.* 2008). The Egr family members are characterised by a DNA-binding domain consisting of three zinc finger motifs which bind to 9-bp GC-rich DNA sequences (Dillon, Brown *et al.* 2007). Figure 1.15 provides a schematic representation of the four members of the Egr family.

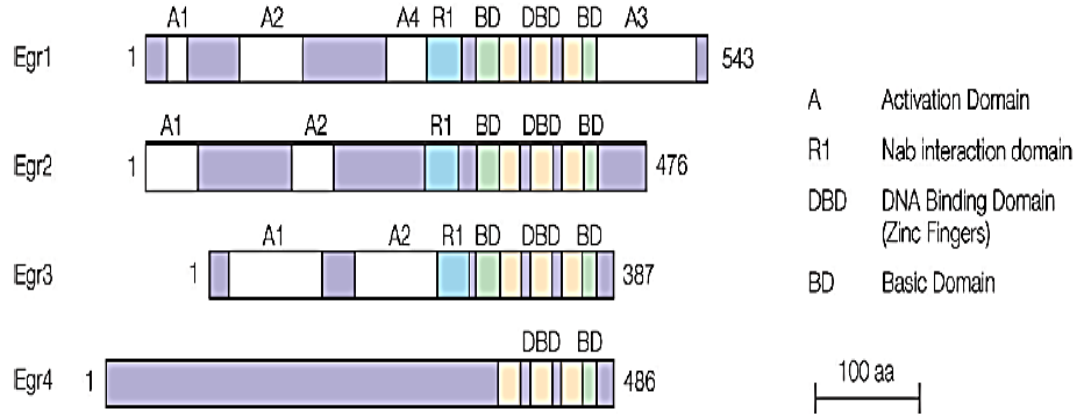

Figure 1.15: The four members of the Egr family (Poirier, Cheval *et al.* 2008).
This schematic representation shows the conserved regions of the different members of the Egr family. The most closely related members are Egr2 and Egr3, followed by Egr1.

The first DNA sequence identified as the Egr1 binding site was GCGGGGGCG, which was discovered within its own promoter in 1989 (Christy and Nathans 1989). Later experiments demonstrated that all four members of the Egr could activate transcription from this sequence; hence this sequence was referred to the consensus binding site for Egr family of transcription factors, even though later GCGTGGGCG was identified as the optimal binding site for Egr2 (Swirnoff and Milbrandt 1995).

1.8.2 Egr family function outside the immune system

The Egr family has found to play a critical role in the formation and maintenance of myelin (Topilko, Schneider-Maunoury *et al.* 1994). Egr2 has been extensively studied in the nervous system. Mutations in Egr2 were identified to correlate with severity of human myelinopathies (Warner, Svaren *et al.* 1999) and Charcot-Marie-Tooth disease (Chung, Sunwoo *et al.* 2005). Studies have shown that

Egr2 transcription factor is tightly regulated during embryogenesis, mainly in the segmented hindbrain and immature myelinating Schwann cells. Egr2 knockout models display disrupted hindbrain segmentation and development, due to rhombomeres r3 and 5 missing in the developing hindbrain (Voiculescu, Charnay *et al.* 2000).

Egr1 deficient animals have provided insight of fertilisation problems in these animals, leading to the discovery that they are deficient in luteinizing hormone β (LHβ), which contains an Egr response element in its promoter. These findings suggested that Egr1 mediates the phasic rise in luteinizing hormone (LH) indicted by luteinizing-hormone releasing hormone (O'Donovan, Tourtellotte *et al*. 1999). Egr1 has also recently indicated that it plays a crucial role in angiogenesis, the formation of new blood vessels from the pre-exiting vasculature (Fahmy and Khachigian 2004; Khachigian 2004).

Egr3 is highly expressed in developing muscle spindles, specialised muscles cells that are used to monitor muscle length and tension (Tourtellotte and Milbrandt 1998). Tourtellotte and Milbrandt, generated Egr3 deficient mice by targeting mutagenesis in embryonic stem cells and found that they displayed severe motor abnormalities, due to the absence of muscle spindles (Tourtellotte and Milbrandt 1998). Recently Egr3 has also been shown to be involved in the sympathetic nervous system, by regulating sympathetic neuron dendrite morphology and terminal axon branching (Eldredge, Gao *et al.* 2008; Quach, Oliveira-Fernandes *et al.* 2013).

Studies have shown that the Egr family have important roles in multiple pathways including development of prostate cancer, hematopoietic cell fate determination and thymic T cell development (Collins, Lutz *et al.* 2008).

Two individual groups in Japan have demonstrated that Egr2 is associated with the apoptosis pathway. One group revealed that Egr2 plays a key role in the PTEN-induced apoptotic pathway. They suggested Egr2 may serve as a tumour suppressor mediator in the PTEN signalling pathway. They found that the exogenous expression of Egr2, with AdCAEGR2 induced apoptosis significantly in various cancer cell lines, such as colorectal cancer and prostate cancer (Unoki and Nakamura 2003). They proposed that Egr2 induces apoptosis in various cancer cell lines by direct transactivation of BNIP3L and BAK (Figure 1.16). However they did not investigate Egr2 in lymphoma or leukaemia cell lines.

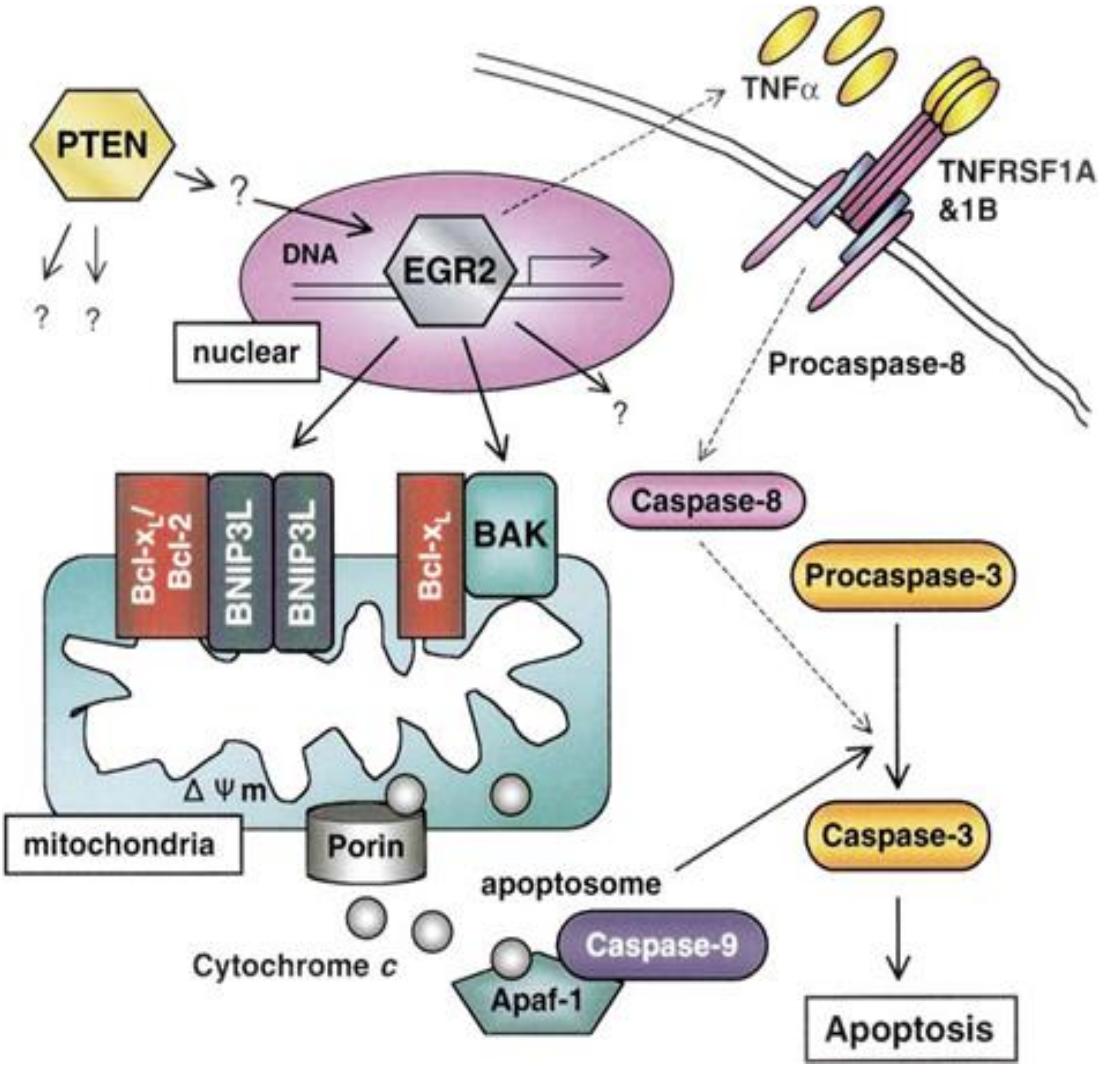

Figure 1.16: Egr2 mediated cell death pathway (Unoki and Nakamura 2003).
This schematic model shows PTEN that activates Egr2 gene. This then initiates the apoptotic pathway.

The other group supporting the findings that overexpression of Egr2 induces apoptosis, Tokini and colleagues, reported that Egr2 expression was up-regulated by DNA damage-induced p53 activity. They also identified a responsive element of p53, Tap63 and Tap73 within Egr2 gene (Yokota, Sasaki *et al.* 2010).

1.8.3 Egr family proteins in B cells

In 1989, it was reported by Seyfert and colleagues, that Egr1 is rapidly induced in mature B cells, upon stimulation by B cell antigen receptor (BCR) crosslinking (Seyfert, Sukhatme *et al.* 1989). Later it was reported that Egr1 expression can be detected already in pre-B cells isolated from bone marrow and in fetal liver-derived pre-B cell cultures (Dinkel, Warnatz *et al.* 1998).

Recently our group demonstrated that together with Egr1, Egr2 is also expressed in selected stages of B cell development and is repressed at pro-B cells (see Figure 1.17). The studies were carried out on Egr2 conditional knockout models (cKO), where Egr2 was specifically knocked out of CD2 positive cells, lymphocytes, and in Egr2 transgenic mice. Very little is known by the mechanism of how Egr2 regulates B cell development but our group was able to demonstrate that the involvement of Her-2 in B cells was supported by the impaired expression of Pax5 in pro-B cells, demonstrated by Egr2 Transgenic mice. Pax5 is exclusively expressed in the B cells from the committed pro-B cells to mature B cell stage and control the commitment of lymphoid progenitors to the B cell pathway (Li, Symonds *et al.* 2011).

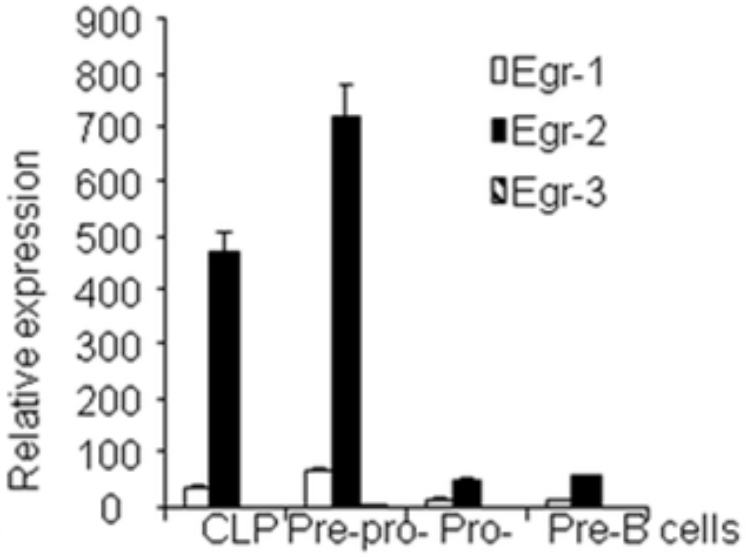

Figure 1.17: Kinetic Expression of Egr in wild type bone marrow B lymphoid lineage (Li, Symonds *et al*. 2011).
Egr2 is shown to be highly expressed at the early stages of B cell development (CLP and Pre-pro stages).

1.8.4 Egr Family proteins in T cells

Earlier studies demonstrated that during the engagement of the T cell receptor (TCR), Egr1, Egr2 and Egr3 are induced. Egr1, Egr2 and Egr3 are all expressed in DN thymocytes after the β-selection checkpoint correlating with pre-TCR signals (Carleton, Haks *et al.* 2002). Egr4 is thought to remain constantly expressed in T cells (Skerka, Decker *et al.* 1997). Li and colleagues performed kinetic expression of Egr1, Egr2 and Egr3 mRNA in the T cell lineage stages (Figure 1.18) and found Egr2 and Egr3 were expressed at significant levels while Egr1 was induced at lower levels.

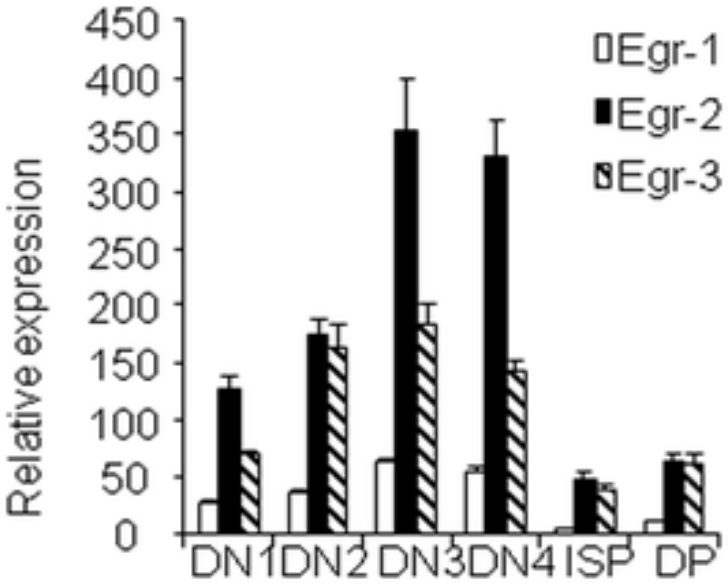

Figure 1.18: Kinetic Expression of Egr genes in wild type T lymphoid lineage (Li, Symonds *et al*. 2011).
Egr2 and Egr3 genes are expressed throughout the development of T cells, especially during DN3 and DN4 stage.

Recent studies have revealed opposing functions among the Egr in regulating T cell activation. Egr1 through the up-regulation of IL-2, TNF, CD154 and IL-2r, is associated with enhancing function. Whereas, Egr2 and Egr3 are emerging as negative regulators of T cell activation (Collins, Lutz *et al*. 2008). The role of Egr2 in the immune compartment is not extensively described, although it has been shown that Egr2 transactivation is dependent on members of the NFAT family in T cells (Harris, Bishop *et al.* 2004).

1.8.4.1 Egr proteins in thymocytes and mature T cell Function

Studies have shown that the activity of the Egr proteins is essential for development of immature DN thymocytes to the DP stage (Carleton, Haks *et al.*

2002). Carleton and his group were able to produce the first report, which demonstrated that the Egr family members are required to enable the pre-TCR complex to promote the differentiation of immature DN thymocytes beyond the β-selection checkpoint (Carleton, Haks *et al*. 2002). Two individual groups also demonstrated that both transgenic over-expression of Egr1 and Egr3 proteins in $Rag2^{-/-}$ and $Rag1^{-/-}$ thymocytes, respectively, enable the T cells to pass through the β-selection checkpoint to the DN4 and in SP stages, due to the lack of pre-TCR signalling (Miyazaki 1997; Xi and Kersh 2004). Hence, demonstrating the involvement of Egr proteins in thymocytes β-selection.

As well as in the thymocytes, Egr proteins are also shown to be expressed in mature T cells. Egr1 has been demonstrated to be involved in the expression of IL-2 (Decker, Skerka *et al.* 1998).

Mouse natural killer T (NKT) cells function in the first line of defence against infectious agents. Recently Lazarevic V., *et al* (2009) demonstrated that a calcineurin-NFAT-Egr2 pathway is essential for the productive selection, survival and maturation of NKT cells (Lazarevic, Zullo *et al.* 2009) (Seiler, Mathew *et al.* 2012).

1.8.4.2 Egr family, T cell tolerance and anergy

Egr2 and Egr3 are important mediators of the negative regulatory program downstream of isolated NFAT signalling (Figure 1.19). Collins and colleagues have also shown that Egr2 is up-regulated by Egr3. Both Egr2 and Egr3 inhibit T cell function by promoting the up-regulation of negative regulators such as Cbl-b and by

inhibiting the expression of the T cell activators Egr1 and NAB2 (Safford, Collins *et al*. 2005; Collins, Lutz *et al*. 2008). Cbl-b has shown to have a major tolerogenic factor.

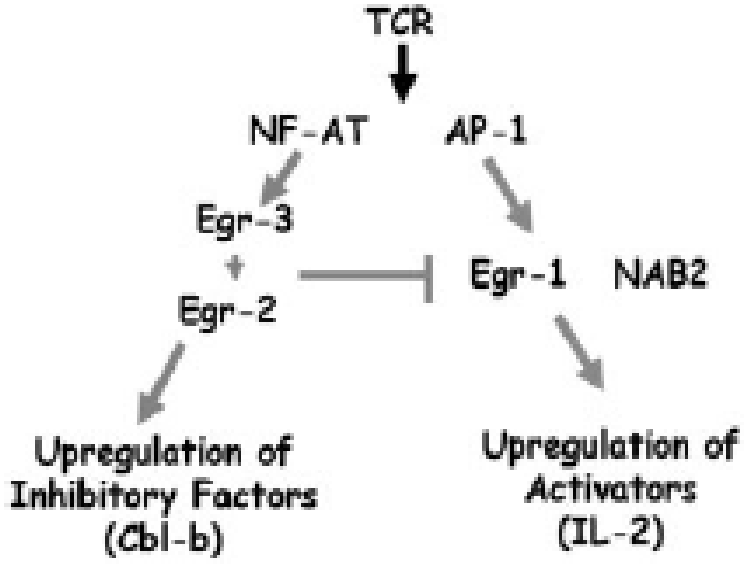

Figure 1.19: Model for the regulation of T cell function by Egr1, Egr2, Egr3 and NAB2 (Collins, Lutz *et al*. 2008).
This schematic representation shows possible routes of TCR activation leading to upregulation of either Cbl-b or IL-2.

Including our group, three individual groups have published their initial findings on the role of Egr2 in tolerance and anergy. Initially in 2004 Dr. Michael P. Czech from University of Massachusetts, USA, silenced the Egr2 expression, by RNAi technology, in an established A.E7 T cell line. They reported that depletion of Egr2 through siRNA-mediated gene silencing disrupts anergy, suggesting that Egr2

expression is required for the full induction of anergy in T cells (Harris, Bishop *et al*. 2004).

Around the same time similar studies were also carried out by another group at John Hopkins School of Medicine, USA. Jonathan, D. Powell's group, carried out microarray analysis to identify Egr2 and Egr3 as negative regulators of T cell activation (Safford, Collins *et al*. 2005). They took an alternative approach to the Czech's group by using lentiviral system to over-express Egr2 , resulting in the reduced expression of IL-2 after T cell activation (Safford, Collins *et al*. 2005).

Both groups performed their studies *in vitro* and had not tested it *in vivo* since Egr2 knockout mice die perinatally due to the defects in the hindbrain development (Schneider-Maunoury, Topilko *et al.* 1993). To assess the function of Egr2 in lymphocytes *in vivo*, our group established Egr2 conditional knockout (CD2-specific $Egr2^{-/-}$) model, in which the Egr2 gene was deleted specifically in $CD2^{+}$ lymphocytes using the Cre/LoxP system. Our group reported that T cells from CD2-specific $Egr2^{-/-}$ mice were hyper proliferative in response to prolonged stimulation and IFN-γ and IL-17 was highly induced, resulting in infiltration of T cells in multiple organs. Hence, the results demonstrated that Egr2 is important for controlling self-tolerance of T cells and preventing autoimmunity (Zhu, Symonds *et al*. 2008).

1.8.5 Egr family and apoptosis

Fas ligand (FasL) is a crucial ligand for the apoptotic pathway. Fas ligand (FasL) is the membrane associated cytokines of the TNF family and is also induced after TCR stimulation and can bind to the Fas receptor. The FasL-triggered

apoptotic death pathway is crucial for T cell homeostasis. A group demonstrated that Egr2, along with previously identified Egr3, is a direct regulator of *fas*L transcription in activated T cells. Ectopically expressed Egr2, like Egr3, was sufficient to induce *fas*L specific transcription (Mittelstadt and Ashwell 1999).

1.9 Aims of the study

Intensive research has been associated with the Egr family in the hindbrain development and myelination but their association with the immune system is still in the early stages. The involvement of Egr2 and Egr3 in T cell tolerance was first suggested less than a decade ago. Our group was the first group to produce Egr2 conditional knockout mice, which had deleted Egr2 expression in $CD2^{+}$ cells, lymphocytes. Previously, it was found that a defect in Egr2 in T cells results in accumulation of IFN-γ and IL-17 in $CD44^{high}$CD4 T cells leading to lupus-like autoimmune disease. Here we have bred CD2-specific $Egr2^{-/-}$ (CD2-$Egr2^{-/-}$) mice with $Egr3^{-/-}$ mice to produce Egr2 and 3 double knockout ($Egr2^{-/-}Egr3^{-/-}$) mice to study the role of Egr2 as previously we proposed that Egr3 compensated the role of Egr2. Therefore, the aim of the project was as follow:

1. To distinguish the phenotype of the CD2-$Egr2^{-/-}Egr3^{-/-}$mice model, as in the CD2-$Egr2^{-/-}$ mice.
2. To characterise the lymphoma developed in CD2-$Egr2^{-/-}$ and $Egr2^{-/-}Egr3^{-/-}$ mice by histology and immunophenotype.
3. To identify the mechanism by which Egr2 and/or Egr3 control the development of lymphoma development.

The hypothesis of my research is that Egr2 and Egr3 act as tumour suppressor genes and are important for the control of tumourgenesis.

Chapter 2 - Materials and Methods

2.1 Experimental Tools and Conditions

All mice used in this study were maintained in the Biological Services Unit, Brunel University, and used according to established institutional guidelines under the authority of the UK Home Office project licence (guidance on the Operation of Animals, Scientific Procedures Act 1986).

2.1.1 Egr2 Conditional Knockout mice

Egr2 conditional knockout (CD2-Egr2$^{-/-}$) mice have a deletion of Egr2 in CD2^{+} lymphocytes. CD2-Egr2$^{-/-}$ were generated by crossing Egr2$^{LoxP/LoxP}$ mice (obtained from P.Charnay, Institute National de la Santé et de la Recherche Médicale, Paris, France) and hCD2-Cre transgenic mice (obtained from Dr D. Kioussis, National institute for Medical Research, London) (Taillebourg, Buart *et al.* 2002; de Boer, Williams *et al.* 2003). The hCD2-Cre transgenic mice induce Cre recombination only in T and B cells (de Boer, Williams *et al*. 2003). Henceforth Egr2 deficient mice will be referred as CD2-Egr2$^{-/-}$ mice.

2.1.2 Egr3 Knockout Mice

Egr3 knockout mice were obtained from Tourtellotte group (Washington University School of Medicine, St Louis, USA), which were generated by targeting mutagenesis in embryonic stem cells by homologous recombination. Tourtellotte, W.G and Milbrandt, J. cloned the entire Egr3 gene into pBluescript vector. They disrupted the Egr3, using a 1.5kb fragment containing a neomycin resistance

selection cassette pMC1NeopA , which deleted a 0.9kb fragment of the gene that encoded the entire zinc finger DNA binding domain and the remaining carboxyl terminus of the protein (Tourtellotte and Milbrandt 1998). From this point forward, these will be referred as $Egr3^{-/-}$ mice.

2.1.3 CD2-specific $Egr2^{-/-}$ and $Egr3^{-/-}$ Mice

Egr2 and Egr3 double knockout mice were generated by interbreeding CD2-$Egr2^{-/-}$ and $Egr3^{-/-}$ mice on the C57BL/6 background. From this point forward these will be referred as $Egr2^{-/-}Egr3^{-/-}$ mice.

2.1.4 Genotyping

All the mice were genotyped prior to any studies carried out. The mice tails were collected and the genomic DNA was extracted using the REDExtract-N-Amp Tissue PCR Kit (Sigma) according to the manufacturer's instructions. Briefly, the DNA was extracted by adding extraction buffer and tissue preparation solution to the tails. For the protease enzyme to react with tissue sample, the samples were heated at 55˚C for 30mins. Both solutions contain protease enzyme, which break down the proteins. The protease enzyme was broken down by heating the samples at 95˚C for 3mins. The samples were neutralised by adding neutralisation buffer and then used for genotyping.

The CD2-$Egr2^{-/-}$, $Egr3^{-/-}$ and $Egr2^{-/-}Egr3^{-/-}$ mice were identified by polymerase chain reaction (PCR). PCR is a technique that was developed by Kary Mullis in the

1980s. It allows the amplification of nucleic acid sequences, with the aid of an *in vitro* enzyme (Mullis, Faloona *et al.* 1986; Mullis and Faloona 1987). The thermostable DNA polymerase from *Thermus aquaticus* (also referred to as taq), is not denatured at the same temperature as the DNA and can withstand the high temperatures required during PCR. PCR amplifies a specific region of the DNA with the aid of primers, which are designed specifically to a particular gene sequence. The Taq enzyme extends the primers with the aid of dNTPs complementary to the pre-existing strand, therefore producing a copy of the DNA. The primers used for genotyping are shown in Table 2.1 with the PCR product size. The wild type PCR product is referred to as WT.

Primer		Sequence (5'-3')	Annealing Temperature	Product Size
Egr2 LoxP	Sense	GTG TCG CGC GTC AGC ATG CGT	65°C	LoxP allele: 210bp WT allele: 150bp
	Antisense	GGG AGC GAA GCT ACT CGG ATA CGG		
hCD2-iCre	Sense	CCA ACA ACT ACC TGT TCT GCC G	56°C	150bp
	Antisense	TCA TCC TTG GCA CCA TAG ATC AGG		
Egr3$^{-/-}$	Sense	CTA TTC CCC CCA GGA TTA CC	57°C	Neo Allele: 800bp WT Allele: 400bp
	Antisense	TCT GAG CGC TGA AAC G		
	Neo	GAT TGT CTG TTG TGC CCA GTC		

Table 2.1: Primer Sequences for genotyping

2.2 Cell Culture and Stimulation

2.2.1 Mouse $CD4^+$ and $CD19^+$ Cells

$CD4^+$ T cells and $CD19^+$ B cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 300mg/L L-glutamine, 10% foetal bovine serum (FBS), 50µM β-mercaptoethanol (2-ME), and 50µg/ml gentamicin (all from Invitrogen). They were maintained in a humidified incubator at 37°C in 5% CO_2. $CD4^+$ T cells were stimulated with 200ng/ml PMA/INO or with plate-bound anti-CD3 at 5µg/ml or anti-CD28 (2µg/ml) antibodies. $CD19^+$ B cells were activated with 5µg/ml of soluble anti-$F(ab')_2$ fragments of goat anti-mouse IgM (anti-TgM $F(ab')_2$, (Jackson ImmunoResearch Laboratories, Inc.) or Lipopolysaccharide (LPS) (Sigma-Aldrich) at 5µg/ml.

2.3 Isolation of primary cells from mouse lymphoid organs

The secondary lymphoid organs were extracted and single cell suspensions were prepared by homogenising the organs. The cells were pelleted by centrifugation at 1300rpm for 5 minutes at room temperature. The erythrocytes were lysed from the cell pellet using 0.8% ammonium chloride lysis buffer at 37°C for 5 minutes. The lysis buffer uses a hypotonic solution to lyse the erythrocytes while maintaining the viability of the lymphocytes. The lysis was stopped by diluting the lysis buffer with phosphate buffered saline (PBS) and centrifugation at 1300rpm for 5 minutes at room temperature.

The naïve $CD4^+$ cells were then isolated by Magnetic Activated Cell Sorting (MACS) using positive selection kit (Miltenyi Biotec) according to the manufacturer's instructions. Briefly, the cells were incubated with anti-CD4 antibodies (L3T4) attached to magnetic beads (Miltenyi Biotec) at 4°C for 20 minutes in beads buffer, composed of PBS containing 0.5% FBS. This labels any $CD4^+$ cells with magnetic beads. The unlabelled anti-CD4 antibody was washed with beads buffer and centrifuged at 1300ropm for 5 minutes at room temperature. The cell pellet was resuspended in beads buffer and run through a magnetic column. $CD4^+$ cells are retained on the column due to the magnetic beads while the unlabelled cells pass through the column into a collecting tube. The column was washed three times with beads buffer to remove any non-specifically bound cells and then the $CD4^+$ cells were eluted by removing the column from the magnetic field and washing with RPMI 1640 medium containing 10% FBS. The naïve $CD19^+$ cells were also isolated by MACS using positive selection kit following the same procedure using the remaining unlabelled cells from the previous step. These were then labelled with anti-CD19 antibodies attached to magnetic beads (Miltenyi Biotec) followed by the same as $CD4^+$ Cells.

2.3.1 Cell Viability

The cell viability assays measures the percentage of a cell suspension that is viable. The cell viability was examined by trypan blue exclusion assay using the Countess™ Automated Cell Counter (Invitrogen). The trypan blue dye is traversed

into cells without an intact membrane (dead cells), whereas cells with an intact cell membrane (live cells) are not coloured by the dye.

The $CD4^+$ and $CD19^+$ cells were isolated from $Egr2^{-/-}Egr3^{-/-}$ and wild type secondary lymphoid organs as described in section 2.3. 2 x 10^6 cells were cultured in RPMI 1640 medium. The initial basal cell viability was examined at day 0 by preparing a 1:1 dilution of the cell suspension using a 0.4% trypan blue solution (Invitrogen, T10282). This was then loaded into the chamber port on the Countess™ cell counting chamber slide (Invitrogen, C10228). The cell viability was analysed on the Countess™ Automated Cell Counter. The cells were cultured for 12 days at 37°C, and cell viability was measured on days 3, 5, 7, 10 and 12.

2.4 Proliferation assay

To analyse proliferation of T and B cells, tritiated thymidine (^{3}[H]-TdR) incorporation was measured. This technique uses the nucleotide thymidine (TdR) that is radioactively labelled with radioactive isotope of hydrogen called tritium (3H). As the cell proliferates and replicates its DNA, the tritium is incorporated in proportional to DNA synthesis, alternatively the proliferation.

The proliferation assay was performed by isolating purified $CD4^+$ T and $CD19^+$ B cells (as described in section 2.3) and 2 x 10^6/ml cells were seeded into 96-well plates. The cells were first incubated for 72 hours in RPMI-1640 medium. The cells were then stimulated with 200ng PMA/INO for 48 hours. 1µCi of ^{3}[H]-TdR was added to each well and cultured for 8 hours at 37˚C, then the DNA was harvested onto glass fibre filters and unincorporated ^{3}[H]-TdR was washed away. Tritium

incorporation was measured by scintillation counting, as tritium emits β radiation which excites scintillation fluid causing it to emit light which can be detected.

Proliferation was measured at days 3, 5 and 10. The scintillation counting was carried out at Barts and London School of Medicine and Dentistry, Queen Mary University of London, by Professor Ping Wang.

2.5 Adoptive Transfer

The adoptive transfer was carried out by Dr Su-Ling Li and Professor Ping Wang at Brunel University. Briefly a total of 10^6 resting B or Naïve $CD4^+$ T cells were isolated from a 4 week old wild type or $Egr2^{-/-}Egr3^{-/-}$ mice and suspended in 100µl of physiological saline and injected into the dorsal tail vein of a 12 week old $Rag2^{-/-}$ female. The phenotype was assessed at two and six weeks after transfer through isolation of the B and T cells from the spleen. The wild type mice were positive for $CD45.1^+$ marker and the $Egr2^{-/-}Egr3^{-/-}$ were positive for $CD45.2^+$ marker.

2.6 RNA Extraction of Cells

Total RNA was extracted using Trizol reagent (Invitrogen). The Trizol reagent is a monophasic solution of phenol, guanidine isothiocyanate, which allows the sequential precipitation of RNA, DNA, and proteins from a single sample (Chomczynski and Sacchi 1987; Chomczynski 1993). RNA extraction was carried out according to the manufactures instructions. Briefly, cells were pelleted and then lysed in 1ml Trizol per 10^7 cells and then incubated at room temperature for 10

minutes. 200µl of chloroform was added to the sample for homogenization and the sample was mixed vigorously, incubated at room temperature for 15 minutes and then centrifuged at 12000rpm for 15minutes at 4°C. The chloroform increases the efficiency of nucleic acid extractions by reducing loss of RNA due to its ability to denature proteins and aid in the removal of lipids, therefore improving separation of nucleic acid into the aqueous phase (Chomczynski and Sacchi 2006).

After centrifugation, the sample was separated into a lower red organic phase, interphase and a colourless aqueous upper phase. The RNA is separated into the aqueous phase while the protein and DNA is located in the organic and interphase. The aqueous phase was transferred into a new eppendorf tube and the RNA was precipitated with 500µl isopropanol, incubated at room temperature for 10 minutes. The sample was then centrifuged at 12000rpm for 10minutes at 4°C. The supernatant was removed and the pellet was washed with 75% RNA free ethanol at 7500rpm for 5 minutes at 4°C. The waste was discarded and the RNA was air dried and then resuspended in RNase-free DEPC water.

2.7 RNA Extraction for Tumour Samples

Total RNA was extracted from the tumour tissue samples using the RNeasy mini kit (Qiagen, UK). Prior to RNA extraction, fresh 1ml RLT buffer (guanidine thiocyanate buffer) was prepared by adding 10µl of 2-ME. Briefly the tumour samples (no more than 30mg) were sectioned into small pieces no less than 0.5cm and homogenised in 600µl of prepared RLT buffer.

The cell and the tissue sample were homogenised using a syringe and a needle. The lysate was passed through a 20-gauge (0.9mm) needle attached to the sterile plastic syringe until a homogeneous lysate was achieved. 600µl of 70% ethanol was added to homogenised lysate; the sample was mixed well by pipetting. 700µl of the sample was transferred into an RNeasy spin column placed in a 2ml collection tube and centrifuged at 10000rpm for 15 seconds. The waste was discarded and the step was repeated for the remaining 500µl sample. 700µl of RW1 wash buffer was added to the column and centrifuged at 10000rpm for 15 seconds. Flow through waste was discarded and the column was washed with 500µl of RPE buffer (to which ethanol was added) by centrifugation at 10000rpm for 15 seconds. The column was washed again with RPE buffer by centrifugation at 10000rpm for 2 minutes. The RNA was eluted in 50ul of RNase-free water into a new eppendorf tube by centrifugation at 10000rpm for 1 minute.

The RNA quantity was measured using the spectrophotometer NanoDrop 2000C (Thermo Fisher Scientific) and the RNA quality was assessed by both the NanoDrop 2000C and confirmed by electrophoresis.

2.8 First-Strand cDNA Synthesis

RNA was converted to first strand cDNA using SuperScript™ III (Invitrogen). The SuperScript™III Reverse Transcriptase is a version of Murine Moloney Leukaemia Virus (MML V-RT). The enzyme catalyses the formation of the complementary cDNA strand using the RNA template. cDNA synthesis was performed in the first step using total RNA with random primers, they act as

primers for reverse transcriptase. Volumes of solutions were chosen to give 1µg RNA per reaction, 50ng of random primers and 500µM each of dATP, dTTP, dCTP and dGTP were added to each tube. These were then heated to 65°C for 5 minutes to denature any secondary structures present in the RNA, facilitating the binding of the oligo (dT) or random primers. The samples were then placed on ice. Reagents were added to give a final concentration of 0.01M DDT (Dithiothreitol), 2 Unit/µl of RNase Inhibitor and 10 Units/µl of SuperScript™ Reverse Transcriptase in 1x First Strand Buffer (all Invitrogen). The reaction was mixed and heated to 25°C for 5 minutes and then to 50°C for 1 hour. The reaction was then inactivated by heating to 70°C for 15 minutes. The samples were stored at -20°C.

