Early Growth Response genes 2 and 3 play a role in chronic inflammation pathology and are essential for the differentiation of T follicular helper cells

A thesis submitted for the degree of Doctor of Philosophy

By

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Declaration

I hereby declare that the work presented in this thesis is my own research, except where otherwise stated. This work has not been submitted for any other degree. Therefore, I hereby submit this work for my examination.

Ane Theodora Ogbe

Abstract

The Early Growth Response genes 2 and 3 (Egr2/3) are zinc finger transcription factors that play an important role in the immune system. These transcription factors have reported functions in T cell receptor signaling, differentiation of effector T cell subsets and the development of lupus-like autoimmune diseases. Using CD2-Egr2$^{-/-}$ Egr3$^{-/-}$ mouse model, I investigate the development of inflammation pathology, differentiation of T follicular helper (Tfh) cells and the formation of germinal centers (GC) following viral challenge within these mice.

The onset of inflammation pathology in CD2-Egr2$^{-/-}$ Egr3$^{-/-}$ mice was discovered to correlate with high levels of pro-inflammatory cytokines in the serum and the development of autoimmune diseases as previously reported by Li et al, 2012. Most importantly, a novel role for the Egr2/3 genes in the differentiation of T follicular helper (Tfh) cells was identified.

Tfh cells are responsible for T cell dependent antibody immune response in the GC. They support the differentiation of GC B cells into plasma cells producing long lived high-affinity isotype-switched antibodies and memory B cells. Tfh cell differentiation is regulated by Bcl6 however; the regulators of Bcl6 during Tfh differentiation remain largely unknown. We have now discovered that Egr2/3 genes are required for Bcl6 expression during Tfh cell differentiation. In the absence of the Egr2 and 3 genes, Tfh cell differentiation is severely impaired and GC formation and functions were defective in response to Vaccinia Virus Western Reserve strain (VV_{WR}) infection. Further investigation revealed that Egr2 regulated Bcl6 expression in a Tfh-specific manner as adoptive transfer of WT CD4^{+} T cells into Egr2$^{-/-}$ Egr3$^{-/-}$ mice was able to rescue Bcl6 expression, Tfh differentiation and GC formation. When the molecular mechanism of how Egr2 regulated Bcl6 was investigated, it was uncovered that Egr2 directly bound to the promoter region of Bcl6 gene in CD4 T cells to regulated Bcl6 expression. Indeed constitutive expression of either Egr2 or Bcl6 in CD2-Egr2$^{-/-}$ Egr3$^{-/-}$ CD4^{+} T cells rescued Tfh cell differentiation and GC formation. Our results inferred that the Egr2/3 genes are essential for Tfh

differentiation and GC formation by regulating Bcl6 expression in CD4 T cells under Tfh condition.

Our studies thus suggest that the Egr2/3 genes are paramount for minimising immunopathology and are also critical for efficient antibody production by regulating Tfh cell differentiation.

Acknowledgement

I have thought about writing this page almost for as long as I have been studying for this degree. However, now that the time is here, words have failed me. I have now starred at this blank page not sure how to express my sincere thanks to everyone who has been a part of this journey. Whatever makes it to the text, I hope you all know that words are limited and this space may not convey it all.

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Dedication

"If I have seen further, it is by standing on the shoulders of giants"
– Sir Isaac Newton

To my Husband, Onuh John Adole and my Mum, Rose Ojoma Ogbe, I stood tall because I stood on your shoulders. This one is for you.

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List of Abbreviations

AA amino acids

ADCC Antibody-dependent cell-mediated cytotoxicity

ANA Anti-nuclear antibodies

AP-1 Activator Protein-1

APC Antigen Presenting Cell

APC Allophycocyanin

BCL-6 B-cell lymphoma 6 protein

BCR B cell receptor

Blimp1 B lymphocyte induced maturation protein 1

BP Base pairs

BSA Bovine Serum Albumin

$C0_2$ Carbon dioxide

CD Cluster of differentiation

CDC Complement dependent cytotoxicity

cDNA Complementary DNA

CFA Complete Freund's adjuvant

ChIP Chromatin immune-precipitation

CLP Common lymphoid progenitor

CMP Common Myeloid progenitor

CTL Cytotoxic T cell

CTLA Cytotoxic T-Lymphocyte Antigen

CVID Combined variable immunodeficiency diseases

DAG Diaglycerol

DBD DNA binding protein

dH2O Distilled Water

DMEM Dulbecco's Modified Eagle Medium

DN Double Negative

DN Double negative

DNA Deoxyribonucleic acid

DP Double positive

DTT Dithiothreitol

EDTA Ethylenediaminetetraacetic acid

Egr Early growth response gene

ELISA Enzyme-linked immunosorbent assay

F(ab') antibody binding fragment

FACS Fluorescence activate cell sorting

FASL Fas ligand

FBS Fetal bovine serum

FBS Foetal Bovine Serum

FITC Fluorescein isothiocynate

FO Mature follicular B cells

FOXO3 Forkhead box O3

FoxP3 Fork head box P3

GATA-3 Trans-acting T-cell-specific transcription factor

GC Germinal centre

GFP Green fluorescent protein

H/E Haematoxylin and Eosin staining

hCD2 Human clusterof differentiation 2

HRP Horseradish Peroxidase

HSC Hematopoietic stem cells

ICOS Inducible T-cell Costimulator

IFN Interferon

Ig Immunoglobulin

IgH Immunoglobulin Heavy chain

IgL Light chain

IL Interleukin

INO Ionomycin

JAK Janus Kinase

KCl Potassium Chloride

LB Luria-Bertani

LDS Lithium dodecyl sulphate

LN Lymph node

LPS Lipopolysaccharide

MACS Magnetic Activated Cell Sorter

MAPK Mitogen Activated Protein Kinase

ME Mercaptoethanol

MgSO4 Magnesium sulphate

MHC Major Histocompatibility Complex

MOI Multiplicity of infection

MOMP Mitochondrial outer membrane permeabilisation

mRNA Messenger RNA

MZ Marginal zone B cells

NaCl Sodium Chloride

NFAT Nuclear factors of activated T cells

NF-κB Nuclear Factor κB

NHEJ Non-homologous end joining

NK Natural killer cells

NKT Natural Killer T cells

OD Optical density

OVA Ovalbumin

PAGE Polyacrymalide gel Electrophoresis

PAMP Pathogen Associated Molecular patterns

PBS Phosphate-buffered saline

PCR Polymerase chain reaction

PD-1 Programmed Cell death-1

PE Phycoerythrin

PFA Paraformaldehyde

PFU Plaque forming unit

PMA Phorbal 12-myristate 13-acetate

PMSF Phenylmethylsulfonyl fluoride

PNA Peanut agglutinin

PRNT Plaque reduction and neutralization test

RAG Recombination activating genes

RBC Red blood cells

RNA **R**ibonucleic acid

ROR Retinoic acid receptor

RPMI Roswell Park Memorial Institute

RT-PCR Real Time PCR

SDS Sodium dodecyl sulphate

SLE Systemic lupus erythematosus

SOCS-1 Suppressor of cytokine signalling-1

STAT Signal transducer and activator of transcription

TAE Tris-acetate-EDTA

Taq Thermus aquaticus

TBE Tris/Borate/EDTA

TBST Tris Buffered Saline with Tween 20

TCR T cell receptor

Tfh T follicular helper cells

TGF-β Transforming growth factor- β

Th T Helper Cell

TMB 3,3',5,5'-tetramethylbenzidine

TNF Tumour necrosis factor

TNF-α Tumor Necrosis Factor alpha

TRAIL TNF-related apoptosis-inducing ligand

UV Ultra Violet

VV_{WR} Vaccinia virus Western Reserve strain

WBC White blood cells

WHO World Health Organisation

WT Wild Type

α alpha

β Beta

γ gamma

δ Delta

ε Epsilon

κ Kappa

λ Lambda

µl microliter

Chapter 1 Introduction

The immune system is a complex network of physical barriers, tissues, cells and serum proteins, which mediate the body's defences against harmful substances. It is composed of the innate and the adaptive immune systems that orchestrate a mechanism known as the immune response. The study of the immune system has expanded a great deal since the time of the great plague of Athens in 430BCE. Today, through the works of immunologists, we have increased our knowledge of the biology of mediators of this system, mechanisms used within the immune system to eliminate harmful pathogens, limitations as well as defects associated with the immune system. Using this knowledge, immunologists have been able to design very successful therapeutics for disease causing pathogens like the small pox vaccine developed by Edward Jenner in 1798. They have also fostered the development of therapeutics in tumour immunology where research is looking into understanding and enhancing anti-tumour immune responses. This review summarises the current literature on the inflammatory responses within the immune system, the regulation of T follicular helper (Tfh) cells, their roles in germinal centre mediated immune responses and the role of the early growth response genes (Egr) 2 and 3 in the regulation of the immune system.

1.1 Innate Immune System

The innate immune system is evolutionarily conserved amongst multicellular organisms and is responsible for immediate defense of the body against danger signals and pathogens (Turvey and Broide, 2010). Although it lacks the ability to discriminate between specific pathogens, innate immune cells are able to use their germ-line encoded pathogen recognition receptors (PRR) to recognise pathogen associated molecular patterns (PAMP) on microbe (Cooper and Alder, 2006; Medzhitov and Janeway, 2000b). PAMPs are often required for the survival of such pathogens. Their recognition leads to initiation of signaling pathways that culminate with the activation of AP-1 and NF-κβ which induce inflammation and proliferation of effector cells (Turvey and Broide, 2010; Medzhitov and Janeway, 2000b). Due to an apparent lack of specificity, anatomical barriers against microbial pathogens including uncompromised skin, lysozyme in saliva and tears, mucus and

PH levels in the gut are often included as constitutive innate defense mechanism (Turvey and Broide, 2010; Medzhitov and Janeway, 2000a). Cellular components active within the innate immune system include the granulocytes and monocytes. Granulocytes comprise of neutrophils, basophils, eosinophils, and mast cells while the monocytic lineage is made up of macrophages and dendritic cells including follicular dendritic cells (FDC). The mechanism of innate immune response includes the following:

1.1.1 Inflammation

Toxic substances released by the activities of virulence factors, allergens, foreign bodies and necrotic cells are stimulants for inflammatory responses (Ashley, Weil and Nelson, 2012; Medzhitov, 2008). Inflammation initiates a physiological response to deregulated homeostasis due to stimulants. This allows the recruitment of non-residential leukocytes to the local sites of infection or tissue injury (Ashley, Weil and Nelson, 2012; Turvey and Broide, 2010). The mediators of inflammation; the cytokines (interleukins, interferons, and chemokines), eicosanoids and vasoactive amines, are triggered when innate immune PRR receptors e.g. transmembrane Toll-like receptors (TLR) and intracellular nucleotide-binding oligomerization-domain proteins (NOD)-like receptors (NLR) on tissue-resident macrophages recognise PAMPs or damage-associated molecular patterns (DAMPs) (Turvey and Broide, 2010; Medzhitov, 2008; Tosi, 2005). In recent years, keratinocytes in the skin have been reported to produce cytokines that initiate active inflammation (Gröne, 2002). Inflammation is marked by;

- Redness (rubor) due to hyperemia
- Swelling (tumor) caused by increase permeability of the blood vessels and protein leakage
- Heat (calor) arising from increased blood flow and the metabolic activities of cytokines
- Pain (dolor) due to changes in perivasculature
- Loss of function (function laesa) due to disruption of normal organ function involved in the inflammatory process

Inflammation may be acute, leading to the resolution of inflammation or chronic with persistent presence of inflammatory cells and molecules (Ryan and Majno, 1977).

1.1.1.1 Acute inflammation

This is a protective well-regulated response mounted by the immune system to eliminate harmful pathogens and toxic substances (DELGADO *et al.*, 2003). Initiation of the acute phase inflammatory response is marked by the enlargement of blood vessels to allow the exudation of polymorphonuclear neutrophils and plasma proteins into extravascular tissues spaces (Ashley, Weil and Nelson, 2012; Medzhitov, 2008). Activated neutrophils eliminate their targets indiscriminately by the release of toxic substances including Reactive Oxygen Species (ROS), reactive nitrogen species, cathepsin G, proteinase 3 and elastase (Medzhitov, 2008). Following pathogen elimination, pro-resolution molecules including lipoxin, an anti-inflammatory agent promotes the recruitment of monocytes (Medzhitov, 2008; Ryan and Majno, 1977). These differentiate into phagocytic macrophages upon activation to promote resolution and repair of damaged tissues (Medzhitov, 2008; Ryan and Majno, 1977). Other lipid mediators like resolvins, protectins and transforming growth factor-β (Tgf-β) are also important in the resolution of inflammation by inhibiting further neutrophil recruitment (Medzhitov, 2008)

1.1.1.2 Chronic inflammation

Control of inflammatory responses is crucial in maintaining homeostasis and avoiding pathological conditions. Hence the balance between pro-inflammatory and anti-inflammatory mediators has to be tightly regulated (DELGADO *et al.*, 2003). Chronic inflammation involves persistent signaling from pro-inflammatory mediators (DELGADO *et al.*, 2003). Chronic inflammation results in the presence of circulating mononuclear macrophages and lymphocytes within the tissues and is often associated with tissue destruction, autoimmunity. Due to the prolonged nature of the chronic inflammatory response, the host attempts to repair damaged tissue by replacing them with either cells of the same type or fibrous connective tissues. In some cases, the host is unable to repair extensively damaged tissue thus leading to fatality. Chronic inflammation has also been known to lead to the

development of cancers and other severe systemic inflammatory diseases (Lu, Ouyang and Huang, 2006; DELGADO *et al.*, 2003; Ryan and Majno, 1977)

1.1.2 Complement system

The complement system is made up of about 30 proteases, found in the serum. They are sequentially cleaved to activate a cascade of other protease which support immune responses (McGeer, Klegeris and McGeer, 2005; Carroll, 2004). These unique proteins are considered components of innate response however; they have receptors on a varied range of cells from leukocytes to stroma cells (Carroll, 2004). They facilitate antibody response in adaptive immune response (Pepys, 1976). There are 3 pathways for complement activation: the classical, alternative and mannan-lectin pathway (McGeer, Klegeris and McGeer, 2005). The classical pathway is driven by antigen:antibody complex formation, the alternative pathway is driven by polysaccharides and the mannan-lectin pathway by mannose or N-acetyl glucosamine bearing microorganisms (Carroll, 2004; Parkin and Cohen, 2001). Activation of any complement pathways initiates cleavage of other complements which climaxes with the formation of the membrane attack complex (MAC) on targeted cells leading to the eventual cell lysis (Parkin and Cohen, 2001)

1.1.3 Phagocytosis

Originally described by Metchnikoff in 1905 (Tosi, 2005), phagocytosis is induced in host innate defenses where it functions in pathogen and dying cell clearance (Henneke and Golenbock, 2004). Phagocytic cells like tissue resident (TR) and circulating macrophages, dendritic cells (DC) and granulocytes are trafficked to the site of infection by cytokine signaling and the upregulation of integrins on endothelial cells (Tosi, 2005). Recognition of PAMPs using TLRs causes them to extend pseudopods which form phagosome that ingests the pathogen (Tosi, 2005). Phagosomes mature by fusion with lysosome to form phagolysosome. This destroy the pathogen via biochemical processes including reduction of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Parkin and Cohen, 2001), hydrolysis, low pH and enzymatic cleavage of bacteria cell walls (Tosi, 2005;

Henneke and Golenbock, 2004). Tagging of invading microorganisms or dying cells by antibodies in a process known as opsonisation, greatly enhances efficiency of phagocytosis

1.1.4 Professional Antigen presentation

Antigen presentation is a process by which processed antigenic peptides are presented on major histocompatibility complexes (MHC) to cluster of differentiation 4 (CD4) or CD8 T cells along with co-stimulatory molecules. All cells express the MHC I for presentation of intracellular pathogens to CD8 T cells via the endogenous pathway. Here, infections by virus result in the translation of non-self proteins in the ribosome (Villadangos and Schnorrer, 2007). These ribosomal products are broken down in the proteasome and then transported by the TAP complex into the endoplasmic reticulum where they are loaded on MHC I prior to transfer to the cell surface (Figure 1.1) (Villadangos and Schnorrer, 2007).

A specialized subset of innate immune cells have the MHC II complex that allows presentation of antigens as peptide fragments to CD4 T cells in the exogenous pathway (Vander Lugt *et al.*, 2014). These specialized cells are known as professional antigen presenting cells (APC) with DC regarded as the most potent of them (Platt *et al.*, 2010; Santambrogio *et al.*, 1999). The exogenous pathway involves the internalization of extracellular antigens via macropinocytosis, receptor-mediated endocytosis or phagocytosis (Figure 1.1) (Villadangos and Schnorrer, 2007). The antigens are tagged for digestion by proteases in the lysosomes. They are then processed and loaded onto the MHC II complex, which is located in the endosomal compartments or MHC II compartments (MIIC) (Platt *et al.*, 2010; Vyas, Van and Ploegh, 2008). From here they are trafficked to the cell membrane and presented to CD4 T cells in secondary lymphoid organs (Platt *et al.*, 2010; Vyas, Van and Ploegh, 2008).

Antigen presentation forms the major link between the innate and adaptive immune response. It functions in the activation of naïve T cells and directing T cell immune response by cytokine secretion thus facilitating transition to antigen specific immunity (Platt *et al.*, 2010; Turvey and Broide, 2010; Lee and Iwasaki, 2007).

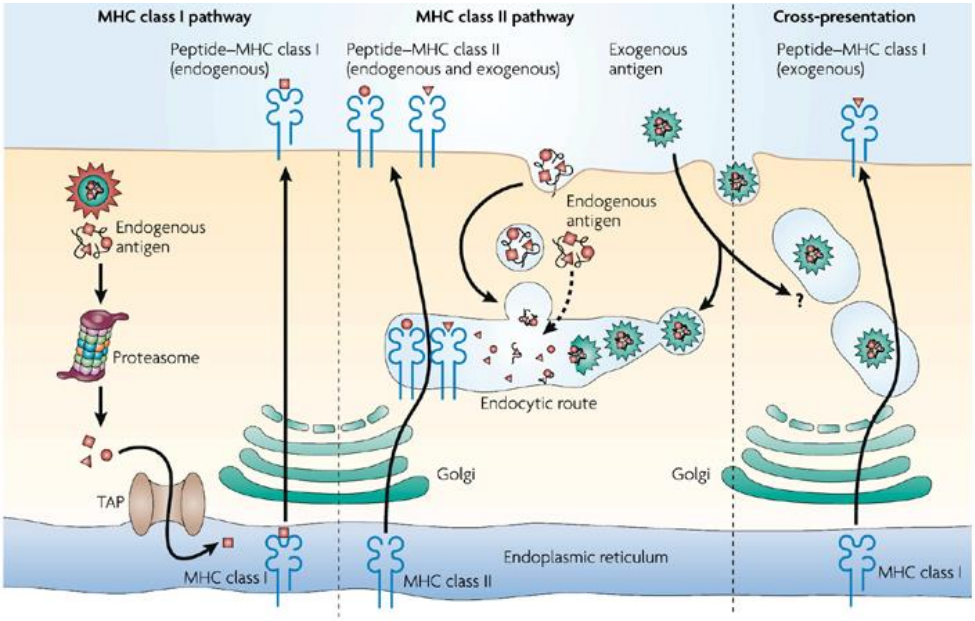

Figure 1.1: Antigen presentation via the MHC class I and II pathways (Villadangos and Schnorrer, 2007).

1.2 Adaptive immunity

Adaptive immune system is sometimes referred to as the acquired immune system, and it is present in all jawed vertebrates (Pancer and Cooper, 2006). It mediates a temporarily delayed immune defense in response to antigens (Palm and Medzhitov, 2009). Its functions span cell-mediated immunity and antibody-mediated humoral immunity and are mediated by T and B cells (LeBien and Tedder, 2008). Some of its characteristics are briefly described below:

1.2.1 Specificity and diversity of receptors

Just as there are many antigens, T and B cells have also evolved a plethora of receptors that can each respond to specific epitopes of an antigen. This increases the range and efficiency of antigen response (Eisen and Chakraborty, 2010). Diversity arises through Recombination Activation Genes (RAG)-mediated somatic recombination of the variable (V), diverse (D) and joining (J) gene segments of the T cell receptor (TCR) on T cells or the heavy (H) and light (L) chains of the immunoglobulin receptors for B cells (Eibel *et al.*, 2014; Medzhitov, 2007; Cooper and Alder, 2006). Once these diverse clones specific for different epitopes have been established in primary lymphoid organs, they exit into the peripheral lymphoid organs where they await recognition by cognate APCs.

1.2.2 Central and Peripheral tolerance

Rearrangement of the VDJ gene segments in order to generate diverse receptors inevitably, leads to the generation of receptors that are responsive to self-antigens (Mathis and Benoist, 2004). In order to prevent destruction of self and the development of autoimmune diseases, the adaptive immune system has evolved mechanism to distinguish between self and non-self antigens (Mathis and Benoist, 2004). These include central and peripheral tolerance.

Central tolerance takes place in the primary lymphoid organs. Newly assembled TCR are tested for MHC binding affinity in a process termed positive selection. Receptors with no affinity for MHC die by neglect. Following that, Immature T lymphocytes are presented with antigens derived from surrounding

thymic medullary epithelial cells (MEC) (Eibel *et al.*, 2014; Xing and Hogquist, 2012; Mathis and Benoist, 2004). Those that bind with high affinity to self-peptides are condemned to 3 fates including clonal deletion leading to cell death by apoptosis or receptor editing to further rearrange the TCR (Eibel *et al.*, 2014; Vale and Schroeder Jr., 2010; LeBien and Tedder, 2008). The third fate for self-reactive T cells is clonal diversion where self-reactive clones develop suppressive functions leading to the generation of natural regulatory T cells (Xing and Hogquist, 2012). For B lymphocytes, assembled IgM are presented with bone marrow antigens, B lymphocytes that interact with high affinity for self-antigens undergo clonal deletion or receptor editing leading to further rearrangement of light chains before they eventually exit the bone marrow to the secondary lymphoid organs (Eibel *et al.*, 2014; Vale and Schroeder Jr., 2010; LeBien and Tedder, 2008)

Due to the limited range of self-antigens presented in the primary lymphoid organs during central tolerance, some self-reactive clones manage to escape and localize in the periphery hence the need for peripheral tolerance (Xing and Hogquist, 2012; Goldrath and Hedrick, 2005). The mechanisms of peripheral tolerance include anergy, peripheral deletion by apoptosis and Treg activity. Anergy arises from the absence of CD28 ligation leading to inhibition of TCR signaling (Xing and Hogquist, 2012; Medzhitov and Janeway Jr, 1998), peripheral deletion by apoptosis is induced by the expression of the death domain receptors FAS upon repeated stimulation by self-antigen while Treg activity results in the production of anti-inflammatory cytokine IL-10 (Xing and Hogquist, 2012).

1.2.3 Clonal expansion

In secondary lymphoid organs, mature but naïve lymphocytes are presented with antigens from their cognate APC (Pancer and Cooper, 2006). Upon receptor engagement, signals are transmitted with cause activation of these naïve cells. Activated CD4 T cells go through an expansion phase where they differentiate into antigen specific effector phenotype and subsequently proliferate (Pulendran and Ahmed, 2006). This stage is referred to as clonal expansion and is promoted by the release of IL-2, a cytokine that promotes T cell proliferation (Stittrich *et al.*, 2010).

Clonal expansion has to be regulated to prevent hyper-proliferation and the development of organ pathology. To that effect, following pathogen clearance, around 90% of these effectors cells die off via programmed cell death in what is understood to be a contraction phase (Gourley *et al.*, 2004). It was reported that blocking B cell clonal expansion mitigated the onset of experimental EAE (Cantor, 2014). Negative regulators of clonal expansion like FOXO1 are important in suppressing hyper-proliferation. MicroRNAs like miR-182 promote clonal expansion by targeting FOXO1 (O'Neill, 2010; Stittrich *et al.*, 2010).

After the contraction phase, the rest of the cells go on to assume a memory phenotype (Gourley *et al.*, 2004). In the absence of pathogenic invasion, lymphocytes numbers are maintained in a state of equilibrium by a process known as homeostasis.

1.2.4 Immunological Memory

The ability to remember previously encountered pathogens in order to protect from re-challenge by stimulating a quicker and more robust immune response is termed immunological memory (Ma *et al.*, 2012). This can last for months, years or even a lifetime (Ahmed and Gray, 1996). For T cells, after the elimination of pathogens, some effector T cells differentiate to become memory T cells. However, in B cells, the current concept is that B cell memory arises from germinal centre (GC) reactions. As this is a T cell dependent pathway, strides are being made to understand the protection from re-infection by T cell independent antigens (Defrance, Taillardet and Genestier, 2011; Pulendran and Ahmed, 2006)

Once memory cell populations have been established they home to different sites of the body. Central memory T (T_{CM}) cells home to the secondary lymphoid organs from where they are recalled (Shenoy *et al.*, 2012; Woodland and Kohlmeier, 2009). Effector memory T cells (T_{EM}) on the other hand are localised to peripheral tissues or site of infections providing prompt response upon re-exposure (Woodland and Kohlmeier, 2009). In the absence of antigenic stimulation, memory cells are maintained by IL-7 and IL-15 driven homeostatic proliferation (Woodland and Kohlmeier, 2009; Pulendran and Ahmed, 2006).

During secondary infection, reports suggest that memory cells can be activated either in an antigen independent manner by the production of pro-inflammatory cytokines such as, IL-12, IL-18 and IFNγ (Raué *et al.*, 2004; Geginat, Sallusto and Lanzavecchia, 2003) or in an antigen dependent manner by DC present in tissues (Gourley *et al.*, 2004).

1.3 T lymphocytes

1.3.1 Development in the thymus

Hematopoietic progenitors cells (HPC) arise in the bone marrow (BM) before migrating to the thymus where development and commitment to the T cell lineage begins (Germain, 2002). These HPCs enter the thymus as double negative (DN) cells bearing neither CD4 nor CD8 receptors (Figure 1.2). DN thymocytes undergo 4 developmental stages – DN1, DN2, DN3 and eventually culminating in DN4 stage based on the expression of CD44 and CD25 (Germain, 2002). The process of DN stage results in the expression of pre-T cell receptor (pre-TCR) that is required for expansion of T cell precursors (Ma, Wei and Liu, 2013; Germain, 2002)

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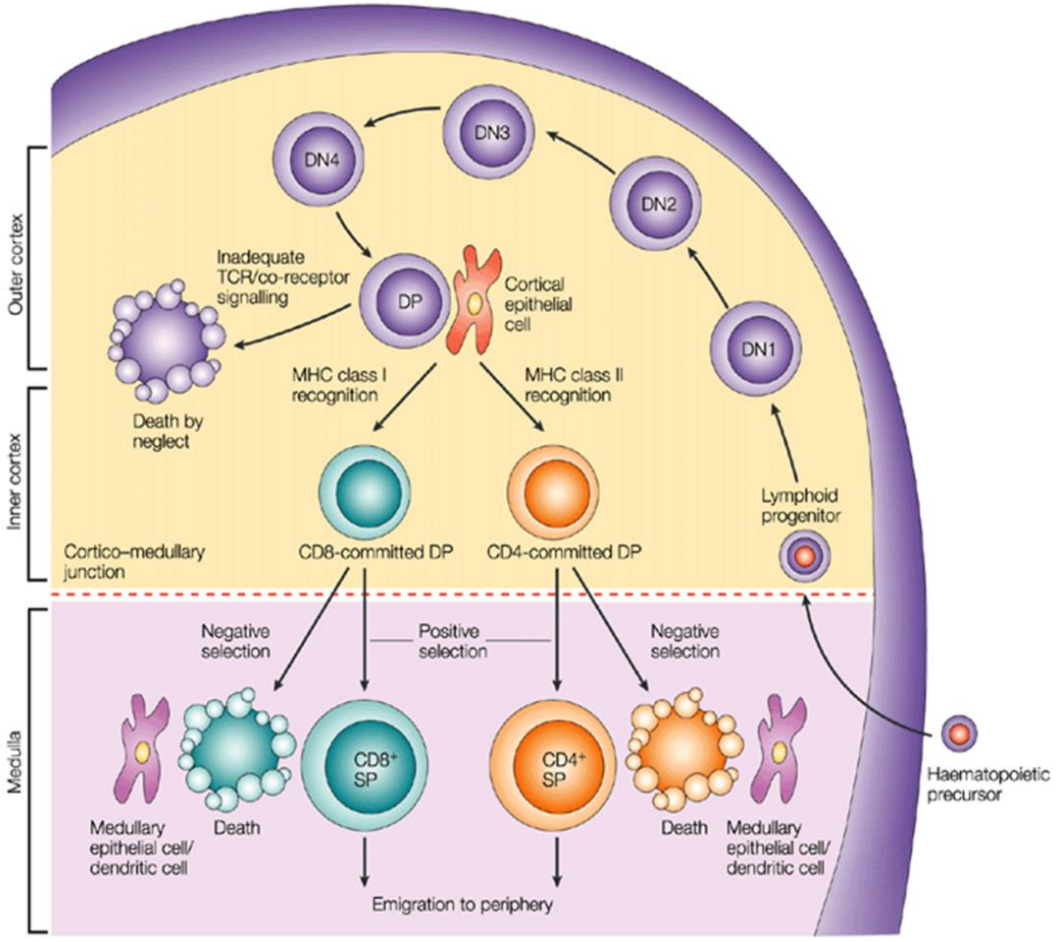

Figure 1.2: T cell development in the thymus (Germain, 2002)**.**

Following pre-TCR signaling, the DN cells then progress to CD4 and CD8 double positive (DP) cells with expression of both TCRα and TCRβ chains and the CD3 co-

receptor (Ma, Wei and Liu, 2013; Germain, 2002). These DP cells carrying somatically rearranged TCR undergo positive and negative selection before developing into mature CD4 or CD8 single positive T cells (Germain, 2002). During selection, DP thymocytes are presented with self-peptides on MHC I or II complexes (Eibel *et al.*, 2014; Xing and Hogquist, 2012; Mathis and Benoist, 2004). Positive selection selects for thymocytes with functional TCR that go on to become single positive (SP) cells (Ma, Wei and Liu, 2013; Xing and Hogquist, 2012). Negative selection selects for cells with a strong binding affinity for self-peptide on MHC complex. These cells undergo clonal deletion, clonal diversion or receptor editing (Ma, Wei and Liu, 2013; Xing and Hogquist, 2012). Thymocytes that have passed these checkpoints have minimum self-responsive ability and are eventually exported from the thymus to the periphery where they respond to specific pathogens. Natural killer T (NKT) cells are also generated from these DP cells (Hu and August, 2008).

1.3.2 T cell Activation and differentiation

3 signals are essential for T cells to become activated and differentiate into effector phenotypes (Figure 1.3). The first signal is initiated upon sustained engagement of TCR with antigenic peptides borne on MHC complexes on APCs (MacDonald and Nabholz, 1986). The TCR recognise a sequence of 8-20 amino acids known as peptides (Savina and Amigorena, 2007). Along with the antigenic complex, APCs also provides the co-stimulatory signal which is the second signal needed for T cell activation. Co-stimulation ensures ligation of the B7 molecules (CD80 and CD86) on the surface of DC with the CD28 receptor on the T cell surface. Several other co-stimulatory molecules have been identified including CD40:CD40L, PD1:PDL1, ICOS:ICOSL. In the absence of co-stimulatory molecules, T cells undergo anergy. Cytokine signaling is reported to be essential for the differentiation of activated CD4 T cells into effector phenotypes. In CD8 T cells, IL-12 and Interferon α/β (IFNα/β) signaling from APC are said to be essential and a lack of these cytokine is said to have abrogated CD8 differentiation and effector function (Curtsinger and Mescher, 2010)

Engagement of the TCR with pMHC complex initiates TCR signaling via receptor associated tyrosine kinase which activates protein kinase C and DAG mediated pathways inducing NF-Kβ, NFAT and MAPK pathways. TCR signaling results in the increased level of intracellular Ca^{2+} (Smith-Garvin, Koretzky and Jordan, 2009). This activates the Ca^{2+} dependent transcription factor NFAT that initiates different transcriptional programmes. Signaling through the NFAT/AP-1 pathway promotes IL-2 dependent T cell proliferation in CD4 and CD8 T cells while NFAT/FOXP3 signaling is necessary for Treg cells (Smith-Garvin, Koretzky and Jordan, 2009; MacDonald and Nabholz, 1986).

In order to carry out their functions, activated T cells are aided by interleukin 2 (IL-2), interferon γ (IFNγ), IL-12, IL-4, IL-17, IL-21 and IL-10. Once activated, T cells release IL-2 which functions in an autocrine manner to upregulate the expression of IL-2 receptors (IL-2R) (Malek and Ashwell, 1985) and send proliferation signals to the T cells (MacDonald and Nabholz, 1986). IL-2 also induces cytotoxic activity of CD8 T cells (MacDonald and Nabholz, 1986).

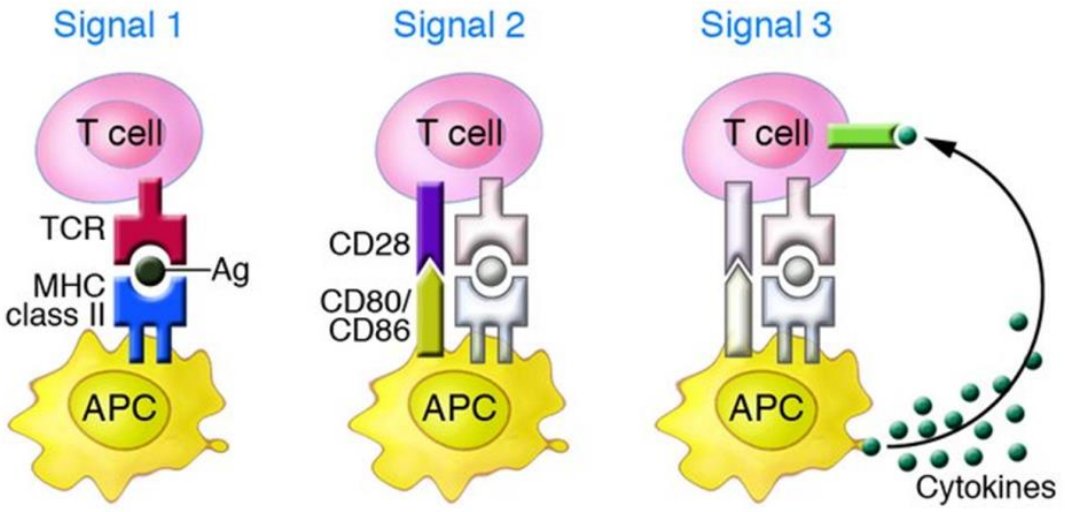

Figure 1.3: 3 signals are required for activation and diffentiation of T cells (Gutcher and Becher, 2007).

1.3.3 Contributions of cytotoxic T cells (CTL) to immune response

This group of T lymphocytes is distinguished from the others by the expression of CD8 co-receptors on their cell surface. They are employed in cytotoxic immunity where they initiate a process that leads to programmed cell death in their target cells (Lieberman, 2010). With the exception of those activated by cross-presentation by APCs, Th1 cells activate cytotoxic T cells. Transcriptional regulators required for cytotoxic T cell differentiation into effector phenotypes include Tbet, Blimp1, ID3 and STAT4 (Kaech and Cui, 2012). Eomesodermin (EOMES), Bcl6, ID3 and STAT3 are reported to promote differentiation into cytotoxic memory T cell phenoytype (Kaech and Cui, 2012). In order to mediate cytotoxic activity, CD8 T cells act by calcium dependent granule exocytosis. This combines the use of perforin to disrupt the cell membrane, granule enzyme (granzyme), a serine protease that pass through perforin channels into target cells and granulysin (Lieberman, 2010; Barry and Bleackley, 2002). Perforin is of utmost importance in cytotoxic immunity as perforin KO mice developed tumours and had difficulty mounting optimal immune response to viruses (van den Broek *et al.*, 1996; Kagi *et al.*, 1994). Other mechanisms employed by CD8 T cells to initiate cytotoxic activity include the FAS pathway and the secretion of TNF and IFN-γ (Lieberman, 2010; Barry and Bleackley, 2002).

1.3.4 CD4 T helper (Th) cells and immune reposnse

CD4 Th cells are aptly named because of the help they provide to activate B cells and cytotoxic T cells. They are very crucial in adaptive immune responses and the regulation of inflammation (Dong, 2006). They express CD4 co-receptor, which binds solely to MHC class II molecules. In addition of CD4, they can be identified by the expression of cell surface markers CD44, CD69 and CD62L, which differ in expression between the naïve and activated states (Hu and August, 2008). CD4 Th cells are heterogeneous. CD4 cells differentiate into different effector phenotypes when activated depending on the nature of antigen, dose received, cytokine signal evoked on the APC by the pathogen, co-stimulatory molecules engaged and genetic modifiers (Liu, Nurieva and Dong, 2013; Li, Zhang and Sun, 2011; Curtsinger and

Mescher, 2010; Romagnani, 1999). The first Th cells discovered were Th1 and Th2 by Mosmann and Coffman in 1989. They discovered that depending on the cytokines environment 2 different T helper subsets developed and they were named T helper 1 (Th1) and T helper 2 (Th2) (Mosmann and Coffman, 1989). These cytokines once expressed are reported to inhibit the differentiation of other Th subsets and moderate signal transducers and activators of transcription (STAT) signaling (Liu, Nurieva and Dong, 2013; Gutcher and Becher, 2007). STATs facilitate the expression of more lineage specific cytokines and of the unique signature transcription for each Th subset (Liu, Nurieva and Dong, 2013; Gutcher and Becher, 2007). Since 1989, more Th subsets have been discovered including Th17, follicular helper T (Tfh) and Treg cells (Figure 1.4). These effector phenotypes are delineated further below.

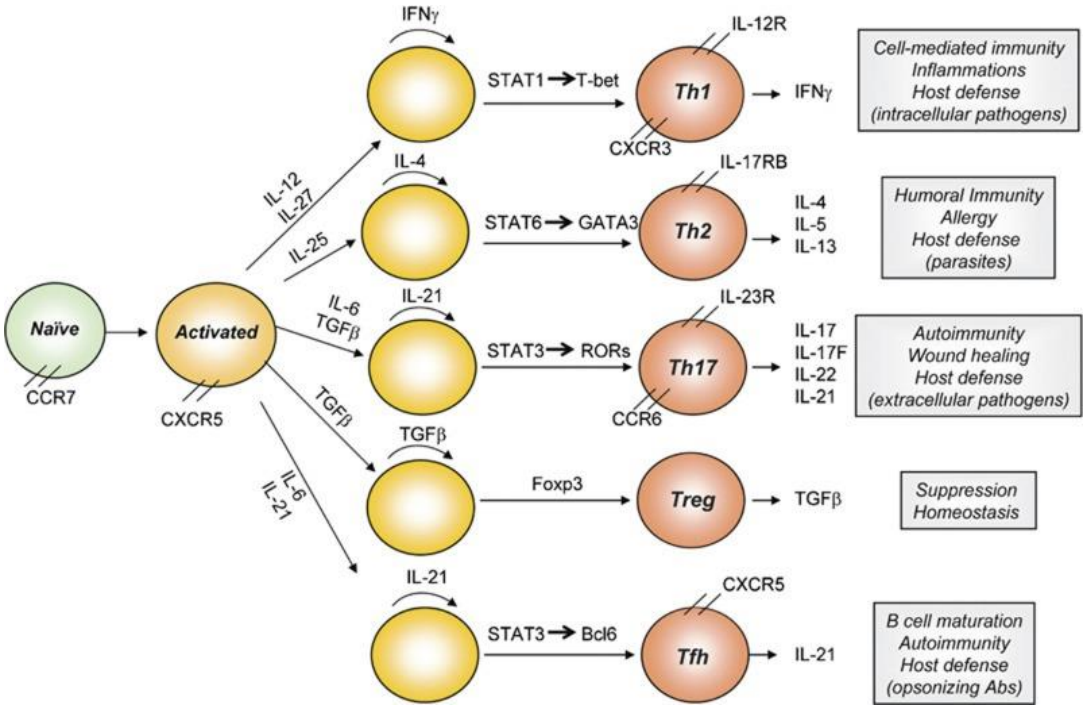

Figure 1.4: Schematic demonstrating effector T helper cell differentiation from naïve CD4 T cells (Nurieva and Chung, 2010).

1.3.4.1 T helper 1 (Th1)

Signature cytokines directing commitment to this lineage are IFNγ and interleukin IL-12 (Schroder *et al.*, 2004). These cytokines induce the expression of STAT1 and 4 which then activates the transcription factor for the Th-1 lineage T-bet (Liu, Nurieva and Dong, 2013; Dong, 2006). Activated Th1 cells secrete IFNγ and IL-

12 and activate cytotoxic CD8 T cells (Liu, Nurieva and Dong, 2013; Li, Zhang and Sun, 2011; Dong, 2006). Th1 has been implicated in autoimmune diseases, organ rejection and spontaneous abortions (Zhu *et al.*, 2008; Gutcher and Becher, 2007; Romagnani, 1999).

1.3.4.2 T helper 2 (Th2)

CD4 T cells activated in the presence of IL-4 will upregulate STAT6 that will in turn activate the transcription factor GATA-binding protein 3 (GATA3) (Liu, Nurieva and Dong, 2013; Dong, 2006). A Th2 response is potentiated by the presence of allergens and parasitic nematodes (Romagnani, 1999). Activated Th2 cells produce IL-4, IL-5, IL-9, IL-13 and IL-25 cytokines that are important in humoral immunity. These effector cytokines help to induce class switching of B cells to generate IgE antibodies that aid in the clearance of parasites, activation of innate immune cells and regulating macrophage (Liu, Nurieva and Dong, 2013; Li, Zhang and Sun, 2011; Schroder *et al.*, 2004; Romagnani, 1999). Th2 immune response is linked to airway inflammation and allergic asthma with studies showing high IgE levels and the presence of Th2 related cytokines at these sites of allergic immune response (Li, Zhang and Sun, 2011).

1.3.4.3 T helper 17 (Th17)

This subset of Th cells were uncovered by Langrish and her colleagues when they discovered a subset of IL-17 producing Th cells that differentiated in a manner independent of Th1 and Th2 pathways(Langrish *et al.*, 2005). IL6, IL-21, IL23 and IL-1 are reported to induce the STAT3 driven transcription of the orphan nuclear regulators RORγt and RORα necessary for the differentiation of CD4 cells into the Th17 lineages (Du *et al.*, 2014; Liu *et al.*, 2014; Miao *et al.*, 2013; Liu, Nurieva and Dong, 2013). IL-17 regulates immune responses by controlling tissue inflammation. Studies involving mouse models demonstrated that IL-17R knockout (KO) mice failed to generate optimal immune response to pulmonary *Klebsiella Pneumonaea* infection and had a mortality rate of 100% 48 hours after challenge (Deenick *et al.*, 2010; Ye *et al.*, 2001). However, overexpression of the same cytokine in lung epithelium induced increased chemokine production and infiltration of the lungs by

leukocytes (Park *et al.*, 2005). Most recently, IL-17 has been linked to the pathology of MS (Miao *et al.*, 2013)

1.3.4.4 Induced Regulatory T cells (iTreg)

As earlier mentioned, Treg cells may arise naturally in the thymus with the expression of the transcription factor fox head box P3 (FOXP3) or be induced in the periphery (Sakaguchi *et al.*, 2009; Miyara and Sakaguchi, 2007) by the IL-10 dependent expression of transforming growth factor β (TGFβ) which leads to the transcription of FOXP3 (Sakaguchi *et al.*, 2008). IL-6 and IL-2 have also been reported to facilitate the differentiation of iTreg *in vivo*. iTreg like their naturally occurring counterparts help to maintain the body's immune homeostasis and self-tolerance, preventing immune mediated inflammation and autoimmunity (Zhou, Chong and Littman, 2009). They are able to do this via a number of mechanisms including the killing of self-reactive T cell subsets, induction of cyclic AMP which inhibits cell proliferation and IL-2 production, antagonizing the CD80 and CD86 receptors on APC and the induction of cytolytic enzymes like indoleamine 2, 3-dioxygenase (IDO) which kill neighbouring T cells (Sakaguchi *et al.*, 2009). Epigenetically, iTreg are reported to be different from natural occurring Treg cells and are also less stable in the expression of defining Treg related molecules (Ohkura, Kitagawa and Sakaguchi, 2013).

1.3.4.5 Other T helper groups

Tr1 cells have regulatory function and thus promote immunological tolerance (Gregori *et al.*, 2012). They are characterized by a distinct cytokine expression profile including the secretion of very high levels of IL-10, normal levels of TGF-β and IFN-γ, low levels of IL-2 and in some reports, very low levels of IL-4 and lL-17 (Gregori *et al.*, 2012; Andolfi *et al.*, 2012). They transiently express FOXP3 and produce granzyme B and Perforin used in the cytotoxic killing of myeloid cells (Gregori *et al.*, 2012; Sakaguchi *et al.*, 2008).

Th3 cells are generally regarded as subsets of Treg cells that are distinguishable from other Treg cells by the large amounts of TGF-β they secrete

(Carrier *et al.*, 2007). They have also been reported to release IL-4 and IL-10 at low levels (Carrier *et al.*, 2007). They can be generated in vitro by culturing CD4 T cells in the presence of TGF-β, IL-4, IL-10 and anti-IL-12 (Sakaguchi *et al.*, 2008; Weiner, 2001).

Th9 cells were initially described in 1994 (Schmitt *et al.*, 1994). They are the major producers of IL-9 cytokine (Nowak and Noelle, 2010). Tgf-β, IL-4 and the transcription factor PU.1 are essential for the generation of this T cell subset in mouse and human cell lines (Chang *et al.*, 2010; Nowak and Noelle, 2010; Dardalhon *et al.*, 2008). Although their functions have not been fully established, they have been implicated in the pathology of autoimmune diseases and allergy (Jäger *et al.*, 2009)

Th22 cells secrete IL-22, TNF-α, FGFs and sometimes IL-10 but not IFN-γ, IL-4 or IL-17 (Eyerich *et al.*, 2009). Their transcription programme is also unique from other Th subsets with the absence of T-bet, GATA3 and RORα/γ but with increased expression of transcription factors BNC2 and FOXO4 (Eyerich *et al.*, 2009). They function in immune defense in keratinocytes and in wound healing in an IL-22 dependent manner (Kim *et al.*, 2012; Eyerich *et al.*, 2009).

1.4 T Follicular helper cells (Tfh)

Follicular helper T cells are so named due to their localization to the B cell follicles after activation. This subset of CD4 T cells were identified by a number of groups between 1999 and 2001 as T helper cells specialized in providing help to GC B cells (Liu, Nurieva and Dong, 2013; Ma *et al.*, 2012; Crotty, 2011; Kim *et al.*, 2001; Breitfeld *et al.*, 2000; Schaerli *et al.*, 2000; Walker *et al.*, 1999). They were described as CXCR5 expressing CD4 T cells with an ability to home to B cell follicles (Kemeny, 2012). They were christened T follicular helper cells in 2004 (Chtanova *et al.*, 2004). As a subset, their differentiation is regulated by Bcl6 and they are identified by the expression of cell surface molecules inducible co-stimulators (ICOS), programmed death 1 (PD-1) and the follicle trafficking cytokine CXCR5 (see table 1.1, Figure 1.6) (Ma *et al.*, 2012).

Gene name	Function(s)	Reference
CXCR5	CXCL-13 gradient dependent localization of T cells at the T-B cell border.	(Liu *et al.*, 2014b)
ICOS	Co-stimulatory signal during cognate T-B cell engagement.Facilitation of Tfh entry into follicular zone.	(Xu *et al.*, 2013; Bollig *et al.*, 2012)
CD40L	Co-stimulatory molecule. Engagement of CD40:CD40L prevents terminal differentiation of GC B cells while driving isotype switching and proliferation.	(Crotty, 2011; Yu and Vinuesa, 2010)
PD-1	Inhibition of excessive Tfh proliferation in the GC. Cytokine synthesis for optimal Tfh function and quality.	(Good-Jacobson *et al.*, 2010)
BTLA	Tfh cell inhibitory receptor.	(Murphy and Murphy, 2010)
SH2D1A	Signal transducing molecule required for maintaining Tfh cell engagement to cognate B cells.	(Crotty, 2011; Cannons *et al.*, 2010; Qi *et al.*, 2008)
SLAM family (CD84)	Maintaining optimal Tfh:B cell interactions. Transmits adhesion signals for T-B cell engagement at border.	(Cannons *et al.*, 2010)

Table 1.1: List of molecules reported to be involved in Tfh cell functions

1.4.1 Transcription programme regulating Tfh differentiation

Tfh differentiation is mediated by B cell lymphoma 6 (Bcl6) and inhibited by B lymphocyte-induced maturation protein-1 (Blimp1), produced by the Prdm1 gene. Other transcription factors that play a role in their development include Acheate schute homologue 2 (Ascl2), Basic leucine zipper transcription factor ATF-like (BATF), mammalian interferon regulatory factor 4 (IRF4) and cellular homolog of avian viral V Maf gene (cMaf) (see table 1.2) (Liu *et al.*, 2014b; Liu, Nurieva and Dong, 2013; Bollig *et al.*, 2012; Gómez-Martín *et al.*, 2011).

Owing to the fact that systemic knockout of the Bcl6 gene leads to growth retardation and severe myocardial injury resulting in early death, the majority of *in vivo* studies of Bcl6 physiological function in lymphocytes involve the transfer of bone marrow cells from Bcl6-/- mice into Rag-/- mice (Tetsuya Fakuda et al., 1997, Ye Bihui H et al, 1997, Dent A. L. 1997, Robert Johnson et al, 2009). Bcl6's role in Tfh differentiation was identified by a number of research groups in 2009 as the master transcription factor for Tfh cell subset (Yu and Vinuesa, 2010; Johnston *et al.*, 2009). It promotes Tfh differentiation via a number of mechanisms. It inhibits Blimp1 expression by actively repressing its transcription (Figure 1.5) (Yu et al, 2009). Bcl6 actively represses genes that code for signature transcription factors of other subsets like Th1, Th2 and Th17 (Liu, Nurieva and Dong, 2013). For instance, Bcl6 represses Th2 differentiation by inhibiting GATA3 transcription (Kusam *et al.*, 2003). It is also reported that Bcl6 inhibits a large number of microRNAs like miR-17-92 that repress the function of the key Tfh molecule CXCR5 (Liu, Nurieva and Dong, 2013; Ma *et al.*, 2012; Yu and Vinuesa, 2010; Yu *et al.*, 2009). Bcl6 is also reported to promote the survival of Tfh cells by minimising apoptosis due to increased PD-1 expression on Tfh cells (Hollister et al., 2013). $Bcl6^{-/-}$ T cells are reported to show defects in the differentiation to Tfh cells and the initiation and sustenance of GC immune responses (Yu and Vinuesa, 2010). Constitutive expression of the Bcl6 gene has been reported to be able to drive Tfh differentiation in *in vivo* studies (Johnston *et al.*, 2009).

Blimp1 acts antagonistically to Bcl6 in a STAT5 dependent manner to promote IL-2 production and the differentiation to non-Tfh CD4 cells and CD8 T

cells (Figure 1.5) (Liu, Nurieva and Dong, 2013; Crotty, 2011; Johnston *et al.*, 2009; Rutishauser *et al.*, 2009). Constitutive expression of Blimp1 in CD4 cells was reported to hinder Tfh differentiation (Johnston *et al.*, 2009). Other transcription factors reported to be involved in Tfh cell development are listed below in table 1.2

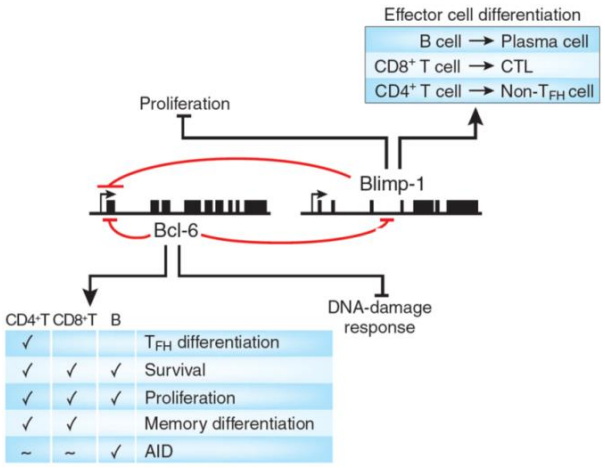

Figure 1.5: Bcl6 and Blimp function in Tfh and GC B cell development (Crotty, Johnston and Schoenberger, 2010).

Transcription factor	Function(s)	References
Bcl6	Tfh cell differentiation and development.	(Yu and Vinuesa, 2010)
Blimp 1 (Prdm1)	Direct inhibition of Bcl6 transcription and Tfh cell differentiation.	(Crotty, 2011)
IRF4	Tfh cell differentiation, plasma cell maturation and class switch recombination.	(Bollig *et al.*, 2012)
Ascl2	Tfh cell migration by upregulation of CXCR5.	(Liu *et al.*, 2014b)
cMAF	Regulation of Tfh-related genes like ICOS, CXCR5, PD-1, CXCR4, IL-21 and IL-4.	(Kroenke *et al.*, 2012)
BatF	Regulating the expression of Bcl6 and cMAF.	(Ise *et al.*, 2011)

Table 1.2: Transcription factors involved in Tfh cell regulation

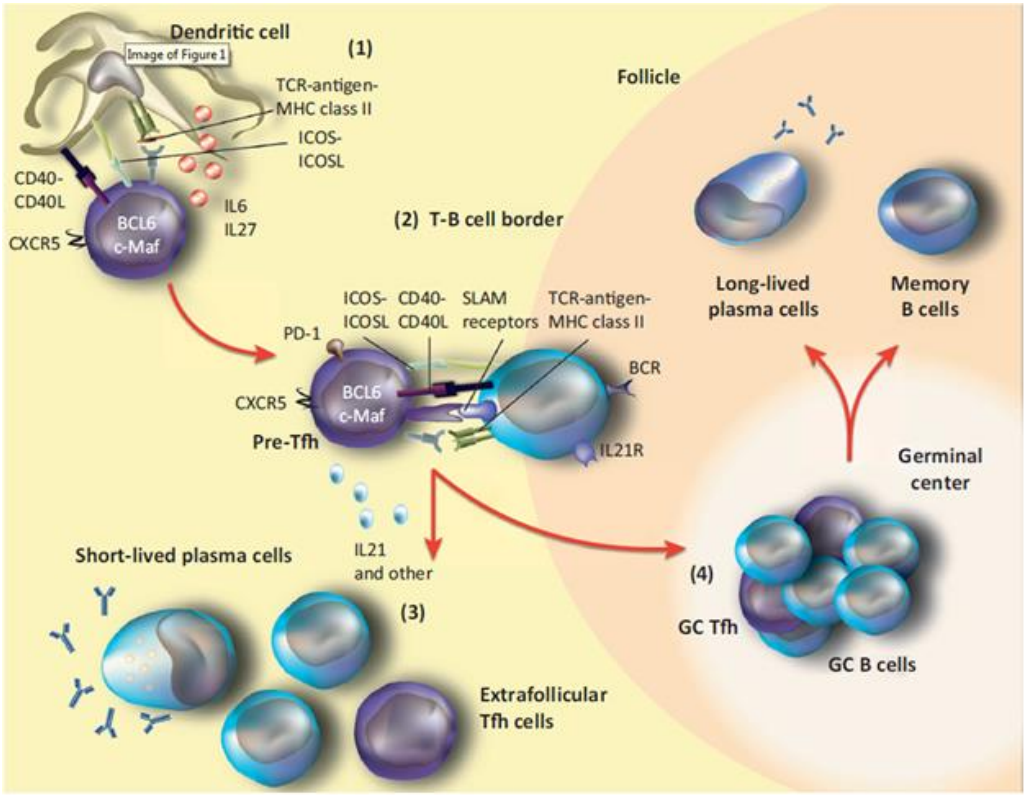

Figure 1.6: Cellular and molecular interaction involved in Tfh cell differentiation (Pissani and Streeck, 2014)

1.4.2 Tfh differentiation models

Several models have been proposed on the ontogeny of Tfh cells. Crotty in 2011 summarised the current literature on Tfh differentiation into 3 models (Figure 1.7). The first model proposed a cytokine-environment driven differentiation for Tfh where IL-6 and IL-21 were essential (Crotty, 2011). The second model was dependent on engagement of activated CD4 cells with cognate B cells. The third described a model where Tfh differentiation was dependent on prior Th1, Th2, Th17 and Treg differentiation, giving rise to Tfh1, Tfh2, Tfh17 and Tfhreg. These models were however not sufficient and were soon disputed (Eto *et al.*, 2011; Eddahri *et al.*, 2008). Some opponents countered the model by showing that cognate B cells interaction was not necessary to drive Tfh differentiation and CD4 T cells will differentiate into Tfh cells in mice with B cell lacking MHC II complex (Deenick *et al,* 2010).

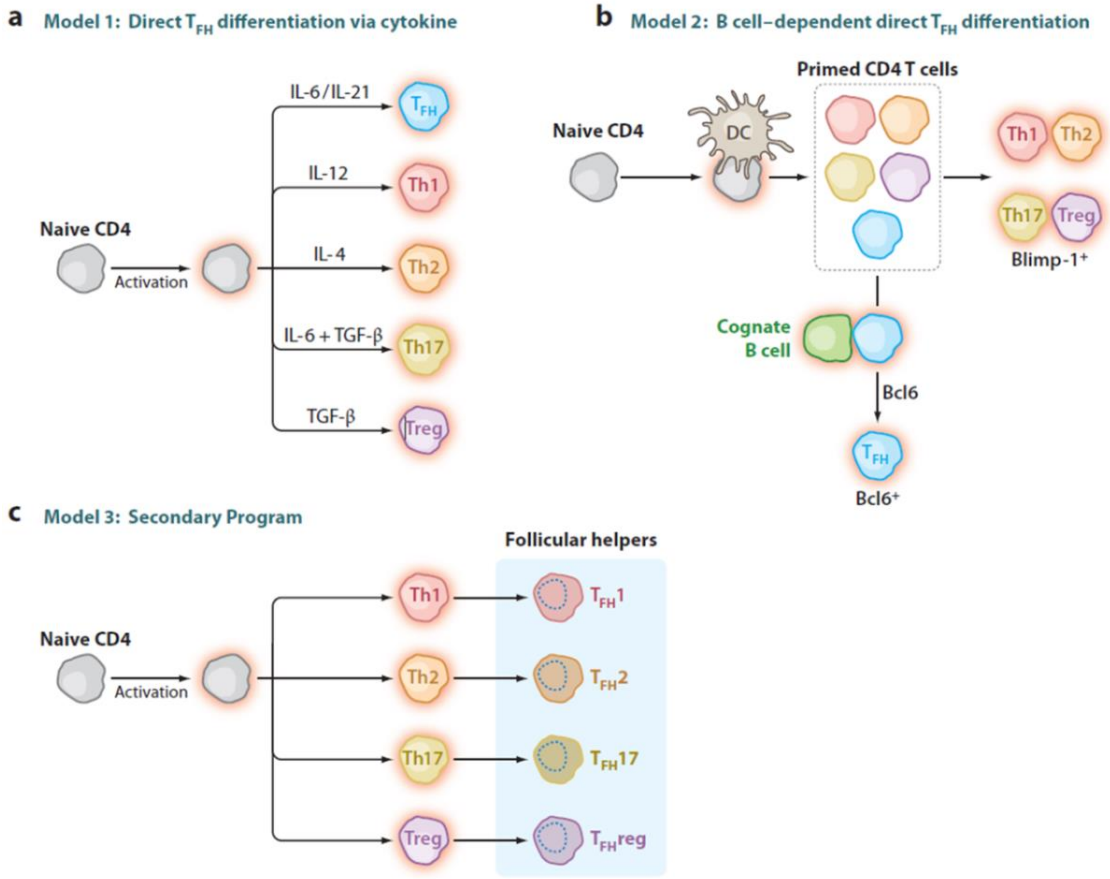

Figure 1.7: The 3 models of Tfh cell differentiation (Eto *et al.*, 2011; Crotty, 2011)

All these posited a need for unified model for Tfh differentiation, which was again proposed by Crotty (2011) (figure 1.8). The prevailing concept in terms of Tfh differentiation is that, upon reception of stimulus, IL6 and IL-21 induce the expression of STAT3. STAT3 in concert with other transcription factors and molecules including co-stimulatory molecule ICOS, will drive the expression of Bcl6, CXCR5 and repress IL-2 production thus generating pre-Tfh cells (Liu, Nurieva and Dong, 2013; Crotty, 2011; Nurieva *et al.*, 2008). At this point, there is a requirement for maintenance of the Tfh phenotype, which is dependent on cognate interaction of Pre-Tfh cell with follicular B cells. CXCR5 allows Tfh cells to migrate in a CXCL13 dependent gradient towards the B cell follicles. At the T-B cell border, it interacts with cognate B cells. This interaction leads to the eventual fully polarized Tfh phenotype localized in the GC where they express highest levels of Bcl6 and CXCR5 (Figure 1.8). This model proposes a fate where GC Tfh and pre-Tfh have a cyclical relationship and can migrate between GC region and follicles and is supported by studies that show that GC Tfh cells migrate from the GC into the surrounding follicular areas regularly depending on engagement of antigen specific GC B cells (Qi *et al.*, 2008).

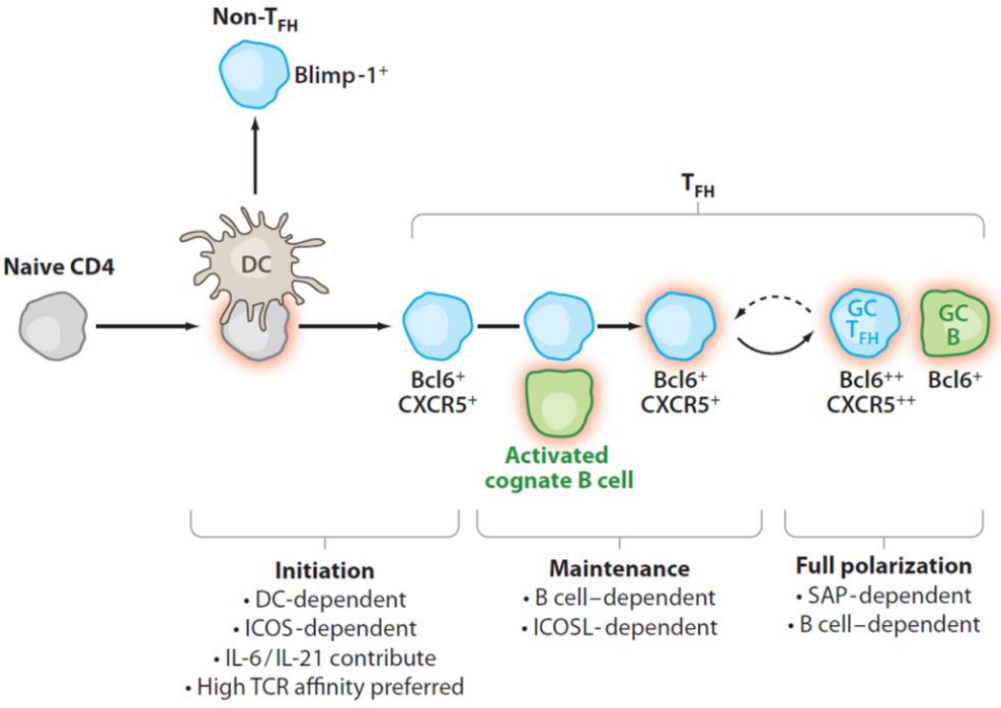

Figure 1.8: Integrated model of Tfh cell differentiation (Crotty, 2011)

1.4.3 Follicular regulatory T (Tfr) cells

3 groups originally described these cells in 2011 as a subset of CD4 T cells arising from nTreg cells in the thymus (Linterman *et al.*, 2011; Chung *et al.*, 2011; Wollenberg *et al.*, 2011). They express Foxp3+, Bcl6, CXCR5, PD-I but not IL-21 (Linterman *et al.*, 2011; Chung *et al.*, 2011). Like nTreg and iTreg cells, Tfr cells maintain tolerance to prevent the development of autoimmunity by suppressing GC dependent immune responses (Sage *et al.*, 2014; Linterman *et al.*, 2011; Wollenberg *et al.*, 2011). Similar to Tfh cells, they are regulated by Bcl6, which promotes their differentiation (Linterman *et al.*, 2011; Chung *et al.*, 2011). Blimp1, CD28 and SLAM proteins are also reported to regulate this subset of T cells (Linterman *et al.*, 2011). Blimp1 is credited with regulating the size of the Tfr population and in the absence of Blimp1, the number of Tfr cells doubled (Linterman *et al.*, 2011). Asides regulating Tfh cell numbers during T cell dependent humoral immune response in the germinal centre, Tfr cells also promote the selection of high affinity B cell clones and regulate the differentiation of plasma cells (Linterman *et al.*, 2011; Chung *et al.*, 2011; Wollenberg *et al.*, 2011).