2.9 Primer Design

PerlPrimer was used to design the primers for the Real Time PCR, using the following criteria (Marshall 2004). Primers were designed to have approximately 20 to 23 nucleotides with around 50% G/C content. The sense and antisense primers were designed so that they anneal to different exons of the sequence of interest. Hence allowing the identification of genomic DNA contaminants as these will contain the intervening introns in addition to the exons so will be larger and therefore detectable via electrophoresis. The melting temperature (Tm) of each primer was estimated using the Wallace rule (Tm = 4 x [G + C] +2 x [A + T]) and the annealing temperature was initially set at Tm - 2°C and altered as necessary. All primers were then checked using NCBI primer blast software. For the endogenous gene, mouse β-actin was used (Table 2.2).

Gene	Primer Strand	Primer Sequence (5'-3')
β-actin	Sense	AATCGTGCGTGACATCAAAG
	Antisense	ATGCCACAGGATTCCATACC
Brca1	Sense	CTGGACAGAAGACAGCAACTGC
	Antisense	ACCAGGTAGGCAACCAGATC
Casp3	Sense	CAGAAGATACCGGTGGAGGC
	Antisense	AGCTTGTGCGCGTACAGCTTC
Ccna2	Sense	CCTTCACCAGACCTACCTCAA
	Antisense	ATGGACTCCGAGCGACTCCT
Ccnb1	Sense	CGTAGAGCATCTAAAGTCGG
	Antisense	ACTGTAGGATAGGTAGTGCTG
Ccnb2	Sense	GAACACCAAAGTACCAGCTC
	Antisense	CCTGTCCTCGTTATCTATGTC
Ccne1	Sense	CCACACCAACAGCTTGGATT
	Antisense	TGCTCTGCTTCTTACTGCTGG
Ccnf	Sense	GATGCACCCAAAGACTACAG
	Antisense	CACAAGTCAGCATAGCTCAG
Cdc25b	Sense	ACAAGGAGTTCTTCCCACAG
	Antisense	GAGTCTTAAGGCGAAAGTTCC
Chek1	Sense	GATACTGGTTGACTTCCGAC
	Antisense	CAGAGCTGACAGCTTCCTCA
Gadd45a	Sense	GAAAGGATGGACACGGTGG
	Antisense	GATCTGCAGAGCCACATCC
E2f1	Sense	TTGGATCCCAGTCAATCCCT
	Antisense	AAGTGATAGTCAAGGGCCTC
E2f2	Sense	GGATCGCAGAGACCATAGAG
	Antisense	CAGCATGTTGTCAGTGGCTT
Cks1b	Sense	ATCTGAATGGAGGAACCTCG
	Antisense	AGAAAGCAACATGGTCACGC

Table 2.2: Real Time PCR primer sequences

Gene	Primer Strand	Primer Sequence (5'-3')
Mad2l1	Sense	ATTCGCTCAGTGATTAGACA
	Antisense	AATTGCGGTCCCGATTCTTC
Mcm2	Sense	GCTGAGCAGGTGACATATCA
	Antisense	TGAAGAGGTCGCTGTCGTAG
Mcm3	Sense	CCGATGATTCTCAGGAGACC
	Antisense	TGGATTCTGTGAGGTGGAGC
Mki67	Sense	CAGCAAGAGGCATGTATCACC
	Antisense	CTTTATCCCGACATTCTCTGC
Mybl2	Sense	GAGAAGCAGAAGAGAAAGCC
	Antisense	ATGTTACCCTCTTTGCTACC
Nek2	Sense	CCTGCTGAAGGAGCACAGGC
	Antisense	AGAATTCTCACTCCTTGCGG
Rad51	Sense	GTCGCAGTGGTAATCACCAA
	Antisense	TTCTCAGGTACAGCCTGGTG
Rad51ap1	Sense	TGAGGCATCAGTGACTTCAG
	Antisense	CCAATGCATTGCTGCTAGAG
Wee1	Sense	AAGAGCGCAGAGCAGTTACG
	Antisense	AGTGGCCATCCGATCTGTGA
Abl1	Sense	CTATGGCATGTCACCTTACC
	Antisense	GTGGATTTCAGCAAAGGAGG
Atm	Sense	AGCAGAGACCAGAAGATGAG
	Antisense	TAAGCAAGTTCACCTGTCCAC
Macf1	Sense	CATCCCTCAGAACATAGACAG
	Antisense	CAAGGACTTTCTGCTACCAC
Pkd1	Sense	CGCTTTACTAGAGTCCAGAG
	Antisense	GCCAAATAGACAGGGTAGAC
Rad9b	Sense	TCTGCAGCTGAAACCAAGAG
	Antisense	CTGTTCACAAGATACAGCTCC
Dstyk	Sense	GCCCTCAAATCAGTTGTTCC
	Antisense	GCTCCATGATAAGCAGTACAG

Table 2.2: Continued

Gene	Primer Strand	Primer Sequence (5'-3')
Rad9b	Sense	TCTGCAGCTGAAACCAAGAG
	Antisense	CTGTTCACAAGATACAGCTCC
Sfn	Sense	TTTCAGTCTTCCACTACGAG
	Antisense	TCCTTGTAGGAGTCCTCAC
sp100	Sense	CCAAACCAAAGCAAAGGAAAGAG
	Antisense	CTGTGGTTGTGAAAGATGAGG
Siglech	Sense	TTCTTCCTCAGCAAGAGATCC
	Antisense	ACCATCATCCATGACTTTCTTGAC
Rbm38	Sense	CTTACAGACGGGCTTTGCTG
	Antisense	CTGGCTGGTGTATACTCAAGG
Prkcq	Sense	TTGTCTACAGGGACCTGAAGC
	Antisense	GGAATGGTTGTACTTCTGACCC
Prdm9	Sense	CACAGCAGGAAGAGAACTAAGG
	Antisense	GATTTCAGTGCGATGATTCCA
Prdm1	Sense	AACCTGAAGGTCCACCTGAG
	Antisense	TGCTAAATCTCTTGTGGCAGAC
Irgm1	Sense	CCCTTTACTATATGACTTCCCGA
	Antisense	GTTGGCTATTCTCTGCTTCC
Irf4	Sense	CAGCTCATGTGGAACCTCTG
	Antisense	GTATTTCTTCTCACTCTTGGATGG
Ipcef1	Sense	GCATTTAAGATCAATCACCCAC
	Antisense	CTCTCACTGTAACATTCTTCATCC
Il9r	Sense	CATCCTTGTAGTTGTGCCCA
	Antisense	CCTGTCCAACTCTGGAAGTC
Ikzf1	Sense	AATTTCTTGGAGACAAGTGCCT
	Antisense	CTGGTACATGGAGCTGATGAC
Ikzf3	Sense	CAGATTAGCAAGCAATGTGG
	Antisense	CATCATCTCGTTCTCCTTCTC
Ifng2	Sense	AATCGAAGAGTATCTAAAGGACCC
	Antisense	CTGGAGAAGAAATAATTGACACGG

Table 2.2: Continued

Gene	Primer Strand	Primer Sequence (5'-3')
Ikzf3	Sense	CAGATTAGCAAGCAATGTGG
	Antisense	CATCATCTCGTTCTCCTTCTC
Ifng2	Sense	AATCGAAGAGTATCTAAAGGACCC
	Antisense	CTGGAGAAGAAATAATTGACACGG
Hjurp	Sense	GGAATACACTCAGGTGATTGAC
	Antisense	CAAGATGGCCAGTTCTCCTC
Hivep3	Sense	CGAAGAAGGAACCAGTGATGAC
	Antisense	CCTCCTCATCTTCATCCAGGT
Hdac7	Sense	GGGTGCACAGGAAATACTGG
	Antisense	GAGGTTCATGGGTTCTTCCTC
Gtf3c5	Sense	CAAGAAGACATCCAACCAGCC
	Antisense	CAACTCCTCCACATTCAAATCAC
Gfi1	Sense	CAAATGCAGCAAGGTGTTCTC
	Antisense	TCTTGCCACAGATCTTACAGTC
Foxo3	Sense	CAAAGCAGACCCTCAAACTG
	Antisense	ATGTTATAGAGTTCTGTTCCACGG
Dgkz	Sense	TGGATCATGCACCTCCAGAG
	Antisense	CTGTGTCTTGAGCCTTCTCAG
Dcun1d4	Sense	CTTCCCTACAATGTGATACAACAG
	Antisense	ACTTGGCAGTGTTTATGTCTAGG
Dcaf6	Sense	CCGTTTGACCCAATTCTAGCC
	Antisense	GGTGTTCCGAGTCTCTTCCA
Chd3	Sense	TAAGCCTGCTACTCCAGCTC
	Antisense	CACTTTGCTGTTCTTCTCTGG
Chd2	Sense	AGTAAATCAAAGCGATCTCAGG
	Antisense	CATCCTCTCCTTACAAATGCTG
Cdk12	Sense	CAGCGGCACTTGATCTATTGG
	Antisense	CAGTGAGGTAGGTCTGGAGG
Btaf1	Sense	AGACTATTACTGTACTCTCAGCCC
	Antisense	ATTGTAACGCCTGGAATACATGAC

Table 2.2: Continued

Gene	Primer Strand	Primer Sequence (5'-3')
Bcl9	Sense	CAGACCACATCAAGTCCCAG
	Antisense	CTTCTGCAGCCTTATTTGCC
Zmynd8	Sense	CTCCAATCAAAGCTCTGTTAGCA
	Antisense	GGGAATGGTACTTCTGGGCT
Zfp36l2	Sense	CACAAGCACAACTTTCCGTC
	Antisense	GGGATTTCTCCGTCTTGCAC
Unc5a	Sense	CTTTAACATCACTAAGGACACGAG
	Antisense	GGTGAAGTTTCTGGGCTAGAG
Ubr3	Sense	AGTTAAGGGCACTGAAGAGG
	Antisense	CAATAGGACTGACCATCGCAC
Ssh2	Sense	GACCAGTCGAAGGCAATCAC
	Antisense	CATGAATGGTGCTCTTCTTTGGT
Spic	Sense	AATGTCACCCACAGAGAACC
	Antisense	TTTCTCCTGCCTCTTCCTCC
Bptf	Sense	CTGTGATCGGTGTCAGAATTGG
	Antisense	TCTTGTGGGCCTGTAAGGAG
mTERT	Sense	TCAAGAATCCAGGAATGACAC
	Antisense	AGCTTTAAGGATGGTCATTGTC

Table 2.2: Continued

2.10 Real Time RT-PCR

Real Time PCR is a highly sensitive technique based on the same principle as normal PCR with the adaptation of allowing the quantitative comparison of gene expression from different samples (Pfaffl 2001). Real Time RT-PCR performs a relative quantification based on the relative expression of a target gene against a reference gene (often referred as 'housekeeping' or 'endogenous' gene). The quantification is simply performed by the detection technique for newly synthesised PCR products in real-time PCR using SYBR Green I fluorescence dye which directly binds to double stranded DNA. After each round of the product synthesis, the amount of product is calculated by the measurement of the fluorescence (Morrison, Weis *et al.* 1998). The Real Time RT-PCR was performed with QuantiTect SYBR Green PCR kit (Qiagen) as per the manufacturer's instructions; briefly using 1µl of cDNA and a final concentration of 0.2µM of relevant sense and antisense primers in 1 x SYBR Green Master Mix. The Real Time RT-PCR was performed on a Rotor-Gene System (Corbett Robotics) using the following program.

95°C for 10 minutes

95°C for 20 seconds

Annealing temperature (50-65°C) for 30 seconds } **40 cycles**

72°C for 20 seconds

Like all techniques, there is a limitation that occurs with RT-PCR; the SYBR Green also binds non-specifically to double strand DNA and can form primer dimer complexes. Therefore, after the Real Time RT-PCR cycles had completed, a melting curve analysis was performed, during which the fluorescence intensity was monitored as the temperature was increased from 50°C to 90°C. The melting curve identifies any non-specific products such as the primer dimers. The Tm is the temperature at which 50% of a particular DNA duplex dissociates and becomes a single stand DNA. The shape and the position of the DNA melting curve are dependent on the GC/AT ratio, with the melting temperature of nucleic acid duplex increases with both its length and width increasing GC content (Ririe, Rasmussen *et al.* 1997). To validate, once the PCR reaction had completed, the products were run on an agarose gel to confirm that there was only one band with the correct product size.

2.10.1 Real Time RT-PCR Data Analysis

The data analysis for the Real Time RT-PCR was carried out using the Rotor-Gene Software. The analysis was performed by choosing a threshold value which was at the exponential phase of the reaction (when the number of copies of DNA molecule doubles, during each cycle). Eventually the reaction comes to a plateau phase, due to a number of factors such as utilisation of primers or nucleotides or thermal inactivation of DNA polymerase. Hence the threshold value is chosen at the exponential phase and the number of cycles (Ct) for the florescence to reach the threshold for each sample is identified. The Ct values of the samples are then

compared to the Ct value of the reference gene (mouse β-actin was used), this is to normalise the results, correcting any variances in the mRNA amount that permits the comparison of different samples. The relative expression was calculated by the $^{\Delta\Delta}$Ct method using the following equation: $2^{(Ct_{(\beta\text{-actin})}-Ct_{(\text{target gene})})}x10000$ (Livak and Schmittgen 2001; Pfaffl 2001)

2.11 Western Blot

Some of the western blots were performed by Dr Tizong Miao at Barts and London School of Medicine and Dentistry, Queen Mary University of London.

2.11.1 Protein Extraction

The cells were harvested and washed once with PBS. The cells were then resuspended in 100µl of CelLytic M lysis buffer containing 1mM PMSF (Phenylmethylsulfonyl fluoride) and protease and phosphatase inhibitors (Roche). The cells were then swelled on ice for 15 minutes. The samples were centrifuged at 13000rpm for 5 minutes at 4°C and the supernatant containing the protein was transferred into a new tube. The protein extraction was aliquoted and stored at 80°C.

2.11.2 Protein Quantification

The protein extraction was quantified using the Bradford Assay. Briefly a standard curve was prepared using a serial dilution series (0mg/ml to 10mg/ml) of

purified BSA 100X (10mg/ml). The standard curve was constructed by plotting the absorbance value against the known BSA concentration. The protein concentrations of the samples were measured against the BSA standard curve.

2.11.3 SDS PAGE Electrophoresis and Western Blotting

The protein samples were mixed with 1x LDS buffer (Invitrogen) and 2-ME and heated to 70°C for 10 minutes to denature and reduce the proteins. The protein samples and 5µl of Novex® Sharp Pre-stained protein standard (Invitrogen) were loaded into the wells of 12% NuPAGE® Bis-Tris Gel (Invitrogen). The SDS-PAGE (Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis) gels were cast and run using the Bio-Rad Western apparatus and electrophoresed in 1X SDS-PAGE running buffer at 200V for 35 minutes at 4°C. SDS-PAGE is a technique that separates proteins according to their electrophoretic mobility (size and charge of protein). The SDS detergent is used to coat the proteins with a negatively charged sulphate group, as there is no relationship between the charge and size of a protein. The SDS treated proteins will then migrate, in an electric field, according to their size and can be separated by electrophoresis using a Polyacrylamide gel.

After separation by electrophoresis, the proteins were transferred onto a nitrocellulose membrane (Amersham). Before the transfer, the membrane was activated by incubating it in methanol for less than one minute, followed by 5 minute incubation in dH2O and finally soaking it in wet-transfer buffer. The gel was placed on top of the nitrocellulose membrane, which was then sandwiched between two pieces of Whatman filter paper. The sandwiched gel and

nitrocellulose paper were place in a cassette in the wet transfer system. The transfer of the protein to the membrane for 1.5 hours at 30V was performed at 4°C. This allowed the negatively charged proteins to move across towards the anode; however it does not allow the proteins to pass through the nitrocellulose membrane and hence are retained on the surface in the same alignment as in the gel.

After the proteins were transferred on to the membrane, the membrane was blocked in 5% w/v milk in TBST (Tris Buffered Saline with 0.1% Tween 20) for 1-2 hours at room temperature, before being incubated with the corresponding primary antibodies (see Table 2.3). The primary antibodies were diluted according to manufacturer's instructions in blocking buffer and incubated at 4°C with agitation. The following day, unbound primary antibody was removed by washing the membrane 3 times in TBST, 15 minutes each at room temperature. The membrane was then incubated in either goat anti-rabbit IgG HRP (Horseradish peroxidase) conjugated or goat anti-mouse IgG HRP conjugated secondary antibody for 1 hour at room temperature (Table 2.3). The secondary antibody was prepared in 5% milk in TBST, at a 1:2000 dilution. The membrane was washed 3 times in TBST for 15 minutes each at room temperature to remove any unbound secondary antibody.

	Primary Antibodies		Secondary Antibodies	
Antibody	**Species(Details)**	**Dilution**	**Antibody**	**Dilution**
pSTAT3	Rabbit (Cell Signalling Technology, 9145)	1:2000	Goat anti rabbit IgG HRP (Biorad, 172-1019)	1:2000
STAT3	Rabbit (Cell Signalling Technology, 9132)	1:1000	Goat anti rabbit IgG HRP (Biorad, 172-1019)	1:2000
IKZF3	Rabbit (Abcam, ab26083)	1:500	Goat anti rabbit IgG HRP (Biorad, 172-1019)	1:2000
FOXO3	Rabbit (Abcam, ab47409)	1:1000	Goat anti rabbit IgG HRP (Biorad, 172-1019)	1:2000
Egr2	Rabbit (Santa Cruz, sc-20690)	1:1000	Goat anti rabbit IgG HRP (Biorad, 172-1019)	1:5000
Egr3	Rabbit (Santa Cruz, sc-191)	1:200	Goat anti rabbit IgG HRP (Biorad, 172-1019)	1:5000
β-actin	Mouse (Cell Signalling Technology, 3700)	1:1000	Goat anti mouse IgG HRP (Sigma, A4416)	1:10000

Table 2.3: List of primary and secondary antibodies used for western blot with relevant dilutions.

2.11.4 Protein Detection with Chemiluminescence

The membrane was transferred onto a cling film where the antibody complexes were detected by ECL (Enhanced Chemiluminescence) plus kit (Amersham) according to the manufacturer's instructions. Briefly Solution A containing 100mM Tris and 30% H_2O_2 was mixed with solution B containing 100mM Tris, 90mM coumaric acid and 250mM luminol was applied to the membrane blot for 5 minutes at room temperature. This reagent contains substances that are oxidised, catalysed by HRP, producing a luminescence signal. All excess solutions were drained and the luminescence was detected by exposing a photographic film (Kodak, Biomax x-ray film) to the membrane and then developing the film.

2.12 Flow Cytometry

Flow cytometry is a technique used to investigate the multiple phenotype and functional parameters simultaneously within a single cell or a mixed population of cells. The protein of interest is identified by the use of antibodies that have a high affinity to it. The antibodies are usually directly conjugated to a fluorescent marker that is detectable by the fluorescence emitted from stained cell. Flow cytometry can also detect the cell complexity and size by the forward and side scattered light.

Single cell suspensions were obtained from the homogenised spleens or lymph nodes, which were then incubated with indicated fluorescently labelled

antibodies on ice for 30 minutes in PBS containing 3% FBS. Fluorescein isothiocyanate (FITC)-conjugated antibodies to B220, CD4, and CD8 were used. Other antibodies that were used were phycoerythrin (PE)-conjugated antibodies to CD3, CD4, CD8, CD19, CD25, CD62L, CD69; PerCP labelled antibodies to CD3, CD19, B220; allophycocyanin (APC)-conjugated antibodies to CD44, CD3, and CD19. All these antibodies were obtained from BD Biosciences. PE-conjugated antibody to Aiolos was obtained from eBioscience (12-5789-80). Anti-Mouse Egr2 PE-conjugated and Anti-Mouse Egr2 APC-conjugated antibodies were obtained from eBioscience. After incubation the cells were washed and resuspended in PBS and run through the flow cytometer, LSRII (BD Immunocytometry Systems) and the data was analysed using FlowJo (Tree Star) at Queen Mary University of London BY Professor Ping Wang.

2.13 Apoptosis Assay

The process of apoptosis brings about a number of changes to the cells specific morphologic features, such as loss of plasma membrane asymmetry, condensation of cytoplasm and nucleus. The loss of the plasma membrane asymmetry occurs at the early stages of apoptosis and causes the membrane phospholipid phosphatidylserine (PS) to translocate from the inner to the outer leaflet of the plasma membrane, hence exposing it to the external cellular environment. To determine the number of apoptotic cells, they were stained with Annexin V (BD Bioscience); a protein that binds to the PS. The cells were also stained in conjunction with Propidium Iodide (PI). This was to exclude the cells that

were already dead, as PI binds to nucleic acids and hence these are only obtainable by the permeable membranes of dead cells. Therefore cells that are negative for both Annexin V and PI are considered viable, while cells that are positive for Annexin V and negative for PI are in their early stages of apoptosis. Cells that are identified positive for both Annexin V and PI are cells in their late apoptosis or already dead.

The staining was carried out as per the manufacturer's instructions. Briefly the cells were resuspended in Annexin V binding buffer containing 100mM HEPES, 140mM NaCl, 25mM $CaCl_2$, pH 7.4 and APC-conjugated Annexin V and PI. The cells were incubated for 15 minutes in the dark at room temperature. After 15 minutes, the sample was further diluted in the binding buffer containing 7-amino-actinomycin (7AAD from BD Bioscience), which is used for the exclusion of nonviable cells. The cells were analysed on a LSRII (BD Immunocytometry Systems) at Queen Mary University of London.

2.14 Identification and characterisation of tumours

2.14.1 Tissue Preparations

All tissue samples collected were recorded and assigned an ID number. All tumours were assigned the name "KO" followed by the next ascending letter, such as 'A', 'B', or 'C'.

The tissue samples were collected and fixed in 4% paraformaldehyde (PFA) overnight at 4°C. The 4% PFA was replaced with 70% ethanol prior to paraffin

processing to wash away the PFA. The organs were secured within the cassettes and placed within the Shandon Hypercenter XP tissue processor which takes the tissue sample through a series of graded alcohol concentration washes to dehydrate the tissues and then into xylene followed by the tissues permeating in hot paraffin (Table 2.4).

Tissues processed into paraffin were melted at 65°C and the paraffinized specimens were placed into a mould and embedded with hot paraffin. The samples were left to cool and stored at room temperature. The tissue samples were sectioned into 7µm sections using a microtome. The cut sections were placed into a 50°C water bath and then mounted onto polysine-coated microscope slides. The slides were incubated at 60°C for approximately 30 minutes and left at 37°C overnight.

Program	Temp	Immersion Time
1 x 70% Alcohol	Room Temp	2 hours
1 x 90% Alcohol	Room Temp	2 hours
1 x 95% Alcohol	Room Temp	2 hours
3 x 100% Alcohol	Room Temp	2 hours
2 x Xylene	Room Temp	2 hours
2 x Paraffin wax	60°C	2 hours

Table 2.4: Tissue processing program. Tissues were dehydrated with alcohol, cleared with xylene and infiltrated with paraffin wax.

2.14.2 Haematoxylin and Eosin Staining

Haematoxylin and eosin staining (H&E) is the most commonly used tissue staining for the histology of animal tissues and routine pathology. Haematoxylin gives a dark purplish blue, which stains the chromatin within the nucleus. Eosin is a pink to red dye that stains the cytoplasmic material, as well as the connective tissues and collagen.

The staining was performed as follow; the tissue sections were deparaffinised and rehydrated by first incubating in 100% xylene and then in decreasing concentrations of ethanol (100%, 95%) for 3 minutes each. The tissue sections were dipped in ddH2O before incubating in Harris acidified haematoxylin (6 minutes), acid alcohol (~15 secs), 0.2% ammonium, solution (40secs), Eosin (45secs). Between each step, the slides were cleansed under running tap water. The tissue sections were then dehydrated in a series of ethanol solutions (95%, 100%) for 2 minutes each, followed by 3 times in 100% xylene. The slides were mounted with Histomount (National Diagnostics, HS-103), covered with coverslips and left overnight to dry. Histomount is a neutral solution of isomers and non-hazardous ingredients in xylene. By applying this solution the coverslips were permanently attached to the slides. The tissue sections were analysed using a conventional microscopy (Axioskop 2). The histology examination of the tissue sections was done in a blind manner.

2.14.3 Immunohistochemistry Staining

All Immunohistochemistry staining were analysed using a Zeiss fluorescence microscope equipped with a CCD camera and the Smart Capture software (Digital Scientific Cambridge, UK).

2.14.3.1 Immunohistochemistry Staining on cells

The cells were harvested and smeared onto Superfrost plus microscope slides (Thermo Scientific). The cells were left to dry at room temperature for 30 minutes and then fixed in acetone for 10 minutes at -20°C. The slides were left dry for approximately 10 minutes. The cells were washed twice in sterile cold 1 X PBS for 5 minutes each followed by blocking in 10% normal goat serum (Invitrogen) for one hour at room temperature, to minimise any non-specific binding of IgG. The cells were incubated with the appropriate primary antibody (Table 2.5) in a humidified chamber at 4°C overnight.

The following day, the cells were washed three times in 1 X PBS for 10 minutes each, at room temperature to remove any unbound primary antibody. The cells were incubated with their respective secondary antibody (Table 2.5) for 2 hours in the dark at room temperature. The cells were washed again three times with 1 X PBS for 10 minutes each at room temperature to remove any unbound secondary antibody. The slides were dried before mounting them in Vectashield® Mounting Medium containing DAPI and covered with a coverslip, removing any excess mounting media and the cells were visualised with a fluorescent microscope.

Negative controls were prepared by omitting the primary antibody during the immunohisotchemsitry procedure.

Primary Antibodies			Secondary Antibodies	
Antibody	**Species(Details)**	**Dilution**	**Antibody**	**Dilution**
Ikaros	Rabbit (Abcam, ab26083)	1:200	Goat anti rabbit Alex Flour 594 (Invitrogen, A11012)	1:2500
FOXO3	Rabbit (Abcam, ab47409)	1:300	Goat anti rabbit Alex Flour 594 (Invitrogen, A11012)	1:4000

Table 2.5: List of primary and secondary antibodies used in IHC on cells.

2.14.3.2 Immunohistochemistry staining on paraffin-embedded tissues.

For the identification of the tumour phenotype, the paraffin embedded tumour tissues were deparaffinised and rehydrated by incubating first in 100% xylene and then in decreasing concentrations of ethanol (100%, 90%, 70%). Antigen retrieval was performed by boiling the slides in 10mM Tris/1mM EDTA, pH 9.0 (or in EDTA) for 20 minutes at 95°C. The slides were cooled at room temperature for 30 minutes and then blocked in 10% normal goat serum in PBS for 1 hour. The slides were then stained with the relevant primary antibody (Table 2.6) prepared in 10% normal goat serum in PBS and incubated overnight at 4°C in a humidified chamber.

After washing with PBS, the sections were stained with the relevant secondary antibody (Table 2.6) for 2 hours at room temperature. The sections were

washed thrice with PBS and counterstained with DAPI and visualised with a fluorescence microscope.

Primary Antibodies			Secondary Antibodies	
Antibody	**Species(Details)**	**Dilution**	**Antibody**	**Dilution**
CD3	Rabbit (Dako, A0452)	1:100	Goat anti rabbit Alexa Flour 594 (Invitrogen, A11012)	1:400
B220	Rabbit (BD, 550286)	1:20	Goat anti rat FITC (Sigma, F-625) Or Rabbit anti rat Texas Red (Abcam, ab6732)	1:200
pSTAT3	Rabbit (Cell Signalling Technology, 9145)	1:200	Goat anti rabbit Alexa Flour 594 (Invitrogen, A11012) Or Goat anti rabbit Alexa Fluor 488 (Invitrogen, A-11008)	1:2000 1:1000
PCNA	Mouse (Santa Cruz, SC-56)	1:100	Rabbit anti Mouse Alexa Flour 488 (Invitrogen, A11059)	1:100

Table 2.6: List of primary and secondary antibodies used in IHC on paraffin tissue sections.

2.14.3.3 Detection of Immunoglobulin deposition in Kidney

The detection of the immunoglobulin deposition in the glomeruli was performed by deparaffinising the kidney sections as mentioned above. The sections were stained with Texas Red conjugated donkey anti-mouse IgG (Jackson

ImmunoResearch Lab, INC) at room temperature for 2 hours. The sections were washed with PBS and counterstained with DAPI.

2.15 Microarray

$CD4^+$ T cells were isolated from CD2-$Egr2^{-/-}$ and wild type mice by MACS, as described in section 2.2, and were stimulated with or without plate bound anti-CD3 for 6 hours. Total RNA was extracted using the Trizol reagent as described in section 2.5. The genome –wild transcriptional profiles were analysed using MouseRef-8 v2.0 BeadChip expression array (Illumina). The BeadChip array consists of 50-mer oligonucleotide probes that targets ~ 25,600 well-annotated RefSeq transcripts.

The probe labelling, array hybridization and data processing were carried out at the Microarray Facility, Barts and London School of Medicine and Dentistry, Queen Mary University of London, according to the manufacturer's instructions. Briefly the fluorescently labelled RNA sequence is hybridised to the array, where the labelled RNA will hybridise to the complementary sequences present on the array. Any unbound RNA was washed away. The array was then scanned to detect the fluorescence intensity, providing the genome wide transcriptional profile for the sample compared to the control.

The data analysis was also performed by Dr Alistair Symonds at Barts and London School of Medicine and Dentistry, Queen Mary University of London, which was returned to us to perform further data analysis. We focused on the gene that showed a difference of at least 2 fold between the wild type and CD2-$Egr2^{-/-}$ $CD4^+$ T cells and excluded the gene that had a detection *p value* greater than 0.05. The

differently expressed genes were first grouped based on self-organisation maps (SOMs) by clustering algorithm method. The SOM clusters were further clustered by a hierarchical clustering program to confirm the different profile. The gene expression profile genes were validated by Real Time RT-PCR and any genes that has a detection p-value greater than 0.05 were excluded.

2.16 Chromatin Immunoprecipitation.

Chromatin Immunoprecipitation (ChIP) is a technique that enables the detection of Protein DNA interaction *in vivo*. The protein of interest is specifically precipitated by an antibody to its associated DNA. The ChIP assays were performed in accordance with the protocol supplied with the kit (cat 9003 from Cell Signalling Technology). This procedure was performed by Dr Tizong Miao.

Briefly, 5 x 10^7 $CD4^+$ T cells from Egr2 transgenic mice were stimulated with plate bound anti-CD3 and anti CD28 for 3 hours at 37˚C. The cells were cross-linked with 4ml of 10% formaldehyde for 10 minutes at room temperature (final concentration of 1%). This chemical binds the chromatin together preventing the dissociation that normally occurs upon cellular lysis. The formaldehyde was quenched with 125 mM glycine and the cells were washed twice with PBS, followed by the cell lysis with lysis buffer containing 1% SDS, 10mM EDTA, 50mM Tris pH8. The cells were transferred into a 15ml tube and the lysed cells were then sonicated to shear the DNA protein complexes into small length fragments. The sonication was verified by taking a small sample of the chromatin and heating it to 65 ˚C overnight, to reverse the crosslinks, and running it on 1% agarose gel. The sample

was centrifuged and the supernatant was dialysed in 200ml dilution buffer and protease inhibitors for 1 hour at room temperature. This was repeated two more times at 4°C for 1 hour each time.

The dialysed chromatin was transferred into a 15ml tubes containing 500µl salmon sperm DNA (ssDNA)/BSA blocked protein A beads (Amersham) and incubated at 4°C for 1hour with rotation to preclear. This step was done to remove any proteins or DNA that binds non-specifically to Protein A beads. Chromatin (500µg) was centrifuged and the supernatant was transferred into a new tube (ensuring beads were present in supernatant). The chromatin was then used as a template for immunoprecipitation. An anti-Egr2 antibody (Covance) or anti-histone 3 antibody as positive control or anti-Ig as a negative control was added and incubated at 4˚C overnight. The anti-Egr2 antibody binds to the Egr2 protein and associated DNA and this antibody-protein-DNA complex can then be precipitated by the addition of ssDNA/BSA-blocked Protein A beads. The antibody-chromatin complex was collected and purified using QIAquick PCR purification kit to purify the DNA, after the final wash the pellet was resuspended in elution buffer containing SDS, which denatures the antibody and Protein A, resulting in the release of the immunoprecipitated chromatin complexes. The crosslinks were then reversed by heating the complexes at 65˚C overnight. The DNA was purified in accordance with the kit and template was used for PCR with ChIP primer sequences shown in Table 2.7.

Gene	Primer Strand	Primer Sequence (5'-3')
SOCS3-1	Sense	CAAGGATTTCACAAACGCCTG
	Antisense	GAGAGGCCTGTAGTACACCA
SOCS3-2	Sense	CCAACTTCTCATTCACACTTTCC
	Antisense	TACATGAGGACCTCGGAGTG
Nab2	Sense	ATAGCTCGGCCTCGGTCAC
	Antisense	GGACTCAAGAATCGGGCTC
IKAROS	Sense	CCCTCTAAGTCCTTCTTTATTCCT
	Antisense	GAAATGAGACACATCAGCCC
FOXO3	Sense	CTGTATTCCAAAGTTGGCCG
	Antisense	CTAAGGAGGAAGGACCAAGAC

Table 2.7: ChIP primer sequences.

2.17 Chromatin Immunoprecipitation Microarray chip

One of the recent techniques performed to identify genes that directly interact with Egr2 transcription factor was Chromatin Immunoprecipitation Microarray chip (ChIP-on-chip).

The procedure follows on from the ChIP protocol. The purified immunoprecipitated sample from the ChIP experiment was amplified using the GenomePlex Whole Genome Amplification (WGA2) kit. The amplified sample was labelled and hybridised to the GeneChip® Mouse Promoter 1.0R Array (a 49 Format Array from Affymetrix). This array studies the protein/DNA interaction in over

26,000 mouse promoters. This was performed by Professor Ping Wang and Dr Tizong Miao.

After hybridisation the chip was analysed using bioinformatics tools by Dr Alistair Symonds. The genes of interest were validated by both Real Time RT-PCR (section 2.10) and ChIP (section 2.16).

2.18 Enzyme-Linked immune Sorbent Assay (ELISA)

All cytokine analysis was performed by Dr T. Miao and Meera Raymond at Barts and London School of Medicine and Dentistry, Queen Mary University of London. The procedures have been briefly explained below. The concentrations of cytokines in the cell culture supernatants and sera were measured using ELISA kits. ELISA kits IFN-γ and IL-3 were obtained from R & D; whereas IL-6, IL-10, IL-17A, IL-17F and GM-CSF were from BD Bioscience. The procedure was performed according to the manufacturer's instructions. The ELISA plates were coated with the relevant 1 X capture antibody at 4˚C overnight. The coating buffer was washed 5 times with wash buffer (1 X PBS and 0.05% tween-20) for 5 minutes each at room temperature. The plate was blocked with 1 X assay diluent (blocking buffer) for 3 hours at room temperature. The plate was washed five times with wash buffer at 5 minutes each followed by the addition of 1 X assay diluent to the control wells. A 2-fold serial dilution was performed using the provided standard for each antibody analysis. To the remaining wells, the serum from $Egr2^{-/-}Egr3^{-/-}$ and wild type samples were centrifuged at 8500rpm for 5 minutes and plated out into the relevant wells. The ELISA plate was wrapped in foil and incubated at 4˚C overnight. The following

day the plate was washed 5 times with wash buffer, as previously mentioned. 100µl of the appropriate detection buffer was added to each well and incubated for 3 hours at room temperature. The wells were washed 5 times with wash buffer. The enzyme Avidin-HRP was added to the plates and incubated for 1 hour at room temperature followed by 7 washes with the wash buffer. The substrate solution was added to the wells for 15 minutes at room temperature followed by the reading the plate at 450nm and the data was analysed.