1.4.4 T helper plasticity

The concept of T helper cell plasticity arose when studies showed that polarised T helper cells might be able to redirect their transcription program in conditions that favour the generation of a different T helper subset (Liu, Nurieva and Dong, 2013; Zhou, Chong and Littman, 2009). This notion challenged the opinion that T helper subsets were terminally differentiated in a mutually exclusive manner and expressed only their signature transcription factors (Nakayamada *et al.*, 2012; Zhou, Chong and Littman, 2009). This was partly because under re-differentiating conditions, these 'terminally differentiated' subsets, expressed cytokines and transcription factors akin to other T helper subsets (Figure1.9) (Nakayamada *et al.*, 2012).

Warren and colleagues were able to demonstrate that in the presence of IL-6, iTreg cells are able to induce the differentiation of CD4 cells into Th-17-like cells and themselves re-differentiated into IL-17 producing cells in a TGF-β dependent manner (Xu *et al.*, 2007). Treg cells in the Peyer's patch of mice have also been reported to differentiate into Tfh upon interaction with B cells (Tsuji *et al.*, 2009). In the presence of helminthic antigens some Th2 cells displayed features of Tfh cells with expression of Bcl6, CXCR5 and PD-1 (Zaretsky *et al.*, 2009). These cells were also GATA3 positive (Zaretsky *et al.*, 2009). Th2 cells can re-programme to express T-bet and IFN-γ in response to Th1 inducing lymphocytic choriomeningitis virus (LCMV) (Hegazy *et al.*, 2010) and Th9 has been reported to be able to be produced from Th2 cells *in vitro* in the presence of TGF-β (Veldhoen *et al.*, 2008). Th17 cells are notoriously unstable and have been reported to re-differentiate in the presence of appropriate cytokines into Th1 or Th2 cells (Lee *et al.*, 2009).

Review of published studies on plasticity indicates intrinsic and extrinsic factors dictate plasticity. Cytokine microenvironment, cell-cell interactions, antigenic stimulants and epigenetic modifications of signature cytokine loci or transcription factors may be linked to the multidirectional differentiation potential of some Th subsets (Zaretsky *et al.*, 2009; Wei *et al.*, 2009; Zhou, Chong and Littman, 2009).

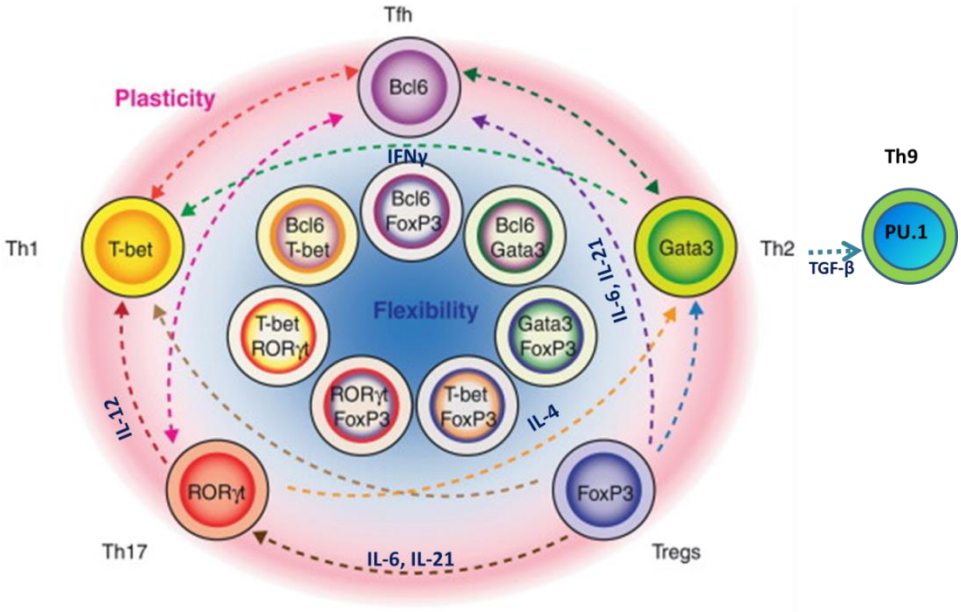

Figure 1.9: Adapted model of T helper cell plasticity showing some cytokines involved in these pathways (Nakayamada *et al.*, 2012)

T helper plasticity, although with beneficial properties including the maximal use of resources for optimal immune response, may also have far reaching detrimental effects. In transplant patients or autoimmune disease patients with high Th-17 inducing cytokine levels, Treg cell therapy may increase inflammation through induced Th17 differentiation (Xu *et al.*, 2007). Conversely a lack of Th17 cells due to plasticity could result in a loss of normal cellular functions of Th17 in protecting the intestinal epithelial integrity (Zhou, Chong and Littman, 2009).

1.5 B Lymphocytes

1.5.1 Development in the Bone marrow

B lymphocytes are so named because of their origin in the bone marrow (BM) where they begin their journey which ends in the peripheral lymphoid organs (LeBien and Tedder, 2008). The fate of Hematopoietic stem cells (HSC) within the BM is committed to the B cell lineage by the expression of transcription factors paired box protein 5 (PAX5), E2A and early B cell factor 1 (EBF1) (Eibel *et al.*, 2014; Vale and Schroeder Jr., 2010; LeBien and Tedder, 2008). Pro-B cells express the Pro-BCR complex Igα and Igβ (LeBien and Tedder, 2008; Monroe *et al.*, 2003) and upregulate the RAG1 and RAG2 genes. This initiates rearrangement of the D-J locus of the heavy chain followed by a V/D-J rearrangement on the same heavy chain (Vale and Schroeder Jr., 2010; LeBien and Tedder, 2008). Nucleotides are added to the rearranged gene segments by an enzyme called terminal deoxynucleotidyl tranferase (TdT) thereby further diversifying the antigen receptor repertoire (Vale and Schroeder Jr., 2010). The Pre-B cell stage follows next. Here, V and J segments of the immunoglobulin light chains are rearranged with the help of RAG genes. This leads to the expression of kappa (κ) or lambda (λ) light chains and a functional BCR receptor (Eibel *et al.*, 2014; Vale and Schroeder Jr., 2010; LeBien and Tedder, 2008). Aiolos, a zinc finger transcription factor is important in regulating light chain rearrangement in the Pre-B cell developmental stage (Eibel *et al.*, 2014). FOXO1, PU.1 (Liu, Nurieva and Dong, 2013), Ikaros and E12/E47 are associated with rearrangements of immunoglobulin gene loci (Eibel *et al.*, 2014; Vale and Schroeder Jr., 2010; LeBien and Tedder, 2008). The expression of IgM hallmarks the immature B cell developmental stage (Vale and Schroeder Jr., 2010). In order to tackle self-reactivity, IgM are tested for affinity to bone marrow antigens, high affinity IgM receptor are either deleted or undergo receptor editing (Eibel *et al.*, 2014; Vale and Schroeder Jr., 2010; LeBien and Tedder, 2008; King and Monroe, 2000; Levine *et al.*, 2000). After this stage, B cells now known as transitional immature B cell may leave the bone marrow for the periphery (Giltiay, Chappell and Clark, 2012). Alternative splicing within the IgM immunoglobulin receptor permits the production of IgD expressing B cells (Eibel *et al.*, 2014). These IgM^+ IgD^+ B cells known as naïve mature

B cells await antigen encounter in the peripheral lymphoid organs (Vale and Schroeder Jr., 2010; LeBien and Tedder, 2008).

1.5.2 B cell activation and differentiation

In the periphery, naïve mature B cells become activated by exposure to antigens carried by APCs or by encounter with antigens themselves (Vale and Schroeder Jr., 2010). Activation is determined by affinity for antigen, dose of antigen and presence of innate signals at site of activation (MacLennan *et al.*, 2003). Depending of the molecular composition of the antigen, primed B cells can function in a T cell dependent (TD) or T cell independent (TI) manner. B cell immune response mounted against non-peptide antigens like lipopolysaccharide (LPS - a composition of cell walls in gram negative bacteria) or lipid based pathogens are done in a TI manner (LeBien and Tedder, 2008). In TD B cell responses, after priming by cognate antigen, B cells will solicit help from MHC II restricted Th2 or Tfh cells (Vale and Schroeder Jr., 2010; LeBien and Tedder, 2008). Antigen experienced B cells then differentiate into marginal zone B cells, extrafollicular B cells or germinal centre (GC) B cells. Absence of Notch 2 receptor expression on B cells is reported to favour the commitment to a follicular fate at the expense of a marginal zone fate where Notch 2 receptor expression is required (Hampel *et al.*, 2011; Pillai and Cariappa, 2009).

1.5.2.1 Marginal zone (MZ) B cells

These are non-recirculating B cells with a less diversified receptor and are capable of recognizing conserved antigenic patterns on pathogens (Cerutti, Cols and Puga, 2013; MacLennan *et al.*, 2003). They express high levels of co-stimulatory molecules, CD21 and IgM (Cerutti, Cols and Puga, 2013; MacLennan *et al.*, 2003). They are strategically placed in the marginal zone of the spleen where they utilise BCRs and TLRs to engage antigens initiating a TI antibody immune response producing IgM, IgG2 and IgA (Cerutti, Cols and Puga, 2013; MacLennan *et al.*, 2003). MZ B cells are also responsible for the production of natural antibodies and are able to respond to pathogen carried in the blood in an innate-like manner before the

onset of conventional adaptive immune response (Cerutti, Cols and Puga, 2013; Vale and Schroeder Jr., 2010).

1.5.2.2 Extrafollicular B cells

Extrafollicular B cells can be found in the marginal sinuses of the spleen, which is enriched with APCs (MacLennan *et al.*, 2003). Shortly after antigen encounter, these extrafollicular B cells downregulate co-stimulatory molecules and MHC II complex while expressing high levels of Blimp1 (MacLennan *et al.*, 2003). Blimp1 promotes their terminal differentiation by the suppression of PAX5 and Bcl6 (MacLennan *et al.*, 2003). Immune response mediated by these cells lead to robust but short-lived antibody production (Pissani and Streeck, 2014; MacLennan *et al.*, 2003). Although the cellular interactions that mediate extrafollicular B cell responses are still being investigated, it has been proposed that, depending on the nature of the antigen, generation of plasmablasts via extrafollicular B cell activity can be T cell dependent or independent (Sweet *et al.*, 2011; MacLennan *et al.*, 2003). It should be noted that T cell dependent extrafollicular response is mediated by Bcl6 expressing CD4 T cells (Lee *et al.*, 2011).

1.6 Germinal Centre (GC) B cells

These subsets of B cells express CXCR5 that enables their localization in the B cell follicles (Bollig *et al.*, 2012; Nutt and Tarlinton, 2011). Here, they engage with follicular dendritic cells (FDC) that present antigens to them. Once FDC signal is received, they seek cognate Tfh cells by migrating to T-B cell border. They interact with Tfh cells using CD40 receptor, ICOS and PDL-1 (Ma *et al.*, 2012; Giltiay, Chappell and Clark, 2012; Nutt and Tarlinton, 2011). Activated GC B cells upregulate Bcl6 that promotes proliferation, survival, inhibits Blimp 1 expression and the expression of genes involved in DNA damage (Nutt and Tarlinton, 2011). In addition to Bcl6, Pax5 and Bach2 are also upregulated to suppress plasma cell differentiation and promote the expression of activation induced (cytidine) deaminase (AID) (Nutt and Tarlinton, 2011; LeBien and Tedder, 2008; Shapiro-Shelef and Calame, 2005). B cells in the GC undergo clonal proliferation, somatic hypermutation (SHM) and class switch recombination (CSR). Molecular regulators of GC B cells are highlighted in figure 1.10 and Table 1.3.

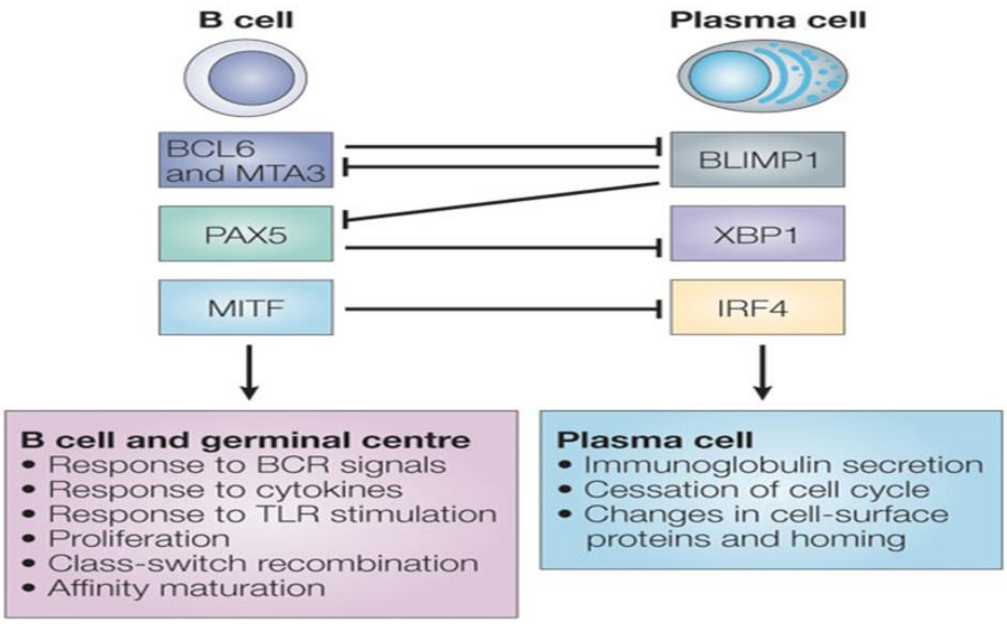

Figure 1.10: Schematic diagram showing genes involved in GC B cell differentiation, maintenance and the terminal differentiation of plasma cells (Shapiro-Shelef and Calame, 2005)

Gene	Function(s)	References
Bcl6	Promoting GC B cell differentiation while inhibiting DNA damage response elements; inhibition of plasma cell differentiation	(Crotty, Johnston and Schoenberger, 2010; Reljic *et al.*, 2000)
Blimp 1 (Prdm1)	Master regulator for plasma cell differentiation and secretion of immunoglobulin from plasma cells. Repressor of Bcl6 and Pax5.	(Shapiro-Shelef and Calame, 2005; Shapiro-Shelef *et al.*, 2003; Turner Jr., Mack and Davis, 1994)
IRF4	Transiently expressed after T:B cell receptor engagement to induce Bcl6 and AID. Sustained expression promotes terminal differentiation of GC B cells into plasma cells by Blimp1 upregulation.	(Ochiai *et al.*, 2013; Mittrücker *et al.*, 1997)
Bach2	Activation of AID to promote SHM and CSR. Repression of Blimp1.	(Nutt and Tarlinton, 2011; Muto *et al.*, 2004)
Pax5	Maintaining mature B cells and activation of AID.	(Gonda *et al.*, 2003)
BatF	Directly regulating the expression of AID to control CSR in GC B cells.	(Ise *et al.*, 2011)
XBP-1	Terminal differentiation of GC B cells to induce plasma cell phenotype and secretion of immunoglobulin by plasma cells.	(Shaffer *et al.*, 2004; Shapiro-Shelef *et al.*, 2003)
MTA3	Preventing plasma cell differentiation by repression of Blimp1 expression.	(Fujita *et al.*, 2004)
STAT3	Induction of Blimp1.	(Reljic *et al.*, 2000)
IL-21	GC B cell differentiation, CSR, antibody secretion.	(Bollig *et al.*, 2012) Kristin Hollister et al, 2013
CD40	Survival and proliferation of GC B cell. It has also been reported to drive class switching and antibody secretion in plasma cells.	(Wykes, 2003)
ICOSL	Promotes GC B cell survival and function.	(Liu *et al.*, 2015)
PDL-1	Enhance B cell survival.	(Good-Jacobson *et al.*, 2010)
Mitf	Inhibit plasma cell differentiation by suppressing IRF4 expression.	(Lin, Gerth and Peng, 2004)

Table 1.3: Molecules reported to be involved in GC B cell regulation and functions

1.6.1 GC Reactions

As mentioned earlier, GCs are birthed as a result of T cell dependent immune response and are manifested a 1-2 weeks after initial infection (Vale and Schroeder Jr., 2010; MacLennan *et al.*, 2003). GC reactions begin with the priming of GC B cells by FDC which bear antigenic immune complexes (Ma *et al.*, 2012; Giltiay, Chappell and Clark, 2012; Vale and Schroeder Jr., 2010). Primed B cells upregulate T cell homing receptor CCR7 while downregulating the follicular homing receptor CXCR5. They migrate to the border of the T cell zone where they solicit for help from follicular helper T cells (Reif *et al.*, 2002). Cognate engagement with Tfh increases GC B cell survival chances through CD40:CD40L signaling and the induction of Bcl6 along with other molecules necessary for GC B cell proliferation in the GC (see table 1.3). Once activated by Tfh cells, GC B cells along with cognate Tfh cells migrate into the follicles to form the GC.

The GC is the site for clonal expansion of activated GC B cells (Ma *et al.*, 2012). B cells entering the GC first migrate into the dark zone as centroblasts where they undergo active proliferation with affinity maturation of immunoglobulin receptors through somatic hypermutation (SHM) (Vale and Schroeder Jr., 2010). An enzyme, AID that is capable of RNA editing catalyzes SHM and class switch recombination (CSR) (Giltiay, Chappell and Clark, 2012). Another enzyme, uracil-DNA glycosylase (UNG), removes the edited nucleotide (Giltiay, Chappell and Clark, 2012; Vale and Schroeder Jr., 2010; Han *et al.*, 2007; Muramatsu *et al.*, 2000). During SHM, AID causes point mutations to occur in the variable region of the immunoglobulin heavy (IgH) and light (IgL) chains permitting the generation of antibodies with a higher affinity for pathogens (Ma *et al.*, 2012; LeBien and Tedder, 2008; Han *et al.*, 2007). Following SHM, immunoglobulin receptors undergo CSR.

During CSR, the constant (C) region of the IgH chain structure is modified through non-homologous recombination to generate other classes of antibodies with a wider range of effector function and location (Eibel *et al.*, 2014; Vale and Schroeder Jr., 2010; LeBien and Tedder, 2008; Teng and Papavasiliou, 2007; Muramatsu *et al.*, 2000). Following SHM and CSR, centrocytes in the light zone interact with FDC and selectively bind antigens based on affinity (Eibel *et al.*, 2014).

B cells that bind with higher affinity to antigen complex are positively selected to compete for help and survival signals administered by Tfh cells while B cells with lower antigen affinity die by apoptosis (Eibel *et al.*, 2014; Nutt and Tarlinton, 2011). The nature of the antigens initiating the immune response determines what class of immunoglobulin would be produced following recombination of switched transcripts (MacLennan *et al.*, 2003). Bacteria would generally initiate an IgG2 response while viruses and protein immunisation generate IgG1 and IgG3 antibody production (MacLennan *et al.*, 2003). Cytokines have been reported to facilitate CSR with studies showing that CSR can be induced in B cells by the presence of IL-4 (Ma *et al.*, 2012). Once centrocytes have been positively selected, they upregulate plasma cell differentiating transcription factor Blimp1 to promote plasmablast generation and memory B cell differentiation (LeBien and Tedder, 2008). Han et al, 2007 reported that SHM and CSR can occur outside of the GC and without the need for T cell help (Han *et al.*, 2007). Indeed CSR have been reported in marginal zone B cells (Cerutti, Cols and Puga, 2013). However, SHM and CSR associated with the production of long lived plasma cells is limited to the GC (Eibel *et al.*, 2014).

B cell immunological memory mainly arises from GC reactions although; there are reports of GC-independent memory B cell generation (Takemori *et al.*, 2014). The memory B cell population predominantly resides in the spleen where they are capable of rapid differentiation into plasmablasts to provide a more robust immune response upon re-exposure (Ma *et al.*, 2012; Tangye and Tarlinton, 2009; LeBien and Tedder, 2008). It has been proposed that memory B cells are maintained by TLR stimulation (Bernasconi, Onai and Lanzavecchia, 2003), Bcl6 expression (Scheeren *et al.*, 2005) and BAFF-R and TACI expression (Salzer *et al.*, 2008), the underlying cellular and molecular mechanism responsible for the maintenance of memory B cell is still very much unclear.

1.6.2 Antibodies as mediators of adaptive humoral immunity

Antibodies are 'Y' shaped structures made up of 2 heavy and 2 light chains. They have a variable region on the top branches of the 'Y' structure and a constant region at the base which serves as an effector site (Figure 1.11) (Hansel *et al.*, 2010). Based on the amino acid composition of the constant region, there are 5 classes of antibodies: IgM, IgD, IgG, IgE and IgA. IgG is the most dominant antibody in the serum mediating cytotoxic activities, complement activation and neutralisation while IgA is found on mucosal surfaces, secretion and skin (Tomasi, 1970). IgE is useful in immunity against helminthic pathogens and also plays a role in allergy (Tomasi, 1970).

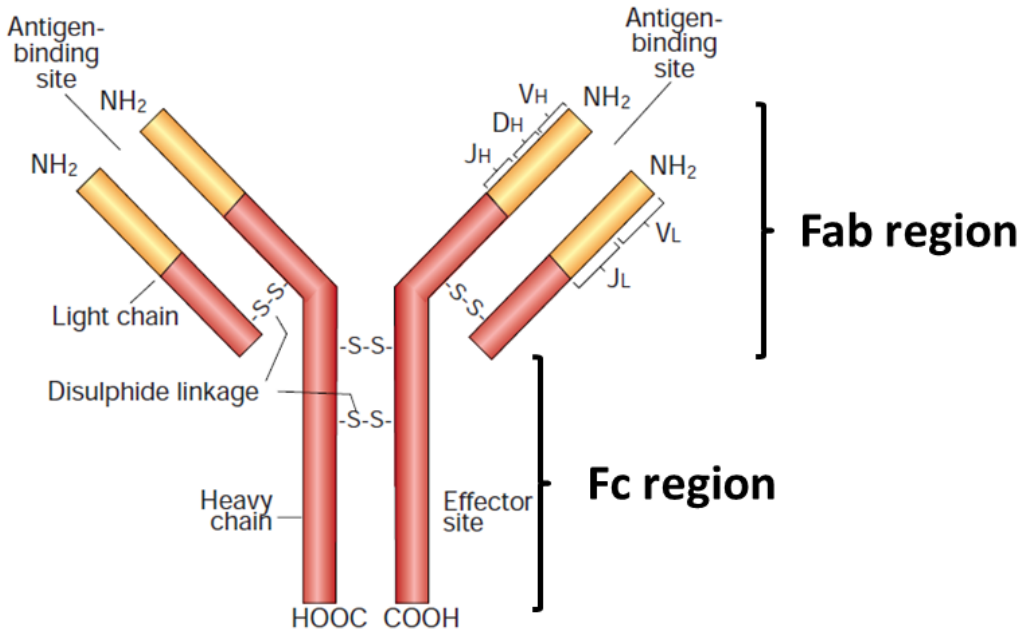

Figure 1.11: Typical structure of a monomer antibody complex showing antigen binding sites and effector site. Image adapted from (Nossal, 2003).

Within the variable region of antibodies, affinity maturation by SHM takes place creating a repertoire of antibody that binds with heightened affinity to a specific epitope (Beck *et al.*, 2010; Wang *et al.*, 2007). Once released from plasma cells, this variable region can then bind to specific antigenic targets (Nossal, 2003). Using the constant region, other effector cells are able to attach to an antibody-antigen complex to initiate antibody dependent cell-mediated cytotoxicity (ADCC) (Wang *et al.,* 2007). Aside cells, complements protein (C1q) is also able to utilise the

Fc region to tag cells for destruction through complement dependent cytotoxicity (CDC) (Hansel *et al.*, 2010). Antibodies are also capable of facilitating traffic of phagocytes to pathogens through opsonisation (Wang *et al.,* 2007 and Hansel *et al.,* 2010). This leads to efficient clearing of pathogens (Wang *et al.,* 2007 and Hansel *et al.,* 2010). Aside ADCC, CDC and opsonisation, another function of antibodies is the neutralisation of pathogens as in the case of viral infection and bacteria toxins (Klasse, 2014; Willey and Aasa-Chapman, 2008). During viral infection, neutralising antibodies (NAb) bind to viral receptors to antagonise viral docking on target cells (Klasse, 2014). If virus has already made contact with cell, NAb binds to viral epitopes to inhibit subsequent steps including replication (Klasse, 2014; Willey and Aasa-Chapman, 2008). These actions abrogate the pathogenic effect of viral invasion (Klasse, 2014; Willey and Aasa-Chapman, 2008). Antibodies are also able to bind to mast cells causing them to release chemical signals that induce allergic reactions like sneezing aimed at expelling immunogen.

1.6.3 Diseases arising from defective host immune responses

Both innate and adaptive arms of the immune system are tightly regulated in order to achieve optimal responses against immunogens while minimising pathology that may arise due to deregulation of the mechanisms mediating immune responses. Dysregulated immune responses have been implicated in the development of autoimmunity, primary and secondary immunodeficiency diseases, cancers and allergies. For the purpose of this review, the focus will be on autoimmunity and immunodeficiency diseases.

1.6.3.1 Autoimmunity

Autoimmune diseases are a consequence of immune responses mounted against harmless self-antigens. In autoimmune diseases, self–reactive immunocytes are constitutively activated in the absence of overt immunization causing the pathological symptoms associated with these diseases (Table 1.5) (Davidson and Diamond, 2001). It is reported that genetics may predispose individuals to the onset of autoimmune diseases. There is increased susceptibility to autoimmunity when genes involved in regulating lymphocyte activity are mutated as observed in CD45 E613R mutated mice that developed lupus like autoimmune diseases (Majeti *et al.*, 2000). Mice with CTLA-4 downregulation have increased T cell activation with Type 1 diabetes and Graves ' disease (Kouki *et al.*, 2000). Loss of regulatory T cell function, environmental triggers, infection with pathogens that mimic self-antigens and hormones are other factors that may contribute to the development of autoimmune diseases (Davidson and Diamond, 2001). Cytokines play a role in the progression of these diseases contributing immensely to the observed pathology (Table 1.4) (Davidson and Diamond, 2001). Th1, Th2 and Th17 cytokines are often associated with autoimmunity (Miao *et al.*, 2013; Damsker, Hansen and Caspi, 2010). Th1 cytokines IFNγ and IL-12 have been linked with the development of multiple sclerosis, lupus like autoimmune diseases, rheumatoid arthritis and Crohns disease (Zhu *et al.*, 2008; Gutcher and Becher, 2007; Romagnani, 1999). Th2 cytokines have been implicated in reactive arthritis following persistent intracellular bacterial infection (Lafaille, 1998). Th-17 and IL-17 have well documented roles in organ pathology and autoimmunity including experimental autoimmune

encephalomyelitis (EAE), inflammatory bowel diseases (IBD), systematic lupus erythematosus (SLE) and rheumatoid arthritis (Miao *et al.*, 2013; Dong, 2006). Tfh responses have been associated with the development of Systemic lupus erythematosus SLE, Sjogren syndrome, and juvenile dermatomyositis (Ma *et al.*, 2012).

Cytokine or Protein	Defect	Result
Tumor necrosis factor α	Overexpression	Inflammatory bowel disease, arthritis, vasculitis
Tumor necrosis factor α	Underexpression	Systemic lupus erythematosus
Interleukin-1–receptor antagonist	Underexpression	Arthritis
Interleukin-2	Overexpression	Inflammatory bowel disease
Interleukin-7	Overexpression	Inflammatory bowel disease
Interleukin-10	Overexpression	Inflammatory bowel disease
Interleukin-2 receptor	Overexpression	Inflammatory bowel disease
Interleukin-10 receptor	Overexpression	Inflammatory bowel disease
Interleukin-3	Overexpression	Demyelinating syndrome
Interferon-γ	Overexpression in skin	Systemic lupus erythematosus
STAT-3	Underexpression	Inflammatory bowel disease
STAT-4	Overexpression	Inflammatory bowel disease
Transforming growth factor β	Underexpression	Systemic wasting syndrome and inflammatory bowel disease
Transforming growth factor β receptor in T cells	Underexpression	Systemic lupus erythematosus

Table 1.4: Deregulated cytokine expression in autoimmune diseases. Reproduced with permission from Davidson and Diamond 2011, Copyright Massachusetts Medical Society. (Davidson and Diamond, 2001).

Disease	Pathogenesis	Reference
SLE	Genetic alterations, environmental factors, hormones and diet promote disease initiation which manifest as Vasculitis, deposition of immune complex in kidney, atherosclerosis and presence of anti-dsDNA.	(Mok and Lau, 2003)
Sjoren Syndrome	Lymphocytic infiltration and subsequent destruction of salivary and lacrimal glands, liver, kidney and lungs. Glomerulonephritis and lymphoproliferative disorders are also reported.	(Goules, Tzioufas and Moutsopoulos, 2014)
Rheumatoid Arthritis	Unknown etiology however disease is presented with Synovitis and synovial hyperplasia, cartilage, joint and bone damage.	(Gibofsky, 2012)
Multiple sclerosis (MS)	A combination of genetic susceptibility and environmental interactions may promote chronic inflammation in the central nervous system resulting in organ damage and severe disability. Th17 cells have also been reported to be involved in MS.	(Loma and Heyman, 2011; Li *et al.*, 2011)

Table 1.5: Some autoimmune diseases and their associated pathology

1.6.3.3 Immunodeficiency

Immunodeficiency diseases are characterised by an inability to mount optimal immune response against invading pathogens and an increased susceptibility to infections. They may arise due to single or multiple mutations on genes (primary immunodeficiency) or due to pathogenic infections (secondary immunodeficiency) (Table 1.6) (Bonilla and Geha, 2003). They manifest in various forms from antibody deficiency to a lack of effector functions in immunocytes and a deficiency in complement proteins (Bonilla and Geha, 2003).

Disease	Pathogenesis	Reference
Severe Combined Immunodeficiency	Group of disease arising due to defects in a number of genes. Leads to an absence of T and sometimes B cell function. Bacterial infection, lung granulomas and lymphoid malignancies develop.	(Maggina and Gennery, 2013; Notarangelo, 2013; Roifman *et al.*, 2012)
X-Linked Agammaglobulinemia (Bruton's Disease)	Defects in B cell development arising from aberrant BTK gene expression. Neutropenia and severe bacterial infections are common in sufferers.	(Notarangelo *et al.*, 2009; Bonilla and Geha, 2003)
Common variable immunodeficiency disorders (CVIDs)	Sufferers present with increased susceptibility to bacterial infections. They may also develop autoimmune diseases, lymphoproliferative and granulomatous diseases.	(Notarangelo *et al.*, 2009)
Wiskott-Aldrich syndrome (WAS)	Mutations in the WAS protein family of genes leading to Lymphopenia, defective actin modelling in cytoskeleton leading to failure in immune synapse formation.	(Nonoyama and Ochs, 2001)
Hyper IgM syndrome	Varied cause. Defective CD40:CD40L signaling and defective AID expression. Pathology include Failure in SHM and CSR leading to an inability to produce highly specific antibody or other antibody isotype and susceptibility to infections.	(Etzioni and Ochs, 2004)

Table 1.6: List of immunodeficiency diseases and their pathogenesis

1.8 The Early Growth Response Genes

The early growth response (Egr) genes are a family of transcription factors. They include Egr1 (NGFI-A), Egr2 (Krox20), Egr3 (Pilot) and Egr4 (NGFI-C). They contain an evolutionarily conserved DNA binding domain (81 – 93% homology) with 3 zinc finger transcription motifs bearing Cys2-Hys2 zinc fingers (Gabet *et al.*, 2010; Gómez-Martín *et al.*, 2010). Each zinc finger motif binds to 3 nucleotide base pairs on G-C rich promoter regions on DNA of target genes (Zheng *et al.*, 2012; Gabet *et al.*, 2010; Gómez-Martín *et al.*, 2010; Poirier *et al.*, 2008). This is known as the Egr response element (ERE). As a family, the Egr genes regulate growth, activation and differentiation of cells in the nervous system and immune system (Gabet *et al.*, 2010; Gómez-Martín *et al.*, 2010; Poirier *et al.*, 2008). Within the nervous system, they control the proliferation and differentiation of Schwann cells (Gabet *et al.*, 2010; Gómez-Martín *et al.*, 2010). Some members of the family are upregulated to mediate the differentiation of precursor lymphocytes, the maturation and activation of B and T cells as well as the induction of tolerance (Li *et al.*, 2011; Gómez-Martín *et al.*, 2010). Egr1 acts as a positive regulator of immune cell activation promoting macrophage cell lineage differentiation (Gómez-Martín *et al.*, 2010; Ramón *et al.*, 2010; Krishnaraju, Hoffman and Liebermann, 2001) and also in the positive selection of single positive CD4 and CD8 cells (Bettini *et al.*, 2002). Egr2 functions as regulators of T cell activation and effector function by the regulation of IFN-γ and IL-17 expression (Zhu et al., 2008, Li et al., 2012). Egr2 also promote T cell anergy and apoptosis (Lawson, Weston and Maurice, 2010; Ramón *et al.*, 2010; Safford *et al.*, 2005). Egr3 is reported to function in the transition from double negative (DN) to double positive (DP) T cells (Li *et al.*, 2011; Gómez-Martín *et al.*, 2010; O'Donovan *et al.*, 1999) and in the induction of tolerance through its interaction with type III histone deacetylase sirtuin 1 (Sirt1) (Gao *et al.*, 2012). Egr4, the last member of the family, is an auto-regulatory transcription repressor of c-FOS protein. Mutations within the Egr family of genes have been linked to the development of lupus-like autoimmune diseases (Gómez-Martín *et al.*, 2010; Zhu *et al.*, 2008), reduction in bone mass (Gabet *et al.*, 2010) neuropathies like Charcot-marie tooth type 1, congenital hypomyelinated neuropathy (Warner *et al.*, 1998),

and sensory ataxia (Tourtellotte and Milbrandt, 1998). Absence of Egr4 is known to cause the appearance of infertile male phenotypes in Egr4 knockout mice due to its expression in male germ cells and involvement in germ cell maturation (Hadziselimovic *et al.*, 2009; Tourtellotte and Milbrandt, 1998).

1.8.1 The Egr2 gene

Egr2 is located on chromosome 10q21.1 in humans and 10qB5.1 in mice. It is a 49kDa protein with 470 amino acids (aa). It binds to a GCGTGGGCG sequence on target genes to regulate their transcription (Swirnoff and Milbrandt, 1995). Functions of Egr2 have been reported in cognitive process and lymphocyte regulation (Miao *et al.*, 2013; Li *et al.*, 2012; Li *et al.*, 2011; Zhu *et al.*, 2008; Poirier *et al.*, 2008; Dong, 2006). Increased Egr2 expression can be seen early in Schwann cell development suggesting a vital role in regulating genes involved in Schwann cell differentiation and proliferation (Warner *et al.*, 1998). The Egr2 gene is also required for the development of the hindbrain with a mutation of this gene being associated with deregulated hindbrain segmentation, development of myelopathies like congenital hypomyelinated neuropathy, Charcot Marie tooth disease and early fatality in neonates (Gómez-Martín *et al.*, 2010; Swanberg *et al.*, 2009; Topilko *et al.*, 1994). Egr2 Function within the immune system includes regulating the development and activation of T and B cells, maintaining immune homeostasis and regulating TCR signaling (Li *et al.*, 2012; Lawson, Weston and Maurice, 2010; Zhu *et al.*, 2008). Egr2 has also been reported to play a role in apoptosis where it upregulates the expression of FasL and induce expression of apoptotic agent BNIP3L and BAK (Gao *et al.*, 2012; Lawson, Weston and Maurice, 2010; Unoki and Nakamura, 2003). Egr2 function is regulated by co-repressors DdX/DP103 (Gillian and Svaren, 2004) or the NAB proteins (Svaren *et al.*, 1998).

1.8.2 The Egr3 gene

Egr3 is the third member of the early growth response genes family of transcription factors. It is closely related to Egr2 in function (Poirier *et al.*, 2008; Safford *et al.*,

2005). It is located on chromosome 8p21.3 in human and 14qD2 in mice (Zhang *et al.*, 2012). It is a 42kDa protein with 387aa. Egr3 is essential for the development of muscle spindle. Egr3 Knockout mice exhibited a phenotype that lacked muscle spindles and show signs of severe muscular abnormalities akin to gait ataxia (O'Donovan *et al.*, 1999; Tourtellotte and Milbrandt, 1998). Egr3 knockout mice also display scoliosis, tremors, ptosis and increased perinatal mortality (O'Donovan *et al.*, 1999; Tourtellotte and Milbrandt, 1998). Egr3 was also identified to function in the negative regulation of T cell activation and the induction of T cell anergic state (Safford *et al.*, 2005). Like Egr2, Egr3 also upregulates FasL and promote apoptosis in Fas bearing cells (Yang *et al.*, 2002; Mittelstadt and Ashwell, 1998). Egr3 bears the closest homology to Egr2 and has been reported to be capable of functionally compensating for the loss of Egr2 (Li *et al.*, 2012; Poirier *et al.*, 2008; Safford *et al.*, 2005). For this study, I would be investigating the role of Egr2/3 in organ pathology and Tfh differentiation (Li *et al.*, 2012; Poirier *et al.*, 2008; Safford *et al.*, 2005; Xi and Kersh, 2004)

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1.8.3 Current knowledge on the role of Egr2 and Egr3 genes in the immune system

1.8.3.1 Induction and Maintenance of peripheral tolerance

Anergy, apoptosis and the action of regulatory T cells are the mechanisms used by the immune system to maintain tolerance to self-antigens and prevent the development of autoimmunity (Schwartz, 2003; Hargreaves *et al.*, 1997). Egr2/3 genes are able to induce anergy in both *in vitro* and *in vivo* conditions (Zheng *et al.*, 2013; Zheng *et al.*, 2012; Collins *et al.*, 2008). ChIP-Seq analysis associated Egr2/3 genes with the direct regulation of genes associated with anergy (Zheng *et al.*, 2013; Zheng *et al.*, 2012; Collins *et al.*, 2008). Global gene expression analysis identified sustained Egr2 and Egr3 expression in anergic A.E7 cells and transient Eg2 expression in fully stimulated A.E7 cells (Safford *et al.*, 2005; Harris *et al.*, 2004). Genetically modified T cells with a knock down of Egr2 were less susceptible to induction of an anergic state whilst overexpression of the Egr2 gene had the opposite effect (Gómez-Martín *et al.*, 2010; Zhu *et al.*, 2008; Safford *et al.*, 2005; Warner *et al.*, 1998). Research has shown that one of the ways by which Egr2 and Egr3 are able to control peripheral tolerance is via their interaction with NFAT leading to the induction of anergy (Müller and Rao, 2010). In anergic cells, NFAT dephosphorylation downstream of TCR engagement leads to the formation of complexes between NFAT and transcription factors such as Egr2 and 3 that promote the expression of proteins, for example the E3 ubiquitin ligase and CBL-B, which are involved in anergy (Martinez *et al.*, 2015; Müller and Rao, 2010; Macián *et al.*, 2004; Macián *et al.*, 2002). NFAT also plays a role in the induction of apoptosis by regulating the expression of the FasL (Rengarajan *et al.*, 2000). NFAT knockout mice have been reported to exhibit hyperproliferation in lymphoid tissues and impaired expression of the death receptor ligand – FasL (Rengarajan *et al.*, 2000). Safford et al in 2005 hypothesized that the E3 ubiquitin ligase, Cbl-b may play a role in anergy induction by Egr2/3 genes (Safford *et al.*, 2005). Upregulation of Cbl-b results in ubiquitinisation of signaling components and inhibition of key pathways necessary for TCR mediated immune response (Safford *et al.*, 2005). Egr2

is also said to be expressed in $CD4^+$ $CD25^-$ $LAG3^+$ Treg cells and may play a role in the maintenance of peripheral tolerance via these cells (Okamura *et al.*, 2009).

1.8.3.2 T and B cell development and activation

Egr2 and 3 regulate the differentiation and maturation of cells of the lymphoid lineage including NKT cells (Li *et al.*, 2011; Gabet *et al.*, 2010; Lazarevic *et al.*, 2009b). Egr2/3 genes are expressed during T and B cell development in the thymus and bone marrow respectively (Li et al, 2011). Development of DN thymocytes post β-selection is very much dependent on Egr2/3 genes upregulation (Xi *et al.*, 2006; Carleton *et al.*, 2002). Overexpression of Egr2 after Pre-TCR signaling results in aberrant numbers of DP thymocytes and immature B cells. Egr2, however, facilitates the transition from double positive (DP) to SP cells and together with Egr3, they can induce phenotypic changes associated with maturation of thymocytes *in vitro* and *in vivo* (Li *et al.*, 2011; Carleton *et al.*, 2002). A similar phenotype was observed in B cells in Egr2 overexpression models where they showed enhanced maturation of B cells in the periphery (Li et al, 2011). This suggests a need for differential expression of these genes during lymphocyte development (Li et al, 2011). Following TCR engagement Egr2 and 3 are transiently induced and are reported to act as negative regulators of T cell activation in the periphery (Collins *et al.*, 2008). T cells from single Egr2 or Egr3 knockout mice show enhanced proliferation and IL-2 production following anti-CD3 or PMA and Ionomycin stimulation (Collins *et al.*, 2008) while overexpression of Egr2 and Egr3 genes using lentiviral constructs significantly reduced IL-2 production in Jurkat cell lines (Safford *et al.*, 2005). Li et al., (2012) suggested that high IL-2 production in single Egr2 knockout mice may be as a result of compensation by Egr3 genes. Recently, we have found that Egr2/3 genes are necessary for the maintenance of homeostasis and to promote antigen receptor induced proliferation *in vitro* (Li *et al.*, 2012). T and B cells from $Egr2^{-/-}$ $Egr3^{-/-}$ mice showed severe defects in proliferation and IL-2 production after antigenic receptor stimulation *in vitro* (Li *et al.*, 2012). This was due to a lack of Egr2 interaction with BATF causing BATF to bind to the AP-1 complex and result in the inhibition of AP-1 activity downstream of TCR signaling (Li *et al.*, 2012). This newly discovered role for the Egr2/3 genes indicates

that they are regulators of not just immune homeostasis but also optimal immune response (Li *et al.*, 2012).

1.8.3.3 Autoimmunity and inflammation

In line with its role in the maintenance of peripheral tolerance, Egr2/3 transcription factors play a monumental role in the regulation of inflammatory responses and the development of systemic autoimmunity (Li *et al.*, 2012; Zhu *et al.*, 2008). Studies in Egr3 null mice found that transfer of Egr3-/- T cells into WT mice led to increase pathology and death due to autoimmune pneumonitis (Collins *et al.*, 2008). Studies conducted on Egr2 conditional knockout mice have shown that a decreased expression of Egr2 in T lymphocytes resulted in the increased levels of STAT 1 and 3, which facilitate the production of pro-inflammatory cytokines, such as IFN- gamma and IL-17 (Miao *et al.*, 2013; Li *et al.*, 2012; Zhu *et al.*, 2008). Double knockout of Egr2 and Egr3 genes in mice also resulted in the presence of an increased level of Th1 and Th17 cytokines IFN-gamma and IL-17, lymphocyte infiltrates into multiple organs including the lungs, kidney and liver and production of autoantibodies and immunoglobulin deposits in the kidney (Miao *et al.*, 2013; Li *et al.*, 2012). Our group reported that the Egr2/3 genes regulate SOCS1/3 to prevent STAT1/3 phosphorylation and the subsequent activation of Th1 and Th17 thus controlling inflammatory responses (Figure 1.15) (Li et al, 2012). The Th1 cytokine, IFN-γ has been implicated in autoimmunity and is reported to acts synergistically with TNF alpha causes necrotizing inflammatory myopathies and damage of tissue morphology (Li *et al.*, 2012; Okamura *et al.*, 2009). Furthermore, Tregs cells expressing Egr2 can actively inhibit the development of intestinal inflammation in mice models (Okamura *et al.*, 2012).

1.9 Aims of the study

In past years, a number of studies, including those done by our group, have looked into delineating the roles of the Egr2 and Egr3 transcription factors. A lot of these studies have highlighted roles in the central nervous system, inflammation, lymphocyte development, and autoimmunity (Li *et al.*, 2012; Gómez-Martín *et al.*, 2010; Topilko *et al.*, 1994; Schneider-Maunoury *et al.*, 1993). We recently found that the $Egr2^{-/-}$ $Egr3^{-/-}$ mice have high levels of Th1 and Th-17 cytokines and showed defective TCR signaling after *in vitro* receptor stimulation (Li et al, 2012). These suggested a possible role for Egr2/3 genes in the maintenance of immune homeostasis and optimal immune responses *in vivo*. So far, using Egr2 conditional knockout mice, 2 studies have looked at the possible role of the Egr2 gene in adaptive immune response after *in vivo* pathogenic challenge (Du *et al.*, 2014; Ramón *et al.*, 2010)

. Though they elucidated some hitherto unknown roles of the Egr2 genes in immune responses, these studies did not take into account the functional compensation by the Egr3 gene in the absence of Egr2. Using our model of CD2-specific $Egr2^{-/-}$ mice crossed with systemic $Egr3^{-/-}$ mice, I further investigated the roles of these genes in inflammation pathology, the differentiation of Tfh cells *in vivo* and the development of germinal centers after antigenic challenge *in vivo*. The aims of this study were therefore to:

- Characterize the phenotype of the $Egr2^{-CD2\ CRE\ -/-}$ $Egr3^{-/-}$ mouse model.
- Investigate the development of systemic inflammation pathology in the $Egr2^{-/-}$ $Egr3^{-/-}$ mouse model.
- Investigate the role of the $Egr2^{-/-}$ $Egr3^{-/-}$ gene in Tfh differentiation and the initiation of GC reactions after *in vivo* antigenic stimulation.
- Identify the molecular mechanism by which Egr2/3 regulate the Tfh cell genetic program.

The hypothesis of my study is that the Egr2/3 genes are essential for the control of systemic inflammatory pathology and are also critical for the differentiation of follicular helper T cells

Chapter 2 Materials and Methods

2.1 Experimental model

All mice used for the purpose of this study including hCD2-Cre-Egr2$^{-/-}$Egr3$^{-/-}$ mice and RAG$^{-/-}$ mice were approved under the U.K Home Office project license authority. They were maintained in the Biological Services Unit at Brunel University according to the Guidance on the Operation of Animals, Scientific Procedures Act 1986.

2.1.1 hCD2-Cre specific Egr2$^{-/-}$ MICE

Systemic knockout of the Egr2 gene results in the birth of lethal phenotypes with defects in hind brain development (Gabet *et al.*, 2010). As these mice die within a few hours after birth, this necessitated the generation of conditional knockout of the Egr2 gene in mice using the Cre-Loxp system (Gabet *et al.*, 2010; Turgeon and Meloche, 2009). Briefly, target genes are floxed with the Loxp gene at both ends and Cre, a recombinase enzyme recognise and cuts at these Loxp sites. hCD2-cre mice (a gift from Dr P. Charnay, Institut National de la Santé et de la Recherche Médicale, Ecole Normale Supérieure, Paris) and Egr2$^{LoxP/LoxP}$ mice (a gift from Dr D. Kioussis, National Institute for Medical Research, London) were crossed to generate the CD2-specific Egr2$^{-/-}$ mice (de Boer *et al.*, 2003; Taillebourg, Buart and Charnay, 2002). As the CD2 promoter is lymphocyte specific, Egr2 is deleted only in T and B cells. All Egr2$^{-/-}$ mice used for the purpose of this study were further backcrossed on a C57BL/6 background to purify the line.

2.1.2 Egr3$^{-/-}$ mice

Egr3$^{-/-}$ mice were a gift from the Tourtellotte group (Washington University School of Medicine, St Louis, USA). They cloned the entire Egr3 gene into a pBluescript vector and then disrupted transcription of the gene using a 1.5kb fragment containing a neomycin resistance positive selection cassette pMC1NeopA. This deleted over 1Kb fragment of the Egr3 gene taking away the DNA binding domain and the carboxyl terminus of the protein (Tourtellotte and Milbrandt, 1998).

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

These mice were generated by crossing hCD2-specific Egr2$^{-/-}$ and Egr3$^{-/-}$ mice. These are then backcrossed on a C57BL/6 background for at least 10 generations. For the purpose of this thesis, they would be referred to as Egr2$^{-/-}$ Egr3$^{-/-}$ mice from here on.

2.1.4 Genotyping of Egr2$^{-/-}$ Egr3$^{-/-}$ mice

Prior to use in any experimental procedure, mice were genotyped to confirm complete excision of the Egr2 and Egr3 genes. DNA was extracted from mouse tail or ears using REDExtract-N-Amp Tissue PCR Kit (Sigma) according to the manufacturer's protocol. Briefly, Extraction buffer and tissue preparation buffer were added to the sample and heated with agitation at 55˚C for 30 minutes. This allows the protease enzyme present in the buffers to break down proteins. This was then followed by heat-controlled denaturing of the enzyme by heating the samples at 95˚C for 3 minutes. Neutralising solution was added to stop the reaction. Extracted genomic DNA was then diluted in water as necessary.

Polymerase Chain Reaction (PCR) was then performed to amplify genomic DNA from mouse samples using 0.5µM of gene specific sense and antisense primers. The Primers are used to amplify a region containing the gene of interest on nucleic acids. The PCR technique uses DNA polymerase from *Thermus aquaticus* (Taq) which is thermostable and hence remains active even at high temperatures. Taq polymerase produces new copies of the gene of interest by adding nucleotides onto newly synthesised single strand DNA using dNTPs. The PCR conditions were as highlighted in table 2.1. PCR products obtained from the amplification were then loaded onto 2% agarose gels which were run in an electrophoresis tank containing 1X TAE Buffer (Sigma), at 100V. Gels were visualized under ultraviolet (UV) light using a BioRad Imager.

Gene	Primers (5' – 3')	PCR Conditions	Product
Cre	Sense: CCA ACA ACT ACC TGT TCT GCC G Antisense: TCA TCC TTG GCA CCA TAG ATC AGG	**Step 1:** 95˚C for 5 min **Step 2:** 94˚C for 40 sec **Step 3:** 56˚C for 40 sec **Step 4:** 72˚C for 40 sec Repeat step 2-4 (30x) **Step 5:** 72˚C for 5 min Keep in 4˚C	150bp
LoxP	Sense: GTG TCG CGC GTC AGC ATG CGT Antisense: GGG AGC GAA GCT ACT CGG ATA CGG	**Step 1:** 95˚C for 5 min **Step 2:** 94˚C for 40 sec **Step 3:** 56˚C for 40 sec **Step 4:** 72˚C for 40 sec Repeat step 2-4 (30x) **Step 5:** 72˚C for 5 min Keep in 4˚C	WT allele: 190bp LoxP allele: 210bp
Egr3	Sense: CTA TTC CCC CCA GGA TTA CC Antisense: TCT GAG CGC TGA AAC G Neo: GAT TGT CTG TTG TGC CCA GTC	**Step 1:** 95˚C for 5 min **Step 2:** 94˚C for 40 sec **Step 3:** 57˚C for 40 sec **Step 4:** 72˚C for 40 sec Repeat step 2-4 (30x) **Step 5:** 72˚C for 5 min Keep in 4˚C	WT allele: 400bp Neo allele: 800bp

Table 2.1: PCR cycles and product sizes for genotyping primers used in the generation of $Egr2^{-/-}$ $Egr3^{-/-}$ mice

2.2 Cell line

Human thymidine kinase deficient 143 (TK143) cell line (Sourced by Dr. Su-Ling Li at Brunel University, London) was used for the propagation of vaccinia virus and for assays requiring *in vitro* virus culture including viral titre and Plaque Reduction Neutralization Tests (PRNT). These TK143 cells show cytopathic activity to vaccinia virus, which is visible by the formation of plaques indicating cell lysis. TK143 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) + GLUTAMAX supplemented with 2% sodium pyruvate, 10% HEPES buffer, 10% Foetal Bovine Serum (FBS), 50µM β-mercaptoethanol (2-ME), 50µg/ml gentamycin (all purchased from invitrogen).

2.3 Vaccinia Virus Western Reserve (VV_{WR}) strain propagation

VV_{WR} (Sourced by Dr. Su-Ling Li at Brunel University, London) is a virus from the pox family of viruses (Poxviridae). Although non-pathogenic in humans, the VV_{WR} is a potent neurovirulent immunogen in mouse capable of generating specific and non-specific immune responses involving NK cells and lymphocytes. VV_{WR} was propagated in TK143 cells. TK143 cells were cultured in 10ml of DMEM in T-75 flasks. When TK143 cells were at 100% confluency, they were infected with 5µl of VV_{WR} and incubated at 37°C, 5% CO_2 for 48-72 hours to allow all cells to show full cytopathic effect. The supernatant containing viruses released from these lysed cells was then collected and used to infect another fully confluent TK143 cells in T-150 flask. These cells were incubated at 37°C, 5% $C0_2$ until all cells were fully lysed. Cells were then harvested using a cell scraper and collected into a 15ml tube in 5ml of DMEM medium. To liberate the virus, these fully lysed TK143 cells were freeze-thawed in liquid nitrogen (Freeze) and 37°C water bath (thaw) 3 times for 5 minutes each. This causes the cell membrane to become perforated and the cells burst open. Viral particles were then collected by high speed centrifugation at 1300rpm (300xg) for 5 minutes. Freshly prepared VV_{WR} were then quantified by infecting TK143 cells with newly propagated VV_{WR} using a pre-titred VV_{WR} with known viral titre as standard. Virus was stored in liquid nitrogen until ready for use

2.4 Virus Infection

Baseline weights of sex, age and weight-matched WT and $Egr2^{-/-}Egr3^{-/-}$ mice were determined on the day of the infection. Mice were then given an intranasal (I.N) injection of sub lethal dose of 2 X 10^5 plaque forming unit (PFU) of VV_{WR}. VV_{WR} Infected mice were observed and weighed daily to assess clinical pathology. Percentage decrease in weights from day 0 (baseline) was determined using the formulae below:

$$\frac{\text{Weight on day 0 – weight at day x}}{\text{Weight on day (0)}} \quad X \quad 100$$

Mice were also observed daily for signs of dehydration, lethargy, fur appearance and agility. Mice were sacrificed between 8 to 70 days post-infection with VV_{WR}. Serum, spleen, lymph nodes and lungs were harvested from culled mice for further analysis.

2.5 Virus plaque assay

Pathogen clearance of VV_{WR} in $Egr2^{-/-}Egr3^{-/-}$ mice was determined by viral plaque assay. Harvested lung tissues from infected and uninfected WT and $Egr2^{-/-}$ $Egr3^{-/-}$ mice were weighed and then homogenised. Supernatant from lung tissue was diluted in DMEM to give a standardised concentration of 200mg/ml lung tissue. Using a 10-fold dilution; serial dilution of lung homogenates were made for fully confluent TK143 cells seeded in 24 well co-star plates (Corning Inc., Corning, NY). These cells were then infected with supernatant from lung tissue and then incubated at 37°C, 5% $C0_2$. After 48 hours, cells were fixed with formalin and stained with 2% crystal violet in 40% methanol. Plaques were counted under a light microscope.

2.6 Plaque Reduction Neutralization Tests (PRNT)

Sera from infected and uninfected WT and $Egr2^{-/-}Egr3^{-/-}$ mice were serially diluted in DMEM medium using a dilution factor of 1/10. Serially diluted sera were then co-incubated with an equal volume of pre-determined TCID50 of 2 x 10^4

PFU/ml for VV_{WR} and allowed to incubate overnight at 37°C, 5% CO_2. The next day, TK143 cells already seeded into a 24-well Co-star plate (Corning Inc., Corning, NY) and rinsed in serum-free medium. 100µl of virus-serum mixture was added to each well in duplicate and left to adsorb for 60 minutes at 37°C with periodic swirling. Following this, wells were washed with serum free medium and then normal growth medium was added and incubated for 48 hours at 37°C, 5% CO_2 to allow plaques to form. After 48 hours, cells were fixed and stained with 0.1% crystal violet in 20% ethanol, and the plaques were quantified over a light microscope.

2.7 Protein immunisation

Baseline weights of sex, age and weight-matched WT and $Egr2^{-/-}Egr3^{-/-}$ mice were determined on the day of immunisation. For protein immunisation, 250ug of OVA protein emulsified in complete Freund's adjuvant (CFA) was administered by intraperitoneal (I.P) injection. OVA immunised mice showed no clinical pathology. Mice were also observed daily for dehydration, lethargy, fur appearance and agility. Serum was collected from mice by bleeding from the tail every 7 days from day 0 through day 54 post OVA protein immunisation. Serum, spleen and lungs were harvested from culled mice for further analysis.

2.8 Enzyme linked immunosorbent assay (ELISA) for antibody isotyping

Sandwich ELISA for antibody isotype was performed using antibody isotyping Kit (Sigma Aldrich) according to manufacturer's instructions. Briefly, 96 well plates (Nunc, MaxiSorb) were coated with IgG1, IgG2a, IgG2b, IgG3, IgM and IgA antibodies diluted in carbonate-bicarbonate coating buffer (Sigma) and incubated overnight at 4°C. The following day, unbound antibodies were washed off the plates 5 times for 5 minutes each using 0.05% Tween 20 in 1X PBS. Plates were then blocked in 10% FBS diluted in 1x PBS for 1 hour at room temperature to minimise unspecific binding and reduce background noise. Following blocking, Plates were washed again and 1/10000 dilution of sera from WT or $Egr2^{-/-}Egr3^{-/-}$ mice were added.

For anti-OVA ELISA, plates were coated with 5ug/ml OVA protein diluted in carbonate-bicarbonate coating buffer (Sigma) and left to incubate overnight at 4°C.the next days, plates were washed in 0.05% Tween 20 in 1X PBS and blocked using 10% FBS diluted in 1x PBS for 1 hour at room temperature. Following blocking, a pre-determined 1/1000 dilution of sera form OVA immunised mice or unimmunised mice for control were then added to the plates and left to incubate at RT for 1 hour. For antibody secreting cell polarisation assay, supernatant from cell culture were added to the wells at this stage. Samples were then allowed to incubate at room temperature for 1 hour.

Following incubation of serum, samples were washed off and wells were incubated with HRP-conjugated rabbit anti-mouse immunoglobulins for 1 hour at room temperature. The wells were then washed and HRP substrate, 3,3', 5,5'-Tetramethylbenzidine (TMB) solution (eBioscience) was added into each well to develop the detection enzyme. Plates were subsequently read on a photometer at 450nm wavelength and optical density (OD) obtained.

2.9 Tissue preparation for histological analysis

Fresh tissue samples were collected from sacrificed mice and placed in 1x PBS. For paraffin embedded sections, tissues were fixed immediately in freshly prepared 4% paraformaldehyde (PFA) at 3:1 w/v of tissue sample to fixative. This is done in order to preserve the tissue. Fixation with 4% PFA crosslinks proteins present within the tissue by covalent bonds. This prevents enzymatic degradation of the basic tissue component. Following overnight fixation at 4˚C, 4% PFA was replaced with 70% ethanol to wash out fixative. Tissues sections were then placed in embedding cassettes and passed through a process of dehydration, clearing and paraffin wax infiltration in a Shandon hypercenter XP tissue processor (table 2.2). Ascending concentrations of alcohol progressively dehydrates the tissue while xylene enables paraffin wax to permeate the tissues and also improve optics for the imaging of tissues. Following embedding, tissue samples were transferred into moulds which was then infiltrated with melted wax to form a cassette block that allows for easy handling and cutting on the microtome. Moulded sections are stored in a -20˚C freezer. 5µm-thick tissue sections are cut using a microtome. Sections were expanded at 45˚C in a water bath, collected and allowed to adhere to slides by heating at 65˚C for about 1 minute.

For frozen sections, tissue samples were snap-frozen in liquid nitrogen. They were embedded in Tissue-Tek™ CRYO-OCT Compound (Thermo scientific) prior to sectioning on a cryostat. 8µm-thick sections were cut and left to air dry for about 30 minutes. This was then followed by fixation in 1:1 acetone: methanol solution at -20˚C for 10 minutes. Sections were allowed to air dry for another 10 minutes before storing in -80˚C freezers.

Program	Immersion Time	Drain time
70% Alcohol	2 hours	15 seconds
90% Alcohol	2 hours	15 seconds
95% Alcohol	2 hours	15 seconds
95% Alcohol	2 hours	15 seconds
95% Alcohol	2 hours	15 seconds
95% Alcohol	2 hours	30 seconds
Xylene	2 hours	15 seconds
Xylene	2 hours	30 seconds
Wax	2 hours	1 minute
Wax	2 hours	2 minutes

Table 2.2: Program used for processing of tissue sections for H and E staining

2.10 Haematoxylin and Eosin (H and E) staining

H and E staining was done on paraffin embedded tissue sections in order to investigate morphology changes within these tissues (Fischer *et al.*, 2008). Haematoxylin stains the chromatin of the nucleus blue while Eosin provides a contrast stain by staining the cytoplasm and other organelles reddish - pink. The procedure for H and E staining involved de-paraffin of tissue sections in xylene for a total of 15 minutes. The tissue sections were then rehydrated in solutions of descending alcohol concentration for 3 minutes each in the following order: 100% X2 and 95% X 2. Following rehydration, slides were then dunked in distilled water before being incubated for 6 minutes in filtered acidified Harris haematoxylin solution. Slides were rinsed under running tap water and then bleached by dipping in acid alcohol. The bluing step was done by incubating sections in 0.2% ammonium solution for 40 seconds. This was followed by counterstaining using Eosin dye for 45 seconds. After Eosin treatment, tissue sections are then dehydrated in ascending concentrations of alcohol and then cleared in xylene before mounting using a xylene based mounting agent, Histomount (National Diagnostics, HS-103). Stained sections were then allowed to dry overnight. Analysis of sections was done using a light microscope (Axioscope). Tissue sections were examined in a blind manner.

2.11 Immunofluorescence

For paraffin sections, tissue sections were de-paraffined by heating at 65°C for 15 minutes followed by xylene treatment for another 15 minutes. Paraffin sections were then rehydrated in descending concentrations of alcohol (100%, 90% and 70%). Antigen retrieval was done by incubating sections in 10mM Tris/1mM EDTA, pH 9.0 for 20 minutes at sub boiling temperatures. This was done in order to release crosslinks formed by fixative and thus expose epitopes for maximal antibody-antigen binding. Following antigen retrieval, sections were blocked for 1 hour at room temperature in 10% normal goat serum (Invitrogen,10379332) or 0.1g skimmed milk in 0.1% Triton X (Sigma,T-9284) diluted in 1X PBS. Primary antibodies were then added and incubated overnight at 4°C. The next day, excess unbound antibodies were washed off thrice in 1X PBS for 10 minutes each. Fluorochrome conjugated secondary antibody was then added to the sections and allowed to incubate for 1 hour at room temperature. This was followed by a wash step done 3 times in 1X PBS for 10 minutes each. Sections were then allowed to air dry and mounted using Vectashield (Vector laboratories, H-1200) which also counter stains the nucleus with DAPI. All antibody used for Immunofluorescence of paraffin embedded sections are listed in table 2.3.