2.19 Telomere Length

2.19.1 Metaphase Preparation

$CD4^+$ T cells and $CD19^+$ B cell were isolated as described in section 2.3. The cells were cultures in medium with or without simulation as described in section 2.2. The cells were treated with colcemid (10µg/ml) (Sigma-Aldrich) for 2 hours at 37°C. Colcemid arrest the cells in metaphase by inhibiting the mitotic spindle formation. The treated cells were transferred into a 15ml tube and centrifuges at 1200rpm for 5 minutes. The decant was discarded and the cells were treated with pre warmed hypotonic buffer (75mM KCl) at 37°C for 15 minutes. This step is critical and had to be performed with a Pasteur pipette with a drop at a time, followed by gentle agitation. A total of 3 ml was added and the incubation time was started from the first drop. The cells were centrifuged and then fixed in freshly prepared fixative containing methanol and glacial acidic acid (3:1) for 10 minutes at room temperature. The fixative process was repeated twice more, with incubation

times of 10 minutes and 30 minutes, respectively. When adding the fixative, care was taken by initially adding the first ml of the fixative slowly and drop wise and then gently followed by the remaining 2 ml. The cell pellet was resuspended in fresh fixative and 20µl of the cells were dropped onto pre-cleaned slides. The cells in the fixative solution ensure that the mitotic cells were spread over the surface of slide effectively. The slides were left to dry and checked under a phase contrast microscope.

2.19.2 Quantitative Fluorescent in situ hybridization (QFISH)

The slides containing the metaphase cells were aged at 54˚C overnight. The slides were washed with PBS for 5 minutes. The slides were then fixed with 4% Formaldehyde for 2 minutes followed by three 5 minute washes with PBS. The slides were further processed with pre-warmed 0.1% pepsin (Sigma) prepared in 50ml acidified waster (49.5ml of ddH20 containing 0.5ml HCl) for 10 minutes at 37˚C. The slides were washed twice with PBS and fixed in 70% Formaldehyde for 2 minutes. The fixative was washed away with three PBS washes followed by dehydration of the slides in ethanol series of 70%, 90% and 100%. The slides were left to air dry at room temperature.

The slides were then hybridised to the synthetic oligonucleotide PNA (peptide nuclei acid) labelled with FITC, which is specific for the telomeric DNA sequence $(CCCTAA)_3$. The slides were heated at 72˚C for 2 minutes and then left at room temperature for 2 hours in a dark humidified chamber for hybridisation.

After hybridisation, the slides were washed twice in 70% formamide solution for 15 minutes at room temperature, followed by three PBS washes. Samples were then dehydrated in ethanol series as before and mounted with Vectashield® Mounting Medium containing DAPI.

Images of both interphase and metaphase cells were acquired using the Smart Capture software (Digital Scientific, Cambridge) and the telomere fluorescence analysed using the IP Lab software.

2.20 Statistical Analysis

Data was expressed as mean± SEM. Statistical analysis was performed using Student's unpaired t-test to analyse the statistical significance of differences between groups. A p-value of <0.05 was considered as significant.

Chapter 3 - Results

3.1 Genotyping of Egr2 and Egr3 Knockout Mice Model

The aim of the project was to understand the mechanism of Egr2 and 3 in the regulation of peripheral lymphocyte function. In order to establish Egr2 and 3 deficient lymphocytes, we cross-bred CD2-Egr2$^{-/-}$ mice, which were established in our group (Zhu, Symonds *et al*. 2008) with Egr3$^{-/-}$ mice (Tourtellotte and Milbrandt 1998) to produce Egr2$^{-/-}$ Egr3$^{-/-}$ mice.

3.1.1 The CD2-specific Egr-2 knockout mice.

Before cross-breeding, the CD2-Egr2$^{-/-}$ mice were genotyped with specific primers that resulted in three PCR products, which were the Egr2 LoxP, Cre and wild type Egr2 bands. The PCR from CD2-Egr2$^{-/-}$ mice showed 210 base pair (bp) LoxP-Egr2 and 150 bp Cre products (Figure 3.1). Wild type mice produced a 190bp band.

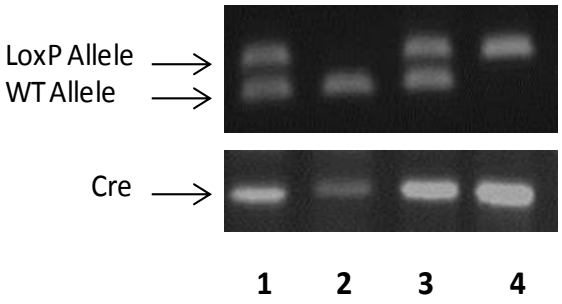

Figure 3.1: Generation of CD2-Egr2$^{-/-}$ mice.
Egr-2$^{LoxP/LoxP}$ mice were crossed with hCD2-Cre transgenic mice to produce CD2-Egr2$^{-/-}$ mice. The genomic DNA was extracted from the mouse tail and the CD2-Egr2$^{-/-}$ mice were identified by PCR. Lane 1 – positive control, lane 2 – WT (CD-Egr2$^{+/+}$), lane 3 - CD2-Egr2$^{-/+}$ and lane 4- CD2-Egr2$^{-/-}$ homozygous mice.

3.1.2 Egr2 and Egr3 knockout mice.

We took F1 littermates from parental breeding to breed F2. From F2, we selected mice with homozygous deletion of Egr3 or Egr2-LoxP and Cre for further breeding. The sequential breeding reached final generation with homozygous deletion of Egr3 and with homozygous positive for Egr2 LoxP and Cre. These mice were then used as breeding pairs to produce experimental mice. The genotyping of mice showed an 800bp PCR product of Neo allele, 400bp of Egr3 wild type allele, 150bp of Cre and 210bp of LoxP (Figure 3.2). Thus, we developed $Egr2^{-/-}Egr3^{-/-}$ mice.

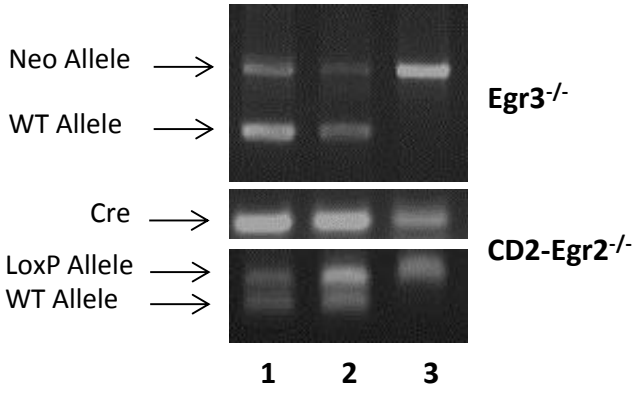

Figure 3.2: Generation of $Egr2^{-/-}Egr3^{-/-}$ mice.
CD2-$Egr2^{-/-}$ mice were crossed with $Egr3^{-/-}$ mice. $Egr2^{-/-}Egr3^{-/-}$ mice were identified by PCR, testing for LoxP, Cre and Egr-3 Neo. Lanes 1 – Positive Control, lane 2 - CD2-$Egr2^{-/+}Egr3^{-/+}$ mice. Lanes 3 - $Egr2^{-/-}Egr3^{-/-}$ positive mice.

3.1.3 Protein Expression of Egr2 and Egr3 in Egr2$^{-/-}$ Egr3$^{-/-}$ mice

The complete deletion of Egr2 and Egr3 at protein levels (by Tizong Miao) was confirmed by western blot and flow cytometry. As expected the level of Egr2 and Egr3 was significantly reduced or undetected in the Egr2$^{-/-}$Egr3$^{-/-}$ mice compared to wild type (Figure 3.3).

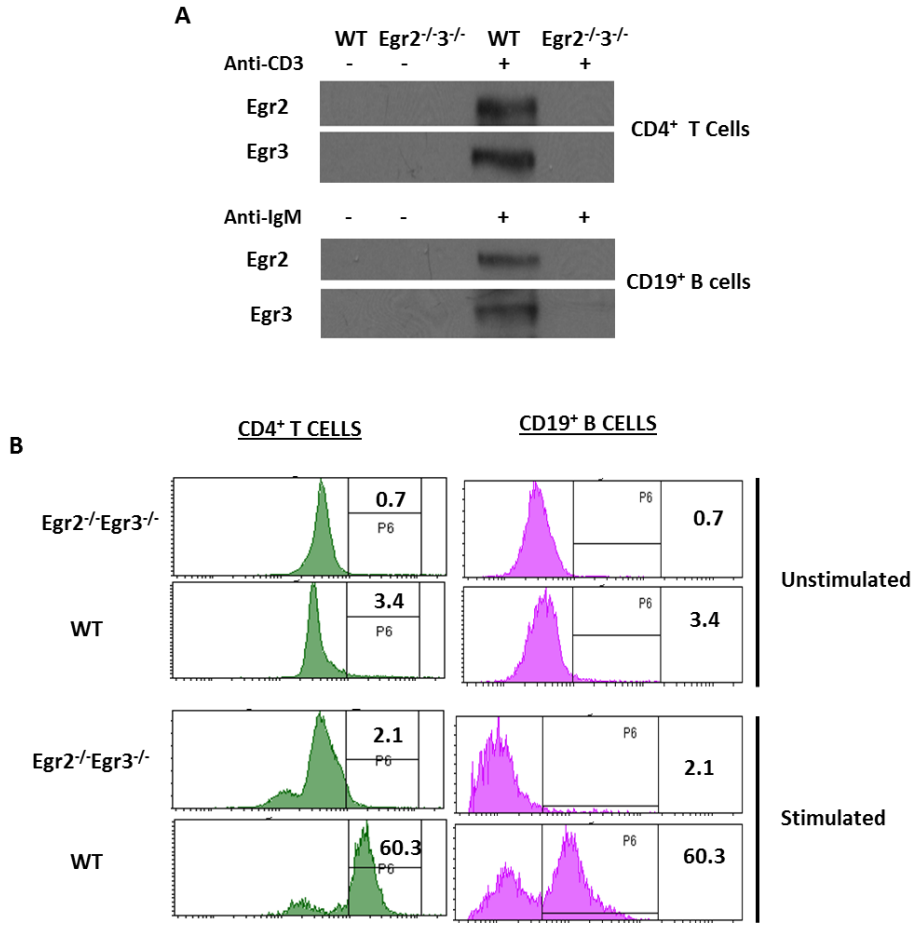

Figure 3.3: Defective Egr2 and Egr3 protein expression in lymphocytes from Egr2$^{-/-}$ Egr3$^{-/-}$ mice.

(A) Western blot analysis of Egr2 and Egr3 in unstimulated (-) and stimulated (+) CD4^{+} T cells and CD19+ B cells(B) Egr2 protein expression validated by flow cytometry. Egr2$^{-/-}$Egr3$^{-/-}$ mice for unstimulated and stimulated CD4+ T cells and CD19+ B cells.

All $Egr2^{-/-}Egr3^{-/-}$ mice were born healthy with no visible symptoms and displayed normal cell surface phenotypes in the spleen for the first month of their lives. The noticeable features of $Egr2^{-/-}Egr3^{-/-}$ mice were the splenomegaly and super-enlarged lymph nodes compared to aged match WT, CD2-$Egr2^{-/-}$ and $Egr3^{-/-}$ mice (Figure 3.4).

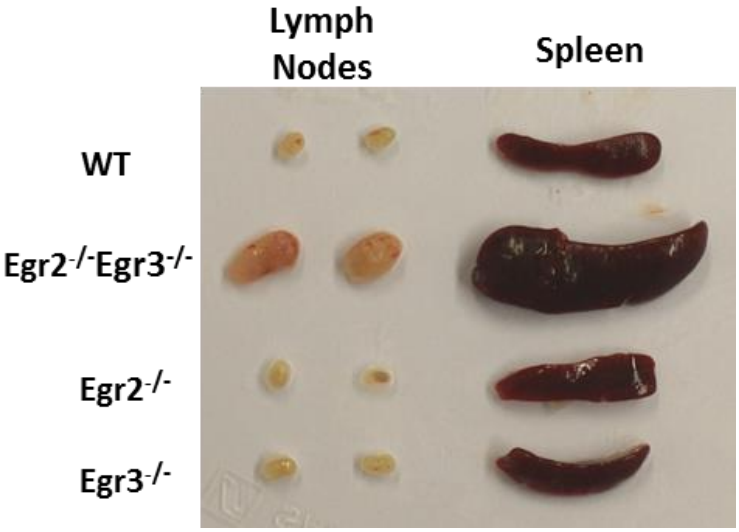

Figure 3.4: Enlarged spleen and lymph nodes in mice lacking Egr2 and Egr3 genes. The spleen from $Egr2^{-/-}Egr3^{-/-}$ mice is visibly enlarged compared to WT, $Egr2^{-/-}$ and $Egr3^{-/-}$ mice.

$Egr2^{-/-}Egr3^{-/-}$ and wild type mice were monitored over a period of 16 months. It was observed that all $Egr2^{-/-}Egr3^{-/-}$ mice became moribund at approximately 8 months of age. However 68% of these mice did not survive beyond the age of 5 months. No deaths were observed in the aged match wild type mice (Figure 3.5).

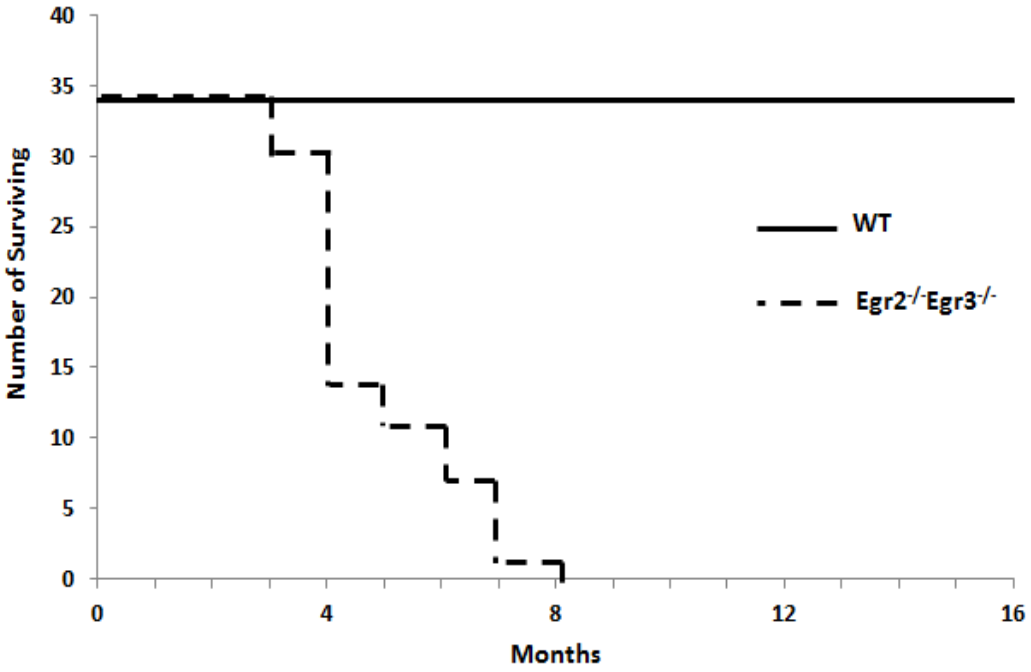

Figure 3.5: Survival Rate of $Egr2^{-/-}Egr3^{-/-}$ and wild type mice.
$Egr2^{-/-}Egr3^{-/-}$ mice became moribund at approximately 8 months of age compared to WT.

Along with the prominent features of splenomegaly and lymphadenopathy, the mice also developed tumours (please refer to section 3.2) and chronic inflammation (please refer to section 3.4).

3.2 Spontaneous Lymphoma Development in Egr2 and Egr3 Double Knockout Mice

3.2.1 Characterisation of lymphomas from Egr2 and Egr3 defective mice.

Surprisingly we observed the development of spontaneous lymphoma in $Egr2^{-/-}Egr3^{-/-}$ mice (Figure 3.6). The initial step was to classify the tumour from the $Egr2^{-/-}Egr3^{-/-}$ mice. All the tumour samples and the organs, such as the spleen, lung, liver, kidney, and various lymph nodes were processed for sectioning. To observe the morphology of the cells, the tissue section slides were prepared and stained with haematoxylin and eosin (H&E). Tissue sections were examined blindly, and classified according to the Bethesda proposal (Morse, Anver *et al.* 2002) (Figure 3.7).

The tumours were characterised by immunostaining using antibodies against T cell, B cell and monocytes specific markers, CD3, B220 and CD11b, respectively. As expected we found the tumours to be of either T cell or B cell origin and negative for monocytes (data not shown). Figure 3.7 shows both B cell positive and T cell positive tumours in the $Egr2^{-/-}Egr3^{-/-}$ mice.

The great majority of the tumours found in our cohort were classified as small B cell lymphoma (SBCL) or small T cell lymphoma (STCL), due to their histological characteristics of small, uniform cell size and round shape and scant, basophilic cytoplasm. The majority of the tumours were of diffuse pattern as suggested in the Bethesda proposal (Morse, Anver *et al*. 2002). It should be mentioned, however, that the mouse SBCL and STCL cannot be considered the counterpart of human small cell lymphoma because of notable differences in histology features (Morse, Anver *et al*. 2002). According to the same classification,

cases of T cell lymphoblastic lymphoma, large cell anaplastic T cell lymphoma and diffuse large B cell lymphoma occurred at very low frequencies.

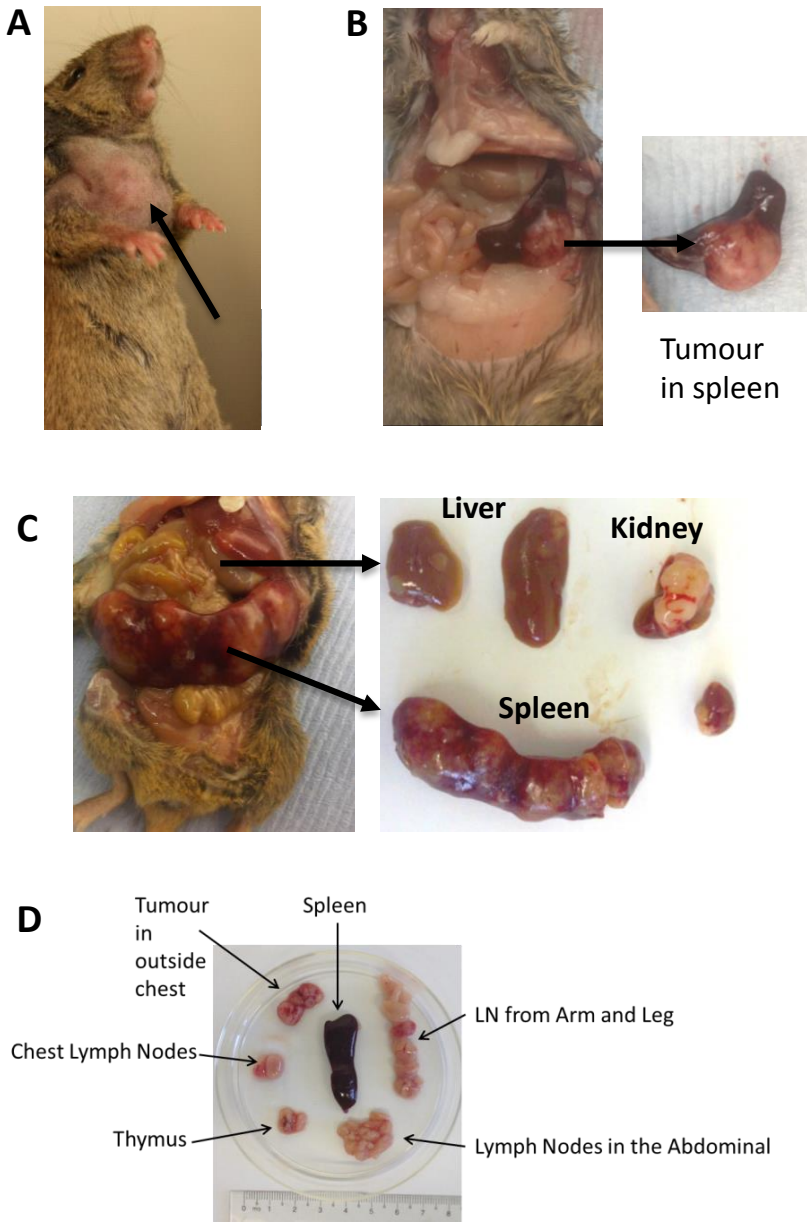

Figure 3.6: Tumour formation in Egr2 and Egr3 defective mice.
(A) Tumour (2 x 1.5 x 1.5cm^2) formed outside of the chest of Egr2$^{-/-}$Egr3$^{-/-}$ mice. (B) Tumour found in the spleen (1cm^2) of Egr2$^{-/-}$Egr3$^{-/-}$ mice. (C) Tumour in spleen (6 X 2 x 2cm^2), kidney and liver of CD2-Egr2$^{-/-}$ mice. (D) Tumour (1cm^2) formed outside of the chest in Egr2$^{-/-}$Egr3$^{-/-}$ mice.

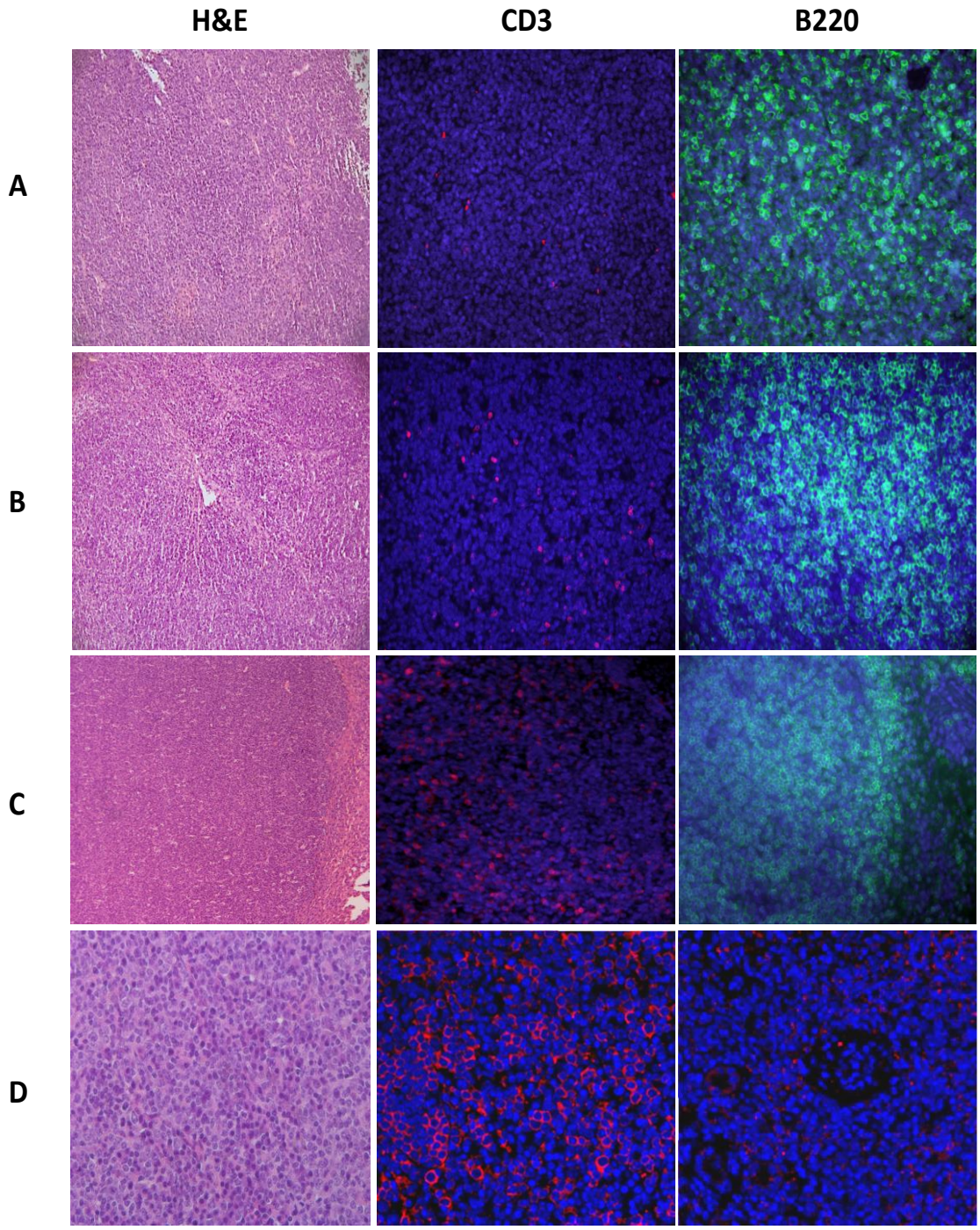

Figure 3.7: B and T Lymphoma Development in $Egr2^{-/-}Egr3^{-/-}$ mice models.
H&E (at x10) and Immunohistochemistry staining of tumour sections with T cell marker (CD3) and B cell marker (B220) (at x20). (A, B and C) B cell lymphoma identified in $Egr2^{-/-}$ $Egr3^{-/-}$ mice. (D) T cell Lymphoma identified in $Egr2^{-/-}Egr3^{-/-}$ mice.

3.2.2 Lymphoma in Egr2$^{-/-}$Egr3$^{-/-}$ mice show malignant features

The lymphoma samples were analysed and a few mitotic cells were identified (

Figure 3.8). This is in line with the Bethesda proposal which characterises SBCL and STCL as having few mitotic cells.

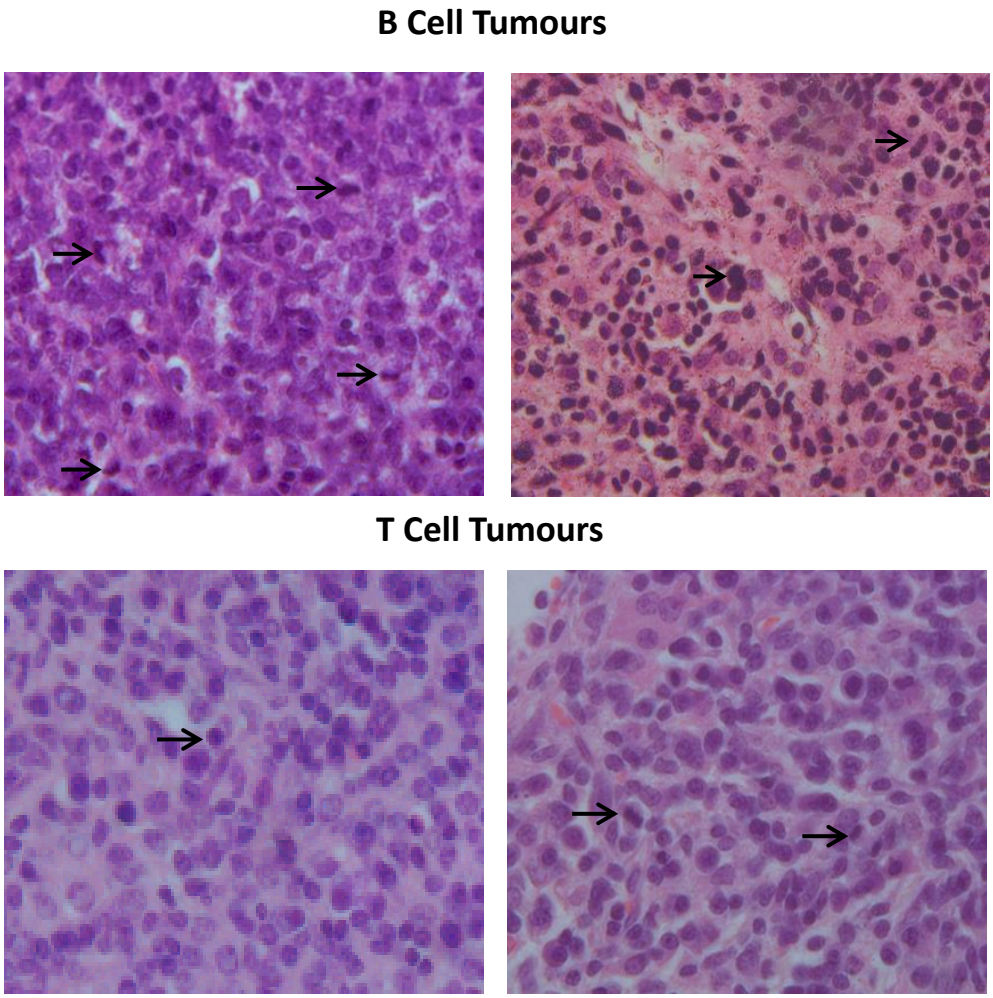

Figure 3.8: Histology of B and T cell Lymphoma Developed in Egr2$^{-/-}$Egr3$^{-/-}$ mice models. Black arrows indicate mitotic cells (x40).

One of the characteristics of malignant tumours is metastasis; hence both lymphoid and non-lymphoid organs were checked for any metastasis. As expected, all the $Egr2^{-/-}Egr3^{-/-}$ mice developed malignant tumours, which also extensively invaded into the non-lymphoid organs. Histology of the organs from $Egr2^{-/-}Egr3^{-/-}$ mice revealed complete effacement of their normal tissue architecture by accumulating lymphocytes (Figure 3.9).

Furthermore, immunostaining of the organs from the tumour bearing mice also demonstrated the same immunophenotype as the tumour tissues. As seen in Figure 3.9, the lymphoma was found outside of the chest and was confirmed as SBCL (Figure 3.7B). The cell morphology and immunophenotype of the spleen, liver, kidney and lung from this mouse were analysed. As seen in Figure 3.9, the normal splenic architecture was obliterated with extensive invasion of the red pulp area by lymphomatous infiltrations. Similar invasion of the lung, liver and kidney by lymphoma cells was also observed. The immunophenotype of the lymphocytes was confirmed to be B cell positive (Figure 3.9).

These results clearly demonstrate that the lymphomas in $Egr2^{-/-}Egr3^{-/-}$ mice are highly malignant.

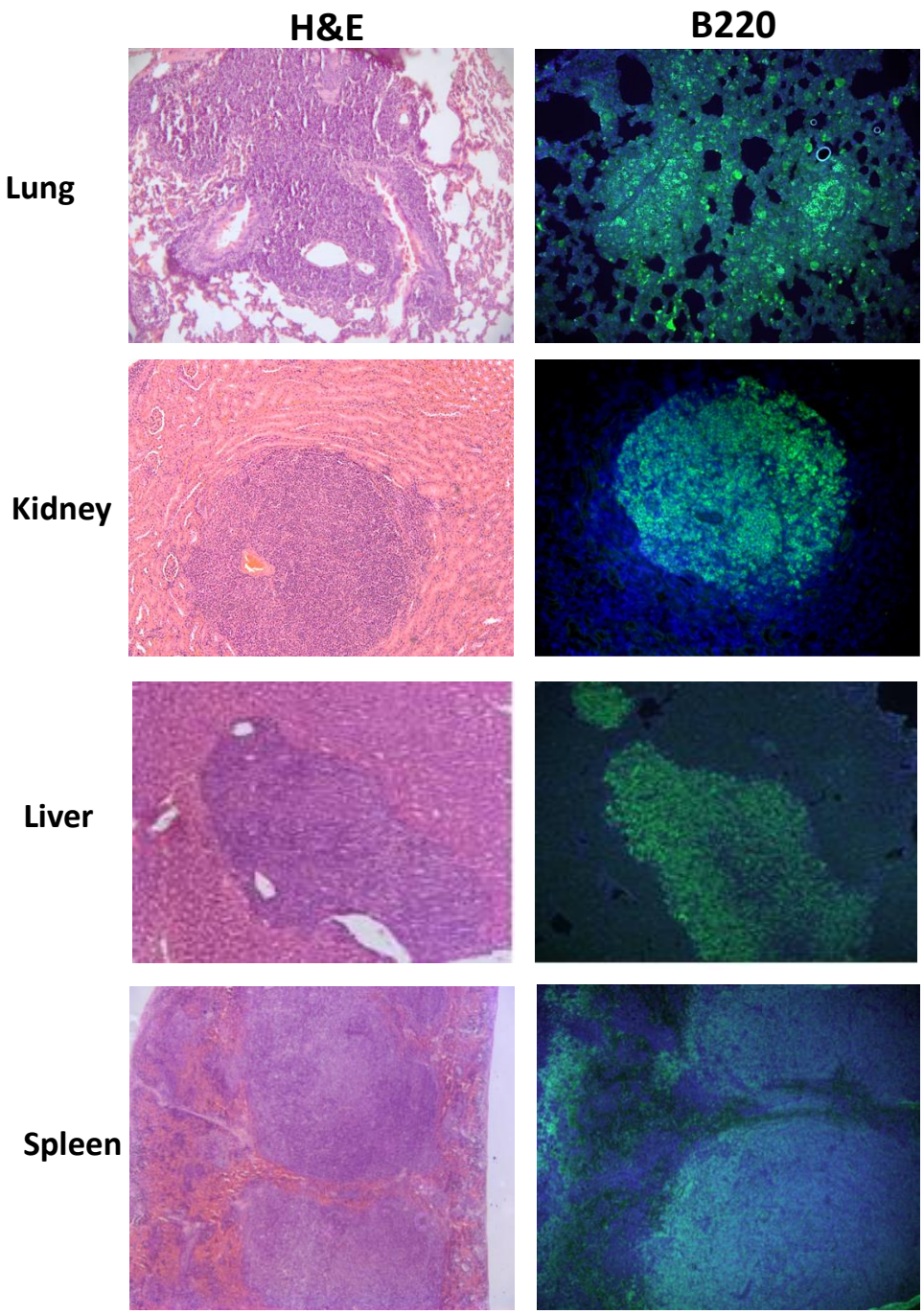

Figure 3.9: Lymphoma in Egr2$^{-/-}$Egr3$^{-/-}$ deficient mice is highly malignant.
Lymphoid and non-lymphoid organs from B cell lymphoma mice stained with H&E (X10) (left panels) and B cell marker B220 (X20) (right panels). All of the organs shown are infiltrated with and positive for B cells.

3.2.3 Genetic dose effect of Egr2 and Egr3 in lymphomagenesis

Interestingly, the lymphoma development is associated with the doses of deficiency of Egr2 and 3. No lymphomagenesis was observed in aged match wild type and $Egr3^{-/-}$ mice (Figure 3.10A). The majority of CD2-$Egr2^{-/-}$ mice had lymphoma formation at later stages of their life. Surprisingly, we found that the $Egr2^{-/-}Egr3^{-/+}$ and $Egr2^{-/-}Egr3^{-/-}$ mice developed lymphoma much earlier, before the age of 5 to 6 months compared to CD2-$Egr2^{-/-}$ mice which were up to 10 months of age. Figure 3.10B and Figure 3.10C clearly demonstrate the genetic dose effect of Egr2 in tumour formation.

All the tumours from the $Egr2^{-/-}Egr3^{-/-}$ mice had metastasised into other organs, specifically in the kidney, liver and lung, whereas only around 70% of the CD2-$Egr2^{-/-}$ showed metastatic tumours. The number of tumour formation in the $Egr2^{-/-}Egr3^{-/-}$ mice was less compared to CD2-$Egr2^{-/-}$, which could be owing to the fact the $Egr2^{-/-}Egr3^{-/-}$ became moribund at a much earlier age, due to the onset of inflammatory autoimmune disease.