Antibody	Company information	Dilution
Rabbit anti-Human/mouse CD3	DAKO, A0452	1/100
Rat anti-Mouse CD45R/B220	BD BIOSCIENCES, 550286	1/20
PNA-Biotin	Sigma Aldrich, L1635	1/100
Extravidin Tritc	Sigma Aldrich, E3011	1/100
Goat anti Rabbit Alexa Fluor 594	Invitrogen A11037	1/400
Goat anti Rat IgG FITC	Sigma Aldrich, F6258	1/200

Table 2.3: List of antibodies used for Immunofluorescence staining of paraffin sections

For frozen sections, tissue sections were incubated for 1 hour at room temperature with directly labeled primary antibodies for CD4-FITC (eBiosciences, 11-0042-86), Alexafluor-647 conjugated IgD (Biolegend, 405708) and PNA TRITC

(Sigma Aldrich, L3766) or PNA-Biotin (Sigma Aldrich, L1635) that was detected using Extravidin TRITC (Sigma Aldrich, E3011). Excess unbound antibodies were washed off thrice in 1X PBS before being left to air dry. Tissue sections were mounted using Vectashield (Vector laboratories, H-1200).

Analysis of all sections from Immunofluorescence staining was done using a Zeiss fluorescence microscope equipped with a CCD camera and the Smart Capture software (Digital Scientific Cambridge, UK).

2.12 Cell Isolation

Secondary lymphoid organs were harvested from mice and homogenized to acquire single cell suspensions. Cells were washed in 1X PBS and collected by centrifugation at 1300rpm (300xg) for 5 minutes at room temperature. Red blood cells (RBC) were depleted by incubating cells in 0.8% ammonium chloride solution at 37°C for 5 minutes. This causes lysis of the RBCs while maintaining the integrity of the white blood cell (WBC) population. The reaction was neutralized by the addition of 1x PBS and cells were then collected by centrifugation at 1300rpm (300xg) for 5 minutes. $CD4^+$ or $CD19^+$ cells were isolated using positive selection on Magnetic Activated Cell Sorting (MACS) kit (Miltenyi Biotec) according to the manufacturer's instructions which are as follows; cells were suspended in beads buffer (PBS + 0.5% RPMI medium) and incubated with magnetic beads conjugated anti-CD4 (clone L3T4) (Miltenyi Biotec,130-049-201) or anti-CD19 (Miltenyi Biotec, 130-052-201) antibodies at 4°C for 20 minutes with periodic agitation. Unlabeled beads were then washed and centrifuged at 1300rpm (300xg) for 5 minutes at room temperature. The cell pellet was then resuspended in beads buffer and passed through a moist LS+ magnetic column (Miltenyi Biotech, 130-042-401). Negative cells flow through the column while magnetic beads bound cells remain within the column, which was then washed. To elute cells, column was removed from magnetic field and passed through fully supplemented Roswell Park Memorial Institute (RPMI) 1640 medium. $CD4^+$ and $CD19^+$ cells were maintained in a humidified incubator at 37°C in 5% CO_2 and grown in RPMI 1640 medium

supplemented with 300mg/L L-glutamine, 10% fetal bovine serum (FBS), 50μM β-mercaptoethanol (2-ME), and 50μg/ml gentamycin (all from Invitrogen).

2.13 Flow cytometry

Flow cytometry is a technique used for the measurement of the physical and chemical properties of a cell including its size, density and complexity based on intensity of signals. The cells are passed through a fluidics system to a laser, which sends the signals to the appropriate detectors. These signals are then translated electronically so that the end user may be able to interpret their experimental data. This is done by the use of antibodies bearing fluorochromes that are able to attach to specific epitopes on the cells of interest and allow the emission of light signals. The antibodies used for this study were usually directly conjugated to a fluorochrome. Single cell suspensions were obtained after homogenising of tissue and isolation of cells of interest as described above (section 2.12). 2 x 10^6 cells were fluorescently labelled with antibodies and then incubated in the dark for 20 minutes at room temperature. This step stains the surface markers and was used for cell surface staining of receptors. Antibodies used for surface staining include Fluorescein isothiocyanate (FITC)-conjugated antibodies to B220, PD-1, GL7 and CD4; eFluor 450 labeled antibody to CD4; phycoerythrin (PE)-conjugated antibodies to CD4, CD19, CD25, CD62L, CD69, IgM; PerCP labeled antibody to CXCR5 and B220; PE-cy7 labeled antibody to B220; and allophycocyanin (APC)-conjugated antibodies to CD44 all obtained from eBioscience. GL7 binds to a neuraminic acid derivative called α2-6-sialyl-lacNac and is commonly used as a marker for germinal centre B cells. Following surface staining, samples were fixed in fixation/permeabilisation (fix/perm) buffer (eBiosciences) for 20 minutes in the dark. Fix/perm buffer was washed away using 1X PBS and was then followed by addition of permeabilisation (perm) buffer to the samples which were incubated at room temperature for 20 minutes in the dark. Perm buffer was then washed off using wash buffer and fluorescently labelled antibodies to nuclear proteins were added. Samples were incubated for 20 minutes in the dark at room temperature. Antibodies used for nuclear staining include allophycocyanin (APC)-conjugated antibodies to Egr2 and

phycoerythrin (PE)-conjugated antibodies to Egr2 and Bcl6 all obtained from eBioscience. Following nuclear staining, samples were resuspended in PBS and passed through the flow cytometer (Canto II, BD Immunocytometry systems). Data obtained from the flow cytometer were analysed using FlowJo software (Treestar Inc.). Cell sorting was performed on a FACSAria sorter with DIVA option (BD Immunocytometry Systems). Professor Ping Wang at Queen Mary University, London, performed flow cytometer runs and cell sorting.

2.13.1 Intracellular cytokine staining

For intracellular cytokine staining, isolated $CD4^+$ cells were stimulated with 0.2ug/ml PMA and 0.5ug/ml Ionomycin in the presence of golgi stopper to inhibit cytokine transport to the cell surface. The cells were then incubated overnight at 37°c, 5% CO_2. Cells were collected the next day and washed in PBS. Intracellular cytokine staining was performed using the Fixation & Permeabilisation Kit from eBioscience according to the manufacturer's protocol. Antibodies specific for cytokines of interest were added to cells and cells were allowed to incubate for 20 minutes at room temperature in the dark. Cells were then either resuspended in PBS and run on flow cytometer or fixed for nuclear staining step as described above (2.13).

2.14 *Invitro* polarization assay

2.14.1 Tfh Polarisation assay

24 well co-star plates (Corning Inc., Corning, NY) were coated with anti-CD3 at 1µg/ml and anti-CD28 (2µg/ml) antibodies for 1 hour at 37°C. Plates were washed with 1X PBS and then 2 x 10^6 $CD4^+$ T cells were seeded into plates for stimulation. For generation of Tfh-like cells, 50ng/ml recombinant IL21, 10µg/ml anti-IFNγ, 10µg/ml anti-IL4 and 20µg/ml anti-TGF-β (1D11) all from R & D systems were added to growth medium. For Th0 conditions, neither cytokine stimuli nor neutralizing antibodies were added. Cells were then incubated at 37°C, 5% CO_2. After 4 days, all cells were collected for flow cytometry and RT-PCR analysis.

2.14.2 Antibody-secreting cell (ASC) Polarisation assay

25 x 10^6 $CD19^+$ positive cells were seeded into 6 well co-star plates (Corning Inc., Corning, NY). Cells were then stimulated using either 1µg/ml anti-CD40 (eBiosciences), 10ng/ml recombinant IL4 (rIL4) (eBiosciences) or 20µg/ml Lipopolysaccharide (LPS) (Sigma-Aldrich) and rIL4 10ng/ml to generate antibody-secreting cells. For control, one group of cells had no cytokine treatment, mitogenic stimulation or CD40 agonist. Cells were then incubated at 37°C, 5% CO_2. After 4 days, cells were collected for RT-PCR analysis while supernatant was collected for antibody isotyping.

2.15 Lentiviral Transduction

Lentiviruses are of the virus family retroviridae and are capable of integrating their genetic material into host's genome. Hence they are widely used as an effective vector system to deliver genetic material into a host. Lentiviral expression utilizes a system whereby viral genes 5'-gag- pol-env-3' are manipulated for the effective transmission of genetic materials. These integrate into the genome of the host without resulting in the production of infectious viral particles within this host (Swift *et al.*, 2001; Pear *et al.*, 1993). Dr. Tizong Miao at Bart's and the London School of Dentistry and Medicine, Queen Mary University of London, carried out generation of lentivirus.

2.15.1 Gene isolation and cloning

Open reading frame (OPF) containing mouse cDNA sequence for Egr2 and Bcl6 were tagged with flag protein (Plasmid construct and cDNA sequence were obtained by Dr Tizong Miao, Barts and the London School of Dentistry and Medicine, Queen Mary University of London). These were then amplified by PCR using Platinum *Pfx* DNA Polymerase kit (Invitrogen, Life Technologies) according to the manufacturer's instructions. The PCR step involved the denaturing, annealing and extension steps which were repeated for between 30-35 cycles. The primers used for Egr2 cloning were sense 5'-

ACTCAGATCTCGAGGCCACCATGGACTACAAAGACGATGACGACAAGACCGCCAAGGCCG TAGAC -3' and anti-sense 5'- AGCTAGCTAGCGAGAATTCCTACAATTCCGG -3', while those for Bcl6 were sense 5'- AAGCTGGCTAGCGCCGCCATGGCCTCCCCGGCTGAC -3' and anti-sense 5'- AGGGGCGGATCCTCAGCAGGCTTTGGGGAGC -3'.

2.15.2 Restriction digestion and ligation of gene and plasmid vector

The cloned cDNA and plasmid vector (pcDNA3.11(-)-IRES EGFP.gb) were then digested with appropriate restriction enzymes. In order to limit self-ligation, linearized plasmid DNA were treated with calf intestine phospahatase (CIP) for 10 minutes at 37˚C. This causes dephosphorylation of 5' and 3' ends of nucleic acids to prevent re-ligation. Digests were run on 0.8% agarose gel and gels were then purified using the QIAquick gel extraction kit (Qiagen) protocol. Purified cDNA and plasmid were then ligated for 2 hours at room temperature.

2.15.3 Transformation of bacteria cells and purification of plasmid DNA

Transformation involved freeze-thawing of bacteria cells to create perforated and tensed cell membrane to allow bacteria take up ligated plasmid DNA containing the gene of interest. 10-beta Competent E. coli (C3019) cells (New England Biolabs) were used for bacteria transformation. Transformation was done by incubating bacteria-Plasmid mixture on ice for 5 minutes, followed by heating at 42°C for 30 seconds and then placed on ice again for a further 5 minutes. The cells were then treated with Super Optimal broth with Catabolite repression (SOCS) medium (Sigma Aldrich) at 37°C for 1 hour at 225rpm. SOCS medium is a nutrient rich growth medium that improves the transformation efficiency. The transformed bacteria cells were then spread on Luria-Bertani (LB) solid Agar plate (10g/L NaCl, 10g/L Tryptone, 5g/L Yeast Extract, 2g/L Agar) supplemented with ampicillin antibiotic at 100µg/µl for antibiotic growth selection. Bacteria were then incubated overnight at 37˚C to grow. Positive bacteria colonies where isolated from agar plate and grown overnight in a small volume of LB broth (10g/L NaCl, 10g/L Tryptone, 5g/L Yeast Extract) supplemented with ampicillin antibiotic. This was incubated at

37°C overnight while shaking. Plasmids were then extracted from selected positive bacteria using the mini-prep kit from Qiagen according to the manufacturer's instruction. Briefly, buffers that lyse the bacteria wall resulting in the release of plasmids were added (buffers P1 and P2). Plasmid DNA and protein is then precipitated in N3 buffer. The lysate was then passed through a silica-gel membrane, coated with PB buffer which binds DNA as it pass through column. The column was then washed with a PE buffer to discard contaminants and plasmid DNA is eluded with EB buffer. Concentration of plasmid DNA was measured using a Nano-drop. Plasmids were sent for sequencing to confirm successful gene incorporation and that the orientation and position of the insertion was correct. Sequencing was done by SourceBioScience.

2.15.4 Transfection of plasmid into lentivirus

Egr2 and Bcl6 containing plasmids were assembled between two long terminal repeats (LTR) regions containing viral genes 5'-gag-pol-env-3' within the lentivirus (P^{RRL}-MCS+gb). Empty lentivirus was used as a negative control. Lentiviral construct was then transfected into packaging cell lines using lipofectamin plus reagent (Gibco). Once the lentivirus vector is inside the cell, it starts to transcribe viral RNA *gag*, *pol*, and *env* along with ψ. This helps it to assemble virions that bud off the cell. The virions, now in the supernatant were then collected by ultracentrifugation and then quantified to 10^9 transducing unit per ml. As Egr2 and Bcl6 expression constructs were labelled with IRES GFP, Egr2 OR Bcl6 expressing cells were isolated by fluorescence activated cell sorting.

For transfection into $Egr2^{-/-}Egr3^{-/-}$ CD4 cells, 1 x 10^6 CD4 cells from $Egr2^{-/-}Egr3^{-/-}$ mice were seeded into each well of a 24 well plate and stimulated with plate bound anti-CD3 and anti-CD28. They were then infected with concentrated lentivirus at a multiplicity of infection (MOI) of 50–100 (~10^5–10^6 transducing units/ng of p24). The infected cells were incubated at 37°C for 7 hours while gently shaking before addition of growth medium. Positive cells were sorted after 24 hours using GFP to select.

2.16 Adoptive transfer experiments

Adoptive transfer experiments were all carried out by Dr Su-Ling Li and Professor Ping Wang at the animal facility in Brunel University. Briefly 2 x 10^6 $CD4^+$ T or B cells were isolated from 4-week old wild type mice and were then suspended in 100µl of physiological saline. This was then injected intravenously into the tail vein of 6 week old $Egr2^{-/-}Egr3^{-/-}$ mice. 24 hours after adoptive transfer of cells, the recipient mice were infected with VV_{WR} intranasally and samples collected for analysis 14 days after infection.

For $RAG^{-/-}$ adoptive transfer experiments, 2.5 x 10^6 WT $CD4^+$ T and 2.5 x 10^6 B cells from $Egr2^{-/-}Egr3^{-/-}$ mice, or 2.5 x 10^6 WT B cells and 2.5 x 10^6 CD4 cells from $Egr2^{-/-}Egr3^{-/-}$ mice were suspended in 100µl of physiological saline. This was then injected intraveneously into the tail vein of $RAG^{-/-}$ mice. Forty days following adoptive transfer, $RAG^{-/-}$ mice were infected intranasally with VV_{WR} as earlier described.

For adoptive transfer of Egr2 and Bcl6 expression construct into $Egr2^{-/-}Egr3^{-/-}$ mice, 10^6 transduced $Egr2^{-/-}$ $Egr3^{-/-}$ $CD4^+$ T cells were suspended in 100ul of physiological saline which was then injected intravenously into the dorsal tail vein of CD2-$Egr2^{-/-}Egr3^{-/-}$ mice. 24 hours after transfer, mice were infected with 2 x 10^5 PFU of vaccinia virus. Analyses of Tfh and GC B cells differentiation as well as GC formation were done 14 days post-infection.

2.17 RNA extraction

Extraction of total RNA from cells was done using Trizol reagent (Invitrogen). Trizol contains phenol and guanidine isothiocyanate, which denatures proteins like RNAse and is capable of separating RNA, DNA and proteins from cells (Chomczynski, 1993; Chomczynski and Sacchi, 1987). RNA extraction was performed according to the manufacturer's protocol. Briefly, cells were collected in a pellet and then lysed in 1ml of Trizol reagent per 10 x 10^6 cells. This was then allowed to incubate at room temperature for 10 minutes. 300µl of chloroform was next added to the samples after which the samples are vortexed thoroughly. Samples were then incubated for 5 minutes after which they were centrifuged at 13000 rpm (300xg)

for 15 minutes at 4°C. The chloroform increases the efficiency of separation by pushing organics to the bottom layer and generating a clearer aqueous phase. After centrifugation, the sample separates into a lower red organic phase, interphase and a colourless aqueous upper phase. The RNA which is contained in the aqueous phase was transferred into a separate tube and precipitated with equal volume of isopropanol. This is because nucleic acids like RNA are insoluble in alcohol and so can be precipitated from the solution. 2µl of glycogen is added as a co-precipitant to ensure maximum RNA recovery. Samples were then incubated at −20°C for 1 hour and then centrifuged at 13000rpm (300xg) for 10minutes at 4°C. RNA pellet formed at the bottom of the tube was then washed in 80% ethanol and re-suspended in RNAse free water and its concentration measured using a Nano-Drop.

2.18 cDNA Synthesis

Isolated RNA was transcribed into cDNA using SuperScriptTM III (Invitrogen). This is a reverse transcriptase enzyme with high thermostability. Reverse transcriptase catalyses the production of new complimentary strands of DNA using isolated RNA as a template. mRNA concentration was normalised to 2ug per sample for cDNA synthesis. Denaturing step was first done with mRNA and 0.5µg oligoDT or random primers which were heated to 70°C for 5 minutes in order to denature secondary structures associated with the RNA and also to optimise binding of nucleotides. OligoDT or random primers have nucleotides that bind to mRNA poly-A tails in order to initiate transcription. Following this, 500µM each of dATP, dTTP, dCTP, dGTP, 0.01M Dithiothreitol (DDT), 2 Unit/µl of RNase Inhibitor and 10 Units/µl of SuperScriptTM Reverse Transcriptase in 1x First Strand Buffer (all Invitrogen) were all added into each tube. The reaction was mixed and heated to 25°C for 5 minutes followed by another round of heating at 50°C for 1 hour. The reaction was then inactivated by heating to 70°C for 15 minutes. The samples were then stored at -20°C for future use.

2.19 Real time PCR

The principle behind Real Time PCR is similar to that of genomic PCR. It differs

in the fact that it allows for the real time quantitative comparison of relative gene expression profiles between experimental samples or conditions against a housekeeping or endogenous gene (Pfaffl, 2001). This is done with the aid of primers and a fluorescent dye called SYBR Green. SYBR green is incorporated into the nucleic acid with each new synthesis. Following this new synthesis, the amount of new copies of a product is then calculated in real time by measuring the fluorescence (Morrison, Weis and Wittwer, 1998).

Primers were designed so as to be between 20-23 nucleotides long and have a G/C content of at least 50%. In addition to these, sense and antisense primers were designed so that they anneal to different exons on the gene of interest. These primers contain the intervening introns in addition to the identified exons thus making them larger and detectable via electrophoresis. The melting temperature (Tm) of each primer was estimated using the Wallace rule (Tm = 4 × [G + C] + 2 × [A + T]) and the annealing temperature was initially set to Tm − 2°C and altered as necessary. All primers were blasted on the NCBI primer blast software. Primers used for Real Time PCR are shown in table 2.4

Real time PCR was performed with the QuantiTect SYBR Green PCR kit (Qiagen) according to the manufacturer's instructions. Briefly, 2µl of cDNA from samples were added to sense and antisense primers of genes of interest to a final concentration of 0.2µM in 1x SYBR Green Master Mix. Real Time PCR was performed using the Rotor-Gene system (Corbett Robotics) and the following programme:

- Step 1: Denaturing of DNA and antibody bound to DNA polymerase at 95°C for 10min
- Step 2: Denaturing at 95°C for 20s
- Step 3: Annealing at 58°C for 30s
- Step 4: Extension at 72°C for 20s

Steps 2-4 were repeated for 30-40 cycles. After each cycle, the level of SYBR green was read. Melting curve analysis was performed at the end of the entire run in which the level of fluorescence intensity was measured as the temperature rises from 50°C to 99°C. As the melting temperature for a DNA molecule is reached, 50%

of that DNA is denatured to become single strand DNA. This is evident by a decrease in the level of fluorescence in the melting curve. Hence, melting curve is able to identify non-specific binding to primers and the formation of primer dimers. After PCR reaction, samples were run on a 0.8% agarose gel electrophoresis to confirm the quality and size of the product.

Gene	Primer	Sequence (5' – 3')
B-Actin	Sense	AATCGTGCGTGACATCAAAG
	Antisense	ATGCCACAGGATTCCATACC
Bcl6	Sense	GCAGTTTAGAGCCCATAAGAC
	Antisense	GCCATGATATTGCCTTCCCT
(Prdm1) Blimp1	Sense	AACCTGAAGGTCCACCTGAG
	Antisense	TGCTAAATCTCTTGTGGCAGAC
Cxcr5	Sense	TATGGATGACCTGTACAAGGA
	Antisense	AGGATGTTTCCCATCATACCC
Egr2	Sense	AGAAGGTTGTGATAGGAGGT
	Antisense	GGATGTGAGTAGTAAGGTGG
Egr3	Sense	CGACTCGGTAGCCCATTACAATCAGA
	Antisense	GAGATCGCCGCAGTTGGAATAAGGAG
Ascl2	Sense	TACTCGTCGGAGGAAAGCA
	Antisense	CTAGAAGCAGGTAGGTCCAC
SH2D1A	Sense	CCTGTAATAGCATCTCGCCTGAT
	Antisense	AGTTTTCCAATCCGCACTTTAAAG
IRF4	Sense	CAGCTCATGTGGAACCTCTG
	Antisense	TTGTTGTCTTCAAGTGGAAACCC
ICOS	Sense	CAAGAAAGGAACCTTAGTGGA
	Antisense	CACTATTAGGGTCATGCACAC
IL-21	Sense	CTCAAGCCATCAAACCCTGG
	Antisense	CATACGAATCACAGGAAGGG

Table 2.4: Primer list for RT-PCR

2.19.1 Real Time RT-PCR Data Analysis

Data obtained from RT-PCR were analysed using the Rotor-Gene Software. During each reaction, the amount of DNA molecules doubles in a phase known as the exponential phase. Eventually, the reaction reaches a plateau phase when the

reagents become limiting and increase is not as significant as the exponential phase. A threshold value was decided at the point when the reaction is still in an exponential phase and the number of cycles (Ct) necessary in order for the florescence to reach this threshold is calculated for each run. Ct values for each gene are then compared against those of reference endogenous genes (mouse β-actin or Gapdh) in order to normalise the results. The relative expression was then calculated with the ΔΔCt method using the following equation: 2^ (-(Ct target gene – Ct Housekeeping gene)) x10000 (Livak and Schmittgen, 2001; Pfaffl, 2001). For consistency and rigour of results, samples were all run in triplicates.

2.20 Microarray

Microarray is a technique that permits genome wide gene expression analysis of cells in order to investigate biological differences. $CD4^+$ T cells from wild type and $Egr2^{-/-}$ $Egr3^{-/-}$ mice were isolated as earlier described in section 2.12. Some cells were then were stimulated using anti-CD3 and anti-CD28. Total RNA was extracted from the cells and purified using RNAeasy kit RNA clean up protocol according to the manufacturer's instructions. Reverse transcription into cDNA was done using the method described in section 2.18. Purified RNA was processed in the Genome centre at Queen Mary University, London. Here, RNA from the cells were fluorescently labeled and hybridized on to Illumina MouseRef-8 v2.0 BeadChip expression arrays. These arrays are made up of polymerized oligonucleotide probes that are able to recognize over 25,000 RefSeq transcripts from mice and thus RNA bind to complimentary sequences. Following hybridization, unbound RNA is washed away and array is then scanned to visualize bound RNA. Array data were analysed by Dr. Alistair Symonds at Barts and the London School of Medicine and Dentistry, Queen Mary University of London using R software for analysis. Briefly, the data was normalized at the 'core genes' level using the robust multi-array average (RMA) method as implemented in the Bioconductor oligo package. The data was filtered to remove transcripts that had more than 50% of probe sets with detection above background p-value greater than 0.05 and transcripts with cross-hybridizing probe sets. Scatterplots were created using the ggplot2. Using the clustering algorithm

method, genes were grouped based on self-organization maps (SOMs). SOMs were then further clustered using a hierarchical-clustering programme. Microarray data are available from the ArrayExpress database (www.ebi.ac.uk/arryexpress) under ArrayExpress accession number: E-MTAB-2432. Genes of interest that are involved in inflammation and the regulation of follicular helper T cell differentiation were further validated using RT-PCR.

2.21 Protein Extraction and Quantification

To extract proteins, cells were isolated as described above (section 2.12) and washed in PBS. 100µl of CelLytic M lysis buffer (Sigma, C2978) was then added to the cells according to manufacturer's instructions. CelLytic M lysis buffer is a mild detergent that lyses the cells to liberate proteins. In addition to lysis buffer, 1mM PMSF (Phenylmethylsulfonyl fluoride) and protease and phosphatase inhibitors (Roche) were also added. The cells were then swelled in a hypotonic buffer for 15 minutes on ice. The cell samples were then centrifuged at 13000rpm (300xg) for 5 minutes at 4˚C to separate cell debris from protein. The protein, which was now in the supernatant, was collected and quantified using Bradford assay. Briefly, a standard curve of absorbance was plotted against the concentration of Bovine serum albumin (BSA) by serially diluting purified BSA from 0mg/ml to 10mg/ml. freshly prepared proteins were the measured against the BSA standard curve. Extracted proteins are then stored in -80˚C for future use. Protein extraction and quantification was done by Dr Tizong Miao at Barts and the London School of Medicine and Dentistry, Queen Mary University, London

2.22 SDS PAGE Electrophoresis and Western blotting

Extracted protein samples were incubated at 95˚C for 5 minutes in 1x LDS buffer (Invitrogen) and 2-β mercaptoethanol. This was done in order to denature the proteins. Protein samples were mixed with 5µl of Novex® Sharp Pre-stained protein standard (Invitrogen) and loaded into wells on a 12% NuPAGE® Bis-Tris Precast Gel (Invitrogen) along with 5µl of Rainbow molecular marker (Amersham). The gel was then run using Bio-Rad Western apparatus in 1X SDS-PAGE running buffer at 200V for 35 minutes at 4°C. 12% NuPAGE® Bis-Tris precast Gel is an SDS-

PAGE (Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis) based gel which is capable of separating a wide range of proteins based on molecular weights. Sodium dodecyl sulphate was used to coat the proteins in order to confer on them the negative charge from the suphate groups. This allows the protein to migrate based on their sizes and electric charge within the matrix of the polyacrylamide gel. Separated proteins on gel were then transferred to transfer buffer-immersed nitrocellulose membrane (Amersham) by placing the gel above a nitrocellulose membrane. Whatman filter paper was placed on either side of the membrane. These were all then placed in a cassette in a wet transfer system. Transfer was done for about 1.5 hours at 30V at 4˚C to allow negatively charged protein to transfer onto nitrocellulose membrane, which traps it while retaining the same alignment as in the gel.

Following transfer, the nitrocellulose 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 in order to prevent non-specific binding. Primary antibodies against Egr2 (Covance) and Egr3 (Santa Cruz) were then added on the membrane and allowed to incubate overnight at 4˚C with agitation. The following day, the membranes were washed 3 times in TBST to remove all unbound primary antibodies. Polyclonal goat anti-rabbit horseradish Peroxidase conjugated antibodies were then diluted in blocking solution and incubated with the membrane for 1 hour at room temperature. Secondary antibody enables the detection of primary antibody bound to the protein of interest. Following incubation with secondary antibody, the membrane was washed 3 times in TBST for 15 minutes each at room temperature to remove unbound secondary antibody and then transferred onto cling film. Detection was done by chemiluminescence using the ECL plus kit (Amersham) according to the manufacturer's instruction. This kit contains a reagent that is oxidised by HRP to produce a luminescent signal, which can be detected by exposing photographic film to the membrane and then subsequently developing this film. Dr. Tizong Miao did western blot as described in Li et al., 2012

2.23 Chromatin Immunoprecipitation

Chromatin Immunoprecipitation (ChIP), as a laboratory technique, allows for the investigation of interactions between proteins and regions of genomic DNA *in vivo (Carey, Peterson and Smale, 2009).* Protein is crosslinked to DNA and then precipitated out of the solution. This is followed by purification and detection of bound DNA via PCR or other methods of choice. Dr Tizong Miao at Barts and the London School of Medicine and Dentistry, Queen Mary University of London, performed chIP. Briefly, 5 x 10^7 CD4 T cells from WT and CD2-Egr2-/- Egr3-/- mice were stimulated with plate bound anti-CD3/anti-CD28 for 16 hours after which protein and DNA were crosslinked in 1% formaldehyde for 10 minutes at room temperature as described by Solomon, Larsen and Varshavsky (Solomon, Larsen and Varshavsky, 1988). Crosslinkage holds protein and DNA together and prevent dissociation caused by cell lysis. Crosslinking was stopped with 125mM glycine followed by washing of the cells in PBS. Cells were lysed in 1% SDS, 10mM EDTA, 50mM Tris pH8 and chromatins were then sonicated to shear them into around 300-1000bp using a Diagenode Bioruptor. Sonication was done on a high output setting with 30 seconds sonication separated by 30 seconds intervals for a total of 10 minutes. To verify sonication, a small volume of chromatin was reverse crosslinked by heating at 65˚C overnight and running on a gel. Sonicated chromatin was then blocked for 1 hour at 4°C using salmon sperm DNA (ssDNA)/BSA-blocked Protein A beads (Amersham) to eliminate any non-specific DNA binding. Anti-Egr2 antibody or anti-Ig (negative control), were then added to the samples and left to incubate overnight at 4˚C. The purpose of adding the antibody is to enable Egr2 antibody to bind to the Egr2 protein and any associated DNA present within the sample. The antibody-protein-DNA complex was then precipitated out using ssDNA/BSA-blocked Protein A beads from *Staphylococcus Aureus* conjugated to agarose beads. Protein A binds to the constant region (Fc) region of immunoglobulin and the insoluble agarose causes these complexes to be found in the pellet after centrifugation. Pellets were then washed in buffers of increasing ionic strengths before resuspension in elution buffer containing SDS, which denatures protein, A beads and release the immunoprecipitated chromatin

complexes. Heating at 65˚C overnight reverses Crosslinks and DNA was purified using phenol chloroform extraction and ethanol precipitation. The DNA was subsequently used as a template for PCR with specific primers which are listed in table 2.5.

Primer	**Sequence 5' – 3'**	
Bcl6 site 1 (Intron 1)	Sense	GAAGATGAACTGGATTCCTCCC
	Antisense	CCCTCAAAGCTCTTAACCGA
Bcl6 site 2 (Promoter-1)	Sense	AAAGGTGAATACAGGGCAGAC
	Antisense	GAAACAAGAGTCTCACTCATCC
Bcl6 site 3 (Downstream -1)	Sense	TGAATCACGGATGCATAAATGG
	Antisense	TGACCGACAGACATTCACAG

Table 2.5: Primer sequences used for Chromatin Immunoprecipitation

2.24 Electrophoretic Mobility Shift Assay (EMSA)

EMSA is a highly sensitive technique that can be employed to investigate the DNA binding affinity of a protein (Hellman and Fried, 2007). The principle behind EMSA is that heavier organic fragments like DNA-Protein complexes migrate through a non-denaturing gel slower and so interactions between protein-DNA can be detected. It involves a simple procedure where a consensus region of DNA is labelled with a radioisotope or a fluorescent dye (hot probe). This is then incubated with a protein of interest and then other DNA site (cold probe) are added in to this reaction to compete for protein binding (Hellman and Fried, 2007). Specificity of protein-DNA complex binding is confirmed by the addition of protein specific antibody which creates a supershift due to heavier complex formation which results in an even greater retardation of migration. EMSA was performed by Dr Tizong Miao at Barts and the London School of Medicine and Dentistry, Queen Mary University of London. For competition, 40µM of sense and antisense oligonucleotides from potential Egr2 binding sites on the Bcl6 gene locus were generated. These were added together and heated at 95°C for 5 minutes before being left to cool at room temperature. Heating at high temperatures is aimed at denaturing secondary structures associated with the oligonucleotides so as to enhance their binding to complementary sequences upon cooling. The Bcl6 oligonucleotides used are; Bcl6-1 (Chr16, 23983443-23983464) 5'-GGAGGGG**GCGGGGGAG**ACAGCT-3'; Bcl6-2 (Chr16, 23990225-23990248) 5'-TTGCC**CTCCTACTC**ATCCCTGGAT -3'; and Bcl6-3 (Chr16, 23964461-23964482) 5'-ACACAG**GAGGAGGTG**GCTGAGT -3'). The consensus probe for Egr2 binding (5' TGTAGGGGCGGGGGCGGGGTTA -3') was labelled with a fluorescent dye cyanine 5.5 (Sigma Aldrich). This consensus probe was then incubated for 40 minutes at room temperature with Egr2 nuclear extracts from CD4 T cells stimulated with anti-CD3 and anti-CD28 for 16 hours, and then re-stimulated for 30 minutes with PMA and Ionomycin in a buffer containing 10mM HEPES pH 7.5, 50mM KCl, 2.5mM MgCl2, 10mM DTT, 1µg poly (dI-dC), 10% glycerol, 0.5mM ZnCl2 and 5mM spermidine. Oligonucleotides from Bcl6 genes locus was then introduced as competition for Egr2 binding. For supershift, anti-Egr-2 antibody (eBiosciences) were added after 10

minutes of incubation and the samples were electrophoresed on 5% non-denaturing polyacrylamide gels composed of 30% Acrylamide in 1ml, 300µl of 10x TBE, 100µl of 10% APS, 20µl TEMED, 4.6ml of water to give a final concentration of 0.5x Tris Borate EDTA (TBE). The gels were scanned using an Odyssey Imager (LI-COR)

2.25 Statistical analysis

All data presented within this study were expressed as mean ±SD. All Statistical analysis for ELISA was done using two tailed Student's unpaired t-test while the frequency of cells from flow cytometry experiments were analysed using Mann-Whitney's U test. Weight loss data was analysed by two-way analysis of variance (ANOVA) using Prism software. These tests determine the level of statistical significance of differences between various groups/samples. Only p-values of <0.05 were considered to be statistically significant. Microarray data was analysed by Dr. Alistair Symonds at Barts and the London School of Medicine and Dentistry, Queen Mary University of London using R statistical software.

Chapter 3 Results

3.1 Identification of the CD2-specific Egr2$^{-/-}$ (CD2-Egr2$^{-/-}$) mice and Egr3$^{-/-}$ mice

This study was carried out using CD2-specific Egr2$^{-/-}$ (CD2-Egr2$^{-/-}$) mice and Egr3$^{-/-}$ mice.

Neonatal fatalities involving hind brain development is experienced in mice with systemic knockout (KO) of the Egr2 gene (Poirier *et al.*, 2008; Topilko *et al.*, 1994; Schneider-Maunoury *et al.*, 1993). Due to this reason, conditional KO of the Egr2 gene was created in our mice to allow for the study of the physiological functions of Egr2 in the immune system including organ pathology, Tfh differentiation and GC formation.

Conditional KO was created using the lymphocyte specific CD2 promoter (TURNER *et al.*, 2006). This promoter, a 50KDa protein, is reported to become activated in the double negative 3 (DN3) stage of thymocyte development (TURNER *et al.*, 2006). It has also been reported to be active in murine mature B cells (Kingma *et al.*, 2002; SINKORA *et al.*, 1998). CD2-specific Egr2$^{-/-}$ mice used for this study were generated within our research group as previously described (Zhu *et al.*, 2008). Briefly, using the Cre-Loxp method, mice with floxed Egr2 gene were cross bred with hCD2-Cre mice. This generates mice with a CD2 specific KO of the Egr2 gene (Zhu *et al.*, 2008). These mice were then further cross bred with Egr3$^{-/-}$ mice (Tourtellotte and Milbrandt, 1998) to establish the CD2-Egr2 $^{-/-}$ and Egr3$^{-/-}$ mice referred to in this thesis as Egr2$^{-/-}$ Egr3 $^{-/-}$ mice.

Before use in any experiment, KO mice were authenticated for the complete excision of the Egr2 and Egr3 genes by Polymerase Chain Reaction (PCR). This was done using DNA extracted from tails of the mice and primers for Cre, Loxp and Egr3 genes. Only mice homozygous for the Loxp allele and retain the presence of Cre gene and Egr3 Neomycin cassette (Neo) were used for further studies (figure 3.1.1).

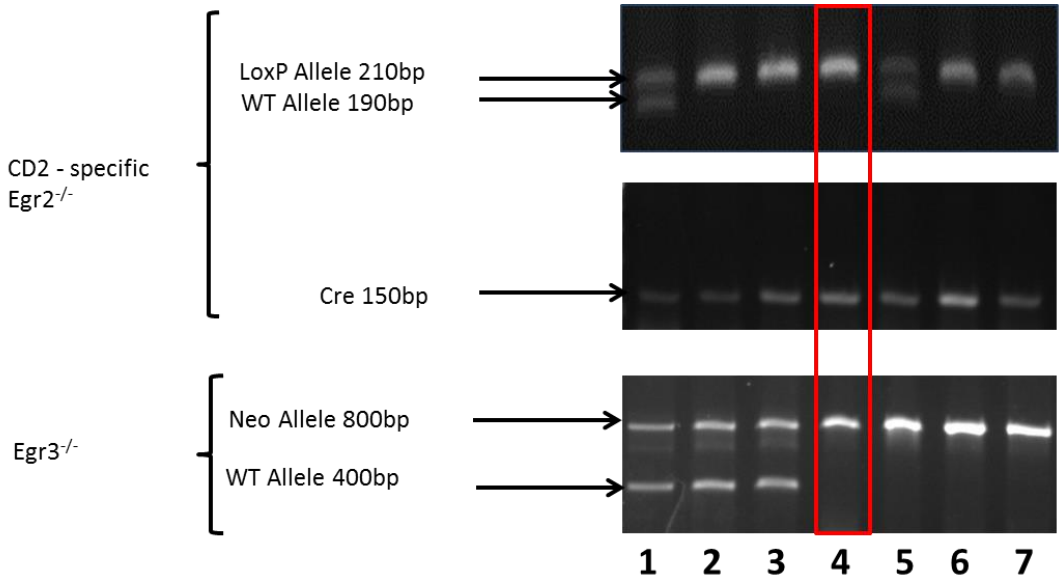

Figure 3.1.1: PCR products obtained from genotyping of CD2-specific Egr2$^{-/-}$ and Egr3$^{-/-}$. The PCR analysis of genomic DNA of transgenic Egr2$^{-/-}$ Egr3 $^{-/-}$ mice show bands for PCR products of Cre, LoxP, Egr3 and Neo alleles in transgenic mice. CD2-specific homozygous deletion of Egr2 was evident in mice expressing only LoxP homozygous bands and Cre PCR product while mice with Egr3 homozygous deletion were those that expressed the neomycin-resistant cassette that disrupts the Egr3 gene. Lane 1 shows Egr2 heterozygous and Egr3 heterozygous, lanes 2 and 3 show Egr2 homozygous and Egr3 heterozygous, lanes 4, 6 and 7 show Egr2 homozygous and Egr3 homozygous and lane 5 shows Egr2 heterozygous and Egr3 homozygous. Only mice with homozygous deletion for both Egr2 and Egr3 allele (lane 4, 6, 7 – lane 4 highlighted in red) were considered for this study.

Deletion of the Egr2 and Egr3 genes were confirmed at protein level in T and B cells by flow cytometry (figure 3.1.2 - performed by Prof. Ping Wang and Dr. Tizong Miao) and western blot (Li et al, 2012).

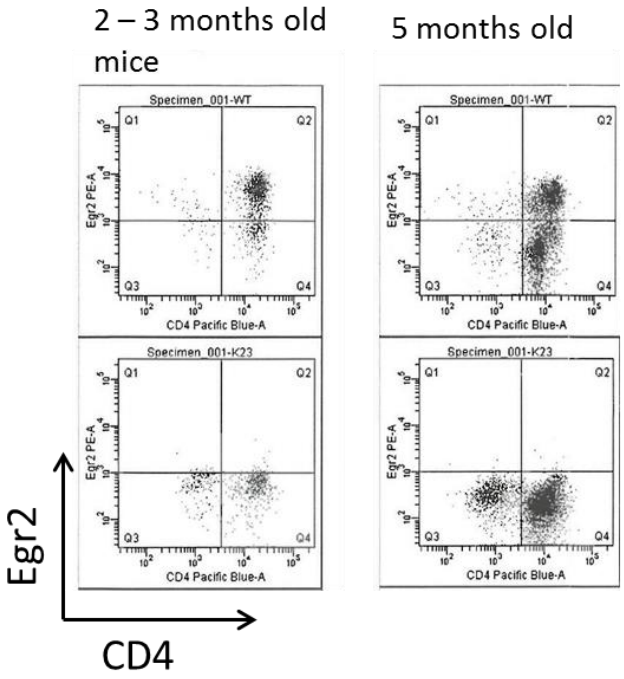

Figure 3.1.2: Absence of Egr2 protein expression in $Egr2^{-/-}Egr3^{-/-}$ mice

Flow cytometry analysis of the Egr2 genes using Egr2 specific antibody confirms the absence of Egr2 protein in CD4 T cells in $Egr2^{-/-}$ $Egr3^{-/-}$ mice. This has also been shown by Li et al (2012) who analysed the Egr2 expression in CD4 T cells and B cell by western blot (Li *et al.*, 2012)

3.2 Inflammation pathology in Egr2$^{-/-}$ Egr3 $^{-/-}$ mice

3.2.1 Histological analysis of Inflammation pathology in Egr2$^{-/-}$ Egr3$^{-/-}$ mice

We have previously reported that Egr2$^{-/-}$ Egr3$^{-/-}$ mice present with chronic inflammation evident by the high levels of serum pro-inflammatory cytokines including GM-CSF, IFNγ, IL-17 and IL-6(Miao *et al.*, 2013; Li *et al.*, 2012). Li et al., 2012 were further able to demonstrate that Egr2 and Egr3 genes control inflammation by the regulation of the suppressors of cytokine signalling (SOCS) 1 and 3 genes. SOCS 1 and 3 are inhibitors of the activity of STAT1 and STAT3. Our group has also previously elaborated how the absence of Egr2 and Egr3 genes posits the rapid development of inflammatory autoimmune syndrome with high levels of serum immunoglobulins (Ig) and lymphoid infiltrates into numerous organs (Li *et al.*, 2012). Although, the development of autoimmunity in Egr2$^{-/-}$ Egr3$^{-/-}$ mice model has already been well established (Li et al., 2012), the link between inflammation and the progressive pathology in the Egr2$^{-/-}$ Egr3$^{-/-}$ mice is yet to be investigated. Therefore, I sought to investigate the correlation between chronic inflammation and the development of systemic inflammation pathology in the Egr2$^{-/-}$ Egr3$^{-/-}$ mice.

To achieve this, morphological analysis of multiple organs including the spleen, lung, liver and kidney from Egr2$^{-/-}$ Egr3 $^{-/-}$ mice was carried out at different ages. Histological analysis showed that young Egr2$^{-/-}$ Egr3$^{-/-}$ mice of about 1 month old did not display any signs of inflammation pathology and the morphology of their organs was very similar to their WT counterpart (figure 3.2.1). However, at 2 months of age, Egr2$^{-/-}$ Egr3$^{-/-}$ mice started to show the presence of lymphocyte infiltrates in the liver (figure 3.2.2). Using macrophage marker CD11b, early organ pathology with a change in cellularity and loss of splenic architecture was observed in Egr2$^{-/-}$ Egr3 $^{-/-}$ mice from this age (figure 3.2.5). This was consistent with previous publications from our group where we reported signs of chronic inflammation and high pro-inflammatory cytokines from this age (Miao *et al.*, 2013; Li *et al.*, 2012). From 3 months of age, other signs of chronic inflammation pathology including increased damage to tissues were observed. There were also changes in the splenic architecture including splenomegaly which was accompanied by the disintegration

of the white pulp area. The liver of 3 - 4 months old Egr2$^{-/-}$ Egr3 $^{-/-}$ mice showed the presence of infiltrating lymphocytes especially around the blood vessels. The alveoli of the lungs were reduced and septa generally showed enlargement and increased cellularity suggesting prolonged inflammatory response (see figure 3.2.3). Lymphocyte infiltration was more remarkable in aged Egr2$^{-/-}$ Egr3$^{-/-}$ mice (figure 3.2.4). After 5 months of age, there was severe white pulp fragmentation as seen by CD11b staining (figure 3.2.5). There were no longer distinctions between white pulp and red pulp areas (figure 3.2.5). Chronic inflammation often results in organ pathology and this may have eventually led to the total splenic derangement and increased cellularity observed in the glomerulus of the kidney in aged Egr2$^{-/-}$ Egr3 $^{-/-}$ mice as well as the massive lymphocyte infiltration into the kidney, liver and lungs of these mice (figure 3.2.4).

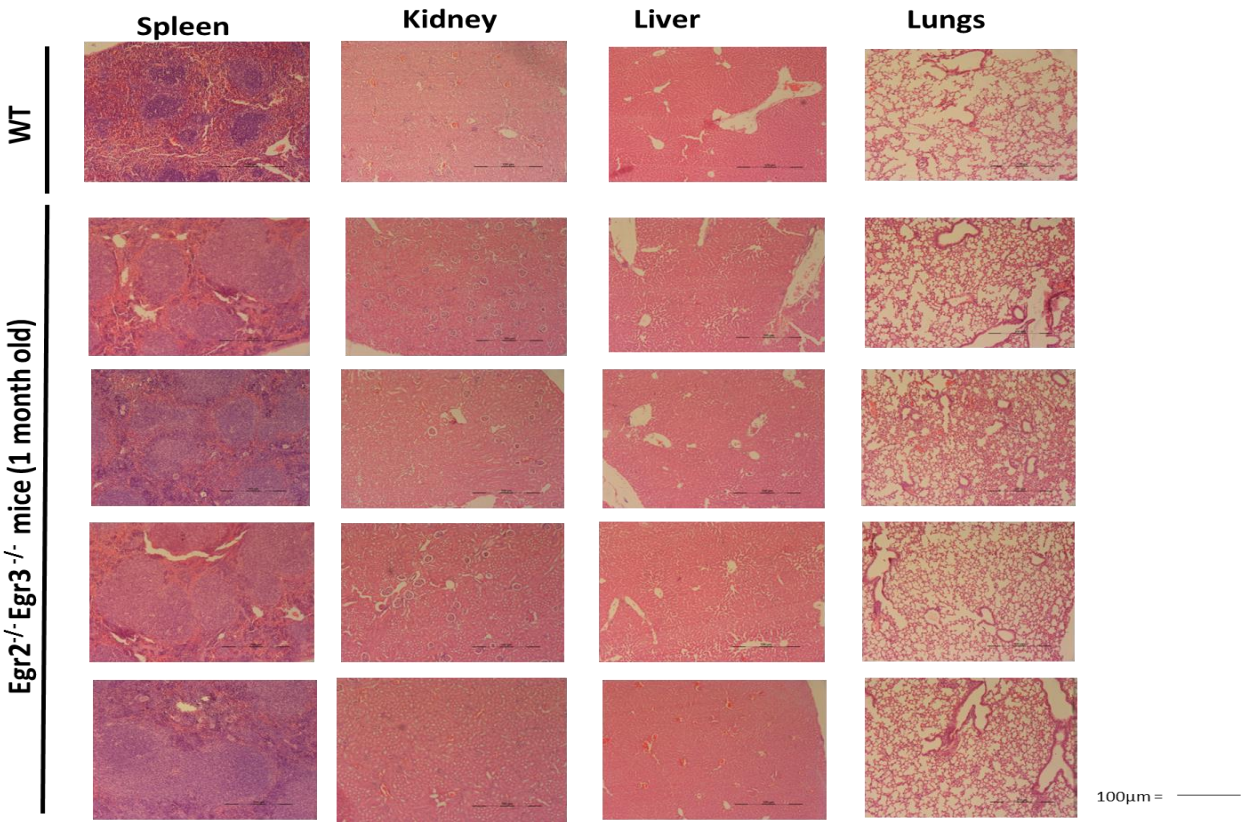

Figure 3.2.1: Organ architecture is preserved in 1 months old Egr2$^{-/-}$ Egr3 $^{-/-}$ mice and it is comparable with WT mice. Heamatoxylin and eosin staining performed on tissue sections from mice that were 1 month old showing an absence of severe pathology in these young Egr2$^{-/-}$ Egr3 $^{-/-}$ mice when compared with WT counterparts. Image taken using Axioscope X10 objective and data was collected from 4 representative mice.

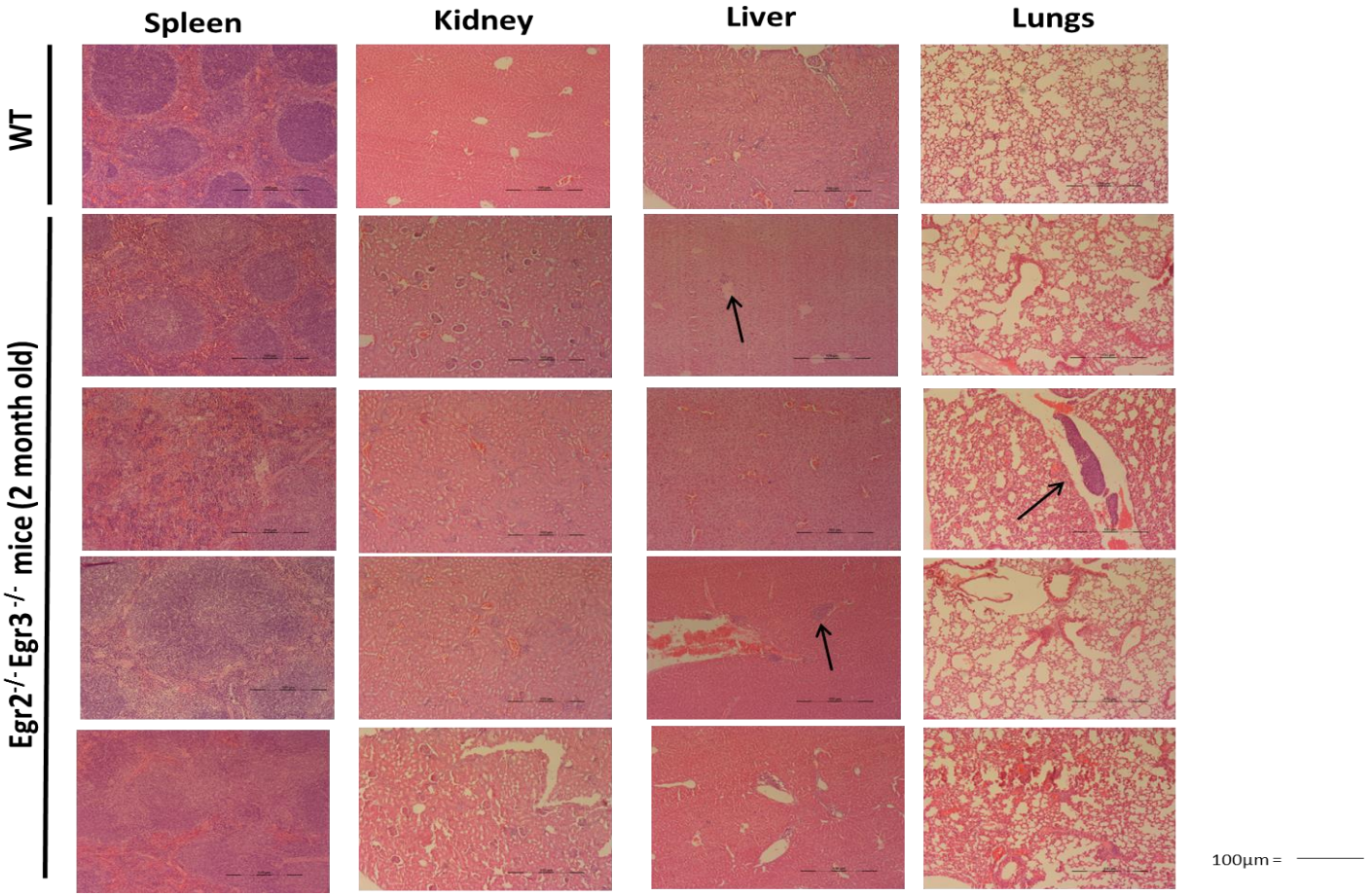

Figure 3.2.2: Haematoxylin and eosin staining of tissue sections from 2 months old Egr2$^{-/-}$ Egr3 $^{-/-}$ mice shows signs of early inflammation pathology with lymphocyte infiltrate in the liver and lungs. Image taken using Axioscope X10 objective and data was collected from 4 representative mice

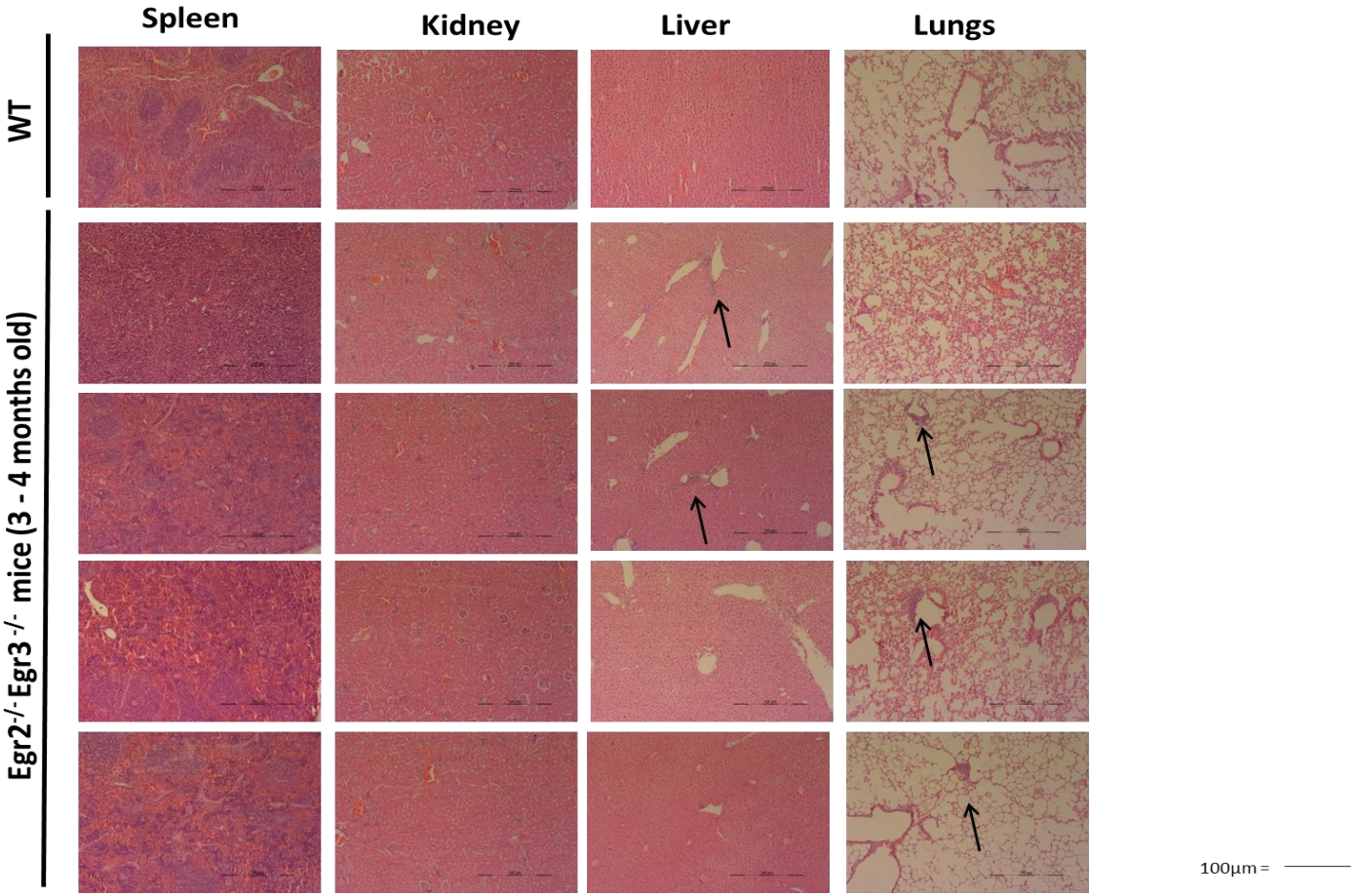

Figure 3.2.3: Analysis of tissue sections using haematoxylin and eosin staining show the presence of lymphocytic infiltrates into the kidney, liver and lungs of 3-4 months old Egr2$^{-/-}$ Egr3 $^{-/-}$ mice. Infiltrations were also accompanied by moderate changes in organ architecture in the lungs and kidney. Image taken using Axioscope X10 objective and data was collected from 4 representative mice

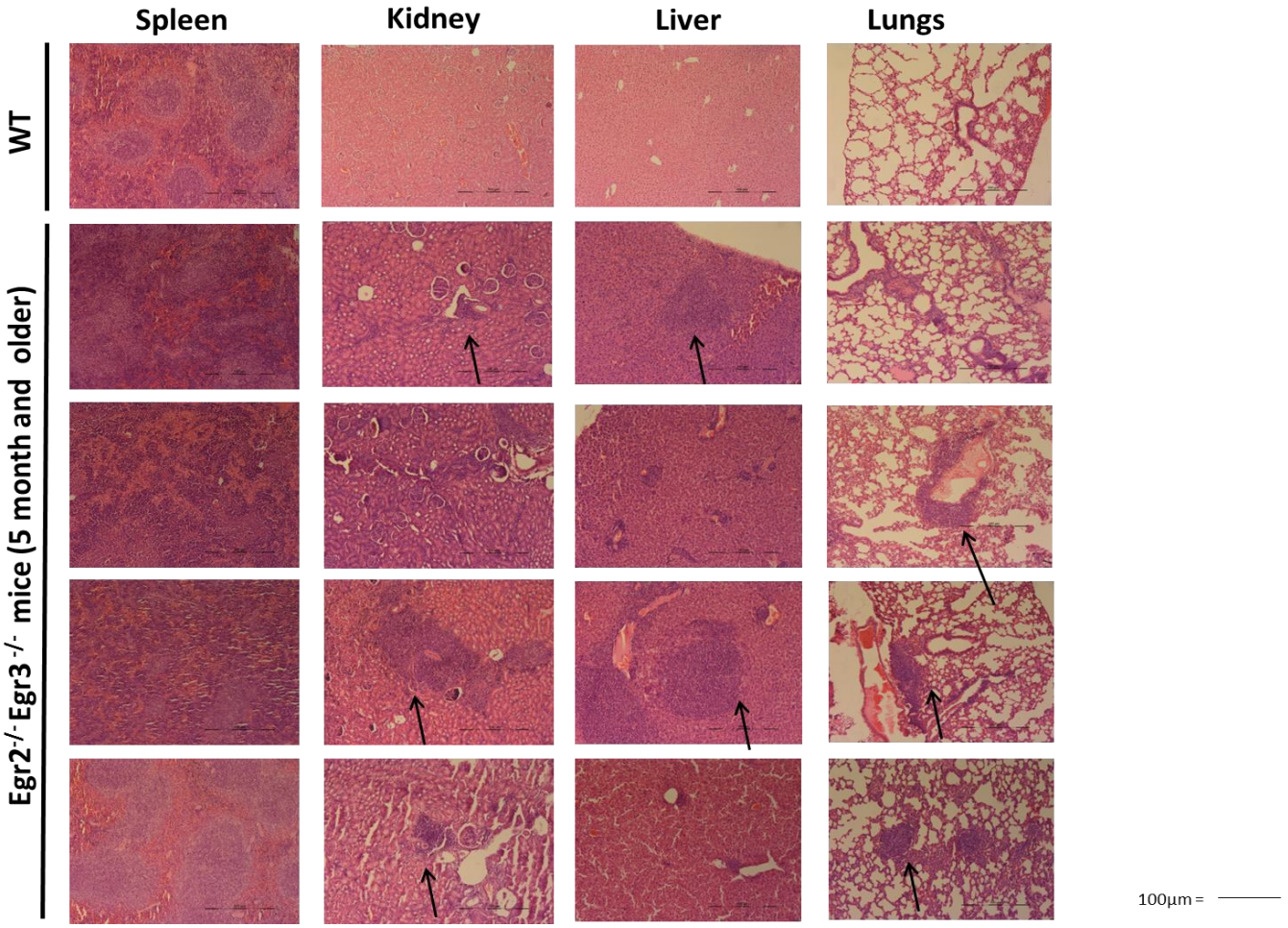

Figure 3.2.4: Haematoxylin and eosin staining show severe organ pathology and lymphocyte infiltrate in Egr2$^{-/-}$ Egr3 $^{-/-}$ mice aged 5 months and older. Image taken using Axioscope X10 objective and data was collected from 4 representative mice

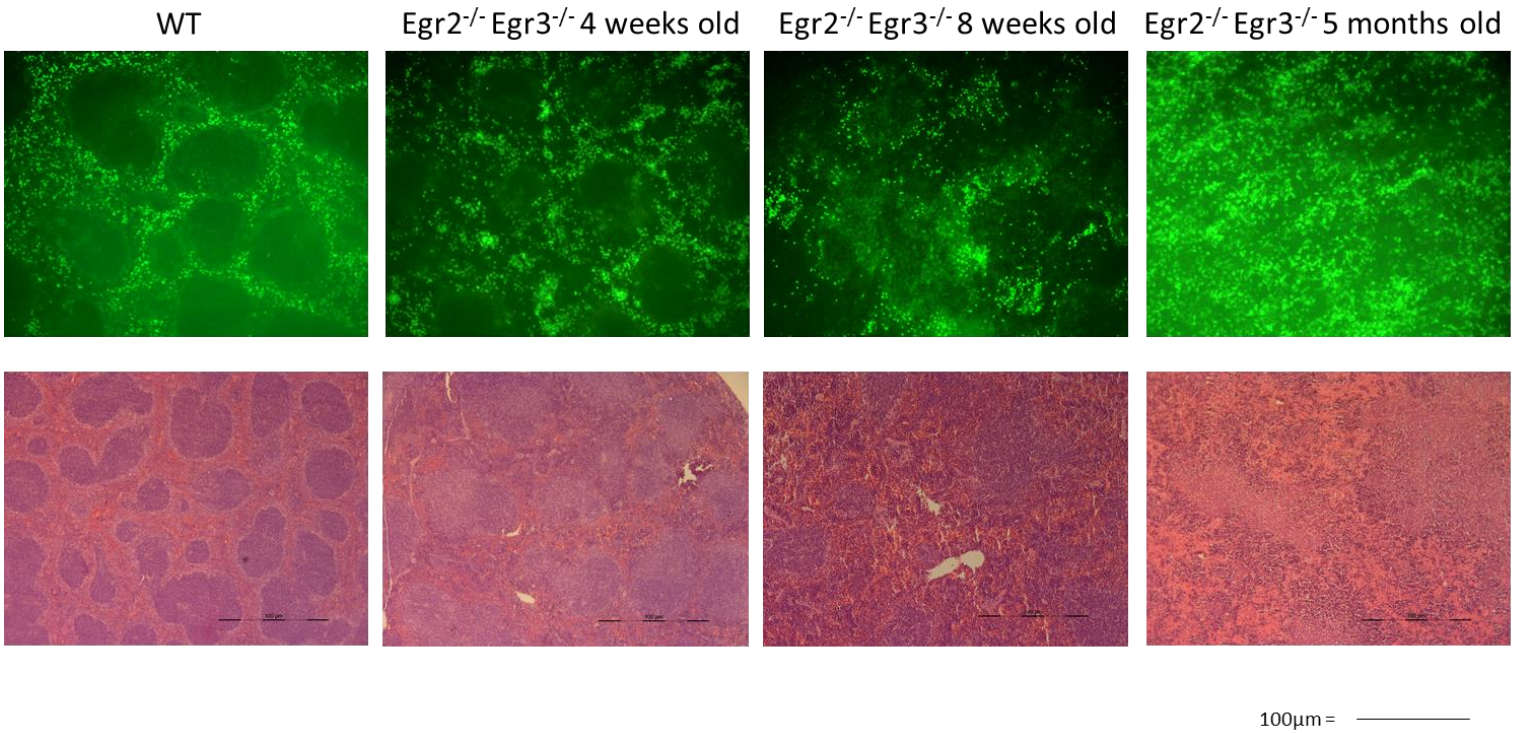

Figure 3.2.5: Pathological analysis show splenic derangement in Egr2$^{-/-}$ Egr3 $^{-/-}$ mice from 2 months of age. Morphology of the organs was analysed using H and E stain and confirmed by Immunofluorescence using anti-CD11b antibody on spleen sections from WT and Egr2$^{-/-}$ Egr3 $^{-/-}$ mice. CD11b stains for an integrin which is upregulated on activated macrophages and facilitates their trafficking to sites of inflammation. It is commonly used as a marker for chronic inflammation. Immunofluorescence and haematoxylin and eosin stained images were taken using a x5 objective

3.2.2 Cytokine profile of CD4 T cells from Egr2$^{-/-}$ Egr3 $^{-/-}$ mice

Cytokines are key players in inflammatory responses and have been implicated in the onset of autoimmune diseases. Microarray analysis carried out on anti-CD3/anti-CD28 stimulated and unstimulated CD4 T cells from WT and Egr2$^{-/-}$ Egr3$^{-/-}$ mice identified cytokines with deregulated expression in the Egr2$^{-/-}$ Egr3$^{-/-}$ mice when compared to their WT counterpart (Figure 3.2.6). When pro-inflammatory cytokines expressions in the WT mice were compared with Egr2$^{-/-}$ Egr3$^{-/-}$ mice, there was an increased expression of interferon ɣ (IFNɣ), IL-17A and F, IL-1A and B, IL-6, IL-3 and IL-21 in total CD4 T cells from Egr2$^{-/-}$ Egr3$^{-/-}$ mice as presented in table 3.1. This data was confirmed by RT-PCR which showed significantly higher levels of IFN-ɣ, IL-21 and IL-1A and B in Egr2$^{-/-}$ Egr3$^{-/-}$ mice (Figure3.2.7). All of this cytokines are known to promote the inflammatory process and the development of autoimmune disease (HELLMICH, CSERNOK and GROSS, 2005). As expected, the data obtained from microarray was consistent with the high levels of pro-inflammatory cytokine reported to be present in the serum of Egr2$^{-/-}$ Egr3$^{-/-}$ mice (Li *et al.*, 2012).