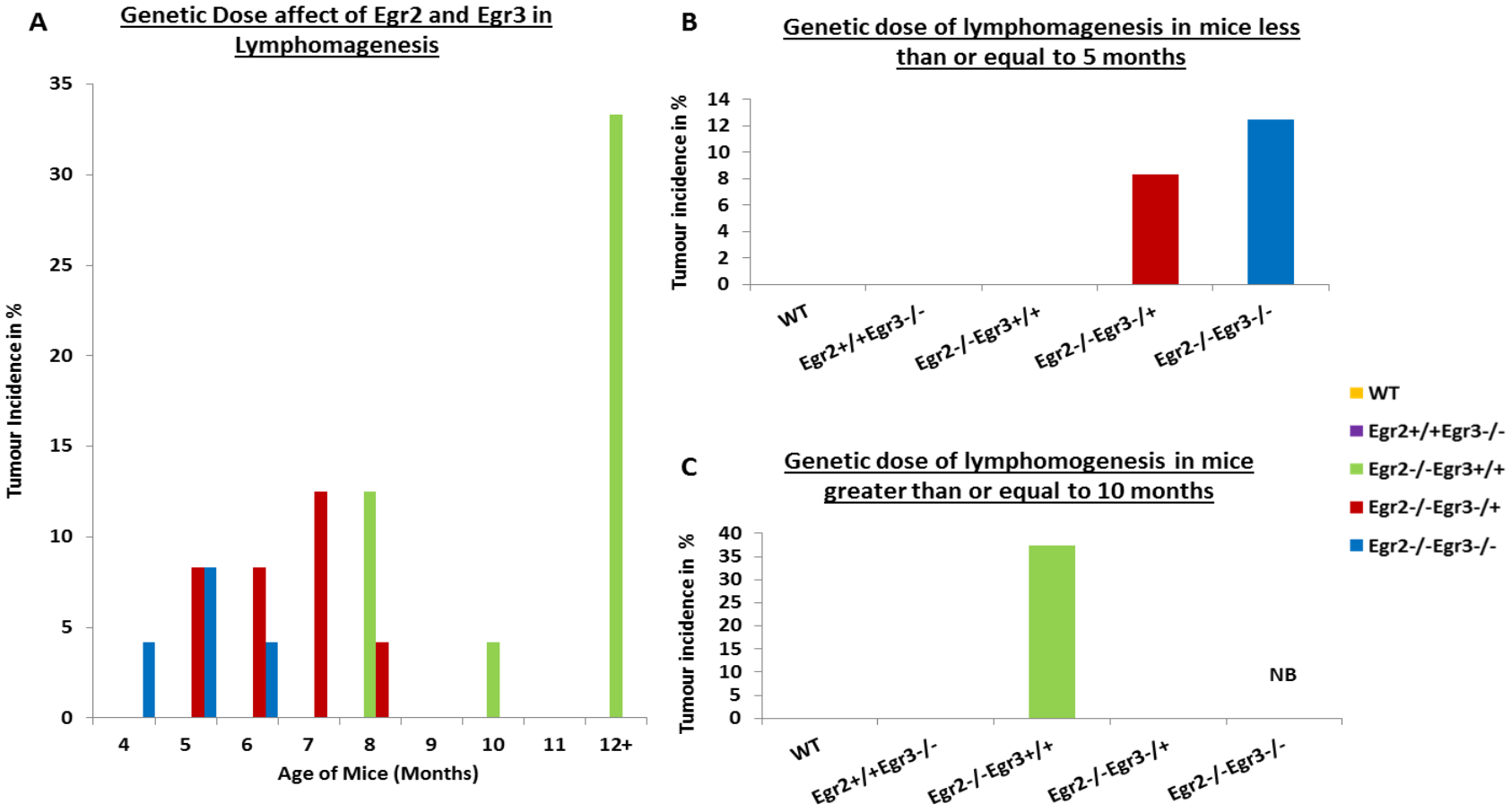

Figure 3.10: Genetic Dose effect of Egr2 and Egr3 in lymphomagenesis.

(A) Tumour induction of all mice from birth and beyond 12 months. (B) and (C) are adapted from (A). (B) Tumour induction in all mice models ages 5 months or younger. (C) Tumour induction in all mice ages 10 months or older. NB: $Egr2^{-/-}Egr3^{-/-}$ mice do not survive beyond 8 months.

3.3 Hyper-Homeostatic Proliferation in Egr2 and Egr3 Defective Mice

3.3.1 Increased lymphocytes in Egr2$^{-/-}$Egr3$^{-/-}$ in lymphoid organs

The noticeable features of Egr2$^{-/-}$Egr3$^{-/-}$ mice, along with lymphoma development were the extremely enlarged spleen and super-enlarged lymph nodes compared to aged match WT, CD2-Egr2$^{-/-}$ and Egr3$^{-/-}$ mice (Figure 3.4). The early onset of spontaneous lymphomas led us to investigate the cell survival of the lymphocytes. Firstly, we quantified the lymphocytes in peripheral lymphoid organs. Consistent with the enlarged lymphoid organs, the total cells in spleen and lymph nodes were significantly increased compared to wild type counter parts. The absolute number of T cells was 7 times that of wild type age matched mice. This was also seen in the B cells, which showed 6 times that of wild type age matched mice. This suggests hyper-homeostatic proliferation in the Egr2$^{-/-}$Egr3$^{-/-}$ mice (Figure 3.11B).

To observe the effect of the enlarged spleen and lymph nodes, we observed the number of lymphocytes from wild type and Egr2$^{-/-}$Egr3$^{-/-}$ mice, in different aged mice. We found that up until 5 weeks the number of CD19^{+}, CD4^{+} and CD8^{+} cells in Egr2$^{-/-}$Egr3$^{-/-}$ mice were similar to aged matched wild type mice. Conversely, from 7 weeks onwards we noticed an increase in the number of lymphocytes in our Egr2$^{-/-}$Egr3$^{-/-}$ mice, with a significant difference at 11 week old mice (Figure 3.11A).

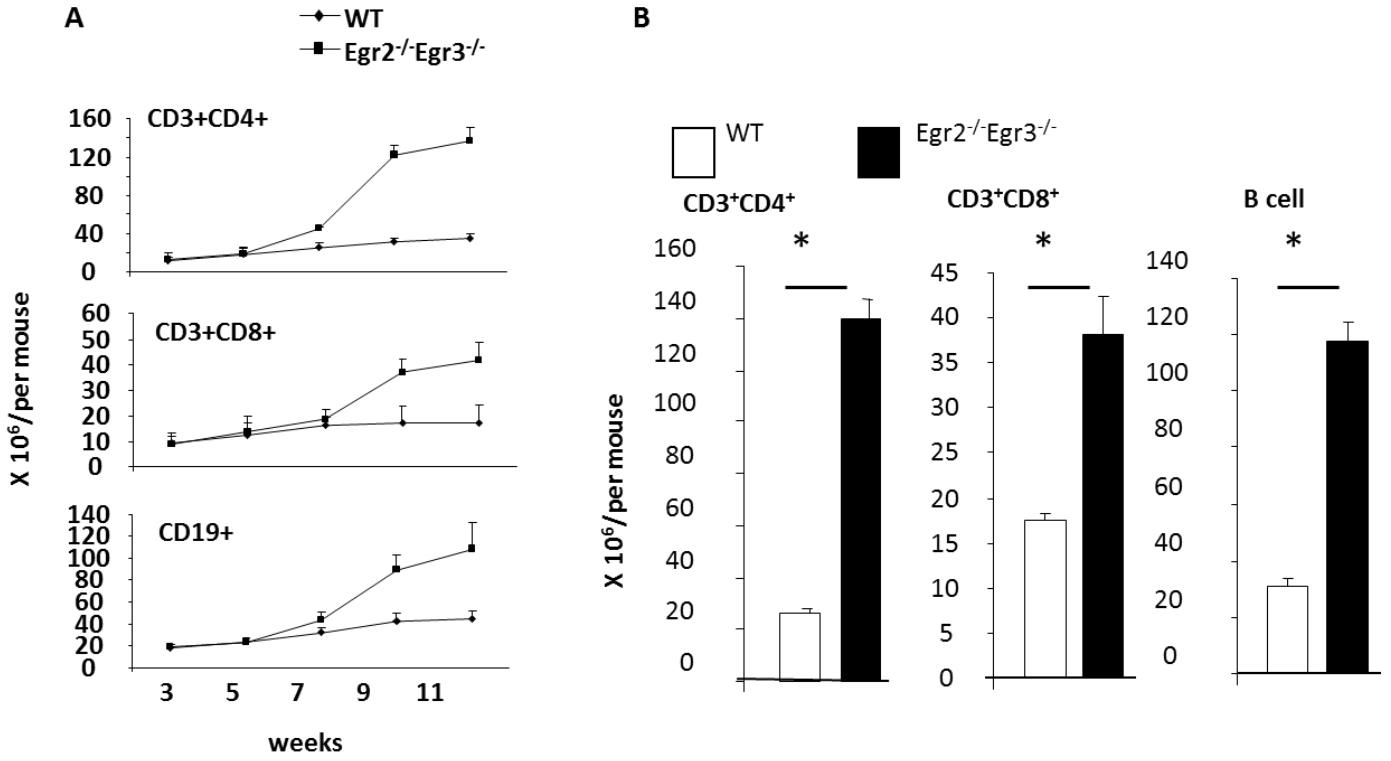

Figure 3.11: Absolute number of B and T cells in Egr2$^{-/-}$Egr3$^{-/-}$ and wild type mice

(A) Absolute numbers of T and B lymphocytes in the spleen of wild type and Egr2 and Egr3 deficient mice at the indicated ages. N = 4 in each group. (B) Absolute number of Splenic B and T cells in 8 week old Egr2$^{-/-}$Egr3$^{-/-}$ and wild type mice adapted from Figure (A). * $p<0.05$

3.3.2 Survival of Egr2 and Egr3 deficient T and B cells

Lymphoma development was observed in our model, as supported by the increased number of lymphocytes in $Egr2^{-/-}Egr3^{-/-}$ compared to wild type; hence we looked at the cell survival. This was performed by studying the cell viability of the lymphocytes from $Egr2^{-/-}Egr3^{-/-}$ mice compared to wild type. Briefly the $CD4^{+}$ and $CD19^{+}$ cells were collected and their cell viability was examined by Trypan blue exclusion assay from day 0 to day 13. As expected, we found both the $CD4^{+}$ and $CD19^{+}$ cells from the $Egr2^{-/-}Egr3^{-/-}$ mice to be more viable compared to the wild type mice. Both $CD4^{+}$ and $CD19^{+}$ cells started with similar cell viability but by day 5, $Egr2^{-/-}Egr3^{-/-}$ $CD4^{+}$ and $CD19^{+}$ cells were around 2 times more viable than wild type mice. This was also seen at Day 10, where CD4 cells in the $Egr2^{-/-}Egr3^{-/-}$ show to be 2 times more viable and $CD19^{+}$ cells show to be 3 times more viable than wild type (Figure 3.12).

These results clearly show that the lymphocytes from $Egr2^{-/-}Egr3^{-/-}$ mice are more viable compared to wild type.

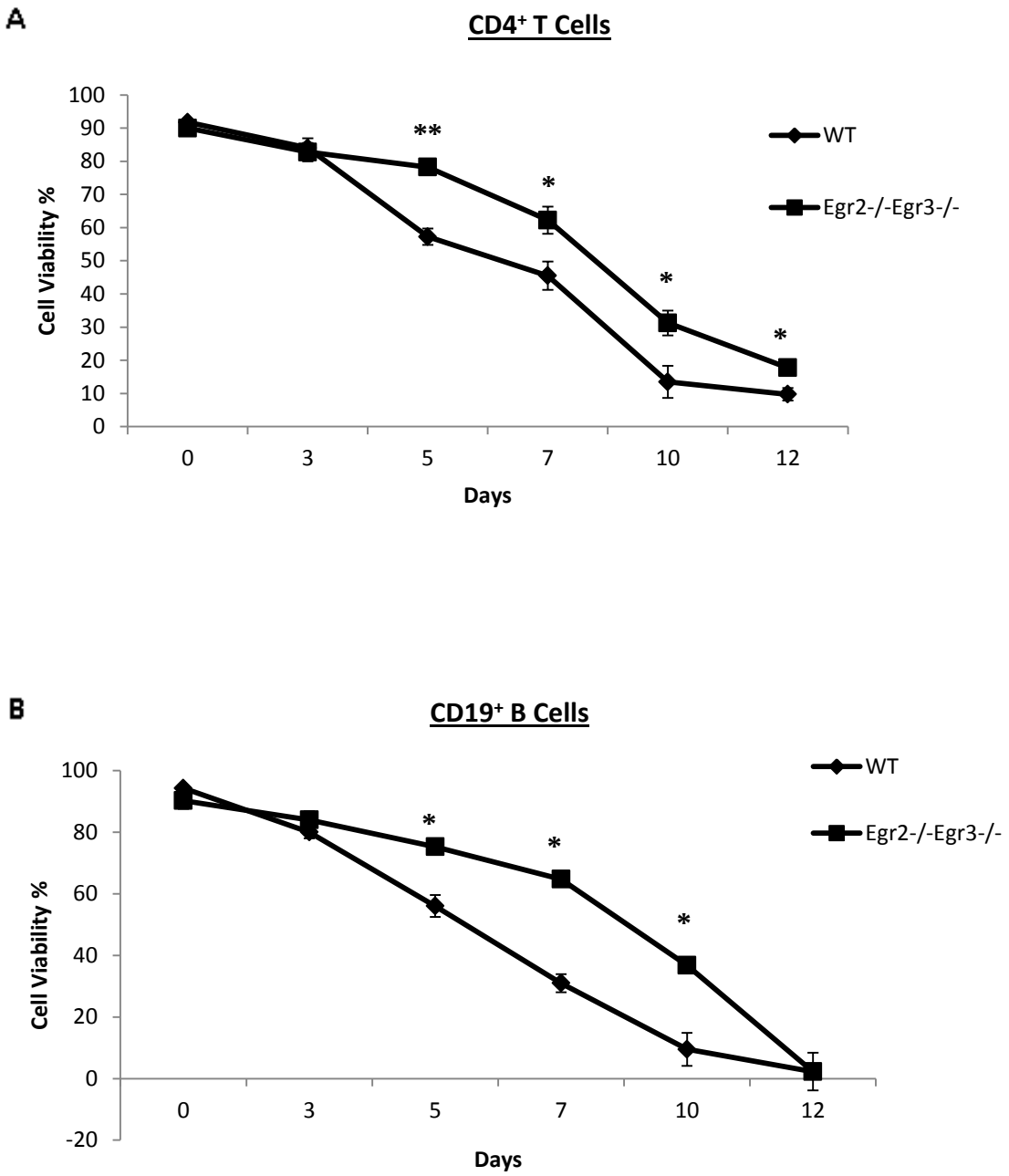

Figure 3.12: Cell Viability of $CD4^+$ and $CD19^+$ cells in $Egr2^{-/-}Egr3^{-/-}$ and wild type mice.

(A) Cell viability of $CD4^+$ T cells from $Egr2^{-/-}Egr3^{-/-}$ and wild type mice for 13 days. (B) Cell viability of $CD19^+$ B cells from $Egr2^{-/-}Egr3^{-/-}$ and wild type mice. Results represent 3 experiments; ** $p<0.01$; * $p<0.05$.

Cell survival was further confirmed in lymphocytes from 5 month old Egr2 and Egr3 defective mice compared to aged matched wild type. Two $Egr2^{-/-}Egr3^{-/-}$ mice with different levels of severity symptoms (labelled K-1 and K-2) were taken and compared with wild type. Enlarged spleen and lymph nodes were observed in the K2-$Egr2^{-/-}Egr3^{-/-}$ mouse but not in the K1-$Egr2^{-/-}Egr3^{-/-}$mouse. A survival recall experiment was performed, where the $CD4^{+}$ T cells and $CD19^{+}$ B cells were isolated and cultured for 3, 5 and 10 days, without the change of medium. On these incubation days, the cells were stimulated with 200ng/ml PMA/INO for 48 hours followed by the incorporation of ^{3}[H]-TdR for 8 hours.

Interestingly, both of the $Egr2^{-/-}Egr3^{-/-}$ mice showed significant levels of proliferating cells compared to wild type at day 3 and 5, whereas at day 10 the level of proliferation was almost at a halt. An unexpected observation was seen in the K2-$Egr2^{-/-}Egr3^{-/-}$ mouse which demonstrated proliferation at a much higher rate than that of K1-$Egr2^{-/-}Egr3^{-/-}$ mouse, suggesting that this mouse was possibly at the early stages of developing lymphoma (Figure 3.13).

This experiment clearly demonstrated that the lymphocytes from $Egr2^{-/-}Egr3^{-/-}$ mice have the ability to survive longer compared to wild type, under homeostatic conditions.

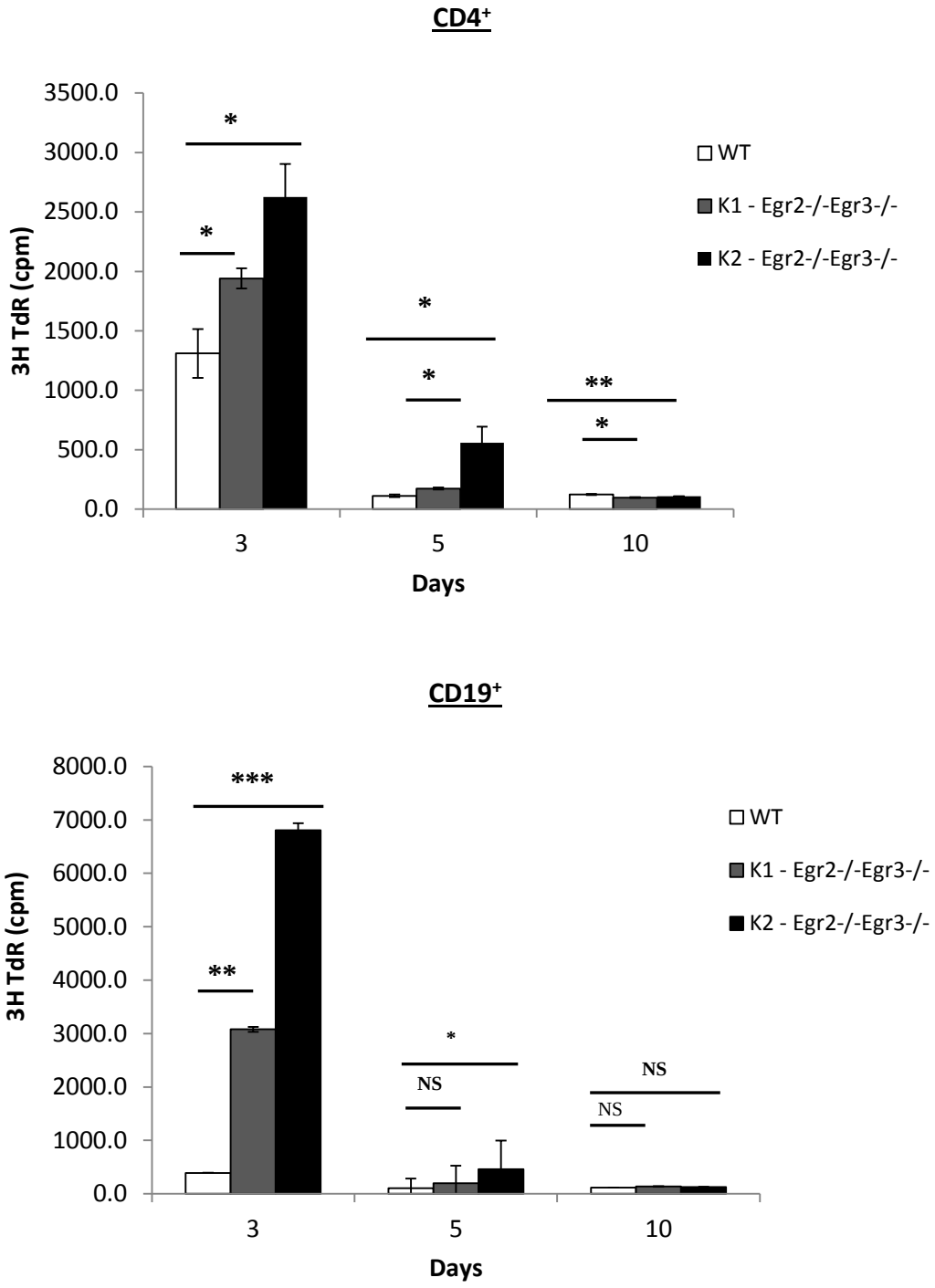

Figure 3.13: Cell Survival of $CD4^+$ and $CD19^+$ cells in $Egr2^{-/-}Egr3^{-/-}$ and wild type mice.

(A) Cell viability of $CD4^+$ cells from $Egr2^{-/-}Egr3^{-/-}$ and wild type mice for 13 days. (B) Cell viability of $CD19^+$ cells from $Egr2^{-/-}Egr3^{-/-}$ and wild type mice. $Egr2^{-/-}Egr3^{-/-}$ cells are more viable than wild type lymphocytes. Results represent 3 experiments; * $p<0.05$, ** $p<0.01$; *** $p<0.001$, NS – Not Significant

3.3.3 Survival of Egr2 and Egr3 deficient B and T cells *in vivo*.

The initial experiments demonstrated that the primary lymphocytes from $Egr2^{-/-}Egr3^{-/-}$ have the ability to survive longer *in vitro*. Hence we investigated the proliferation ability of the lymphocytes in $Egr2^{-/-}Egr3^{-/-}$ mice *in vivo*. To determine whether the function of Egr2 and Egr3 in lymphoma development is intrinsic or extrinsic, we looked at the population of $Egr2^{-/-}Egr3^{-/-}$ lymphocytes in $Rag2^{-/-}$ mice. $Rag2^{-/-}$ mice carry a germ line mutation, resulting in a large portion of Rag2 coding region being deleted. This results in these mice lacking mature B and T cells, with a severe combined immune disorder (SCID) phenotype (Shinkai, Rathbun *et al.* 1992). We transferred either resting B or naïve $CD4^+$ T cells from a 4 week old wild type and $Egr2^{-/-}Egr3^{-/-}$ mice into the $Rag2^{-/-}$ mice, before the onset of lymphadenopathy and splenomegaly. Six weeks after the transfer, $CD19^+$ B cells and $CD4^+$ T cells were isolated from the spleen and the phenotype of the cells were analysed. As expected, the wild type B and T cells displayed a resting phenotype in the $Rag2^{-/-}$ recipient mice. However the $Egr2^{-/-}Egr3^{-/-}$ B and T cells were seen to be highly activated in their recipient $Rag2^{-/-}$ mice (Figure 3.14) allied with high levels of Ig and cytokines respectively (see section 3.4 for further details on cytokines in $Egr2^{-/-}Egr3^{-/-}$ mice).

We also transfected 50:50 B and $CD4^+$ T cells from a 4 week mice old CD45.1 wild-type and CD45.2 $Egr2^{-/-}Egr3^{-/-}$ mice into a $Rag2^{-/-}$ mice. After 2 and 6 weeks we sacrificed the mice and isolated the $CD19^+$ B cells and $CD4^+$ T cells from the spleen. As expected we identified a similar activated phenotype in the $Egr2^{-/-}Egr3^{-/-}$ deficient but not the wild type $CD4^+$ T and B cells from the $Rag2^{-/-}$ mice.

Additionally, when we looked at the absolute number of B and $CD4^+$ T cells, we found that the percentage of $Egr2^{-/-}Egr3^{-/-}$ deficient mice were increased after 6 weeks of transfer. We identified 71% of B cells from $Egr2^{-/-}Egr3^{-/-}$ compared to 21% from wild type. Similarly we found 68% of $CD4^+$ T cells derived from $Egr2^{-/-}Egr3^{-/-}$ compared to 38% from wild type (Figure 3.14). Consequently, Egr2 and Egr3 regulate the homeostasis of both B and T cells in a cell intrinsic manner.

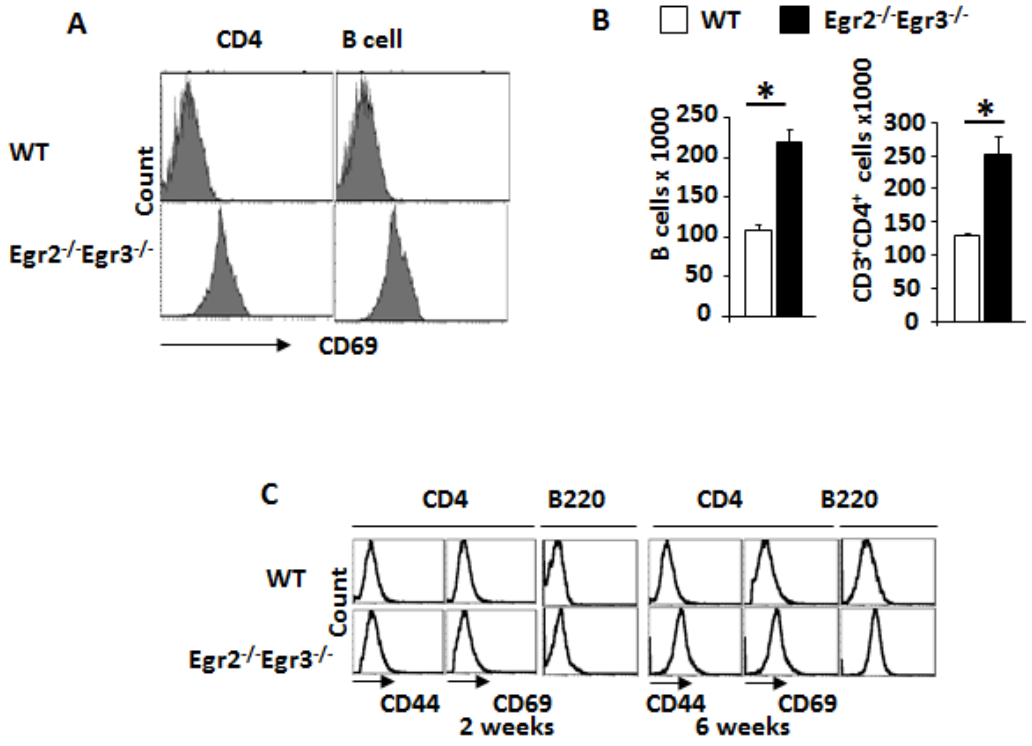

Figure 3.14: Egr2 and Egr3 regulate the homeostasis of lymphocytes.
(A) Naïve B or $CD4^+$ T cells from spleen of 4 week old WT and $Egr2^{-/-}Egr3^{-/-}$ mice were adoptively transferred into $Rag2^{-/-}$ mice. After six weeks the cells harvested from spleen and lymph nodes and surface marker expression analysed. (B) Cell number was analysed. (C) 50:50 mixture of wild type ($CD45.1^+$) and $Egr2^{-/-}Egr3^{-/-}$ ($CD45.2^+$) lymphocytes, from 4 week old mice were transferred into $Rag2^{-/-}$ mice. 2 and 6 weeks after transfer activation marker expression were analysed. The data are representative of two independent experiments and N=3 in each.

To support the hyper homeostatic proliferation observed in the $Egr2^{-/-}Egr3^{-/-}$ mice, we stained the spleen and tumour bearing tissue sections from $Egr2^{-/-}Egr3^{-/-}$ mice with a proliferating marker, proliferating cell nuclear antigen (PCNA). The spleen from the wild type mice was also stained as control (Figure 3.15). PCNA is associated with the cell cycle and immunocytochemical detection of PCNA represents a useful tool for the study of tumour proliferation activity, whereby a correlation can be detected between PCNA index and the lymphoma grading (Czader, Porwit *et al.* 1995; Rabenhorst, Burini *et al.* 1996). We found that the lymphocytes from both lymphoid organs and invaded non-lymphoid organs from the tumour bearing mice were highly proliferative.

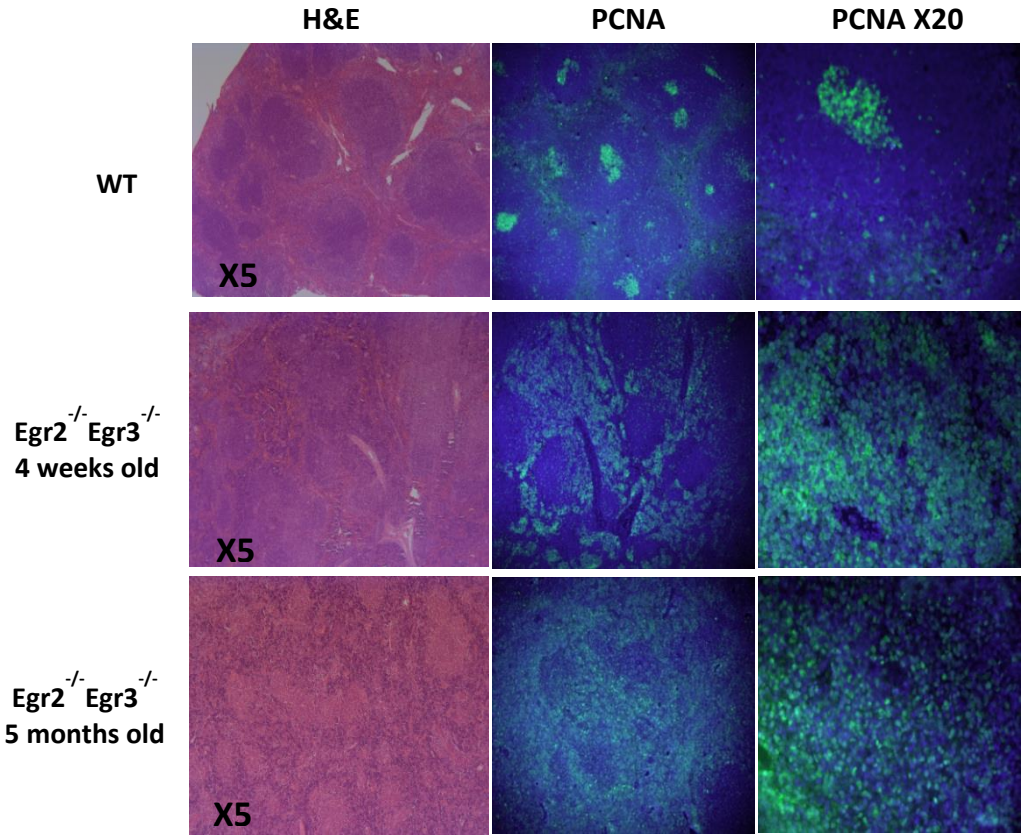

Figure 3.15: Enhanced proliferation of lymphocytes in $Egr2^{-/-}Egr3^{-/-}$ mice.
H&E staining of spleen tissue and PCNA staining on the same tissue section. Shown at magnitude X5 and X20. The PCNA staining shows that the lymphocytes are proliferating.

As seen in Figure 3.15, there is increased proliferation in both 4 week old and 5 month old $Egr2^{-/-}Egr3^{-/-}$ mice compared to wild type. Supporting the *in vivo* cell survival mentioned in section 3.2.5, the level of proliferation is significantly increased in the older mice compared to the 4 week old mice, resulting in the normal splenic architecture being eradicated with extensive invasion of the red pulp area by lymphocyte infiltrations. The extensive proliferation in the $Egr2^{-/-}Egr3^{-/-}$ mice affects the splenic architecture and results in fragmentation of the follicular structure, whereas in young $Egr2^{-/-}Egr3^{-/-}$ mice we still see a certain level of follicular structure.

The level of proliferation was also checked in the lymphoma tissues and in organs metastatically invaded by tumour cells. As expected the lymphoma cells also carried a proliferating phenotype. Both the tumour tissues and the organ with metastasis from B and T cell positive lymphomas were positive for the PCNA marker (Figure 3.16).

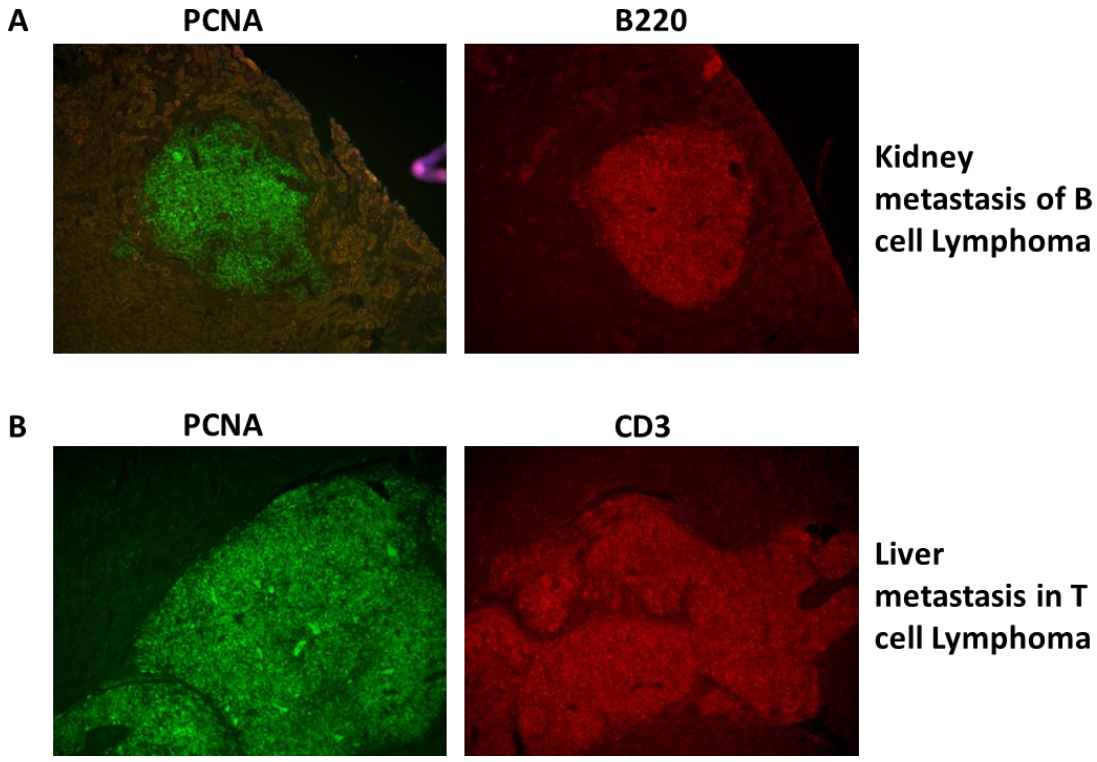

Figure 3.16: Enhanced proliferation in both T and B cell lymphomas in $Egr2^{-/-}Egr3^{-/-}$ mice.
(A) B cell lymphoma metastatically spread into the kidney. Sample stained for B cells, B220 (red) and for proliferation marker, PCNA (green). Staining shows the B cells to be highly proliferative. (B) T cell lymphoma metastatically spread into the liver. Sample stained for T cells, CD3 (red) and for proliferation marker, PCNA (green). Staining shows the T cells to be highly proliferative.

It is evident that the $Egr2^{-/-}Egr3^{-/-}$ mice are hyper proliferating, leading to lymphoma development. Both Egr2 and Egr3 are transcription factors and have the potential to regulate a number of other genes. The question arises as to which regulation process causes the development of lymphoma in the absence of Egr2 and Egr3.

Cancer is a multi-step, genetic disease (Hanahan and Weinberg 2000). As the normal cells progress to a neoplastic state, they acquire a succession of the hallmarks of cancer. Initially six hallmarks of cancer were suggested by Hanahan

and Weinberg in 2000; however since then they have identified a number of emerging hallmarks that also contribute to the development of cancer, these include tumour-promoting inflammation, genomic instability and mutation (Hanahan and Weinberg 2011).

Uncontrolled proliferation is the main hallmark of all cancers and we were able to demonstrate that the lymphocytes in our model were hyper-proliferative in physiological conditions.

The second hallmark of cancer is the evasion of apoptosis; hence we looked at the apoptosis in our model.