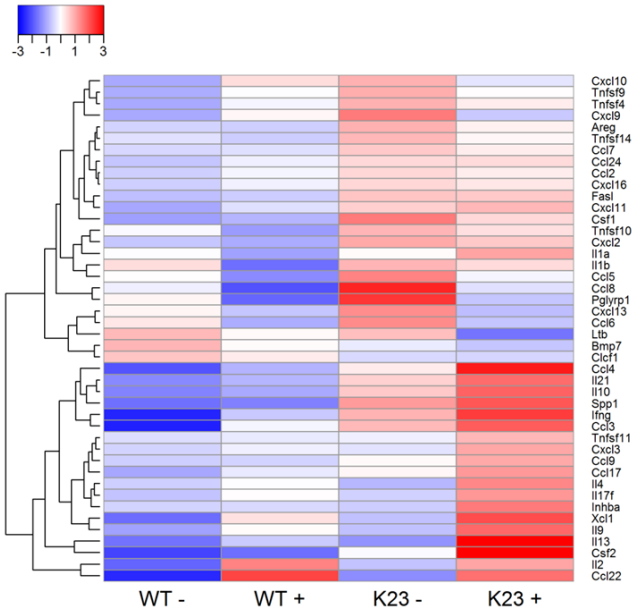

Figure 3.2.6: Microarray data analysis show deregulated expression of cytokines in stimulated and unstimulated Egr2$^{-/-}$ Egr3$^{-/-}$ CD4 T cells when compared with its WT counterpart.

Gene name	Expression in Egr2$^{-/-}$ Egr3$^{-/-}$ mice	Fold change between WT and Egr2$^{-/-}$ Egr3$^{-/-}$ (Unstimulated)	Fold change between WT and Egr2$^{-/-}$ Egr3$^{-/-}$ (stimulated)
IFNγ	Upregulated	12.58	7.89
IL-17a	Upregulated	1.12	1.8
IL-17f	Upregulated	1.19	3.48
IL-6	Upregulated	1.5	0.58
IL-3	Upregulated	1.42	46.1
IL-10	Upregulated	5.26	6.91
IL-1a	Upregulated	1.26	5.24
IL-1b	Upregulated	1.34	5.02

Table 3.1: Microarray data was analysed for fold difference in cytokine expression. Data shows higher expression levels of pro-inflammatory cytokines in CD4 T cells from Egr2$^{-/-}$ Egr3$^{-/-}$ mice.

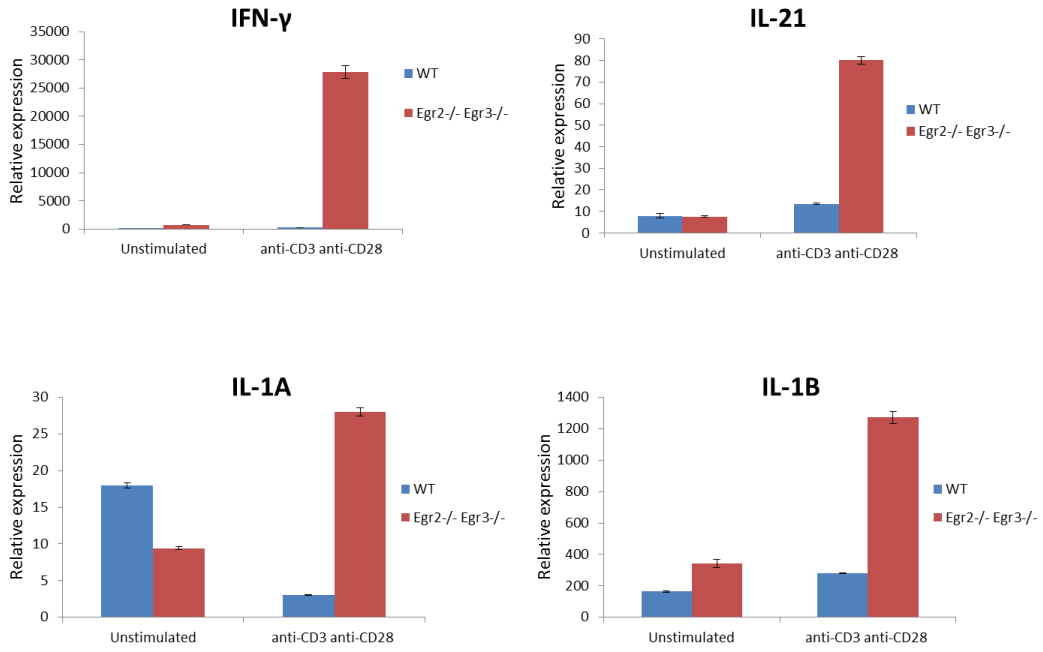

Figure 3.2.7: High levels of pro-inflammatory cytokines in Egr2$^{-/-}$ Egr3$^{-/-}$ mice.

RT-PCR on PMA-Ionomycin stimulated and unstimulated CD4 T cells shows significantly higher expression of pro-inflammatory cytokines IFN-γ, IL-21, and IL-1A and B in Egr2$^{-/-}$ Egr3$^{-/-}$ mice.

3.2.3 Autoimmune disease development in Egr2$^{-/-}$ Egr3 $^{-/-}$ mice

From observations in this study, Egr2$^{-/-}$ Egr3$^{-/-}$ mice exhibited severe inflammations with lympho-proliferative disorders and developed symptoms related to autoimmune diseases like splenomegaly and swollen limbs (figure 3.2.8). This is in line with previous reports from our group stating that the Egr2$^{-/-}$ Egr3$^{-/-}$ mice develop autoimmune diseases (Li et al., 2012). Autoimmune diseases are characterised by high levels of pro-inflammatory cytokines and are often diagnosed by the presence of antibodies that react to self-antigens termed anti-nuclear antibodies (ANA) (LYONS *et al.*, 2005). Li et al in their 2012 paper compared total immunoglobulin and ANA levels between WT and Egr2$^{-/-}$ Egr3$^{-/-}$ mice. They reported significantly higher levels of total immunoglobulin (Ig), anti-histone and anti-dsDNA in the serum of 8 weeks old Egr2$^{-/-}$ Egr3$^{-/-}$ mice (Li et al, 2012). This is consistent with the age from which early signs of chronic inflammation pathology were observed in Egr2$^{-/-}$ Egr3$^{-/-}$ mice model (figure 3.2.2). In addition to high ANA levels, Egr2$^{-/-}$ Egr3$^{-/-}$ mice were also reported to produce significantly more proteinuria (Li et al., 2012). This is in line with a phenotype consistent with organ pathology.

Morphological analysis of the kidney identified lupus-like autoimmune symptoms including increased glomeruli cellularity, reduction in bowman space due to infiltrates and immunoglobulin deposits in the kidney (figures 3.2.9). The results presented here suggest that chronic inflammation accompanied by high levels of pro-inflammatory cytokines in the Egr2$^{-/-}$ Egr3$^{-/-}$ mice may contribute to systemic organ pathology that may play a pathogenic role in the development of systemic inflammatory autoimmune disease observed in the Egr2$^{-/-}$ Egr3$^{-/-}$ mice.

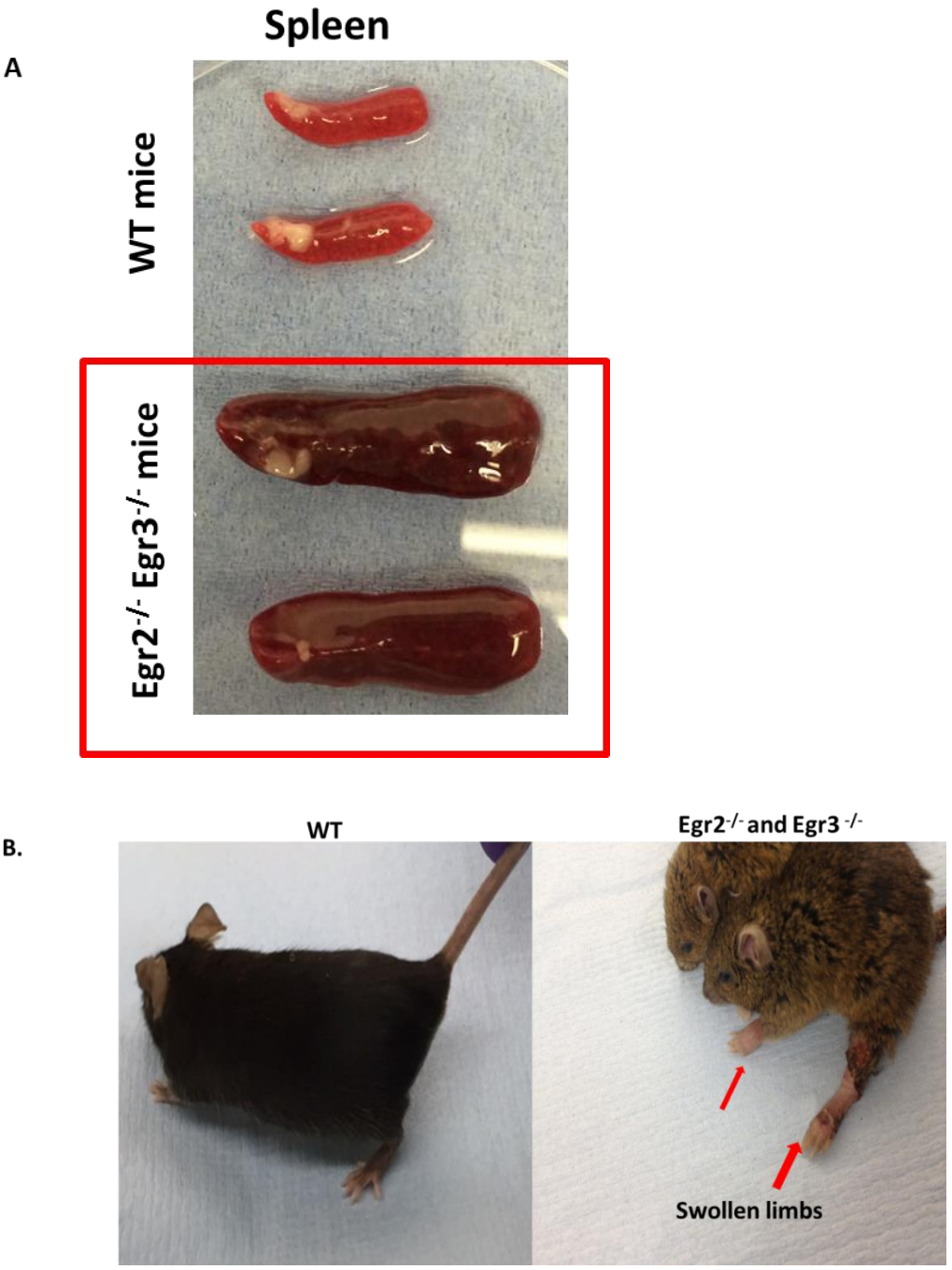

Figure 3.2.8: Egr2$^{-/-}$ Egr3$^{-/-}$ mice developed symptoms associated with inflammation pathology and autoimmunity. Figure above shows pathologically enlarged Spleens and swollen ankles in 2 months old Egr2$^{-/-}$ Egr3$^{-/-}$ mice (A and B). These symptoms can be associated with an autoimmune phenotype.

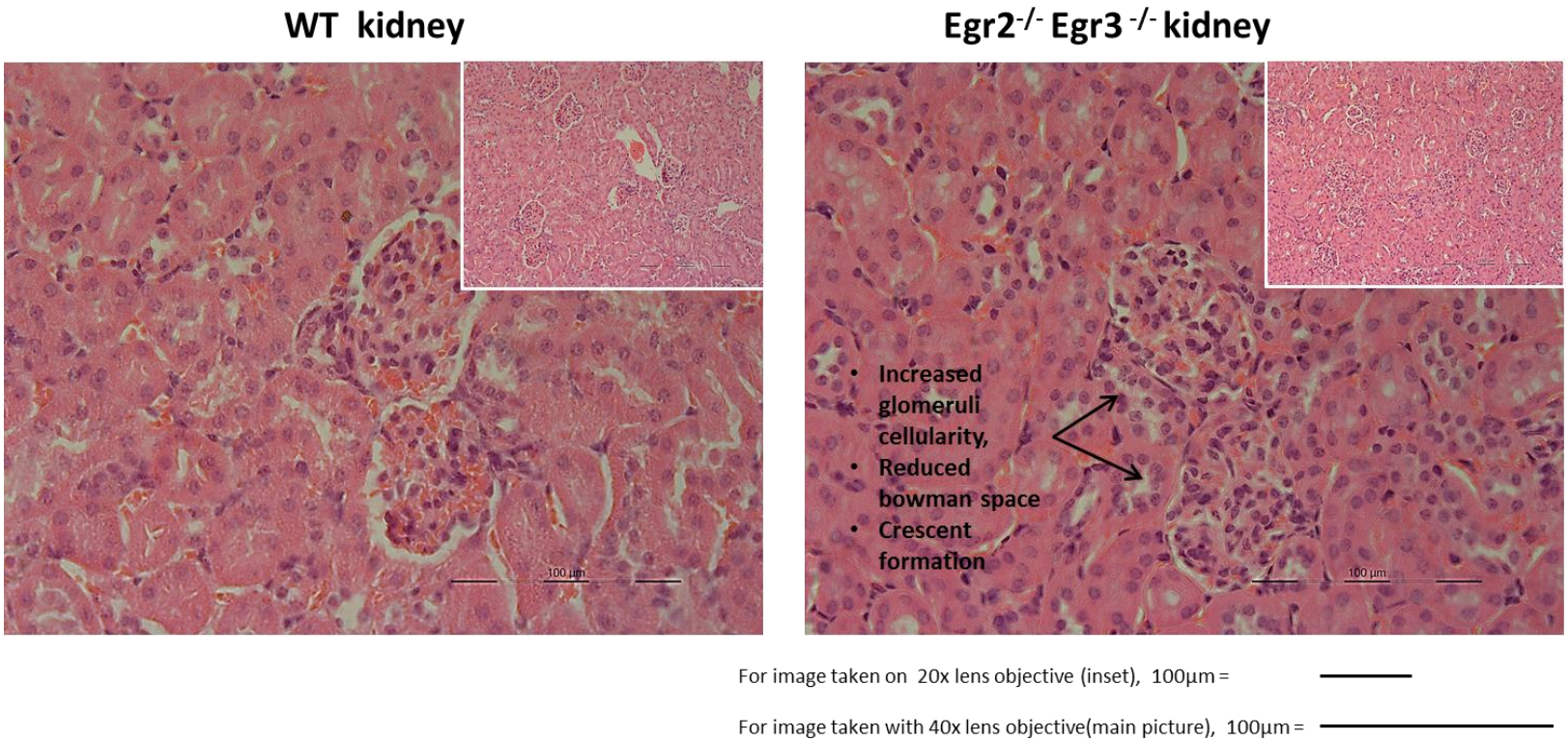

Figure 3.2.9: Morphology of Kidney from Egr2$^{-/-}$ Egr3$^{-/-}$ mice shows glomerulonephritis with increased cellularity of glomeruli and reduced Bowman space. H and E staining of Kidney section from WT and 3 month old Egr2$^{-/-}$ Egr3$^{-/-}$ mice show pathology in the kidney in line with an autoimmune phenotype. This is in line with a publication from Li et al, 2012 showing Egr2$^{-/-}$ Egr3$^{-/-}$ mice had Immunoglobulin (Ig) deposits in the glomeruli of the Kidney (Li et al, 2012).

3.3 Egr2$^{-/-}$ Egr3$^{-/-}$ mice fail to develop germinal centres

3.3.1 Histological analysis show that GCs are absent in Egr2$^{-/-}$ Egr3 $^{-/-}$ mice

The spleen, much like the lymph nodes, tonsils and payers patch, is a secondary lymphoid organ and home to mature lymphocytes. It is divided into 2 main areas: the white pulp containing the T and B cell zones and red pulp which are blood filled sinuses made up of macrophages, DC and granulocytes (Mebius and Kraal, 2005). Splenic architecture is optimal for the generation of immune responses (Mebius and Kraal, 2005). Spleens were harvested from 6-8 weeks old WT and Egr2$^{-/-}$ Egr3$^{-/-}$ mice for morphological analysis. It was found, that the germinal centre (GC), which is responsible for the generation of T cell dependent humoral immune response was conspicuously absent from the Egr2$^{-/-}$ Egr3$^{-/-}$ mice (figure 3.3.1). This was further confirmed by Immunofluorescence staining using the GC B cell marker peanut agglutinin (PNA). An absence of GC suggests that T cell dependent humoral immune responses could be severely abrogated.

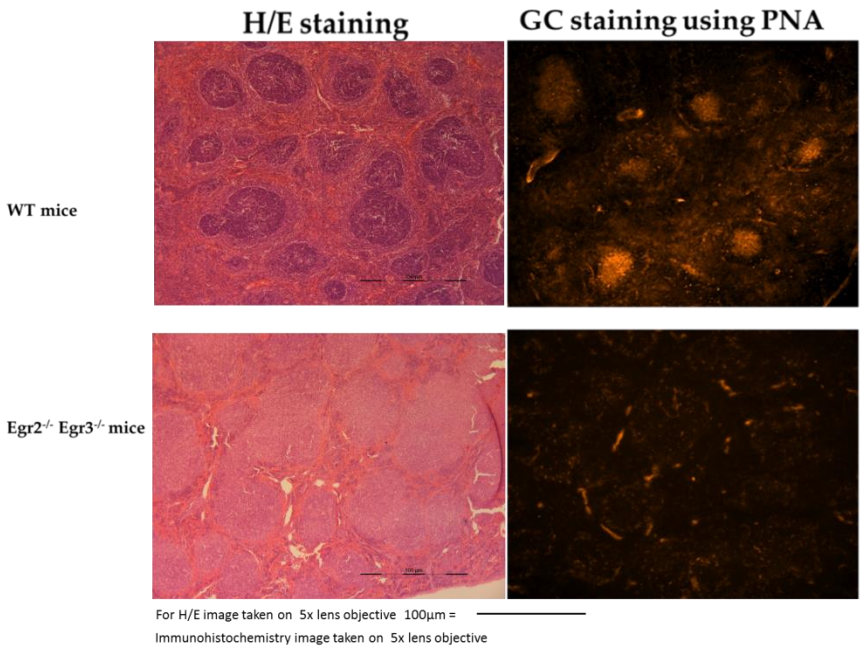

Figure 3.3.1: Morphological analysis of the spleen of WT and Egr2$^{-/-}$ Egr3$^{-/-}$ mice highlight an absence of GC in the Egr2$^{-/-}$ Egr3$^{-/-}$ mice. (H and E images were acquired using an X5 objective on an Axioscope light microscope while Immunofluorescence images were acquired using a X5 objective on an Zeiss fluorescence microscope).

3.3.2 Egr2$^{-/-}$ Egr3 $^{-/-}$ mice show severe clinical pathology and high viral load

Given the importance of the spleen in immune response and the observed absence of GC in Egr2$^{-/-}$ Egr3$^{-/-}$ mice, it became important therefore, to assess the immune response in these mice and determine what clinical implications the absence of GC may present upon antigenic challenge *in vivo*. Mice were then infected with sub lethal doses of Vaccinia Virus Western Reserve strain (VV_{WR}) and mice weights were used as an indication of clinical pathology.

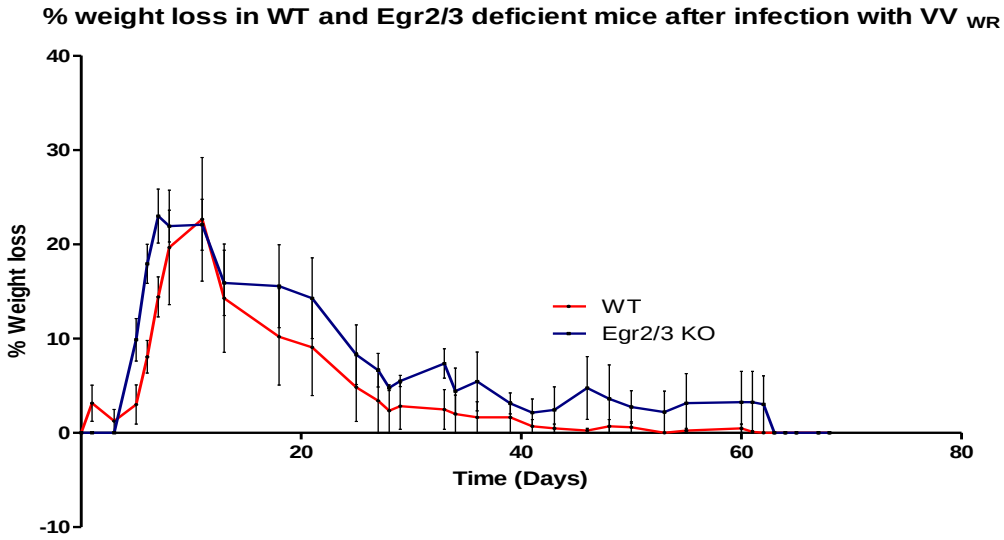

Figure 3.3.2: Egr2$^{-/-}$ Egr3$^{-/-}$ mice show severe clinical symptoms in response to VV_{WR} challenge *in vivo*. This graph is representative of over 10 experiments done using different ages and sexes of mice. 3-4 mice were used per group for each of these experiments. Statistical significance calculated using a 2 way ANOVA showing a p value of <0.001 in % weight loss between the WT and Egr2/3 deficient mice over the course of the infection.

The figure presented above shows that after viral challenge, Egr2$^{-/-}$ Egr3$^{-/-}$ mice showed rapid and more severe clinical pathology compared to its WT counterpart. This was consistent over the course of the infection. In terms of recovery from infection, which was determined as the day mouse weights was equal to or more than baseline weights (day 0 weights), Egr2$^{-/-}$ Egr3$^{-/-}$ mice took

twice as much time as the WT mice to recover from VV_{WR} antigenic challenge. WT recovered 27 days after infection whilst $Egr2^{-/-}$ $Egr3^{-/-}$ mice recovered on day 53 post infection (figure 3.3.2). Interestingly, it was discovered that the $Egr2^{-/-}$ $Egr3^{-/-}$ mice did not show any clinical pathology when re-challenged using same dose of VV_{WR} antigen indicating that these mice may have developed immunological protection from re-challenge (figure 3.3.2). It is important to note also that there was no difference in clinical pathology in response to VV_{WR} among the different ages and sexes of $Egr2^{-/-}$ $Egr3^{-/-}$ mice studied. The rest of this study was focused on mice aged 8-10 weeks old.

Viral pathogens cause the immune system to initiate immune responses. Humoral immune response via ADCC, CDC, antibody opsonisation and viral neutralisation enhances viral clearance and allows for the processing and presentation of viral antigens by phagocytic cells (Carrasco, 2009). In order to investigate why $Egr2^{-/-}$ $Egr3^{-/-}$ mice showed such severe clinical pathology and delayed recovery after primary challenge with VV_{WR}, the lung viral load was measured 8 days after infection for the clearance of virus. It was observed that the $Egr2^{-/-}$ $Egr3^{-/-}$ mice had about 100 times higher viral load in its lungs compared to its WT counterpart (figure 3.3.3). Severe clinical pathology and high viral load suggests defective immune response in $Egr2^{-/-}$ $Egr3^{-/-}$ mice.

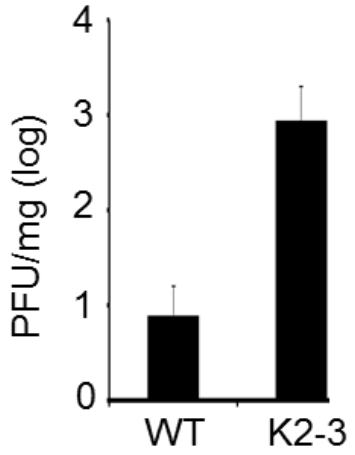

Figure 3.3.3: Impaired VV_{WR} clearance in $Egr2^{-/-}$ $Egr3^{-/-}$ mice following viral infection. Analysis of Lung viral titre done 8 days after infection show that the **$Egr2^{-/-}$ $Egr3^{-/-}$** mice had about 100 times higher viral load compared to their WT counterparts

3.3.3 Egr2$^{-/-}$ Egr3$^{-/-}$ mice failed to generate GC in response to VV$_{WR}$ infection

During infection, the GCs expand and increase in number in order to mount optimal humoral immune responses. Within the GC, T follicular helper (Tfh) cells provide help to cognate GC B cells causing them to expand and proliferate. This in turn will lead to the production of plasma cells that generate long-lived, high affinity, isotype-switched antibodies and the development of B cell immunological memory. Due to the relatively weak anti-viral immune response observed in mice lacking the Egr2/3 genes, spleens were again harvested from WT and Egr2$^{-/-}$ Egr3$^{-/-}$ mice before and after infection with VV$_{WR}$ and assessed for development of GC. These spleens were then stained using GC B cell marker peanut Agglutinin (PNA) and markers for B and CD4^{+} T cells

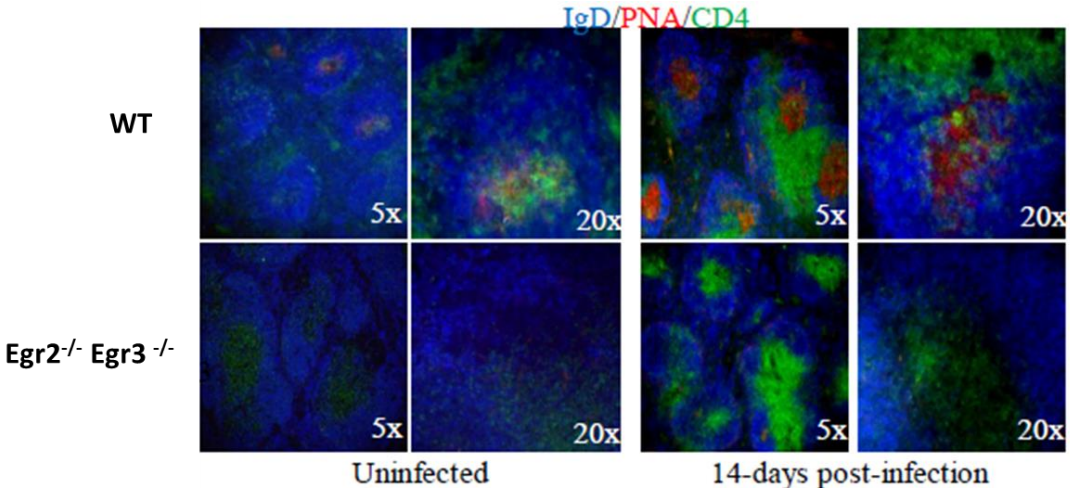

Figure 3.3.4: Egr2$^{-/-}$ Egr3$^{-/-}$ mice failed to develop GC in response to VV$_{WR}$ infection. Immunofluorescence staining for GC B cells using PNA (red), CD4^{+} T cells (green) and non-GC B cells stained with IgD (blue) on WT and Egr2$^{-/-}$ Egr3$^{-/-}$ mice show lack of GC in the Egr2$^{-/-}$Egr3$^{-/-}$ mice before and 14 days after VV$_{WR}$ challenge. This data is representative of 3 experiments. Magnification indicates objective used to acquire images

From the figure above (figure 3.3.4), it can be seen that in uninfected WT mice, there was sporadic GC in the spleen, which as expected, became enlarged and increased in number after viral challenge. In the Egr2$^{-/-}$ Egr3$^{-/-}$ mice however,

there was an absence of these structures in these mice both uninfected and 14 days after primary challenge with VV_{WR} (Figure 3.3.4). Although $Egr2^{-/-}$ $Egr3^{-/-}$ mice did contain normal T and B cell areas confirming inflammation pathology had not set in, they, however, failed to generate GCs. The absence of GCs in these mice indicates a defect in normal immune response and demonstrates that the Egr2/3 genes may play a role in the development of GC during T cell dependent humoral immune response.

3.3.4 Single Egr2 or Egr3 deficient mice showed normal GC development

I had earlier reviewed the compensatory function of the Egr3 gene in the absence of Egr2 gene in section 1.8.2. When the spleens from VV_{WR} infected CD2-Egr2 single deficient mice were analysed for the development of GC 14 days after infection, It was found that in CD2-Egr2$^{-/-}$ mice, GC development was normal (figure 3.3.5A). Analysis of the LN of Egr3 deficient mice also showed normal GC development when compared to its WT counterpart (figure 3.3.5B). This validates the compensatory role of the Egr3 gene in the absence of Egr2 and points to an overlapping function of the Egr2 and Egr3 genes in the regulation of Tfh differentiation and GC development.

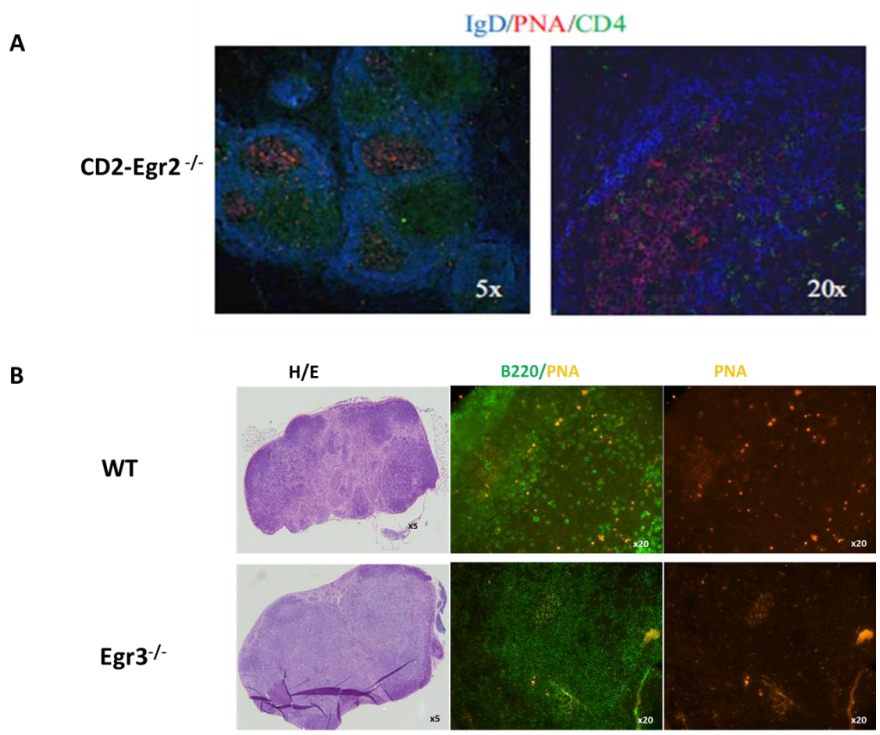

Figure 3.3.5: Single CD2-Egr2$^{-/-}$ and single Egr3$^{-/-}$ mice show no defects in germinal centre formation. A. immunofluorescence on spleen of 3 month old Egr2 conditional knockout mice showing normal germinal centre formation. B. H and E and immunostaining of LN of Egr3 deficient mice and WT mice showed normal GC development

3.3.5 GC function is defective in Egr2$^{-/-}$ Egr3$^{-/-}$ mice

As earlier stated, the GC is responsible for the production of high affinity antibodies, immunoglobulin class switching of these antibodies and B cell memory. Having observed an absence of GC in Egr2$^{-/-}$ Egr3$^{-/-}$ mice, we next investigated GC functions in these mice. To do this, 2 systems were employed. They are the VV_{WR} infection system and the OVA protein immunisation system. The merits and de-merits of the two methods are highlighted below (table 3.2). Both Viral and OVA protein systems are able to generate T dependent immune responses (Choi *et al.*, 2011; Johnston *et al.*, 2009; Yu *et al.*, 2009).