3.3.4 Apoptosis not affected in lymphocytes from Egr2$^{-/-}$Egr3$^{-/-}$ mice

A number of functional studies over the years have shown that programmed cell death by apoptosis serves as a natural barrier to cancer development (Lowe, Cepero *et al.* 2004). The evasion of the apoptosis pathway is also one of the hallmarks of cancer. In 2003, Unoki and Nakamura demonstrated that Egr2 induced apoptosis significantly in a number of cancer cell lines by directly inducing the expression of two proapoptotic proteins of the Bcl-2 family. They carried out their study mainly on different Human Carcinoma and Glioblastoma cell lines (Unoki and Nakamura 2003). Yokota and colleagues have identified Egr2 as a direct target of the p53 family in human cancer cell lines. They also confirmed the Egr2 expression is induced in mouse and rat fibroblast cells by DNA-damage-induced p53 activity (Yokota, Sasaki *et al*. 2010).

Here we studied the effects of apoptosis in our Egr2$^{-/-}$Egr3$^{-/-}$ lymphocytes to understand if suppression of apoptosis could play a role in the lymphoma development. Naïve CD4^{+} T cells were isolated from 3 month old wild type, Egr2$^{-/-}$Egr3$^{-/-}$, CD2-Egr2$^{-/-}$ and Egr3$^{-/-}$. The CD4^{+} T cells were stimulated with plate-bound anti-CD3 and anti-CD28 for 16 hours and stained for Annexin V and 7AAD. The cells were measured by flow cytometry. As seen in Figure 3.17, there was a slight but not significant difference in the percentage of apoptotic cells from the Egr2$^{-/-}$Egr3$^{-/-}$ CD4^{+} T cells compared to wild type.

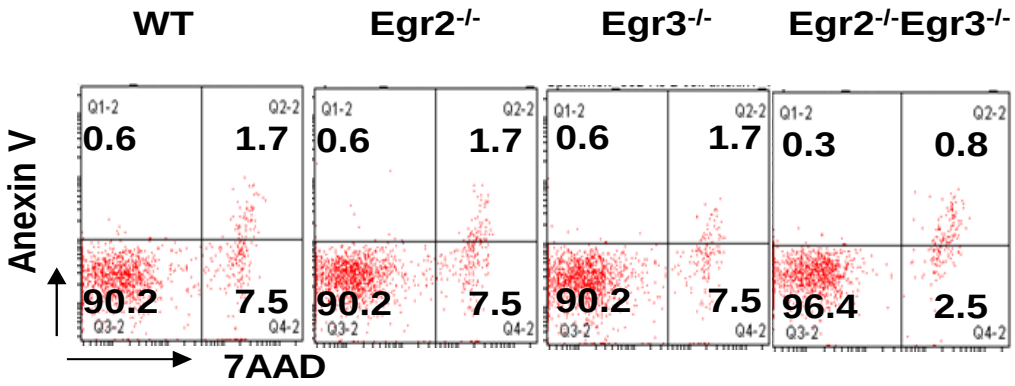

Figure 3.17: Apoptosis of activated T cells.
(A) Resting CD4 T cells from indicated mice were stimulated with anti-CD3 and anti-CD28 for 16 hours and Annexin V positive cell were measured. The data are representatives of three experiments.

There was half the amount of early stage apoptotic steps (Annexin V positive and 7-AAD negative cells) in the $Egr2^{-/-}Egr3^{-/-}$ $CD4^{+}$ T cells compared to wild type, $Egr3^{-/-}$ and CD2-$Egr2^{-/-}$. The same fold change was seen for cells at the late stage of apoptosis (Annexin V and 7-AAD positive).

Clearly it is shown that apoptosis is not affected in the $Egr2^{-/-}Egr3^{-/-}$ mice model and possibly not the direct pathway causing the lymphoma development in our model.

3.3.5 Shorter Telomere Length in Egr2$^{-/-}$Egr3$^{-/-}$ mice

One of the hallmarks of cancer cells is that they acquire an infinite replication potential (Negrini, Gorgoulis *et al.* 2010). Telomeres, a repetitive DNA sequence are based at the ends of the chromosomes which functions as the molecular counter of the cell's replicative potential. The telomeres are composed of several thousand repeats of the sequence TTAGGG bound by a set of specific protein. As the cell divides the telomeres shorten, eventually causes the cell to undergo senescence, once it has reached its limit (Allsopp, Vaziri *et al.* 1992). It has been proven that in cancer cells, telomeres are extremely short due to the intense proliferation properties of the cells (Artandi and DePinho 2000).

We examined the telomere lengths of lymphocytes in Egr2$^{-/-}$Egr3$^{-/-}$ and wild type mice. The lymphocytes were collected from secondary lymphoid organs. Half of the lymphocytes were stimulated with PMA/INO for 48 hours (hrs). The other half was cultured without stimulation for 48hrs. Metaphase spread were prepared and fixed onto slides. The telomere length was examined by Q-FISH, which measures the fluorescence intensity of the telomere repeats (Wong and Slijepcevic 2004) (Figure 3.18).

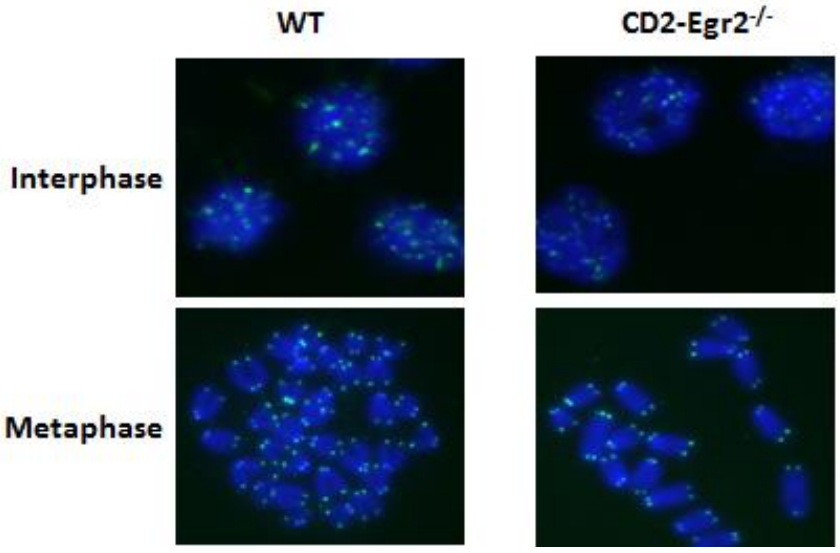

Figure 3.18: Q-FISH image of interphase and metaphase $CD4^+$ T cells from wild type and CD2-$Egr2^{-/-}$ mice

The telomere length of interphase $CD4^+$ T cells and $CD19^+$ B cells were measured with the aid of IPLab software. The telomere length shortening was compared between CD2-$Egr2^{-/-}$ and wild type. The telomere length of CD2-$Egr2^{-/-}$ T and B cells were significantly shorter than wild type under homeostatic conditions (unstimulated) (Figure 3.19).

We also tested the telomere length in fibroblast cells from both wild type and CD2-$Egr2^{-/-}$ mice as a negative control because Egr2 was specifically removed from the lymphocytes. As expected there was no significant difference in the telomere length from both wild type and CD2-$Egr2^{-/-}$ fibroblast cells.

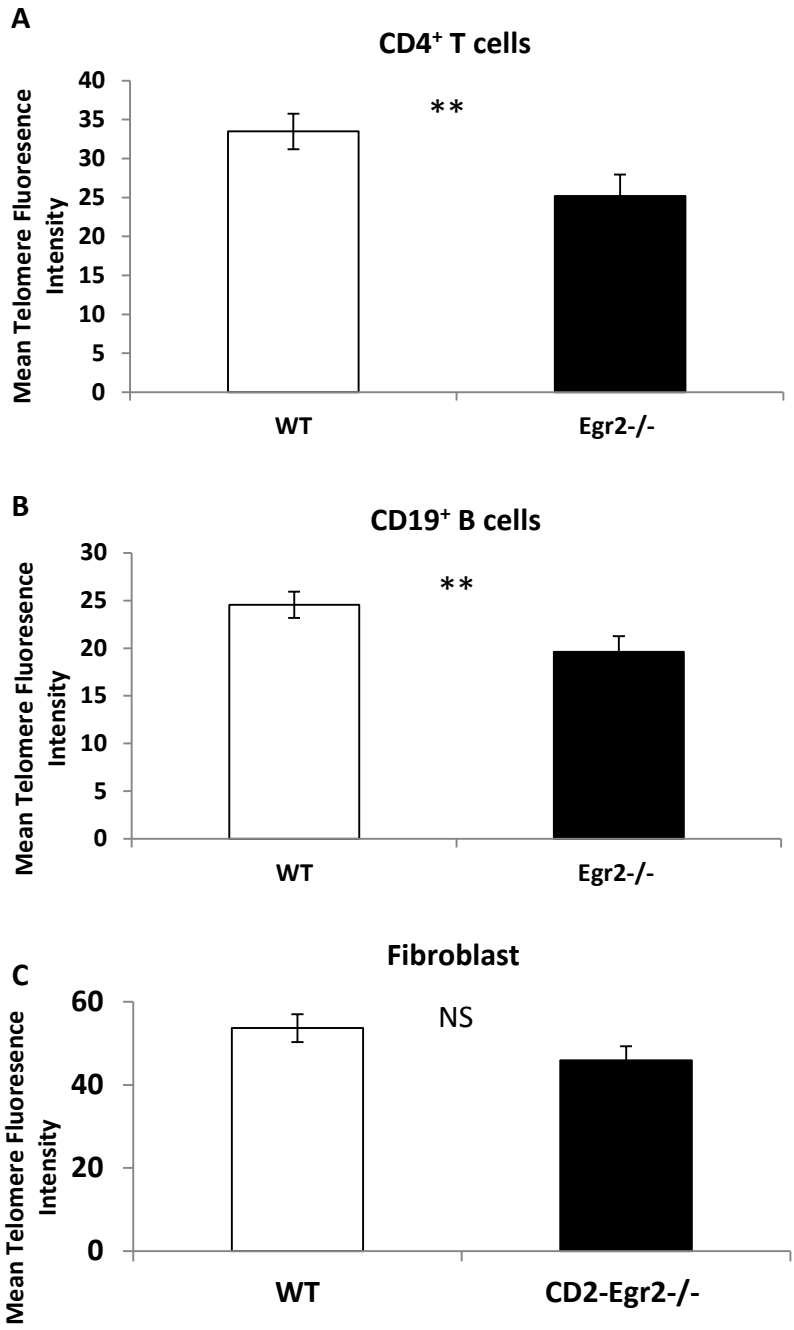

Figure 3.19: Telomere Length of Lymphocytes.
(A) Telomere length in $CD4^+$ T cells of CD2-$Egr2^{-/-}$ and wild type. (B) Telomere length in $CD19^+$ B cells of CD2-$Egr2^{-/-}$ and wild type. The telomere length is shorter in both T and B cells compared to wild type. (C) Telomere length in fibroblast cells from wild type and CD2-$Egr2^{-/-}$. Standard error bars indicate SEM, ** P<0.01, NS-Not Significant.

In line with telomere length, it is also known that the telomere length shortening is maintained by telomerase enzyme (Meyerson 2000). We then looked at the telomerase enzyme activity within the lymphocytes. RNA was extracted from the isolated $CD4^+$ T cells and $CD19^+$ B cells before and after stimulation with PMA/INO. RNA expression was determined by real-time PCR for mTERT gene. Consistent with our findings for the telomere length, the expression of mTERT was increased in the CD2-$Egr2^{-/-}$ model compared to wild type.

There was no difference in the expression of telomerase enzyme in the unstimulated $CD4^+$ T cells or $CD19^+$ B cells, as no or very little telomerase activity is detected in mature resting $CD4^+$ and $CD8^+$ T lymphocyte (Weng, Levine *et al*. 1996; Weng, Granger *et al*. 1997). There was a 2 fold difference in the stimulated $CD4^+$ T cells and a 4 fold difference in $CD19^+$ B cells.

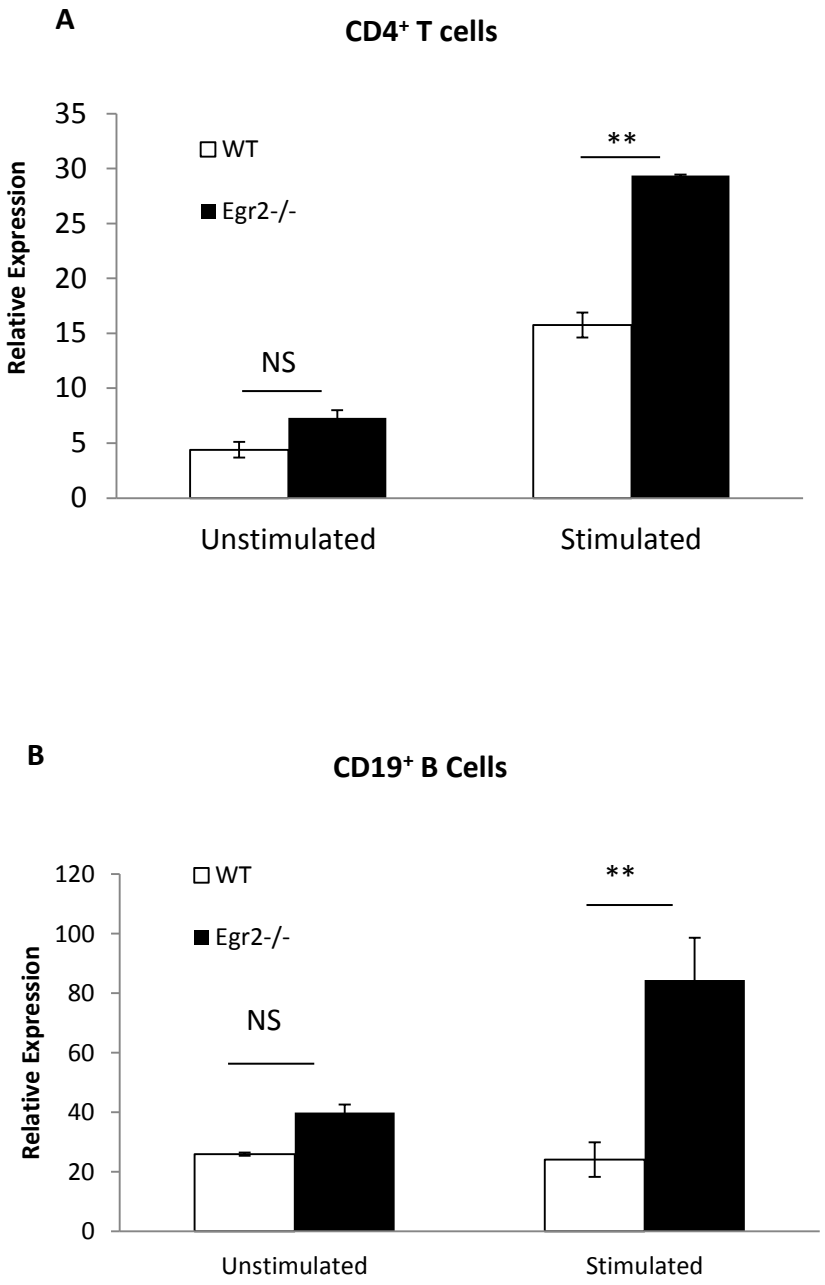

Figure 3.20: Telomerase Enzyme Expression.
(A) mTERT expression in $CD4^+$ T cells from both wild type and CD2-$Egr2^{-/-}$. (B) mTERT expression in $CD19^+$ B cells from both wild type and CD2-$Egr2^{-/-}$. Standard error bars indicate SEM ** $p<0.01$, NS – Not significant difference.

3.4 Molecular Mechanism of Egr2 and Egr3 in Preventing Tumour Development

To understand the mechanism of Egr2 and Egr3 in tumourgenesis two approaches were taken.

1. The possible effect of the inflammatory microenvironment in the support of tumour development was investigated in the $Egr2^{-/-}Egr3^{-/-}$ mice.
2. Using functional genomic tools to identify the transcriptional program regulated by Egr2 in the control of tumourgenesis.

3.4.1 Possible effect of inflammatory microenvironment supports the tumour development in $Egr2^{-/-}Egr3^{-/-}$ mice.

Inflammation has long been associated with the development of cancer (Rakoff-Nahoum 2006). The effect of microenvironment for the tumour developments was investigated in the $Egr2^{-/-}Egr3^{-/-}$ Mice.

Previously, our group found that Egr2 deficient T cells are highly active *in vivo* under homeostatic conditions, leading to the development of autoimmune diseases in the CD2-$Egr2^{-/-}$ mice in later age (Zhu, Symonds *et al*. 2008). However no autoimmune symptoms were found in the $Egr3^{-/-}$ mice. An early onset of the pathological phenotypes was seen in the $Egr2^{-/-}Egr3^{-/-}$ mice. At 2 months of age, both male and female $Egr2^{-/-}Egr3^{-/-}$ mice developed chronic inflammation. Zhu, *et al* (2008) demonstrated the inflammatory autoimmune disease in adult CD2-$Egr2^{-/-}$ mice, with lymphocyte infiltration and severe glomerulonephritis (Zhu, Symonds *et*

al. 2008). Here we report similar symptoms in the $Egr2^{-/-}Egr3^{-/-}$ mice but onset much earlier in age. In 3 month old $Egr2^{-/-}Egr3^{-/-}$ mice the kidney histology revealed deposition of immune complexes in the glomeruli and infiltration of lymphocytes in non-lymphoid organs, but these symptoms were not seen in wild type mice (Figure 3.21).

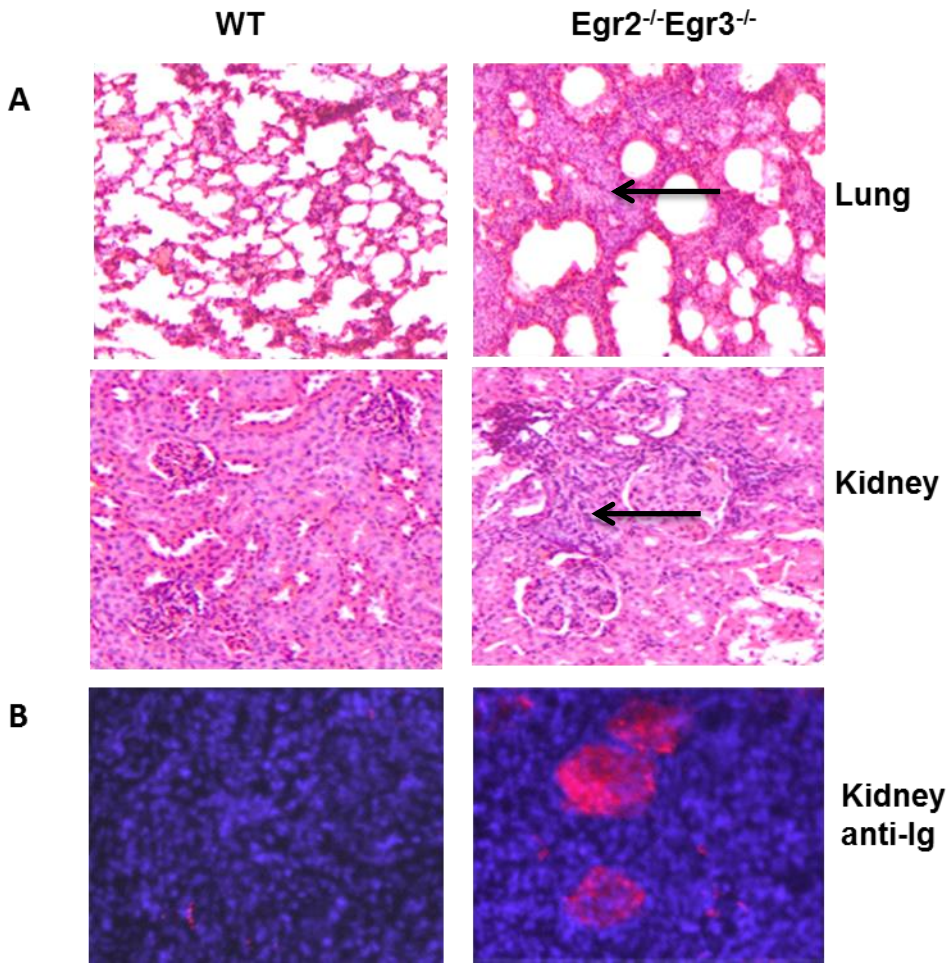

Figure 3.21: Mice lacking Egr2 and Egr3 develop chronic inflammation disease.
(A) H&E analysis of the lung and kidney, from a 3 month old wild type and $Egr2^{-/-}Egr3^{-/-}$ mice. lymphocyte infiltration seen in the $Egr2^{-/-}Egr3^{-/-}$ mice (marked by an arrow) compared to wild type (B) Glomerular Ig deposits in 3 month $Egr2^{-/-}Egr3^{-/-}$ mice (Texas red labelled anti-mouse Ig).

The $CD4^+$ and $CD8\ T^+$ cells expressed high levels of activation markers such as CD44, CD69 and CD25, while CD62L positive T cells were significantly reduced compared with wild type (Figure 3.22A) suggesting that the Egr2 and 3 deficient T cells are hyper-active. The activation markers, CD86 and CD69, were also analysed for the splenic B cells. Both CD86 and CD69 were expressed significantly at high levels (especially CD69) compared to wild type, suggesting that the B cells were highly activated in the spleen (Figure 3.22A). The same active phenotype was seen the LN too (Figure 3.22).

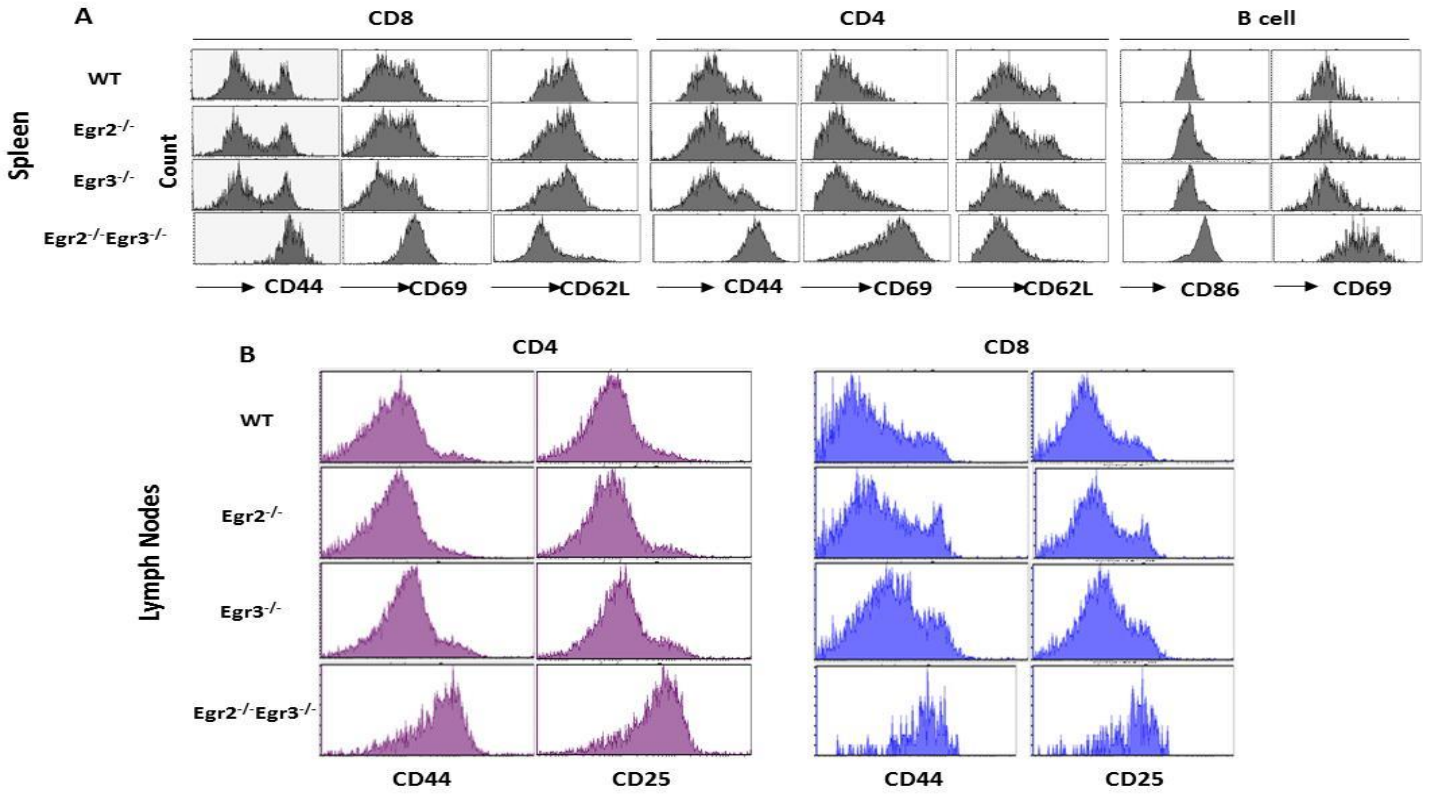

Figure 3.22: Functional analysis of peripheral lymphocytes.

(A) Surface marker expression on splenic lymphocytes from 8-week-old wild type and $Egr2^{-/-}Egr3^{-/-}$ mice. (B) Surface marker expression on lymph nodes lymphocytes from 8-week-old wild type and $Egr2^{-/-}Egr3^{-/-}$ mice. Results represent 3 experiments.

Chronic inflammation observed in the $Egr2^{-/-}Egr3^{-/-}$ mice provides the inflammatory microenvironment for the tumour to progress. Tissue inflammation is also brought upon due to a deregulated production of cytokines, such as IL-6, IL10 and IL-17 (Ohl and Tenbrock 2011). It was observed that the $Egr2^{-/-}Egr3^{-/-}$ mice became moribund at around 8 months of age, due to the multi-organ inflammation, which is known to be associated with high levels of serum inflammatory cytokines. We, therefore, checked the presence of cytokines in the $Egr2^{-/-}Egr3^{-/-}$ mice compared to wild type and as expected, we found that there was high levels of inflammatory cytokine expressions of IL-17, IL-17F, IL-10, IL-6 and IL-3, granulocyte macrophage colony stimulating factor (GM-CSF) and IFN-γ in the serum of $Egr2^{-/-}Egr3^{-/-}$ mice (Figure 3.23).

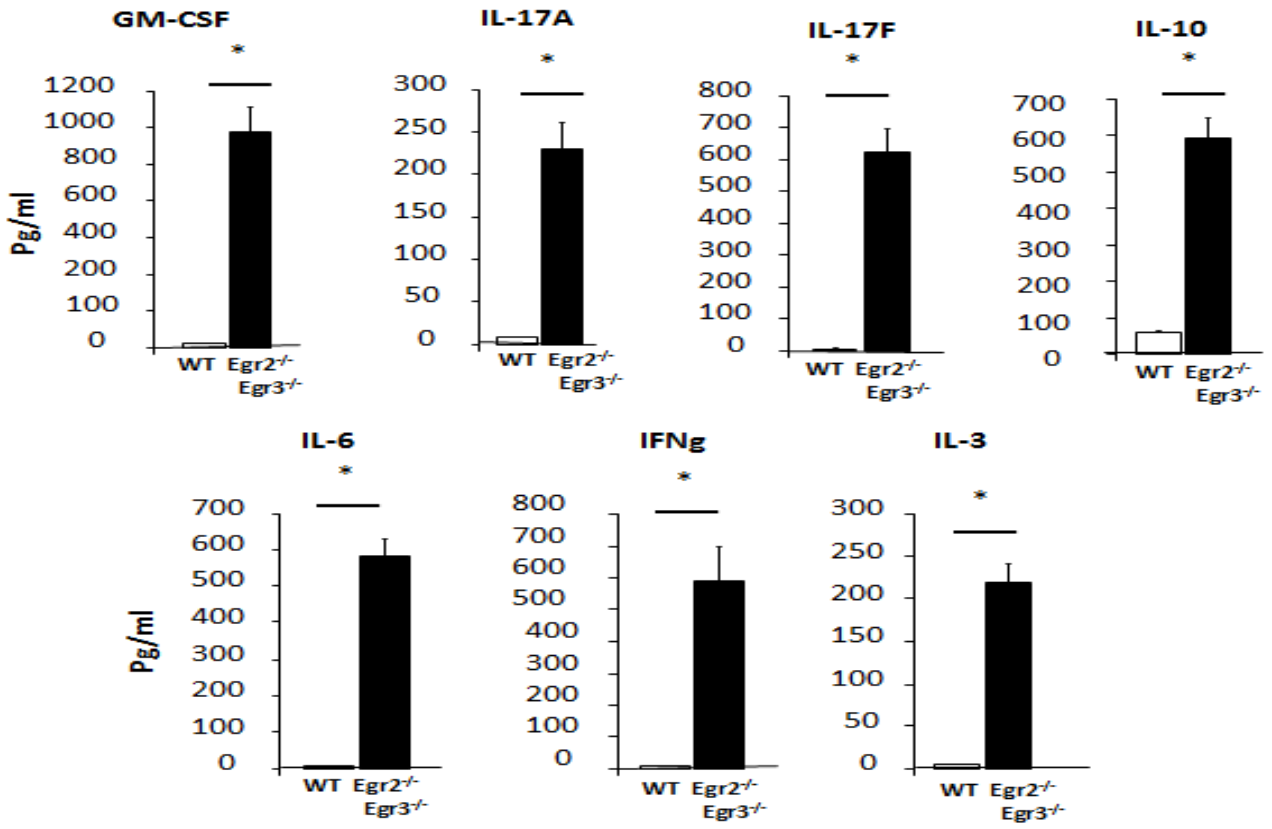

Figure 3.23: Egr2 and Egr3 control the homeostasis of inflammatory cytokines.

Serum cytokine levels in 3 month old wild type (WT) and $Egr2^{-/-}Egr3^{-/-}$ mice. High levels of inflammatory cytokines observed in $Egr2^{-/-}Egr3^{-/-}$ mice compared to WT. * $p<0.05$

3.4.2 Constitutive activation of STAT3 oncogene expression detected in Egr2$^{-/-}$Egr3$^{-/-}$ mice.

Cytokines play an essential role in the development and differentiation of lymphoid cells, which include interleukins and interferon. In section 3.4.1, cytokines were described to be significantly increased in the Egr2$^{-/-}$Egr3$^{-/-}$ serum compared to those in wild type mice. Cytokines bind to their specific receptor chain, the hematopoietin receptor, to initiate the formation of a functional cytokine receptor complex and the intracellular signalling pathway (Kishimoto, Taga *et al.* 1994). The cytokine signalling pathway induces proteins, such as Signal transducers and activators of transcription (STATs). STATs function as transcription factors to regulate gene expression of target genes, such as c-Myc. Importantly the cytokine signalling pathways are negatively regulated by suppressors of cytokine signalling (SOCS) proteins, which have also be profound to play additional roles in many immunological processes (Kubo, Hanada *et al*. 2003).

Our group have earlier shown that SOCS1 and SOC3, suppressors of STAT1 and STAT3 activation, are induced in T cells by TCR stimulation (Anderson, Sundstedt *et al.* 2003). Hence we reviewed the cytokine signalling pathways in B and T cells. We focused our investigation on STAT3 as it is a known oncogene and shown to be constitutively active in nearly 70% of solid and haematological tumours (Avalle, Pensa *et al.* 2012). STAT3 protein expression was analysed in both T and B cells from Egr2$^{-/-}$Egr3$^{-/-}$ and wild type mice. As expected, STAT3 was highly activated in the lymph nodes from 3 month old Egr2$^{-/-}$Egr3$^{-/-}$mice. This was further confirmed

by western blot, where $CD4^+$ T cells and $CD19^+$ B cells were isolated from both $Egr2^{-/-}Egr3^{-/-}$ and wild type mice. The endogenous STAT3 was used as the endogenous control. The pSTAT3 was significantly increased in both unstimulated and stimulated $CD4^+$ T cells and $CD19^+$ B cells from the $Egr2^{-/-}Egr3^{-/-}$ mice compared to aged match wild type mice

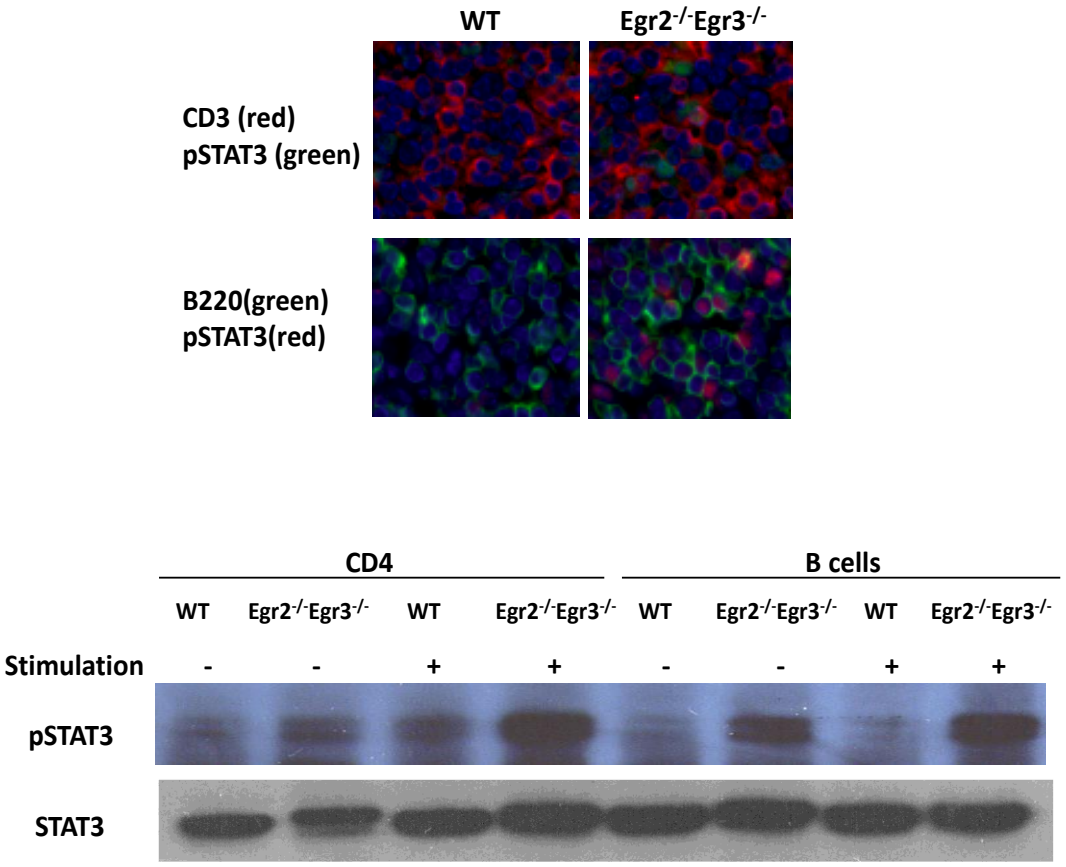

Figure 3.24: Hyper activated STAT3 in Egr2 and Egr3 deficient B and T lymphocytes.

(A) Phosphorylation of STAT3 in lymphocytes in both wild type and $Egr2^{-/-}Egr3^{-/-}$ lymph nodes at 3 months of age. (B) Western Blot analysis of phosphorylation of STAT3. Results demonstrate high expression of pSTAT3 in $Egr2^{-/-}Egr3^{-/-}$ compared to WT.

Further, the expression of STAT3 mRNA was confirmed to be upregulated in both B and T cell lymphomas, as represented in Figure 3.25. The level of relative mRNA expression was significantly increased in all representing B and T cell lymphomas compared to normal T and B cell isolated from wild type lymphocytes. Constitutive activity of STAT3 is essential for proliferation and survival in many established primary tumour cells and its inhibition impairs tumour growth (Avalle, Pensa *et al*. 2012).

To further investigate whether Egr2 and/or Egr3 can regulate the expression of SOCS3, we analysed the expression of SOC3 in naïve B and $CD4^+$ T cells from a 4 week old mice before and after antigen receptor stimulation. Supporting the findings of hyper activated STAT3, the SOCS3 were highly deficient in the $Egr2^{-/-}Egr3^{-/-}$ mice in comparison to wild type mice (Figure 3.26A).

To investigate whether Egr2 regulates the expression of SOC3 we looked at proximal promoters of SOCS3 for potential Egr2 binding sites with the aid multiTF utility (http://multitd.dcode.org) (Figure 3.26B). By ChIP we demonstrated that Egr2 directly interacts with SOCS3 promoters and a conserved intergenic element in the SOCS3 locus (Figure 3.26C). Hence the impaired function of SOCS3 leads to the hyper-activation of STAT3 in the $Egr2^{-/-}Egr3^{-/-}$ B and $CD4^+$ T cells.

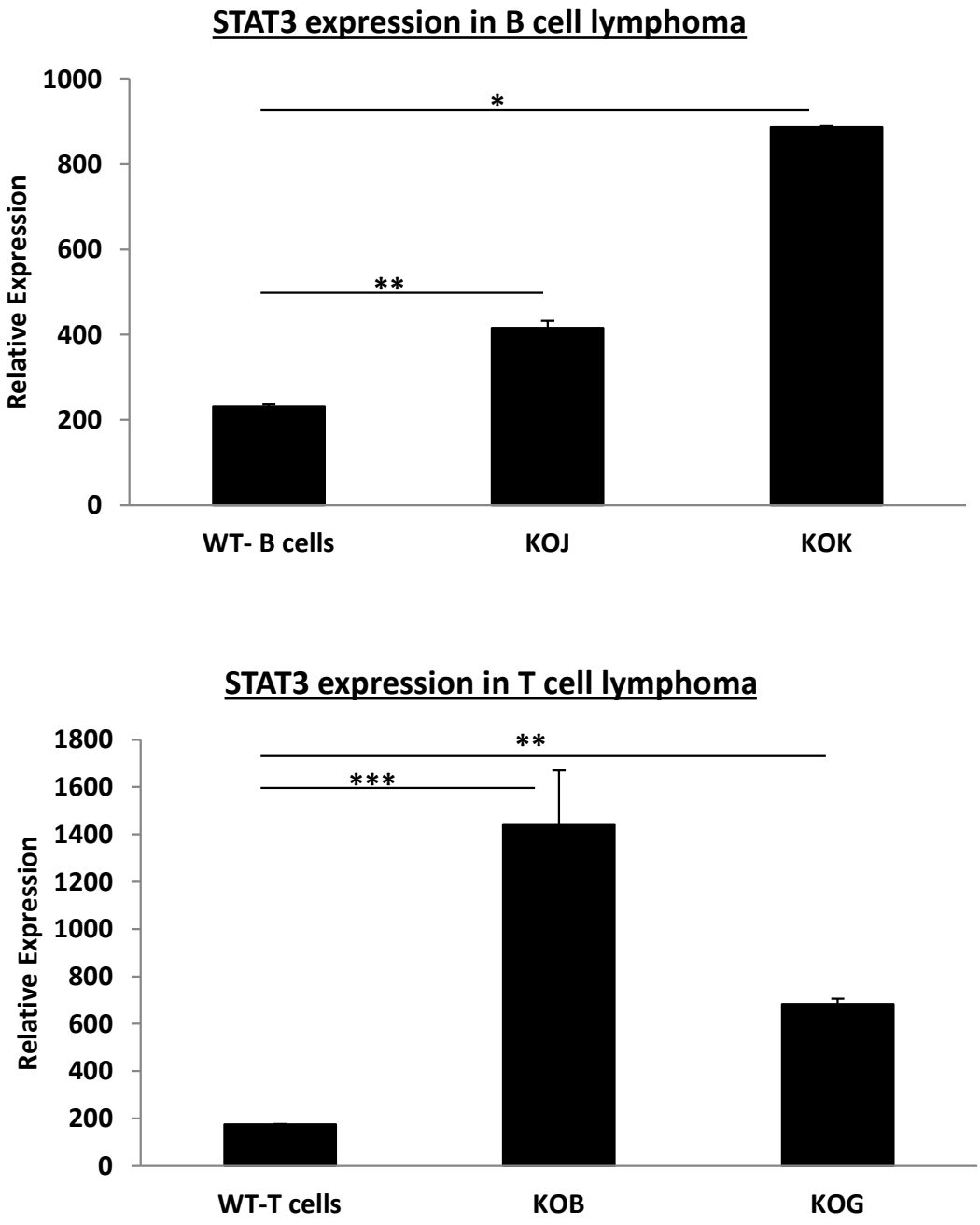

Figure 3.25: Hyper activated STAT3 in B and T lymphoma from Egr2$^{-/-}$Egr3$^{-/-}$ mice. (A) STAT3 mRNA expression in B cell lymphomas (labelled KOJ and KOB) from Egr2$^{-/-}$Egr3$^{-/-}$ mice. (B) STAT3 mRNA expression T cell lymphomas (labelled KOB and KOG) from Egr2$^{-/-}$ Egr3$^{-/-}$ mice. The expression was compared to normal STAT3 expression in wild type B and T cells. Standard error bars indicate SEM. *p<0.05, **p<0.01 and ***p<0.001.

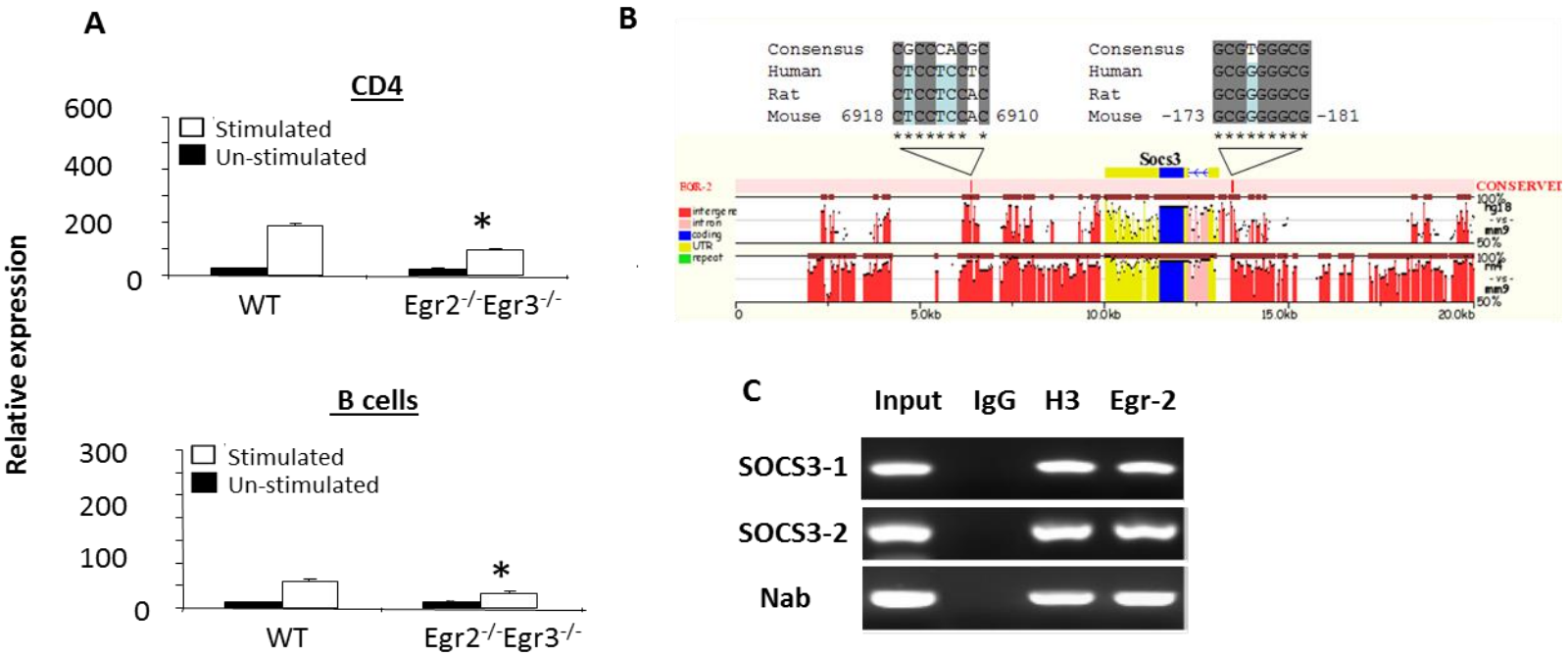

Figure 3.26: Egr2 regulated the mRNA expression of SOCS3

(A) mRNA expression of SOCS3 in naïve B and $CD4^+$ T cells from a 4 week old wild type and $Egr2^{-/-}Egr3^{-/-}$ mice before and after antigen receptor stimulation. (B) Identification of potential Egr2 binding sites in the proximal regions of SOCS3. (C) Binding of Egr2 to the proximal regions of the SOCS3 promoters. CD4+ T cells from Egr2 Transgenic mice (Egr2cTg) were stimulated with anti-CD3 and anti-CD28 for 3 hours and used in the ChIP with primers flanking Egr2 binding sites in the promoters of SOCS3, and Nab. Total input DNA and anti-H3 precipitates served as positive control and anti-IgG precipitates as negative control. Standard error bars indicate SEM. * $p<0.05$.

3.4.3 Transcriptional profile of Egr2 in the control of tumourgenesis.

Since Egr2 and Egr3 are transcription factors, they could have the potential to regulate many other genes, any of which might be involved in preventing tumour development. Therefore to observe the effects of Egr2 and Egr3 in lymphocytes, we assessed the genome-wide transcriptional profiles of unstimulated and anti-CD3 stimulated cells for both wild type and CD2-Egr2$^{-/-}$ CD4^{+} T cells using MouseRef-8 v2.0 BeadChip expression arrays (Illumina). These arrays consistent of oligo-nucleotide probes directed against ~24,000 well annotated RefSeq transcripts.

CD4^{+} T cells from CD2-Egr2$^{-/-}$ and wild type mice, were isolated and stimulated with anti-CD3 or left unstimulated and the RNA was extracted and purified as described in the chapter 2. RNA labelling, array scanning and data normalisation was performed by the Microarray facility, Barts and London School of Medicine and Dentistry, according to the manufacturer's instructions. Upon receipt of the normalised data, we first examined the expression of Egr2. Consistent with the previous findings, Egr2 had very weak expression in unstimulated CD2-Egr2$^{-/-}$ CD4^{+} T cells and further reduced expression in stimulated CD2-Egr2$^{-/-}$ CD4^{+} T cells compared to wild type (data not shown).

To distinguish the gene expression profiles in CD2-Egr2$^{-/-}$ CD4^{+} T cells from wild type CD4^{+} T cells the microarray data was carefully evaluated, focusing on genes that showed difference of at least 2-fold between wild-type and CD2-Egr2$^{-/-}$ CD4^{+} T cells and excluded genes that had a detection *p*-value >0.01, anything with a

p-value greater than 0.01 was considered background. The genes that were deferentially expressed were first grouped based on self-organizing maps (SOMs) by clustering algorithm method. To confirm the differential profile of the expressed genes, the SOM clusters were further clustered by hierarchical clustering program (Figure 3.27).

The full set of microarray data is available from the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress) under the accession number E-MEXP-1698.

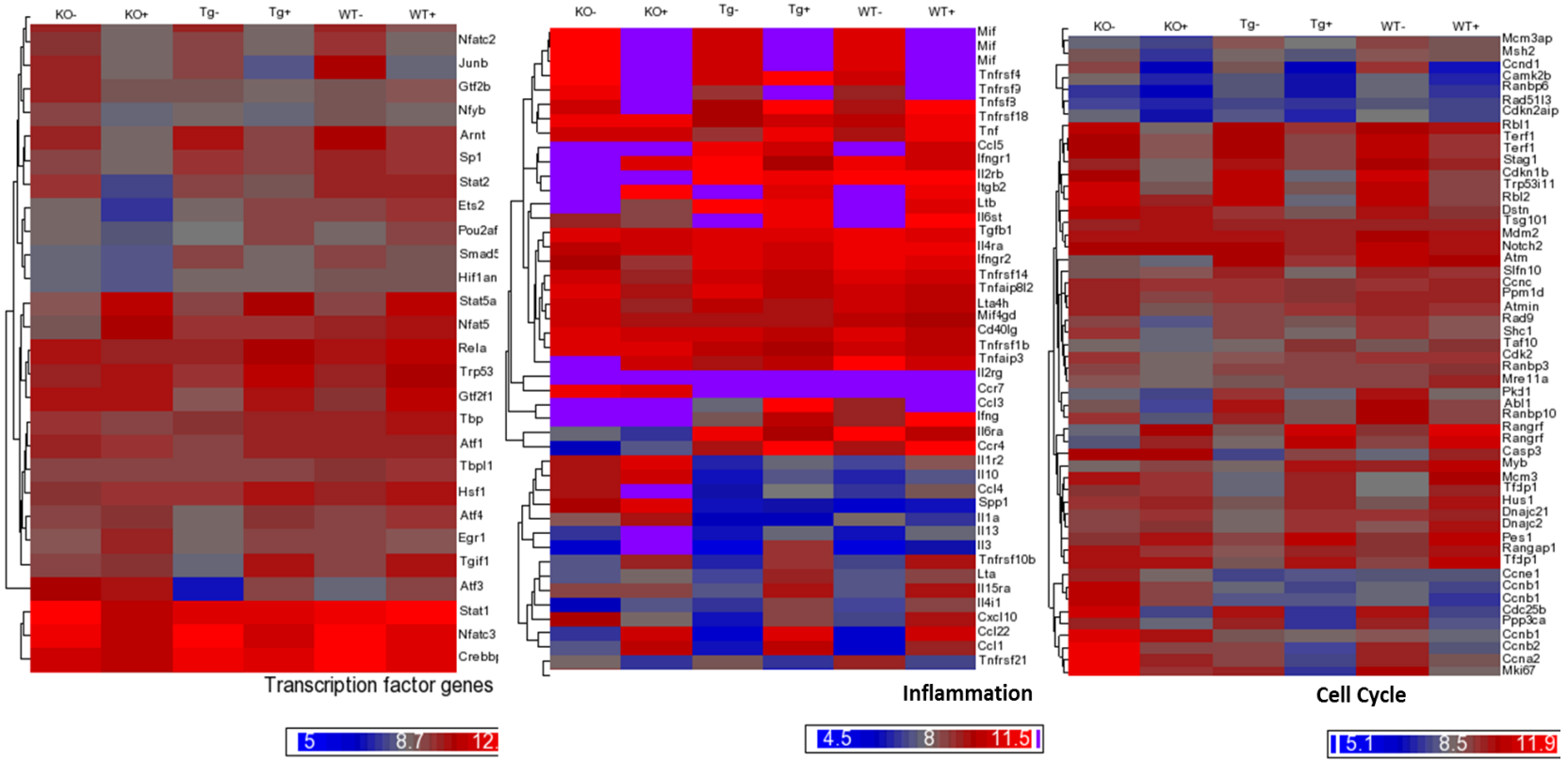

Figure 3.27: Gene expression profile.

Altered expression of transcription factors, inflammation and cell cycle genes in CD2-Egr2$^{-/-}$ (represented as KO) CD4^{+} T cells as assessed by genome-wide transcriptional analysis. The SOM cluster of transcription factors, inflammation and cell cycle genes were further clustered by hierarchical clustering program to show the genes with differential expression profile. Note: Tg represents Egr2 Transgenic mice, which were not included as part of this study.

3.4.3.1 Egr2 regulates the gene expression of cell cycle genes

Cancers results from multiple molecular events that essentially amend the normal properties of the cell. In the cancer cells, the cell cycle control, a process of regulating normal cell division is disrupted. Cancer cells differ from normal cells, such as they have loss of differentiation and increased invasiveness. However other characteristic of cancer cells includes a category of genes that, when mutated, increases the genetic instability and accelerates the cancer growth. These are the genes that regulate the cell cycle control (Hartwell and Kastan 1994).

From our microarray analysis we looked at possible cell cycle genes that were differently expressed in $CD4^+$ T cells from $Egr2^{-/-}Egr3^{-/-}$ model compared to the wild type. We identified 23 genes that were 2 fold or more upregulated in the $CD4^+$ T cells from $Egr2^{-/-}Egr3^{-/-}$ compared to wild type. We also identified 7 genes that were 2 fold downregulated in the $CD4^+$ T cells from $Egr2^{-/-}Egr3^{-/-}$ compared to wild type (Table 3.1).

32 genes, categorised from cell cycle control function were validated by Real-Time RT-PCR; of which only 7 did not correlate with the microarray data (Table 3.1).

Gene	Expression	Microarray Fold Change	RT-PCR Fold Change	p-value from RT-PCR
Brca1	Upregulated	2.44	2.55	< 0.01
Casp3	Upregulated	3.06	6.43	< 0.01
Ccna2	Upregulated	3.89	3.81	> 0.05
Ccnb1	Upregulated	3.67	0.77	> 0.05
Ccnb2	Upregulated	3.08	0.34	< 0.01
Ccne1	Upregulated	2.78	2.92	< 0.05
Ccnf	Upregulated	3.13	1.89	< 0.01
CDC25B	Upregulated	2.05	1.41	> 0.05
Chek1	Upregulated	3.25	4.08	< 0.05
Cks1b	Upregulated	4.41	3.01	> 0.05
E2f1	Upregulated	2.18	0.75	> 0.05
E2f2	Upregulated	2.36	2.66	< 0.05
Itgb1	Upregulated	2.8	6.85	< 0.05
Mad2l1	Upregulated	2.74	4.76	< 0.05
MCM2	Upregulated	2.01	2.06	< 0.05
Mcm3	Upregulated	2.55	3.78	< 0.05
Mki67	Upregulated	2.57	4.48	< 0.01
Mybl2	Upregulated	2.05	0.41	> 0.05
Nek2	Upregulated	4.21	4.01	< 0.05
Pmp22	Upregulated	2.06	3.03	< 0.05
Rad51	Upregulated	4.34	5.76	< 0.01
Rad51ap1	Upregulated	2.31	5.27	< 0.01
Wee1	Upregulated	2.34	1.81	> 0.05
Abl1	Downregulated	2.17	4.51	< 0.05
Atm	Downregulated	2.74	2.77	< 0.01
Dstyk	Downregulated	2.33	3.41	< 0.01
Macf1	Downregulated	3.06	5.98	< 0.01
Pkd1	Downregulated	2.62	2.83	< 0.05
Rad9b	Downregulated	2.62	4.09	< 0.05
Sfn	Downregulated	4.66	2.81	< 0.01

Table 3.1: Validation of Microarray Expression Profile with Real-Time PCR.

While the pattern of expression usually related fairly well with those from the microarray, the relative levels of expression sometimes differed. For example, Figure 3.28, compares the expression of three differently expressed genes, Brca1, Ccna2 and SFN, in unstimulated CD2-Egr2$^{-/-}$ and wild type CD4^{+} T cells, as detected by the two different methods. The validated Real-Time expression shows a similar pattern to the normalised microarray expression. Brca1 and Ccna2 both showed exactly the same fold change ratio of 2.5 and 3.8 respectively. SFN showed a slight decrease in the microarray expression compared to Real-Time PCR. This quantitative difference may be due to the different sensitivity of the two techniques.

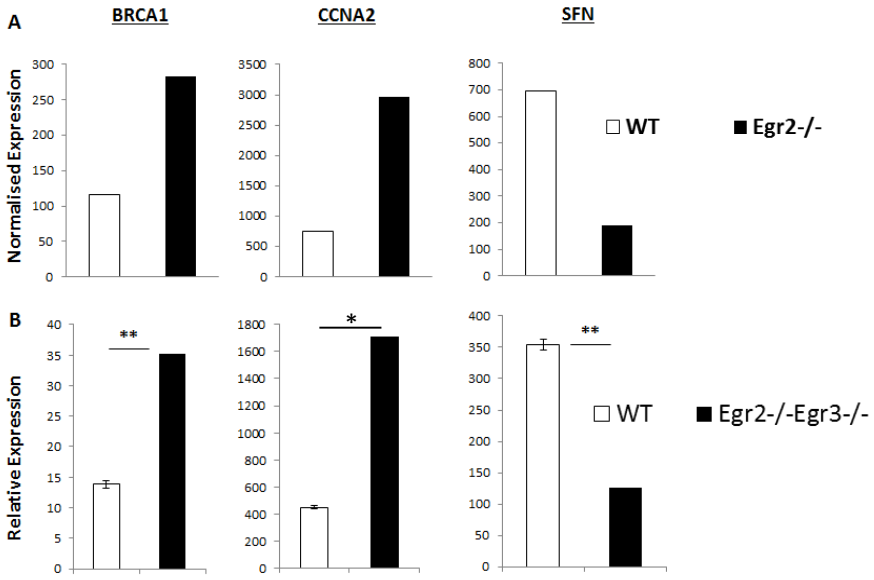

Figure 3.28: Brca1, Ccna2 and SFN mRNA expression in CD2-Egr2$^{-/-}$ CD4^{+} T cells.
Splenic CD4^{+} T cells were isolated from the CD2-Egr2$^{-/-}$ and wild type mice. (A) Normalised expression from the microarray. (B) Relative Expression from the Real-Time RT-PCR validation. Data represents 3 experiments. Standard error bars indicate SEM. *p< 0.05 and ** p< 0.01.

The validation of the microarray results revealed number of genes that were significantly ($p<0.01$) up-regulated in the CD2-Egr2$^{-/-}$ CD4^{+} T cells than in wild type CD4^{+} T cells, which play an important role in the cell cycle control. Some of the interesting genes were RAD51, RAD51AP1, and MKI67 (Figure 3.29).

Both *RAD51* and *RAD51AP1* participate in the DNA response pathway associated with the activation homologous recombination and double-strand break repair. The microarray data of normalised expression showed a 4.97 and 1.1 fold increases in Rad51 and Rad51ap1 respectively. The Real Time RT-PCR showed a 5 fold increase for both Rad51 and Rad51ap1 confirming the increased expression of these two DNA repair genes. A number of studies have found that overexpression of RAD51 in cells leads to increased resistance to chemotherapeutic drugs (Klein 2008). Overexpression of RAD51 genes results in the overstimulation of homologous recombination and chromatid exchange mechanisms, this is predicted to lead to genomic instability and eventually contributing to carcinogenesis (Raderschall, Stout *et al.* 2002).

Mki67 (also known as Ki67) is a known marker for proliferation, with evidence of high expression of this gene in diffuse large B-cell lymphoma and mantle cell lymphoma (Ek, Bjorck *et al.* 2004; Li, Huang *et al.* 2012). The microarray indicated that CD2-Egr2$^{-/-}$ CD4^{+} T cells expressed around 3 fold more Mki67 than WT CD4^{+} T cells. A similar correlation was also seen with the Real Time RT-PCR data with around 4 fold increase in CD2-Egr2$^{-/-}$ CD4^{+} T cells. This data also supports the enhanced expression of PCNA marker in Egr2$^{-/-}$Egr3$^{-/-}$ spleen and tumour samples (section 3.3).

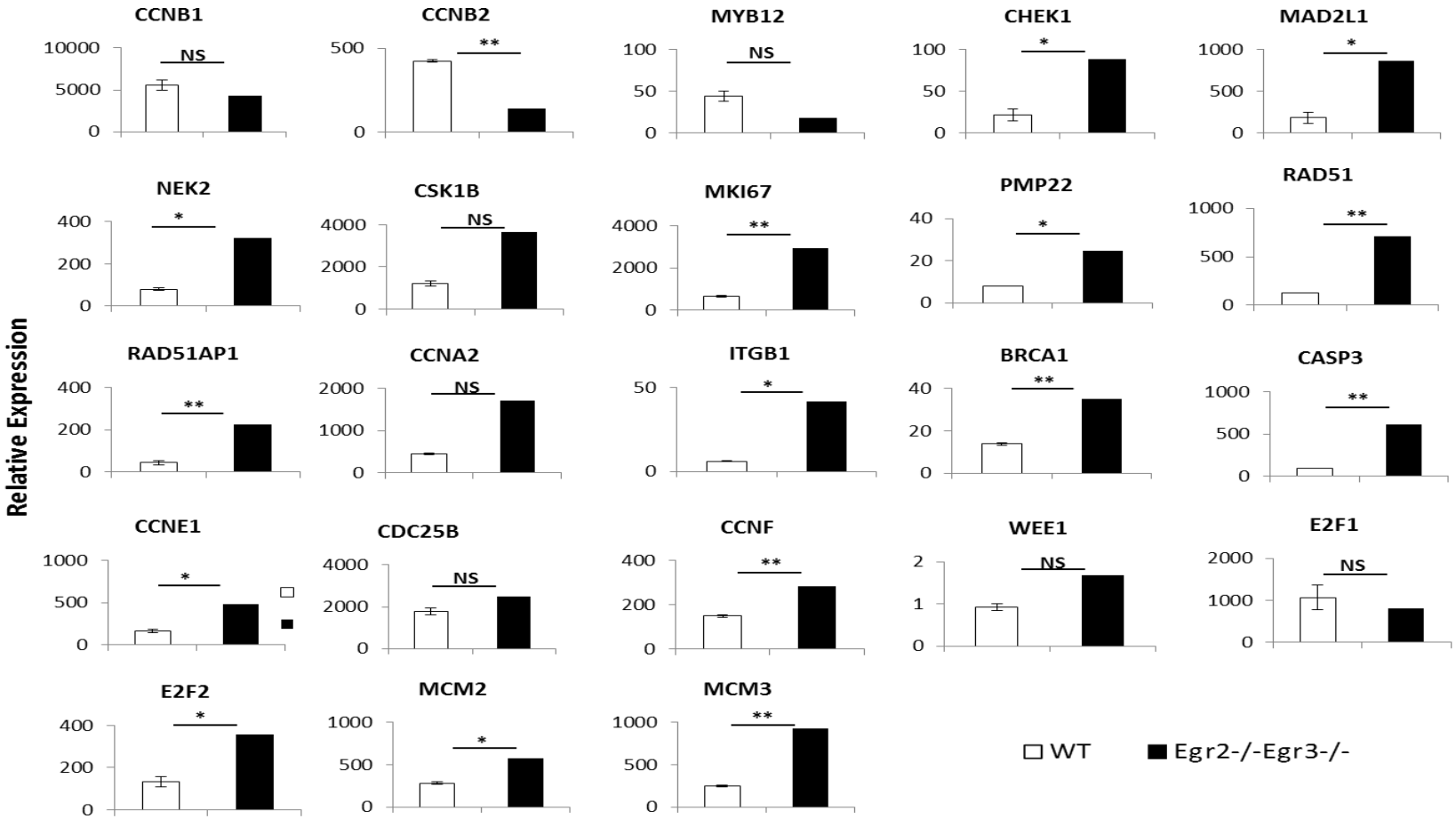

Figure 3.29: Cell cycle genes mRNA expression upregulated in CD2-Egr2$^{-/-}$ CD4^{+} T cells.

Relative expression of cell cycle genes analysed by Real Time RT-PCR for wild type (white) and CD2-Egr2$^{-/-}$ CD4^{+} T cells. Standard error bars represent SEM. * P<0.05, ** p<0.01 and NS – Not Significant.

7 genes were identified to have a 2 fold decrease gene expression from the microarray data. All 7 genes were validated by Real Time RT-PCR and the results correlated with the microarray data. Some of the interesting genes were Stratifin (SFN) and Ataxia Telangiectasia Mutated (ATM) (Figure 3.30).

SFN, commonly known as 14-3-3, regulates a number of cellular processes that are crucial for tumour biology, such as cell cycle regulation and apoptosis (Dong, Kang *et al.* 2007). Here we have shown, from our microarray data, that *SFN* is significantly reduced (by 4 fold) in the CD2-Egr2$^{-/-}$ CD4^{+} T cells. This correlation was also demonstrated by Real Time RT-PCR, with a 3 fold reduction in the CD2-Egr2$^{-/-}$ CD4^{+} T cells compared to the wild type.

There are seven isomers of 14-3-3, of which 14-3-3σ (sigma) is the most researched and has shown to be directly linked to cancer (Hermeking 2003). 14-3-3σ acts in support of their relevance to tumour biology by negatively regulating cell cycle control. 14-3-3σ has been shown to inactivate CDC25 proteins by cytoplasmic sequestration, which prevents CDC25C and CDC25B from dephosphorylating and activating cyclin-dependent kinase CDC2, hence the cells become arrest at G2 (Graves, Lovly *et al.* 2001; Bulavin, Higashimoto *et al.* 2003). Interestingly our microarray data reported a 3 fold increase of CDC25C gene in our CD2-Egr2$^{-/-}$ CD4^{+} T cells compared to wild type (data not shown), showing a correlation between the reduction of SFN gene expression and the increase of CDC25C gene expression.

Both the microarray and Real-Time RT-PCR reported a 2.7 fold reduced expression in the ATM gene in CD2-Egr2$^{-/-}$ CD4^{+} T cells compared to wild type

(Figure 3.30). ATM is a pleiotropic protein kinase that is activated in response to a double-stranded DNA breaks (Rotman and Shiloh 1997). This leads on to ATM rapidly activating many factors that aid DNA repair and regulate cell cycle control (Yan, Kuang *et al.* 2002). Deficiencies in ATM has been linked to a number of deficiencies, including thymic lymphoma (Taylor, Metcalfe *et al.* 1996). Zha and colleagues have provided compelling evidence of ATM deficiency and tumour development. They showed that ATM-deficient mice developed a striking predisposition to lymphoid malignancies, particularly thymic lymphoma, due to V(D)J recombination errors at Tcrd, as opposed to Tcra locus (Zha, Bassing *et al.* 2010).

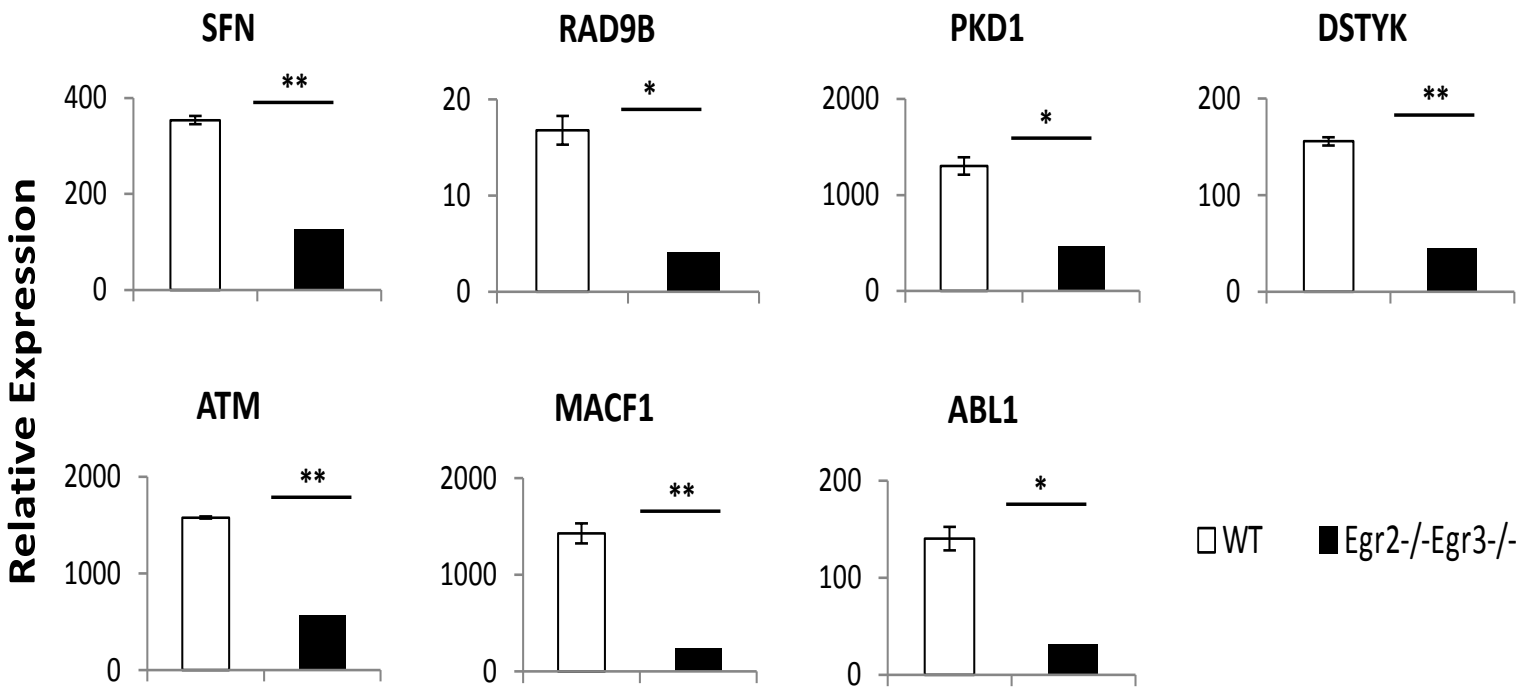

Figure 3.30: Cell cycle genes mRNA expression downregulated in CD2-Egr2$^{-/-}$ CD4^{+} T cells.

Relative expression of cell cycle genes analysed by Real Time RT-PCR for wild type and CD2-Egr2$^{-/-}$ CD4^{+} T cells. Standard error bars represent SEM.
* P<0.05, ** p<0.01 and NS – Not Significant

The microarray gene expression profile was conducted on $CD4^+$ T cells, which were also validated by Real Time RT-PCRs. However our group has shown that Egr2 regulated both T and B cell development (Li, Symonds *et al*. 2011), supported by the B cell lymphomas identified in the Egr2 deficient mice model. This lead us to look at the genes, which demonstrated a significant difference in $CD4^+$ T cells, and further investigated in $CD19^+$ B cells, by Real-Time RT-PCR.

From the 23 up-regulated genes, 18 showed a significant mRNA expression increase in CD2-$Egr2^{-/-}$ $CD4^+$ T cells. Interestingly, when the gene expression of the 23 genes was examined in $CD19^+$ B cells, 57% of them also showed a significant up-regulation in CD2-$Egr2^{-/-}$ cells compared to wild type (Figure 3.31).

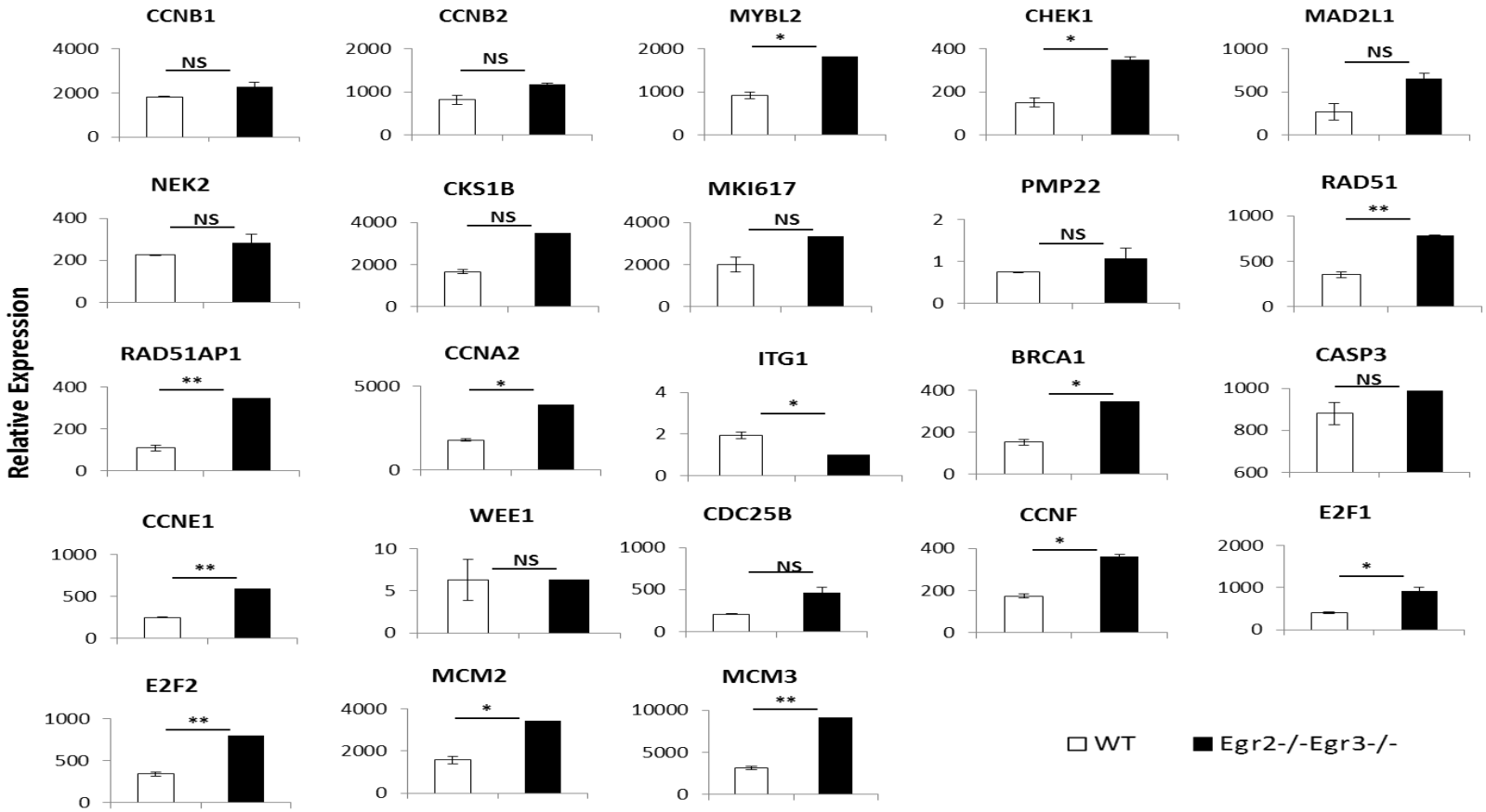

Figure 3.31: Cell cycle genes mRNA expression upregulated in CD2-Egr2$^{-/-}$ CD19^{+} B cells.

Relative expression of cell cycle genes analysed by Real Time RT-PCR for wild type and CD2-Egr2$^{-/-}$ CD19^{+} B cells. Standard error bars represent SEM.
* P<0.05, ** p<0.01 and NS – Not Significant

Unlike $CD4^+$ T cells, where all 7 down regulated genes showed a significant reduction in the CD2-$Egr2^{-/-}$$Egr3^{-/-}$ mice compared to wild type, all, except ABL1, showed an up-regulation in CD2-$Egr2^{-/-}$$Egr3^{-/-}$ compared to wild type. However, only three of the genes showed a significant difference (SFN, PKD1 and MACF1) (Figure 3.32).

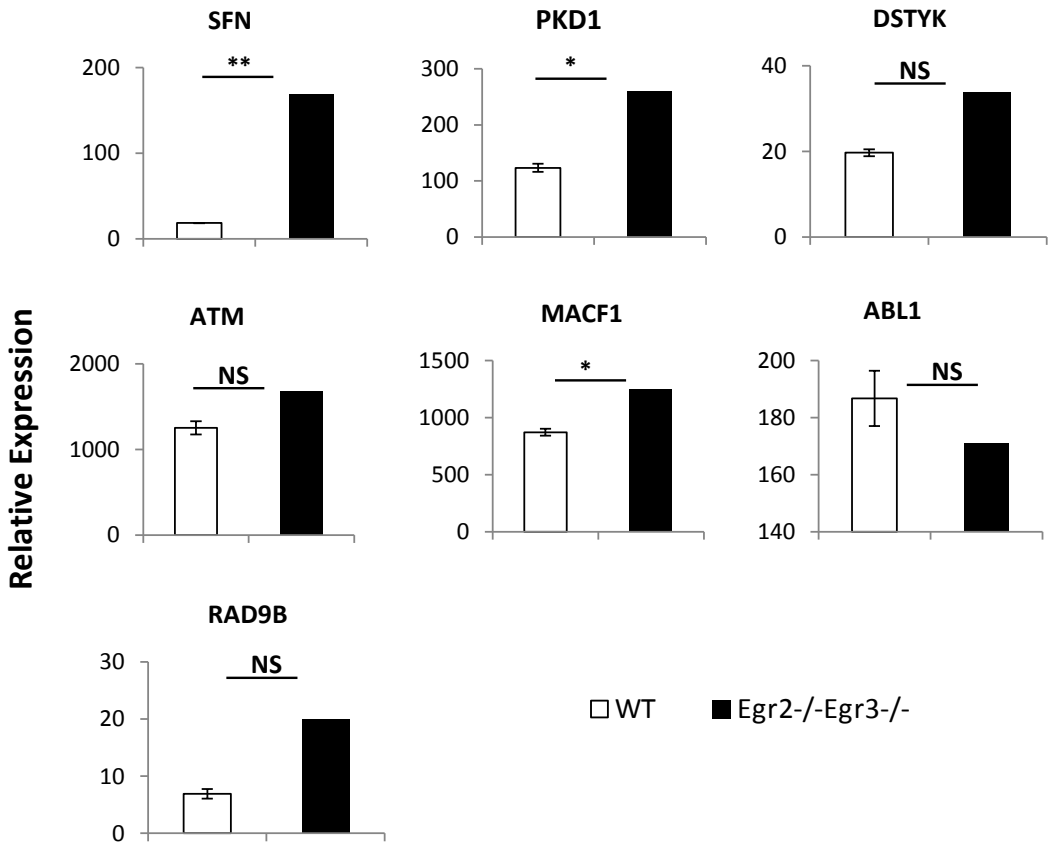

Figure 3.32: Cell cycle genes mRNA expression downregulated in CD2-$Egr2^{-/-}$ $CD19^+$ B cells.
Relative expression of cell cycle genes analysed by Real Time RT-PCR for wild type and CD2-$Egr2^{-/-}$ $CD19^+$ B cells. Standard error bars represent SEM. * P<0.05, ** p<0.01 and NS – Not Significant

3.4.4 Identification of target genes for Egr2 by ChIP-on-chip

With microarray gene expression alone we were unable to identify key genes that demonstrate the effect of Egr2 and Egr3 leading to tumour development, as Egr2 and Egr3 are transcription factors and play a role in transcribing other genes. To understand the mechanism between the loss of Egr2 and tumour development we need to identify the genes that are regulated by Egr2 directly. The microarray gene expression provided us with an insight of which genes might be involved under the influence of Egr2, however it does not provide direct evidence that Egr2 directly regulates the expression of these genes.

Hence, we carried out ChIP-on-chip. In ChIP-on-chip the Eg2 protein was covalently cross-lined to the DNA by formaldehyde, cells were lysed, the chromatin was immunoprecipitated with an Egr2 antibody and then fragmented DNA was fluorescently labelled and hybridised to the GeneChip® Mouse Promoter 1.0R Array.

The data generated, consisting of the intensity, which measures the relative quantity of DNA at the probe's genomic position in the immunoprecipitated material, was analysed. We looked at both ChIP-on-chip data and microarray expression data, to identify possible genes which are controlled or interact with Egr2. The criteria used to identify these genes was set to include the genes that were enriched with a *p*-value more than 0.01 in the ChIP-on-chip compared with the genes that were 2 fold down regulated in the CD2-Egr2$^{-/-}$ CD4^{+} T cells from the microarray analysis. The ChIP-on-chip suggests promoter regions of genes to which

Egr2 binds to. The microarray 2 fold downregulated genes in CD-Egr2$^{-/-}$ CD4^{+} T cells identified possible genes which are expressed in the presence of Egr2. From this data mining we identified 137 genes, of which a number of these genes were found to be involved in cell regulation, chromatin remodelling, etc. (Figure 3.33).

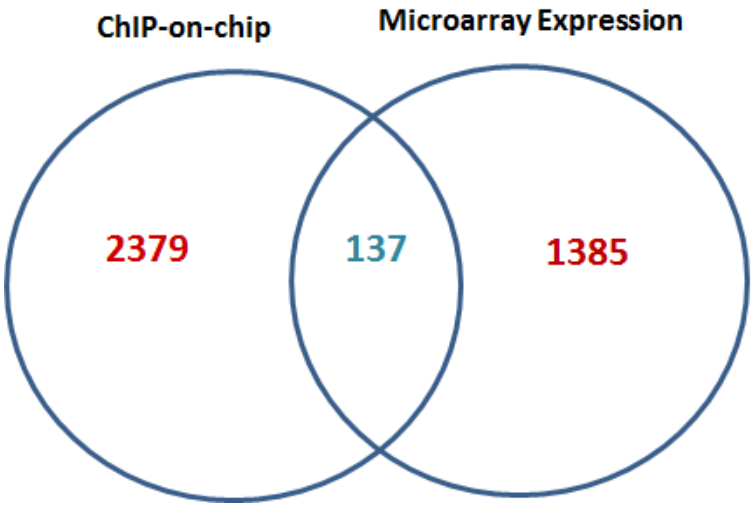

Figure 3.33: ChIP-chip Vs. Expression comparison.
137 genes enriched with a *p*-value greater than 0.01 in ChIP-on-chip and with a 2 fold change in the microarray gene expression.

From the 137 genes identified, 33 of the genes were validated, which had a known function in cell regulation, chromatin remodelling or tumour development. These genes were validated by Real Time RT-PCR. From the 33 genes, three of the genes; Ikaros, Aiolos and FOXO3, demonstrated a 2 fold downregulation in the microarray expression as well as a DNA binding site for Egr2. These genes are also known to function as tumour suppressor genes.

3.4.4.1 Egr2 regulates the expression of Ikaros and Aiolos in lymphocytes

Ikaros family, consisting of Ikaros (IKZF1), Aiolos (IKZF3), Helios (IKZF2), Eos (IKZF4) and Pegasus (IKZF5) - are zinc finger nuclear proteins that play critical role in T and B cell development and differentiation. These proteins are essential for the normal development of lymphocytes and also function as tumour suppressor genes (Clevers, Oosterwegel *et al*. 1993; Georgopoulos, Bigby *et al*. 1994; Molnar and Georgopoulos 1994).

The comparison of the ChIP - on- chip and microarray gene expression identified IKZF1 and IKZF3 as two possible target genes for Egr2 and their role in preventing lymphoid tumour development. Inspection of the microarray data revealed that the mRNA expression of IKZF1 and IKZF3 was reduced in CD2-Egr2$^{-/-}$ CD4^{+} T cells compared to wild type. Under unstimulated (homeostatic conditions) there was a 1.5 fold and 3 fold decreases in IKZF1 and IKZF3, respectively. After stimulation a 2 fold and a significant 11 fold reduction was seen in IKZF1 and IKZF3, respectively (Figure 3.34).

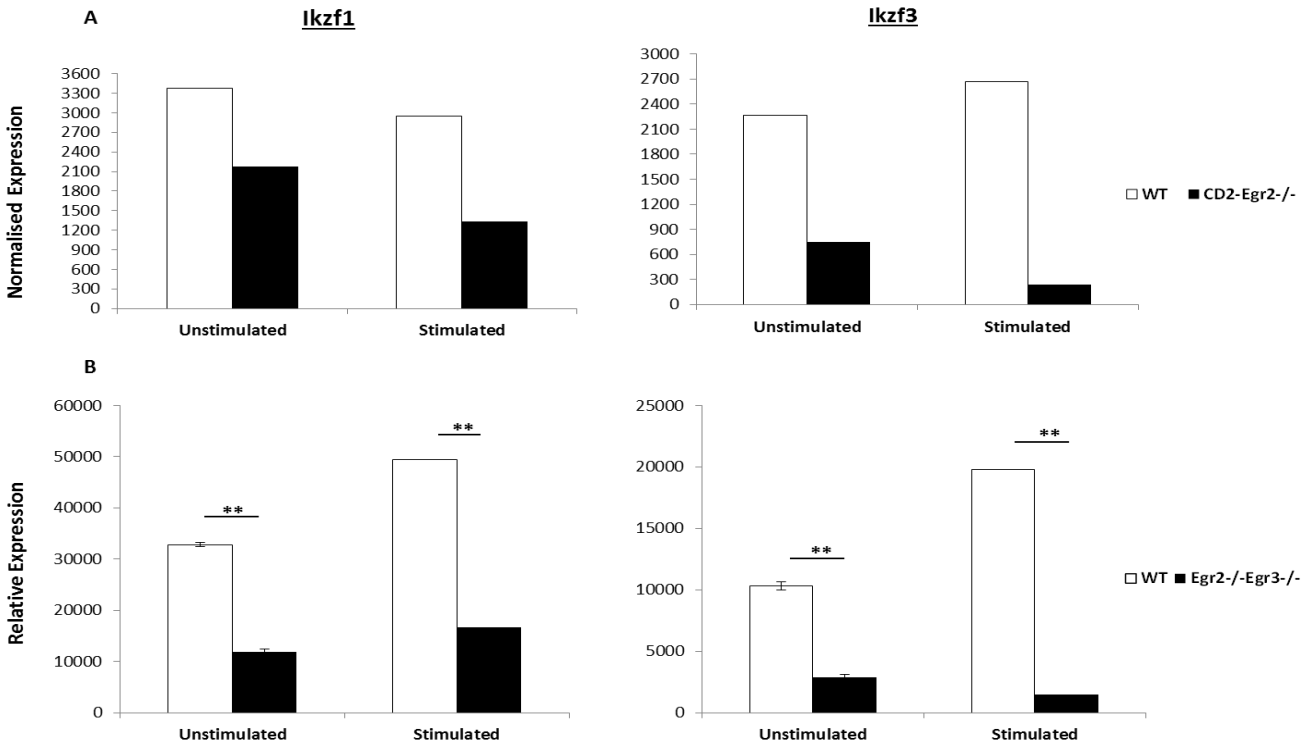

Figure 3.34: mRNA expression of IKZF1 and IKZF3 in CD2-Egr2$^{-/-}$ CD4^{+} T cells.

(A) Microarray normalised expression of Ikzf1 and Ikzf3 in splenic CD4^{+} T cells isolated from the CD2- Egr2$^{-/-}$ and wild type mice. (B) Relative expression measured by Real-Time PCR in splenic CD4^{+} T cells isolated from the Egr2$^{-/-}$Egr3$^{-/-}$ and wild type mice. Standard error bars indicate SEM. ** p< 0.01, NS - Not Significant.

A lot of evidence has reported that a defect in IKZF1 and IKZF3 expression in lymphocytes leads to lymphomas. Winandy and Colleagues found that the loss of Ikaros activity at the late stages of thymocyte maturation leads to uncontrolled lymphoproliferation and rapid development of malignant T cell leukaemia and lymphoma (Winandy, Wu *et al*. 1995). More surprisingly the phenotype of the Ikaros heterozygous mice was similar to that found in the $Egr2^{-/-}Egr3^{-/-}$ mice, with a lymphadenopathy and splenomegaly (Winandy, Wu *et al*. 1995). The same group also studied the Aiolos gene and found that in a similar fashion to Ikaros in T cells, lack of Aiolos in B cells leads to uncontrolled proliferation, causes the breakdown of B cell tolerance and facilitates the development of B cell malignancies (Wang, Avitahl *et al*. 1998).

Here at mRNA gene expression level we have also shown a reduced level of IKZF1 and IKZF3 in the $Egr2^{-/-}Egr3^{-/-}$ mice model and that there is protein-DNA interaction, which we further validated by ChIP assay. Below is a graph of the enriched regions for the IKZF1 locus and possible binding sites for Egr2 (analysed by Dr Alistair Symonds). This was further validated by ChIP, which demonstrated that Egr2 directly interacts with the IKZF1 promoters (Figure 3.35).

At protein level we found that the Ikaros protein was significantly defective in our $Egr2^{-/-}Egr3^{-/-}$ $CD4^{+}$ T cells. From the western blot there is a significant reduction of Ikaros after stimulation but not in the unstimulated cells (Figure 3.36 B and C). However, when the Ikaros was studied by immunohistochemistry we found that the Ikaros was also reduced significantly in the $CD4^{+}$ unstimulated T cells (Figure 3.36A).

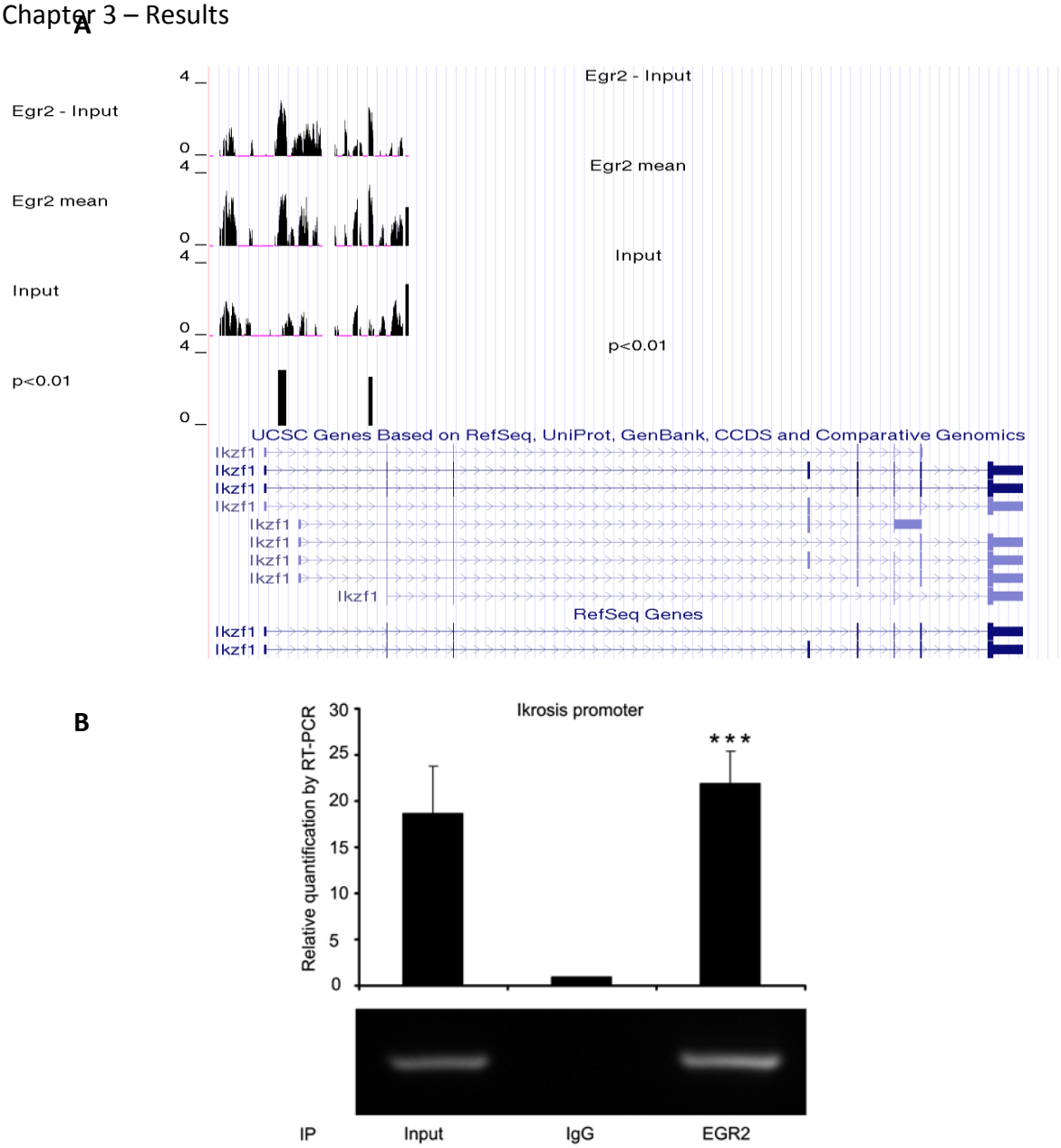

Figure 3.35: Graph of MAT scores and enriched regions for the IKZF1 locus.
(A) Data were exported into bedGraph format using the *rtracklayer* package (Lawrence *et al.*, 2009) and imported into the UCSC Genome Browser. "Egr2 - Input" is the difference in MAT score between the Egr2 and Input samples and was used to calculate enriched regions (p<0.01). (B) Binding of Egr2 to the IKZF1 promoters. Total input DNA and anti-H3 precipitates served as positive controls and anti-Ig precipitates as negative control. Standard error bars indicate SEM. ***p<0.001 as compared to IgG.

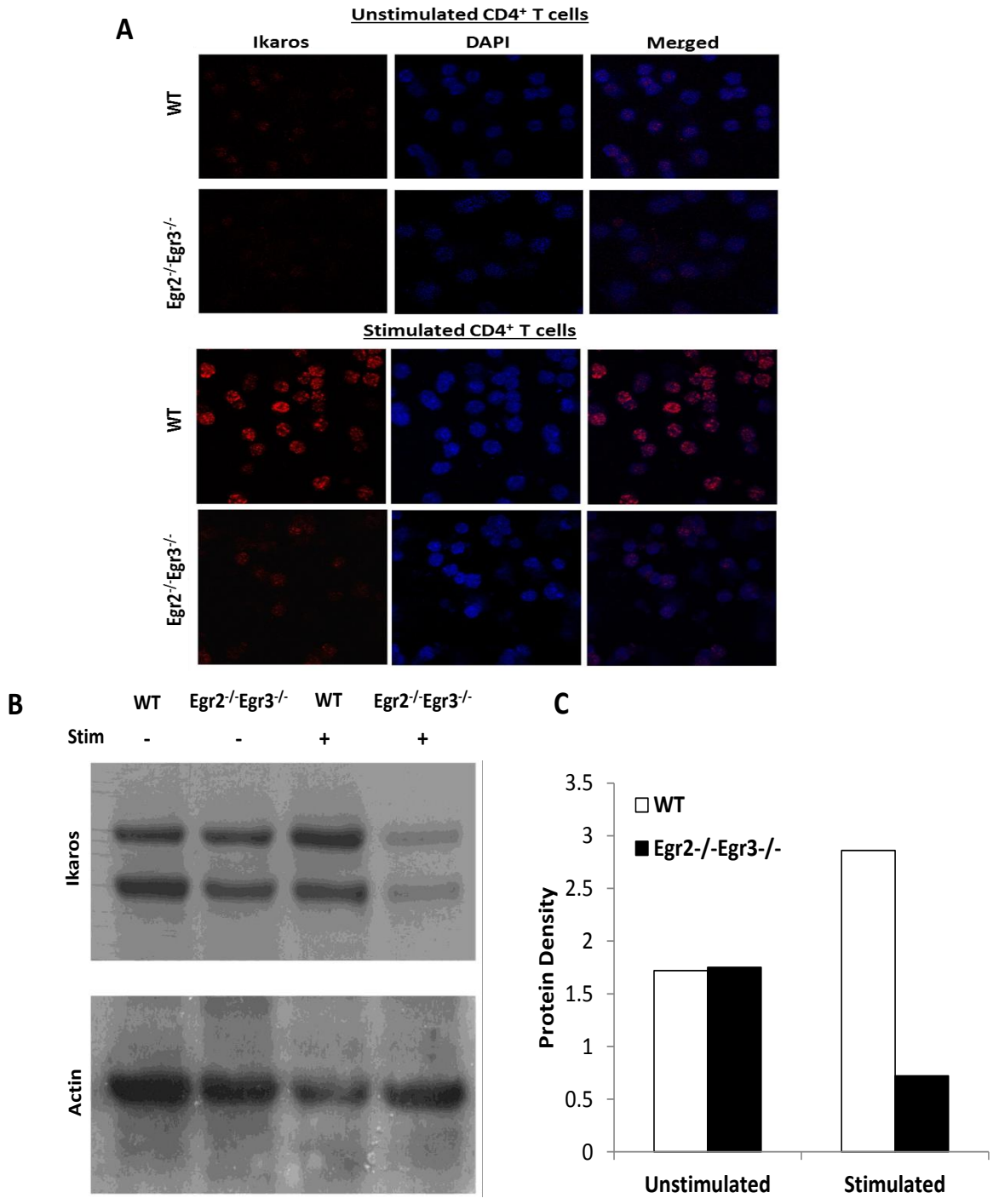

Figure 3.36: Lack of Egr2 Reduced the Protein Expression of Ikaros.
(A) Reduced Ikaros expression in $Egr2^{-/-}Egr3^{-/-}$ unstimulated $CD4^{+}$ T cells. (B) Western Blot analysis of Ikaros protein expression in WT and $Egr2^{-/-}Egr3^{-/-}$ $CD4^{+}$ T cells. Ikaros is significantly reduced in $Egr2^{-/-}Egr3^{-/-}$ after stimulation. (C) Protein Density measured from the western blot.

Aiolos protein expression was also studied in both B and T cells and as expected there was no major difference in the protein expression in $Egr2^{-/-}Egr3^{-/-}$ $CD4^{+}$ T cells but a significant reduction in $CD19^{+}$ B cells. Interestingly when the expression of Aiolos was measured in Egr2 Transgenic mice (TG), which has an over expression of Egr2 protein, there was a significant increase in the AIolos expression (Figure 3.37). These results are in line with the Georgopoulos group, who also reported that a defect in *Ikaros* results in T cell malignancies and defects in Aiolos results in the B cell malignancies (Maxwell, Li *et al.* 2009; Zha, Bassing *et al*. 2010).

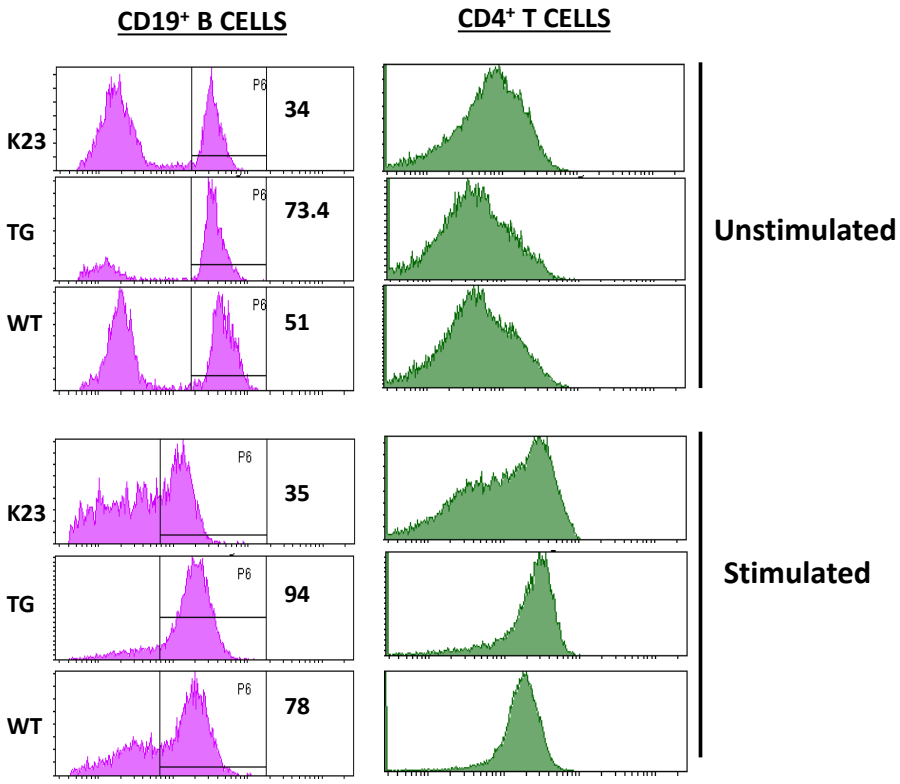

Figure 3.37: Aiolos Protein Expression.
Aiolos protein expression was measure in WT, $Egr2^{-/-}Egr3^{-/-}$ and Egr2 TG B and T cells by flow cytometry. A significant reduction in Aiolos protein observed in $Egr2^{-/-}Egr3^{-/-}$ B cells but not in T cells.

3.4.4.2 Egr2 regulates the expression of FOXO3 in lymphocytes

The Forkhead box, class O (FOXO) family are transcription factors that function as tumour suppressors that control cell fate, induce quiescence or cell cycle arrest (Fu and Tindall 2008). Four subfamily members have been identified in mammals, FOXO1, FOXO3 (previously known as FKHRL1; (Anderson, Viars *et al.* 1998)), FOXO4 and FOXO6.

FOXO3 is the prime member of the FOXO family in lymphoid peripheral tissues. The inactivation of FOXO3 is required for the lymphocytes to proliferation, this has been shown in both B and T cells (Stahl, Dijkers *et al.* 2002; Yusuf, Zhu *et al.* 2004). It has also been shown that the constitutively inactive form of FOXO3 also leads to leukaemia and lymphomas (Obrador-Hevia, Serra-Sitjar *et al.* 2012).

FOXO3 was identified as one of the targets for Egr2 from the ChIP-on-chip and microarray gene expression profile. The normalised expression was validated by Real-Time RT-PCR and confirmed a significant reduced expression of FOXO3 in $Egr2^{-/-}Egr3^{-/-}$ $CD4^{+}$ T cells compared to wild type. For unstimulated $CD4^{+}$ T cells both the microarray and Real Time RT-PCR showed a 3 fold reduced expression in CD2-$Egr2^{-/-}$. The microarray normalised expression for stimulated cells demonstrated a 4 fold reduction in FOXO3 gene expression however when validated by Real Time RT-PCR there was only a 2 fold reduction in gene expression, which statistically was not significantly different due to a p value of 0.06 (Figure 3.38).

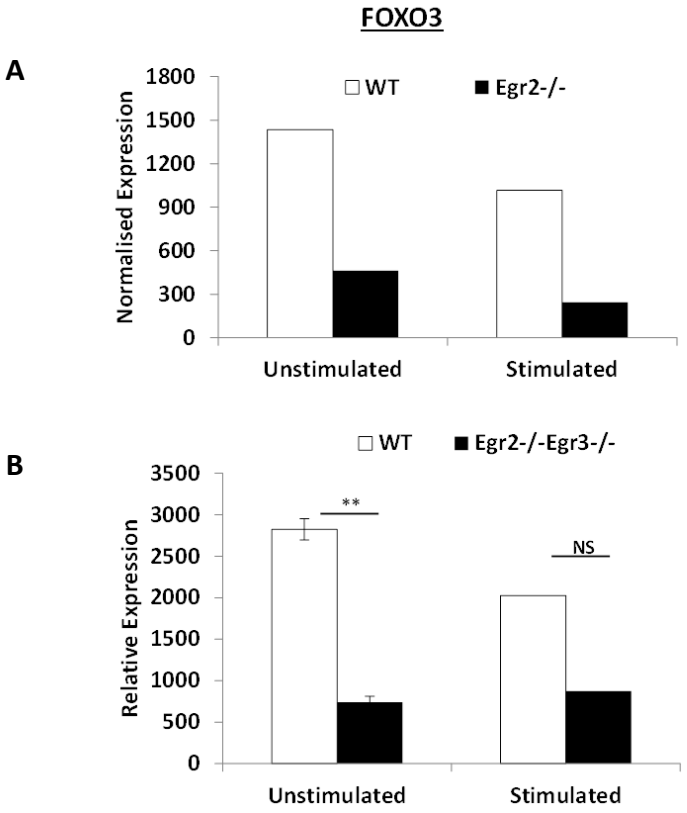

Figure 3.38: mRNA expression of FOXO3 in CD2-Egr2$^{-/-}$ CD4^{+} T cells.

(A) Microarray normalised expression of Ikzf1 and Ikzf3 in splenic CD4^{+} T cells isolated from the CD2-Egr2$^{-/-}$ and wild type mice. (B) Relative expression measured by Real-Time PCR in splenic CD4^{+} T cells isolated from the Egr2$^{-/-}$Egr3$^{-/-}$ and wild type mice. Standard error bars indicate SEM. ** p< 0.01, NS - Not Significant.

To assess whether Egr2 proteins associates with the proximal regions of the mouse FOXO3 promoter (analysed by Dr Alistair Symonds) , a ChIP assay was performed. The results demonstrated that Egr2 binds to the promoter region of FOXO3 and as expected, no signal above background was seen with irrelevant IgG (Figure 3.39). At protein level no difference in FOXO3 expression was seen in unstimulated $CD4^+$ T cells from both wild type and $Egr2^{-/-}Egr3^{-/-}$. However a significant 4 fold difference was seen in both the immunohistochemistry staining and western blot for stimulated $Egr2^{-/-}Egr3^{-/-}$ $CD4^+$ T cells (Figure 3.40).

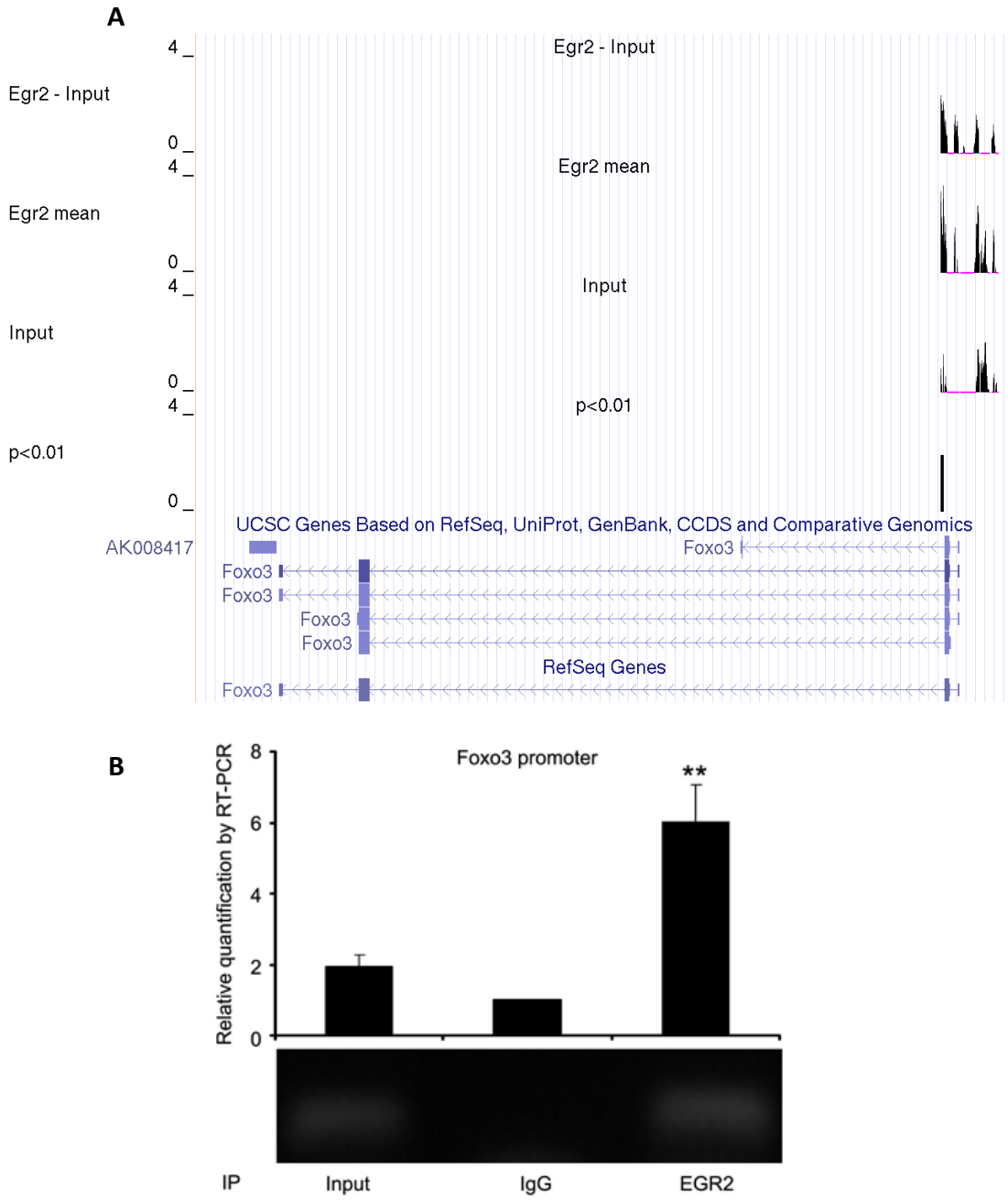

Figure 3.39: Graph of MAT scores and enriched regions for the FOXO3 locus.
(A) Data were exported into bedGraph format using the *rtracklayer* package (Lawrence *et al.*, 2009) and imported into the UCSC Genome Browser. "Egr2 - Input" is the difference in MAT score between the Egr2 and Input samples and was used to calculate enriched regions ($p<0.01$). (B) Binding of Egr2 to the FOXO3 promoters. Total input DNA and anti-H3 precipitates served as positive controls and anti-Ig precipitates as negative control. Standard error bars indicate SEM. **$p<0.01$ as compared to IgG.

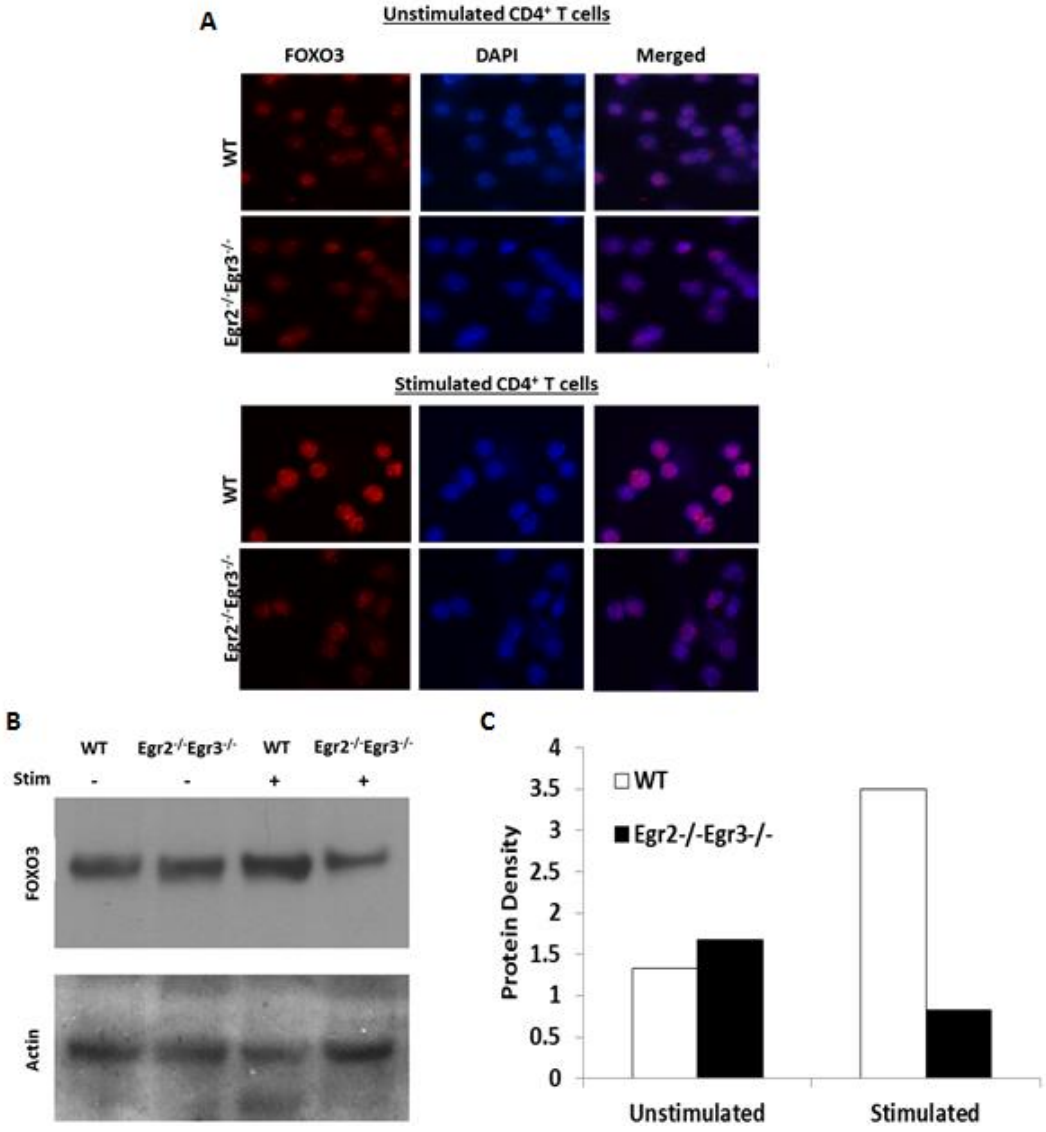

Figure 3.40: Lack of Egr2 Reduced the Protein Expression of FOXO3.
(A) Reduced FOXO3 protein expression in $Egr2^{-/-}Egr3^{-/-}$ after stimulation. (B) Western Blot analysis of FOXO3 protein expression in WT and $Egr2^{-/-}Egr3^{-/-}$ $CD4^{+}$ T cells. FOXO3 is significantly reduced in $Egr2^{-/-}Egr3^{-/-}$ after stimulation. (C) Protein Density measured from the western blot.

Chapter 4 Discussion

The study of Egr family in the immune system is still in its young ages. Egr2 and Egr3 transcription factors are members of the Egr family and have been demonstrated to be involved in a number of different processes. To investigate the role of Egr2 and Egr3 in lymphocytes we developed Egr2 and Egr3 double knockout mice, where Egr2 was specifically deleted in lymphocytes with the aid of CD2 promoter.

In this study, we have discovered that Egr2 and Egr3 have major regulatory functions in lymphocytes and the deficiency of these genes in B and T cells results in the development of spontaneous lymphomas. The novel findings of this thesis have been categorised into two sections.

Firstly, deficiency in Egr2 and Egr3 in B and T cells results in lymphoma development. We demonstrated that the lymphocytes from the $Egr2^{-/-}Egr3^{-/-}$ mice were hyper-proliferative in all lymphoid tissues and also invade all major organs. Egr2 transcription factor was found to interact with the promoters of Ikaros and FOXO3 tumour suppressor genes, all of which are crucial for lymphocyte development, as well as controlling lymphocyte proliferation and homeostasis. We also demonstrated that the absence of Egr2 and Egr3 genes impaired the expression of Ikaros, FOXO3 and Aiolos. Taken together, these observations clearly demonstrate that Egr2 and Egr3 are essential regulators of Ikaros, Aiolos and FOXO3 tumour suppressor genes.

Secondly, deficiency in Egr2 and Egr3 genes in the lymphocytes resulted in inflammatory autoimmune disease. There was an overproduction of inflammatory cytokines by the lymphocytes in $Egr2^{-/-}Egr3^{-/-}$ mice, which we found was associated

with hyper-activation of STAT3. We found that Egr2 and Egr3 play an important role in the control of inflammatory cytokine signalling under homeostatic conditions by directly regulating the expression of SOCS3, which is an essential regulator of STAT3.

4.1 Defective Egr2 and Egr3 lymphocytes lead to rapid development of lymphoma.

Egr2 and Egr3 deficiency in lymphocytes lead to spontaneous lymphoma, bearing the immunophenotype of either T or B cells. The hyper-homeostatic lymph-proliferation in $Egr2^{-/-}Egr3^{-/-}$ mice and the accumulation of active lymphocytes led to the development of spontaneous lymphoma in mice as young as 5 months old. Interestingly, spontaneous lymphoma development was also seen in the CD2-$Egr2^{-/-}$ mice models, but at a much later age. Importantly, no tumours were observed in aged match wild type mice or $Egr3^{-/-}$ mice. These observations demonstrate that Egr3 compensates for the functions of Egr2 in its absence, hence the late onset of lymphoma development. This is a novel finding, as to date no reports have been published on linking Egr2 and Egr3 knockout mice with the development of lymphoma.

The hyper-homeostatic proliferation of lymphocytes in $Egr2^{-/-}Egr3^{-/-}$ mice initiates spontaneous lymphoproliferation, which manifests as splenomegaly and lymphadenopathy at an early age. This may be due to an accumulation of active lymphocytes in the periphery caused by the increased production of cytokines.

Notably, several models that have perturbed lymphocyte cell homeostasis also develop an onset of chronic inflammation and lymphoma (Ohashi 2002; Suarez, Lortholary *et al.* 2006; Zhang, Wang *et al.* 2007) as observed in $Egr2^{-/-}Egr3^{-/-}$ mice model.

Similar to our model, Hagenbeek and Spits (2008) developed T cell specific PTEN deficient mice, using either proximal Lck promoter (Lck-Cre mice) or CD4 promoter (CD4-Cre mice). They found that both PTEN Lck-Cre deficient and PTEN CD4-Cre deficient mice developed T cell lymphomageneis in the thymus, as early as 1 month of age, much earlier than what we observe in the $Egr2^{-/-}Egr3^{-/-}$ mice. However these mice had a much shorter lifespan of 3 months in comparison to $Egr2^{-/-}Egr3^{-/-}$ mice which survived to 4-8 months in age. Similar to the $Egr2^{-/-}Egr3^{-/-}$ mice, the T cells from PTEN T cell deficient mice reflected a constitutive state of activation and infiltration of lymphocytes into non lymphoid organs (Hagenbeek and Spits 2008). Interaction between PTEN and Egr2 was first described in the hindbrain study. Suzuki, *et al*., 1998 used Egr2 as a hindbrain marker in PTEN deficient mice embryos, where they found cephalic region was severely overgrown and abnormal Egr2 expression occurred on one side of the prospective forebrain. This indicated that Egr2 expression was regulated by PTEN (Suzuki, de la Pompa *et al.* 1998). Similarly Unoki, and Nakamura, (2003) identified Egr2 as an important mediator of the PTEN growth suppressor signalling pathway. They found exogenous expression of Egr2, with AdCAEGR2 significantly induced apoptosis in various cancer cell lines (Unoki and Nakamura 2003). In contrast we did not see any effect in the level of apoptosis in the$Egr2^{-/-}Egr3^{-/-}$ mice in our model. However Unoki, M., *et al*.,

2003 carried out the analysis of apoptosis only in carcinomas cell lines and not in lymphoma/leukaemia cell lines. PTEN expression was evaluated in our microarray data and we found that the normalised expression was reduced, but not significantly in CD2-Egr2$^{-/-}$ CD4^{+} T cells compared to wild type.

Although a number of spontaneous lymphoma models have been proposed, here we have found a novel model which demonstrates aggressive spontaneous B or T cell lymphoma development in the absence of Egr2 and Egr3.

As it is well known that tumourgenesis results from a multiple molecular events that amend the normal properties of a cell. We looked at the possible hallmarks of cancer formation and to our surprise we identified Egr2 and/or Egr3 are essential for the regulation of tumour suppressor genes; Ikaros, Aiolos and FOXO3, which are important for lymphoid lineage development and homeostasis.

4.1.1 Ikaros family in haematological malignancies

We found Egr2 regulates the expression of Ikaros protein, which is a zinc finger transcription factor encoded by the IKZF1 gene. The Ikaros gene was discovered 20 years ago and is part of the Ikaros family, which also consists of Helios, Aiolos, Eos and Pegasus. Ikaros and Aiolos are highly conserved and are expressed in the lymphoid tissues (Molnar and Georgopoulos 1994; John, Yoong *et al.* 2009). Aiolos was identified as a homologue to the largest Ikaros isoform with similarities in the DNA binding activation and dimerisation domains. Aiolos forms homo and heterodimers with Ikaros proteins in T and B cells (Rebollo and Schmitt

2003). Ikaros was first identified in 1990s by Georgopoulos' team, they proposed it as a master regulator of haematopoiesis and found it to be essential for lymphocyte development (Georgopoulos, Moore *et al.* 1992). The importance of Ikaros as a tumour suppressor in murine T cell leukaemia has been known for about 20 years and more recently the Ikaros inactivation in human T-ALL is becoming clearer.

The role of Ikaros in lymphoid development was illustrated by the complete lack of lymphoid cells following gene inactivation by targeted deletion of exons 3-5, which generate dominant negative Ikaros isoform in mice. Mice homozygous to the mutation fail to prosper after birth and completely lack T and B cells and their earlier progenitors (Georgopoulos, Bigby *et al*. 1994; Nichogiannopoulou, Trevisan *et al.* 1999). In contrast, mice heterozygous for this mutation have a normal lymphoid cell distribution after birth but after a few months the activity at the late stage of thymocyte maturation leads to uncontrolled lymphoproliferation and rapid development of malignant T cell leukaemia and lymphoma, with the lymphocytes being highly activated (Winandy, Wu *et al*. 1995), as seen in the $Egr2^{-/-}Egr3^{-/-}$ mice. Interestingly, the phenotype of the heterozygous mutant of Ikaros mice was very similar to our model. They also demonstrated splenomegaly and lymphadenopathy in their Ikaros heterozygous mice, with 10-fold increase in the number of splenocytes and circulating lymphocytes in the peripheral blood, supporting the increase in absolute T and B cells in secondary lymphoid organs found in $Egr2^{-/-}Egr3^{-/-}$ mice. Increase in the white pulp and decrease in the red pulp areas of the spleen was consistently observed in their Ikaros heterozygous mice, in align with the structure of our spleen and destruction of the follicular structure of the

lymph nodes due to the hyper-proliferation of the lymphocytes (Winandy, Wu *et al*. 1995). They observed a general lymphoproliferative lead to T cell leukaemia and lymphoma.

In contrast, in human disease, Ikaros mutations are rare in T cell leukaemia. One group studied the Ikaros mRNA and protein expression in 25 human T-ALL samples and found only one sample harboured non-functional Ikaros (Davis 2011). In contrast to the findings in mice, deregulation of Ikaros function in humans is far more common in primary B progenitor ALL (B-ALL). Notably, dominant negative isoforms of Ikaros proteins are present in about a quarter of high-risk paediatric B-ALL cases (Mullighan, Su *et al.* 2009). Ikaros mutations are also associated with a poor prognosis in terms of overall survival and frequency of relapse, most of which was studied in paediatric B-ALL but has also shown true for adult B-ALL (Kuiper, Waanders *et al.* 2010; Dupuis, Gaub *et al.* 2013).

Ikaros has been shown to regulate expression of multiple genes that are essential for T cell differentiation. One of the pathways that are essential for T cell development is the Notch pathway. Activation of the Notch-1 gene has been found in over 50% of T-cell ALL (Weng, Ferrando *et al.* 2004; Chiaramonte, Basile *et al.* 2005). Synergism between the loss of Ikaros and the Notch activation in T cell leukemogenesis has been demonstrated by Beverly and Capobianco (Kleinmann, Geimer Le Lay *et al.* 2008). Ikaros directly bind to the upstream regulatory element of a Notch target gene Hes-1 and down regulates its expression. High expression of HES-1 is found in T-cell ALL cells (Chiaramonte, Basile *et al*. 2005; Kleinmann, Geimer Le Lay *et al*. 2008).

Ma *et al*, have demonstrated that Aiolos and Ikaros suppress c-Myc expression in pre-B cells, by directly binding to its promoter *in vivo*. c-Myc as well as cyclin D3 is crucial for the expansion of pre-B cells, which are driven by signals from the IL-7 receptor. The downregulation of c-Myc by Ikaros and Aiolos precedes p27 induction and cyclin D3 suppression (Ma, Pathak *et al.* 2010). However in our model, we did not find any difference in the normalised expression of p27 and c-Myc.

Along with Ikaros, Aiolos has also been shown to act as a tumour suppressor gene. The inactivation of the Aiolos gene in knockout mice produced an increase in B cell precursors and spontaneous production of autoantibodies, hence causing the breakdown of B cell tolerance and facilitating the development of B cell lymphoma (Wang, Avitahl *et al*. 1998). In the Aiolos null mice the marginal zone B cells were severely deleted, hence the enhanced maturation of the follicular B cells. In addition, those mice that were heterozygous for null mutation in Ikaros and homozygous for a null mutation in Aiolos developed T cell lymphomas more rapidly than mice with a homozygous knockout of Ikaros (Rebollo and Schmitt 2003). This also supports the interaction of Aiolos and Ikaros, together function to inhibit pre-B-BCR signalling, as the loss of these two genes results in continued or even increased signalling (Thompson, Cobb *et al.* 2007).

Elevated expression of Bcl-xL (part of the Bcl-2 family) has also been associated with the loss of Ikaros function. It is suggested that Ikaros downregulates Bcl-xL expression (Yagi, Hibi *et al.* 2002; Kano, Morimoto *et al.* 2008), hypothesising that Ikaros regulated apoptosis. However this hypothesis still remains speculative

due to lack of evidence to define the mechanism of this action. Bcl-xL normalised gene expression was not effected in our Egr2 deficient mice.

The observations of all the studies and analysis in both mouse and human lymphomas have always concluded Ikaros and Aiolos as tumour suppressor genes. The findings in this study have identified Egr2 and Egr3 to regulate the mRNA and protein expression of Ikaros and Aiolos in lymphocytes, supporting the hypothesis of Egr2 and/or Egr3 functioning as a tumour suppressor genes.

4.1.2 FOXO3 in haematological malignancies

FOXO3 is a transcription factor that is crucial for the haematopoietic cell fate and controls proliferation and apoptosis, amongst other processes. FOXO3 is the predominant member of the FOXO family in lymphoid peripheral tissues (Lin, Hron *et al.* 2004). The deactivation of FOXO3 is associated with proliferation of B cells (Yusuf, Zhu *et al*. 2004) and T cells (Stahl, Dijkers *et al*. 2002).

A study looked at the key FOXO genes, in particular FOXO3 in a panel of MCL cell lines and primary MCL cultures. The analysis of the MCL cell lines and patient derived cells found the phosphorylated form of FOXO3 in the cytoplasm, representing that the FOXO3 was in the inactive state. (Obrador-Hevia, Serra-Sitjar *et al*. 2012). A similar expression of phosphorylated and cytoplasmic FOXO3 was also reported in acute myeloid leukaemia (Kornblau, Singh *et al.* 2010). The phosphorylation of FOXO3 causes the recruitment of 14-3-3 proteins; microarray expression of $CD4^+$ T cells in $Egr2^{-/-}Egr3^{-/-}$ mice and the validation of mRNA

expression by Real-Time PCR confirmed the significant reduction of 14-3-3. However, it is unknown if the 14-3-3 protein is reduced due to the downregulation of FOXO3 protein or vice versa.

Interestingly, one group studied the role of FOXO3 in lymphoid homeostasis. They derived mice deficient in FOXO3a by generating animals bearing a disabled FOXO3 allele. They observed that the FOXO3a deficiency leads to spontaneous lymphoproliferation, which was also associated with inflammation of several key organs. Immunohistochemical examination revealed a mixed infiltration of $CD4^+$ and $B220^+$ cells, suggesting a lymphoid infiltration (Lin, Hron *et al*. 2004). Similarly we also found chronic inflammation in the $Egr2^{-/-}Egr3^{-/-}$ mice and hyper activated T and B cells. Surprisingly, same as the lymphocyte phenotype in our model, they also observed that the T cells were highly activated, but they did not observe any autoantibody production.

Tsai group have demonstrated that FOXO3a DNA-damage pathway is mediated by protein complexes such as ATM and its downstream mediators to sense DNA damage and control damage induced cell cycle checkpoints and DNA repair (Tsai, Chung *et al.* 2008). This is in align with our results which show a reduction in FOXO3 expression and significant downregulation of ATM mRNA in $CD4^+$ T cells.

Collectively, the observations on FOXO3 function as a tumour suppressor gene, by inducing apoptosis and inhibiting proliferation in lymphocytes, supports the findings of Egr2 regulating the expression of FOXO3. However the exact mechanism of action is still unknown.

4.1.3 Egr2 and Egr3 essential for the control of homeostatic proliferation

Notably, several models that have disturbed T cell homeostasis also develop late onset of inflammation disease (Salvador, Hollander *et al.* 2002; Doyle, Gee *et al.* 2003; Arias, Ballesteros-Tato *et al.* 2007). Deficiency in both Egr2 and Egr3 in lymphocytes resulted in chronic inflammatory disease. The $Egr2^{-/-}Egr3^{-/-}$ mice developed symptoms similar to those seen in systemic lupus erythematosus, with lymphocyte infiltration in multiple organs, severe glomerulonephritis, and deposits of immune complexes in the glomeruli. Egr2 and Egr3 deficient B and T cells induced excessive production of inflammatory cytokines, despite the impaired production of IL-2 (data not shown). The transfer of either naïve B or $CD4^{+}$ T cells from $Egr2^{-/-}Egr3^{-/-}$ mice into $Rag2^{-/-}$ mice resulted in the development of inflammation in recipient mice and importantly an increase in hyper-homeostatic proliferation was observed. These observations validate that Egr2 and Egr3 function via cell-intrinsic mechanisms to regulate inflammatory autoimmune response and homeostasis of lymphocytes.

In our $Egr2^{-/-}Egr3^{-/-}$ mice model the lymphocytes resulted in hyper-homeostatic proliferation, coinciding with the splenomegaly and super-enlarged lymph nodes. Previously in our CD2-$Egr2^{-/-}$ mice no effect was seen on the development of the major thymocyte subsets, regulatory T cells, and major sub-groups of peripheral T and B cells. This was due to the incomplete excision of the

floxed Egr2 gene in the B cells from specific $Egr2^{-/-}$ mice affected by the heterozygous phenotype of Cre-transgene (Zhu, Symonds *et al*. 2008).

Egr2 and Egr3 are induced in both naïve and tolerant T cells (Harris, Bishop *et al*. 2004; Anderson, Manzo *et al*. 2006). The B and T cells from $Egr2^{-/-}Egr3^{-/-}$ mice were hyper-proliferative, with hyper-activated phenotypes and produced excessive levels of proinflammatory cytokines.

4.1.4 Egr2 and Egr3 essential for the control of inflammation and cytokine microenvironment

The over-production of inflammatory cytokines by B and T cells in $Egr2^{-/-}Egr3^{-/-}$ mice is allied with the hyper activation of STAT3, the major inflammatory cytokine pathway controlling the development of Th17 cells and expression of inflammatory cytokines. This indicates that Egr2 and 3 play vital roles in the control of inflammatory cytokine signalling under homeostatic conditions.

Several key effector cytokines were altered in the serum of $Egr2^{-/-}Egr3^{-/-}$ mice, compared to wild type mice, such as IL-6 and IL-3. We also found increased expression of the anti-inflammatory cytokine IL-10 in our mice model. Although IL-10 is an anti-inflammatory cytokine, IL-10 has been implicated as having a pathogenic role in lupus (Moore, de Waal Malefyt *et al.* 2001).

Interestingly, we detected high levels of IL-17 producing $CD4^{+}$ T cells in diseased Egr2 and Egr3 mice, in which half of them also produced IFNγ. Hence we put forward that Th17 play major roles in inflammatory pathology of diseased

$Egr2^{-/-}Egr3^{-/-}$ mice. Li, *et al*, 2012 have demonstrated that Egr2 and 3 directly regulate SOCS3 expression and deficiency in Egr2 and 3 results in reduced expression of SOCS3 (Li, Miao *et al.* 2012). SOCS3 is a critical regulator for the control of STAT3 mediated cytokine expression and differentiation of Th17 cells (Yang, Panopoulos *et al.* 2007; Tamiya, Kashiwagi *et al.* 2011).

In unstimulated cells, STATs are inactive and typically the SOCS genes are not expressed. Upon binding of cytokines, receptor aggregation occurs and the STATs are activated by phosphorylation, which allows their dimerisation and translocation to the nucleus, where they stimulate the transcription of cytokines responsive genes, as well as those that encode for SOCS proteins. The SOCS proteins bind to Janus Kinase (JAK) or cytokine receptors, thereby suppressing further signalling events (Alexander 2002).

SOCS3 is a major negative feedback regulator of STAT3 activating cytokines. SOCS3 is crucial for the control of Th17 differentiation by desensitising STAT3 to activating cytokines, such as IL-23, thus governing the expression of Th17 cytokines (Chen, Laurence *et al.* 2006; Tanaka, Ichiyama *et al.* 2008). Therefore the absence of SOCS3 has a dramatic proinflammatory effects by promoting TH17 development. However, T cell specific SOCS3 defect showed a reduced immune response, and produced significant levels of transforming growth factor (TGF)-β1 and IL-10, but less of IL-4, hence inhibiting Th1 and Th2 development (Kinjyo, Inoue *et al.* 2006; Taleb, Romain *et al.* 2009; Yoshimura, Suzuki *et al.* 2012). Deficiency of SOCS3 specifically in lymphoid and myeloid cells results in the development of inflammatory diseases (Chong, Metcalf *et al.* 2005; O'Sullivan, Liongue *et al.* 2007).

These findings put forward that inflammatory pathology, which normally results from pathological functions of Th17 cytokines, is controlled by SOCS3. We have now established that SOCS3 are expressed in B and T cells in response to antigen receptor stimulation. The excessive production of Th17 cytokines and activation of STAT3 in Egr2 and 3 deficient B and T cells propose that regulation of SOCS3 expression by Egr2 and 3 is vital, not only for averting the development of autoimmune diseases, but similarly for preventing immunopathology during productive adaptive immune responses.

STAT3 has been implicated in tumourgenesis in both mice and humans. STAT3 is considered as a powerful oncogene, as it plays an important role in cell growth and its activation has been described in nearly 70% of haematological and solid tumours (Frank 1999). Studies have found STAT3 to be constitutively activated in human cutaneous T cell lymphoma and diffuse large B cell lymphoma (Sommer, Clemmensen *et al.* 2004; Ding, Yu *et al.* 2008; Wu, Song *et al.* 2011). STAT3 is an important downstream target of IL-6. It has been demonstrated that the Il-6 mediated T cell survival is impaired in T cell specific STAT3 deficient mice (Takeda, Kaisho *et al.* 1998). In the $Egr2^{-/-}Egr3^{-/-}$ model, we also demonstrated, *in vivo*, an increase in IL-6 expression as well as an increased STAT3 expression, coinciding with the findings of Takeda K. *et al*., 1998.

4.2 Conclusion

Our work has confirmed a role for Egr2 and/or Egr3 in the control of inflammation and lymphocyte homeostasis. The study revealed hitherto unknown roles of Egr2 and/or Egr3 in regulating the expression of tumour suppressor genes, Ikaros, Aiolos and FOXO3, controlling the formation of lymphoma.

Based on our findings presented here, I have proposed two possible pathways for the development of lymphoma in defective Egr2 and Egr3 mice. Firstly, the defect of Egr2 and 3 results in the downregulation of important tumour suppressor genes, Ikaros and Aiolos, as well as of FOXO3. Secondly, the chronic inflammation in the mice provides the microenvironment for the tumour growth by activating STAT3 oncogenes (Shacter and Weitzman 2002; Rakoff-Nahoum 2006).

Defects in Ikaros and Aiolos may suppress the target genes involved in cell proliferation; however we have not studied any of the Ikaros or Aiolos target genes in our model. FOXO3 is known to aid DNA repair by interacting with ATM. Defects in these tumour suppressor genes allow the cells to proliferate rapidly and continuously, without crucial controls in place. In parallel, Egr2 and/or Egr3 are unable to control oncogene STAT3, by unable to bind to their suppressors SOCS3, hence activating their target genes, such as CDC25B. This leads to lymphoma development. The inflammation caused by Egr2 and 3 defect increases the cytokines, in the microenvironment, hence increasing the availability of growth factors for the tumours to expand. This has been illustrated in Figure 4.1.

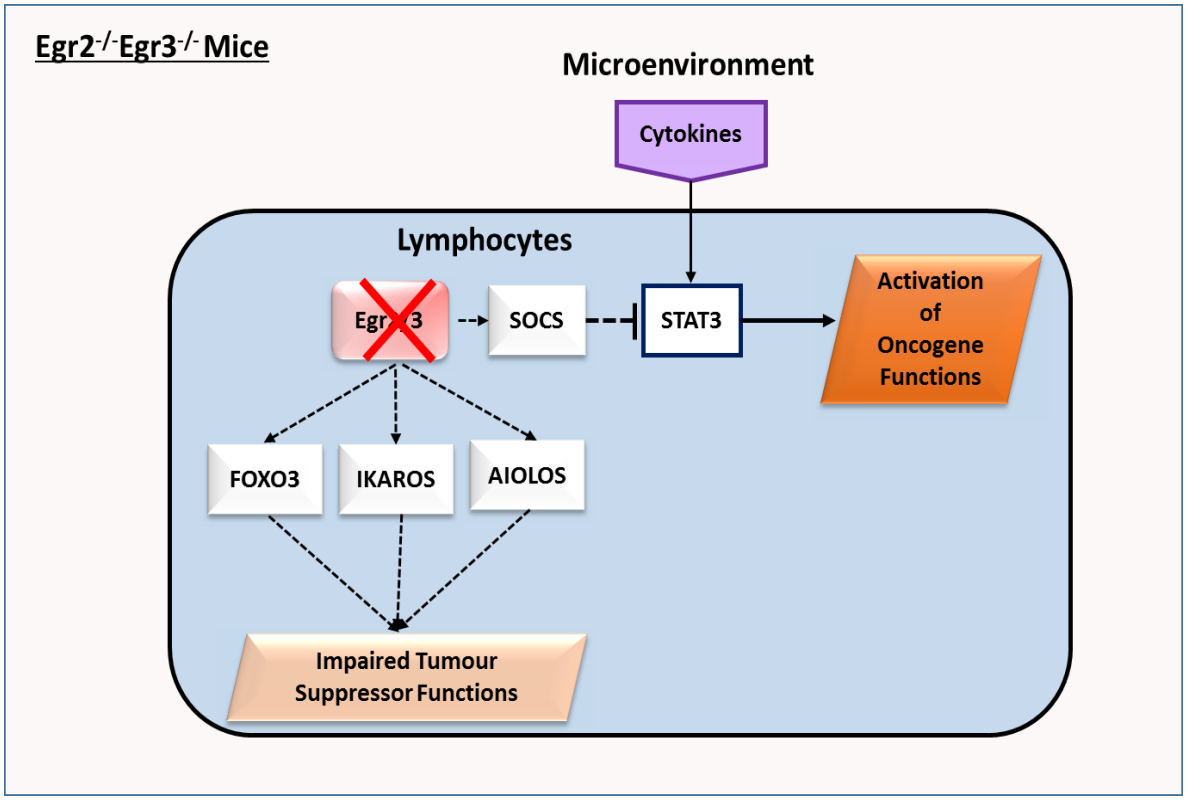

Figure 4.1: Proposed role of Egr2 and/Egr3 in preventing lymphoma development. This figure demonstrates that the absence of Egr2 and Egr3 in lymphocytes is unable to activate the expression of Ikaros, Aiolos and FOXO3 genes, which results in the dysregulations of the tumour suppressor functions of these genes. Similarly Egr2 and Egr3 are unable to bind to and activate the expression of SOCS3 gene, which is important for inhibiting the STAT3 oncogene. This results in the activation of oncogene function. Both the evasion of growth suppressors and activation of oncogenes are key hallmarks of cancer development.

To summarise our model Egr2 and Egr3 act as tumour suppressor genes. They retard proliferation and prevent tumourgenesis by positively regulating other transcription factors and inhibiting oncogenes. Currently there has already been research on possible STAT3 inhibitors for cancer therapy. The novelty of the findings in this thesis provides new insights of cancer therapy. Here we put forward that Egr2 and Egr3 can be investigated as potential cancer therapy targets.

4.3 Future Work

The ultimate aim of tumour suppressor genes is to repress the effect of cell cycle or promote apoptosis, which can be performed in a number of ways, such as by repressing gene that are essential for promoting cell growth or by coupling the cell cycle DNA damage. We have shown that Egr2 and/or Egr3 act as a tumour suppressor gene by regulating the expression of other transcription factors; Ikaros, Aiolos and FOXO3. The absence of Egr2 and/or Egr3 results in a defect expression of these genes, which may result in lymphoma development.

To date the exact mechanism and key target genes of Ikaros, Aiolos and FOXO3 is still not clear. It is therefore important in future development to identify the exact pathway/mechanism of how these transcription factors function with the presence of Egr family proteins and what target genes are activated or suppressed to prevent lymphoma development.

Proposal of using ChIP-sequencing to systemically investigate all possible genes involved in tumour development in relation to Egr2 function.

Many lymphomas are a result of chromosome deletions. These deletions are thought to contain the tumour suppressor genes within the lost region. However, most loss of function mutations that occur in the tumour suppressor genes are recessive mutations hence both of the cells tumour suppressor genes must be mutated or lost (‘two-hit’ theory) for cancer to progress. Here in this study chromosome deletion has not been investigated, which should be considered for

further investigation to support the function of Egr2 and or Egr3 as tumour suppressor genes.

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Conference Presentation

The work from this thesis was represented at the following conferences:

Bhullar P.K, Miao T, Symonds A.L, Ghaffari E, Li S and Wang P (2013) Early growth response gene 2 and 3 are essential for the regulation of tumour suppressor genes, Ikaros, Aiolos and FOXO3 ***– oral presentation at "15th International Congress of Immunology – ICI", Milan, Italy.***

Bhullar P.K, Miao T, Symonds A.L, Ghaffari E, Li S and Wang P (2013) Early growth response gene 2 and 3 are essential for the regulation of tumour suppressor genes, Ikaros, Aiolos and FOXO3 – ***poster presentation at "NCRI Cancer Conference", Liverpool, UK.***

Publications

During my PhD, I have also been involved in the following publications:

Li S, Miao T, Sebastian M, **Bhullar P**, Ghaffari E, Liu M, Symonds A.L.J and Wang P (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(4-2): 685-696.

Miao T, Raymond M, **Bhullar P**, Ghaffari E, Symonds A.L.J, Meier U.C, Giovannoni G, Li S and Wang P (2013) Early Growth Response Gene-2 Controls IL-17 Expression and Th17 Differentiation by Negatively Regulating Batf. *The Journal of Immunology*, 190(1): 58-65.

Appendix

Genotype	Age (Months)	Gender	CD3	B220	Metastasis
$Egr2^{-/-}$	12+	Female	+	-	NO
$Egr2^{-/-}$	12+	Female	+	-	NO
$Egr2^{-/-}$	12+	Female	-	+	NO
$Egr2^{-/-}$	8	Female	+	-	YES
$Egr2^{-/-}$	8	Female	+	-	YES
$Egr2^{-/-}$	10	Male		+	YES
$Egr2^{-/-}$	8	Female	+	-	YES
$Egr2^{-/-}$	12	Female	-	+	YES
$Egr2^{-/-}Egr3^{-/+}$	7	Female	-	+	YES
$Egr2^{-/-}$	12+	Female	-	+	YES
$Egr2^{-/-}Egr3^{-/-}$	5	Male	-	+	YES
$Egr2^{-/-}Egr3^{-/+}$	6	Female	-	+	YES
$Egr2^{-/-}Egr3^{-/+}$	6	Male	-	+	YES
$Egr2^{-/-}Egr3^{-/-}$	4	Female	-	+	YES
$Egr2^{-/-}$	12	Female	-	+	YES
$Egr2^{-/-}Egr3^{-/-}$	5	Female	+	-	YES
$Egr2^{-/-}Egr3^{-/+}$	5	Female	+	-	YES

Table A.1 Summary of tumour development in $Egr2^{-/-}Egr3^{-/-}$ mice models

Note: around 80% of the tumours were characterised as small cell lymphoma.*