	Advantage	**Disadvantage**
OVA Protein system	• More refined. Not lethal, safer for mice (Ethically more viable) • Mice can be studied over a longer period of time • No inflammation involved	• Cannot monitor clinical signs as OVA is none pathological
VV_{WR} system	• Clearly shows clinical symptoms • Humoral immune response to virus and viral neutralisation assay can be determined	• Mice cannot be studied past humane end point

Table 3.2: Advantages and disadvantages of the 2 systems employed for antigenic challenge *in vivo.*

Somatic hypermutation leads to the production of antibodies with high affinity for pathogens. 21 days after VV_{WR} infection, Serum was collected from infected mice. This was then analysed for the production of anti-VV_{WR} neutralising antibodies. Results obtained showed there was significantly higher plaque number in plated TK143 cells containing serum from Egr2$^{-/-}$ Egr3$^{-/-}$ mice. This higher number of plaques in Egr2$^{-/-}$ Egr3$^{-/-}$ mice compared to WT mice indicates less production of neutralising antibodies against the VV_{WR} 21 days after infection (figure 3.3.6). Using the OVA protein immunisation system, we further confirmed the results obtained from neutralisation assay by evaluating the production of total anti-OVA specific antibodies following immunisation with OVA protein. In line with the failure to generate GC in Egr2$^{-/-}$ Egr3$^{-/-}$ mice and the impaired production of VV_{WR} neutralising antibodies, there was a significantly lower production of anti-OVA antibodies from

the serum in the Egr2$^{-/-}$ Egr3$^{-/-}$ mice. This was consistent over the period of primary immunisation (figure 3.3.7).

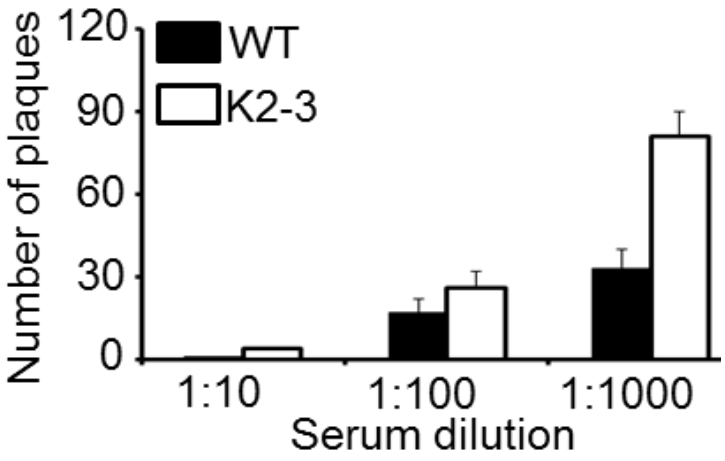

Figure 3.3.6: Production of neutralising antibody is impaired in Egr2$^{-/-}$ Egr3$^{-/-}$ mice in response to VV$_{WR}$ infection. Following co-incubation of VV$_{WR}$ and sera from infected mice, TK143 cells were then cultured in VV$_{WR}$-sera mix overnight and plaques indicating cell lysis were counted after 48 hours.

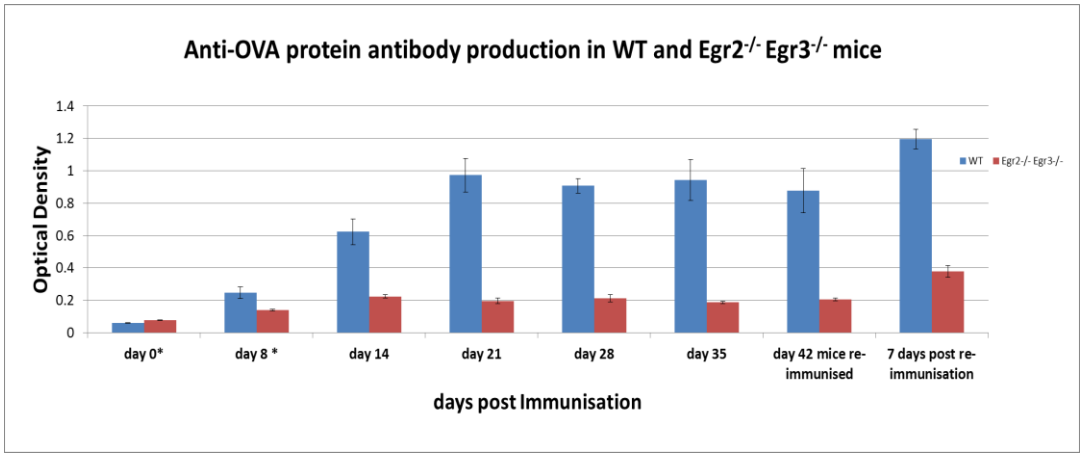

Figure 3.3.7: Defective production of anti-OVA specific antibody in Egr2$^{-/-}$ Egr3$^{-/-}$ mice. (* = day of immunisation). WT and Egr2$^{-/-}$ Egr3$^{-/-}$ mice were immunised with OVA protein emulsified in complete Freund's adjuvant (CFA) and mice were bled every seven days. Total anti-OVA specific immunoglobulin was then measured in these mice. From the data presented above, it was determined that the Egr2$^{-/-}$ Egr3$^{-/-}$ mice showed impaired production of antigen-specific antibody against OVA protein.

Another function of the GC is class switch recombination (CSR). This occurs in order to generate other antibody isotypes with different effector functions. As IgG1 is produced during class switching in T cell dependent humoral immune response (Liu *et al.*, 2014b; Ise *et al.*, 2011), class switching from IgM to IgG1 antibody in response to viral challenge in the WT and Egr2$^{-/-}$ Egr3$^{-/-}$ mice was investigated. IgG1 antibody levels were found to be significantly higher in the serum of WT mice from 21 days after viral challenge when compared with the Egr2$^{-/-}$ Egr3$^{-/-}$ mice at same time points (p=0.000137 and 0.006772 respectively) (Figure 3.3.8).

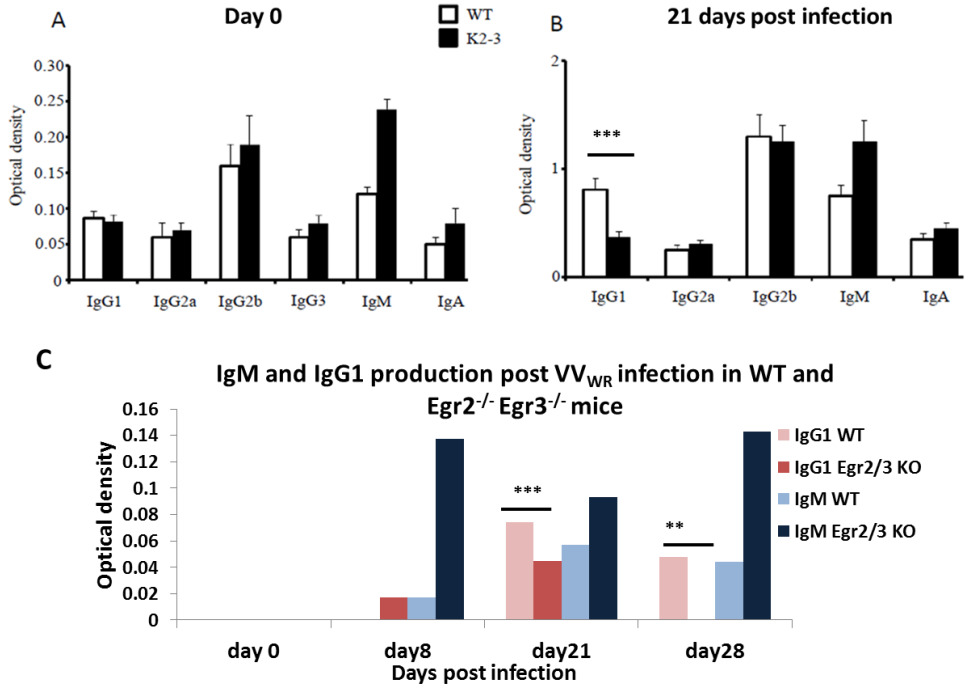

Figure 3.3.8: Impaired IgG1 titres in serum pooled from Egr2$^{-/-}$ Egr3$^{-/-}$ mice 21 days after VV$_{WR}$ infection.

A and B: IgG1 antibody isotype levels analysed by ELISA in Egr2$^{-/-}$ Egr3$^{-/-}$ mice was significantly less than its WT counterpart 21 days after infection with VV$_{WR}$. As measured by sandwich ELISA **C)** Kinetics shows that IgG1 levels were consistently higher in WT mice and significantly different from that of Egr2$^{-/-}$ Egr3$^{-/-}$ mice 21 and 28 days after VV$_{WR}$ infection. Data presented here have been normalised by subtracted baseline values for day 0 from that of subsequent days. This result is representative of serum from 2 independent experiments each with at least 4 mice per group. Student T test used P<0.001 = ***; P<0.01 = **; P<0.05 = *

Humoral immune response during secondary immunisation was next investigated. To do this, the OVA system was used. WT and Egr2$^{-/-}$ Egr3$^{-/-}$ mice were re-immunised 42 days after the first dose. In line with defective GC functions, production of OVA-specific antibodies in the serum of Egr2$^{-/-}$ Egr3$^{-/-}$ mice 7 days after re-immunisation was found to be significantly lower (Figure 3.3.9). When antibody production during primary and secondary immune responses in WT and Egr2$^{-/-}$ Egr3$^{-/-}$ were compared, it was observed that WT mice had 3 folds higher antigen-specific antibody in their serum compared to their Egr2$^{-/-}$ Egr3$^{-/-}$ counterparts during both primary and secondary immune response indicating aberrant GC function during re-immunisation (Table 3.3).

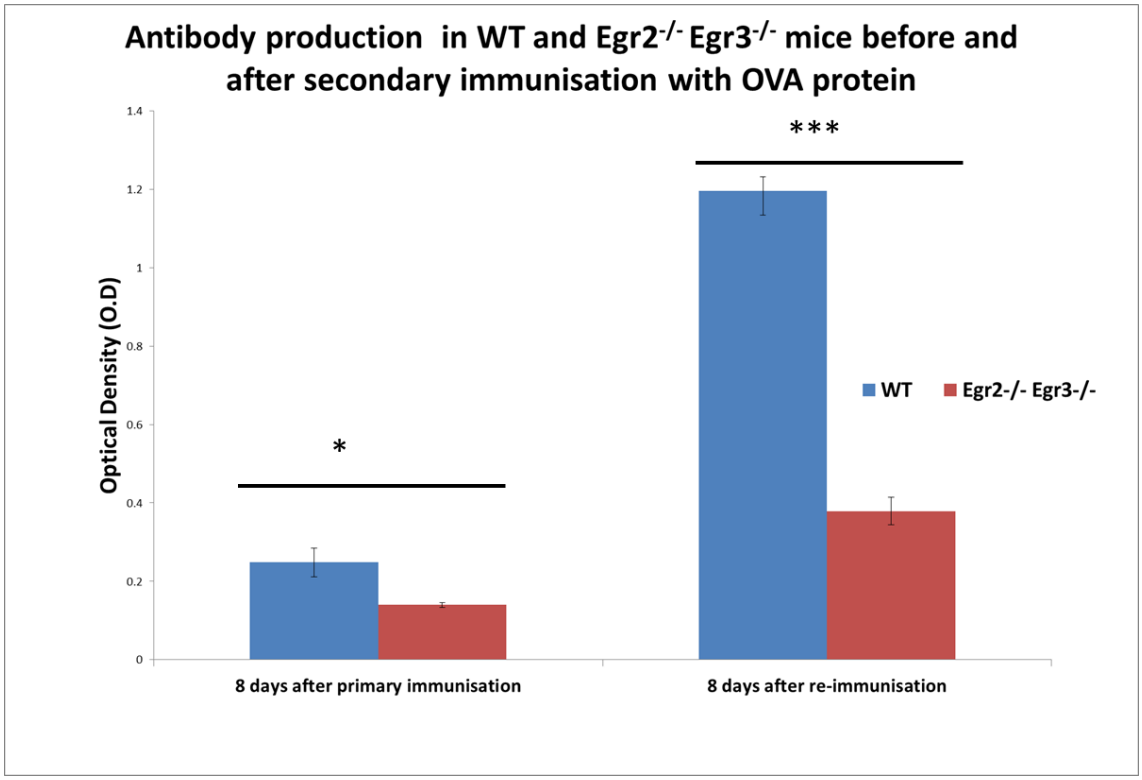

Figure 3.3.9: Defective anti-OVA antibody production during primary and secondary immune response in Egr2$^{-/-}$ Egr3$^{-/-}$ mice. This was analysed by ELISA. Student T test used P<0.001 = ***; P<0.01 = **; P<0.05 = *

	WT	KO	Fold change between WT and KO
Day 0	0	0	
8 days after primary immunisation	0.188667	0.060333	3.127071823
8 days after re-immunisation	1.136333	0.299667	3.791991101

Table 3.3: Fold change in anti-OVA antibody production between WT and Egr2$^{-/-}$ Egr3$^{-/-}$ mice during primary and secondary immune response.

Taken together, these results indicate that GC functions like somatic hypermutation, isotype class-switching and generation of antibody during memory immune responses are impaired in Egr2$^{-/-}$ Egr3$^{-/-}$ mice consistent with a lack of GC. This suggests that the Egr2/3 genes may play a role in the generation of GC and GC functions.

3.4 Egr2/3 regulate the differentiation of follicular helper T cell (Tfh) development

3.4.1 Bcl6 expression is defective in Egr2$^{-/-}$Egr3$^{-/-}$ mice

We have established that in the absence of the Egr2/3 genes, mice did not develop GC and lacked GC functions. We then sought to investigate the reason this occurred by looking into the regulation of Tfh cells, a cellular mediator of GC immune response. Tfh cell differentiation and development much like any physiological process within any biological entity is regulated by a number of molecules including transcription factors, chemokines and cytokines. Using microarray, the gene expression profile of Tfh-related genes in anti-CD3/anti-CD28 stimulated and unstimulated total CD4^{+} T cells from WT and Egr2$^{-/-}$ Egr3$^{-/-}$ mice was analysed. It was observed that the expression of Tfh-related genes, Bcl6 and Prdm1 (the gene that codes for Blimp1), were altered (figure3.4.1)

Microarray data were further analysed by scatter plot analysis. The result showed a 2 fold down regulation of Bcl6 in both stimulated and unstimulated Egr2$^{-/-}$ Egr3$^{-/-}$ CD4^{+} T cells while Prdm1 (Blimp1) had at least 2 fold upregulation in the stimulated and unstimulated conditions in the CD4^{+} cells from Egr2$^{-/-}$ Egr3$^{-/-}$ mice (Figure 3.4.2A). To further confirm this microarray data, mRNA was extracted from anti-CD3/anti-CD28 stimulated and unstimulated total CD4^{+} T cells for RT-PCR and indeed the results showed a similar expression pattern for Bcl6 and Blimp1 in the WT and Egr2$^{-/-}$ Egr3$^{-/-}$ CD4^{+} T cells (Figure 3.4.2B).

Next, Tfh and non-Tfh cells were sorted and the mRNA expression levels of Bcl6 and Blimp1 were measured. Again, there was a downregulation of Bcl6 and an upregulation of Blimp1 specifically within these CD4^{+} CXCR5^{+} PD-1^{+} Tfh cells (figure 3.4.2C). Bcl6 is the signature transcription factor responsible for Tfh cell differentiation while Blimp1 (Prdm1), another transcription factor, promotes the differentiation of CD4 T cells into other T cell lineages by repressing Bcl6 (Johnston *et al.*, 2009). Differential expression of these genes in Egr2$^{-/-}$ Egr3$^{-/-}$ mice in relation to their WT counterpart suggest that Egr2/3 genes play a role in the regulation of Tfh cell differentiation and function.

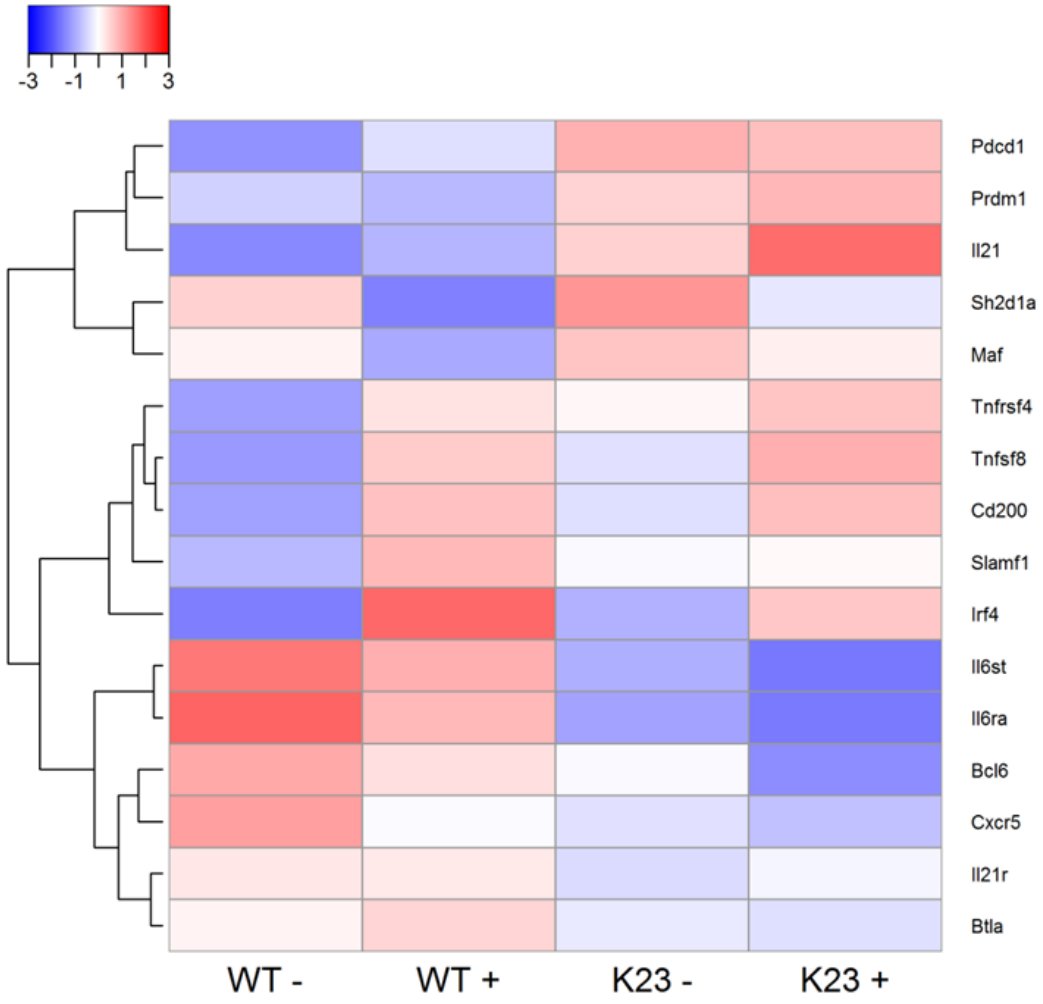

Figure 3.4.1: Microarray expression profile show altered gene expression of Bcl6 and Blimp1 in CD4 cells from Egr2$^{-/-}$ Egr3$^{-/-}$ mice. RNA was extracted from 2-month-old WT and Egr2$^{-/-}$ Egr3$^{-/-}$ mice to perform microarray. Data acquired from microarray show deregulated expression of Tfh related genes Bcl6 and Blimp1.

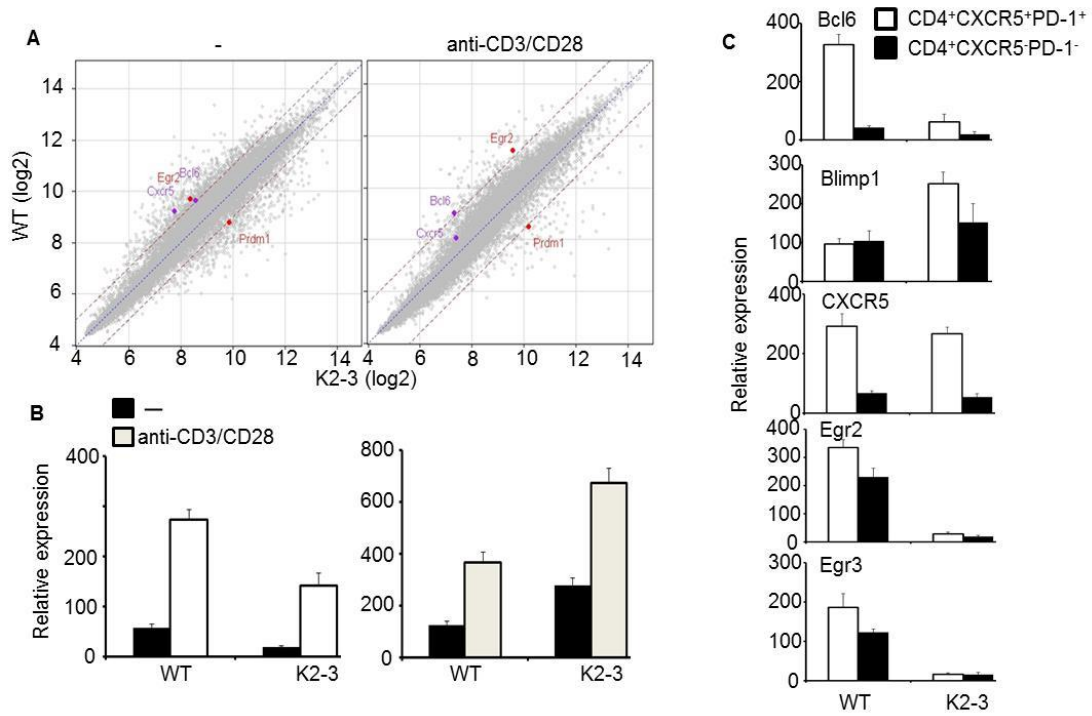

Figure 3.4.2: The expression of Tfh cell-related genes is defective in CD4 cells from Egr2$^{-/-}$ Egr3$^{-/-}$ mice.

A: Scatterplot analysis of data obtained from microarray show Tfh cell-related genes with at least 2 fold change in Egr2$^{-/-}$ Egr3 $^{-/-}$ CD4 T cells in stimulated and unstimulated conditions. **B:** Validation of the microarray data was done at mRNA level in total CD4 T cells from WT and Egr2$^{-/-}$ Egr3 $^{-/-}$ mice. **C:** CD4 T cells were stained for Tfh markers CXCR5 and PD-1 and then sorted for CXCR5+ PD-1+ CD4 cells and CXCR5- PD-1 CD4 cells. mRNA was then extracted from these cells and used for RT-PCR.

3.4.2 Aberrant Tfh cell differentiation in Egr2$^{-/-}$ Egr3$^{-/-}$ mice

In order to ascertain the effect of defective expression of Tfh-related genes in the Egr2$^{-/-}$ Egr3$^{-/-}$ mice, CD4 and B220 cells were isolated before and 14 days after infection with VV_{WR}. The differentiation of both Tfh and GC B cells were then analysed based on the expression of surface markers and signature transcription factors. In line with an absence of GC, levels of CD4^{+} CXCR5^{+} PD-1^{+} Tfh cells from the Egr2$^{-/-}$ Egr3$^{-/-}$ mice was significantly lower at day 0 when compared with WT mice (figure 3.4.3A). Analysis of the Bcl6^{+} cells in uninfected Egr2$^{-/-}$ Egr3$^{-/-}$ CD4 T cells was also severely diminished when compared to its WT counterpart (figure 3.4.4A). 14 days after infection with VV_{WR}, the expression levels of CD4^{+} CXCR5^{+} PD-1^{+} Tfh cells in WT mice was significantly increased compared to Egr2$^{-/-}$ Egr3$^{-/-}$ mice and more than half of these CD4^{+} CXCR5^{+} PD-1^{+} Tfh cells in WT mice expressed the Bcl6 gene. However, in the Egr2$^{-/-}$ Egr3 $^{-/-}$ mice, VV_{WR} infection did not increase the expression of Bcl6 within these Tfh cells (figure 3.4.3B).

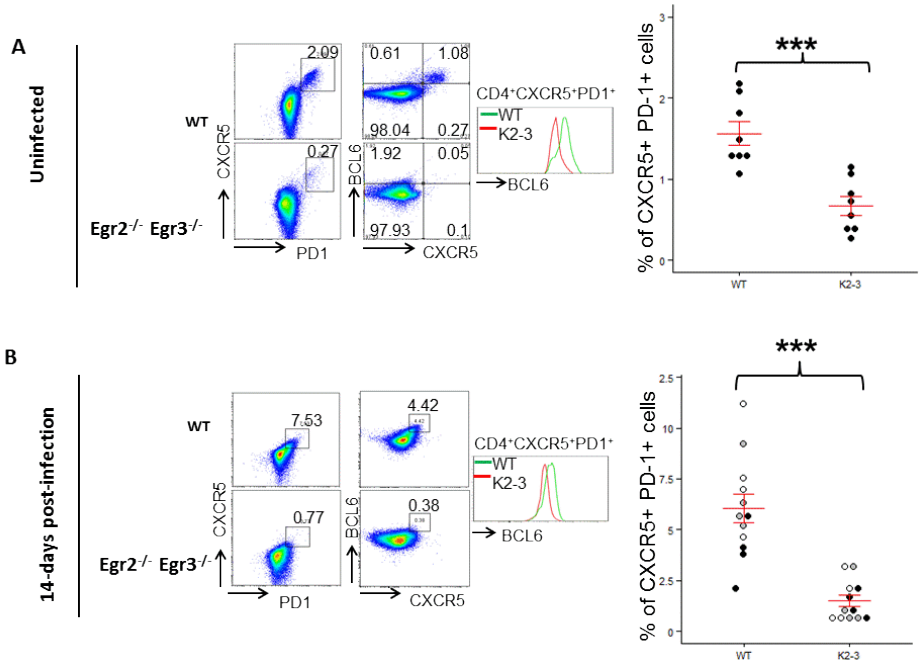

Figure 3.4.3: Deregulated Tfh cell differentiation in Egr2$^{-/-}$ Egr3$^{-/-}$ mice after VV_{WR} infection. Each dot presents an individual mouse from different experiments; Mann-Whitney's U test was used to evaluate statistical significance. P<0.001 = ***; P<0.01 = **; P<0.05 = *

Next, GC B cell differentiation was analysed in these mice before and after infection. Prior to infection, GC B cell numbers were found to be significantly higher in the WT mice (figure 3.4.4A). As expected, this increased in response to VV_{WR} challenge and was significantly more than the percentage number of GC B cells from $Egr2^{-/-}$ $Egr3^{-/-}$ mice after infection (figure 3.4.4B). Interestingly, the levels of Bcl6 in GC B cells from WT and $Egr2^{-/-}$ $Egr3^{-/-}$ mice were found to be similar after VV_{WR} challenge (figure 3.4.5).

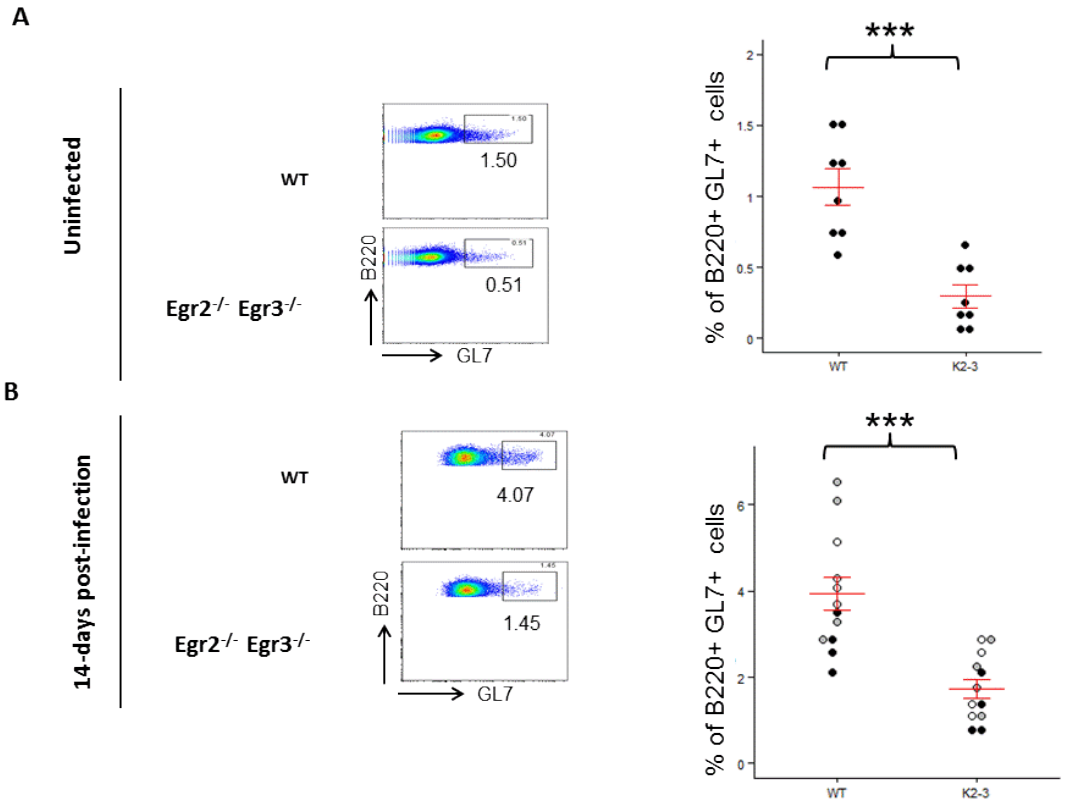

Figure 3.4.4: GC B cells numbers are reduced in the absence of Egr2/3 genes. Each dot presents an individual mouse from different experiments; Mann-Whitney's U test was used to evaluate statistical significance. P<0.001 = ***; P<0.01 = **; P<0.05 = *

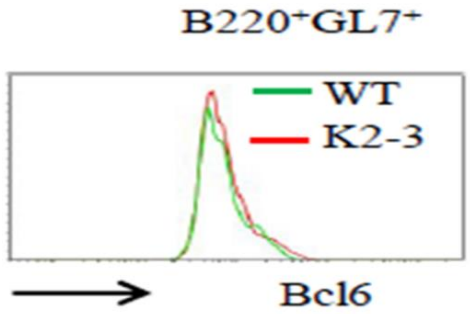

Figure 3.4.5: Bcl6 levels in GC B cells were similar in WT and Egr2$^{-/-}$ Egr3 $^{-/-}$ mice following VV$_{WR}$ infection. Histogram plots of Bcl6 expression from WT and Egr2$^{-/-}$ Egr3 $^{-/-}$ B220+ GL7+ cells following infection show similar expression of Bcl6 in these 2 mice groups

These results suggest that Egr2/3 genes may play a role in maintaining the basal numbers of Tfh cells and the differentiation of these Tfh cells after viral challenge. As there was no difference in Bcl6 expression in GC B cells from WT and Egr2$^{-/-}$ Egr3$^{-/-}$ mice, we considered a possibility that the Egr2/3 may not play a role in Bcl6 regulation of GC B cells. This suggested that the decrease in the number of GC B cells may be due to a lack of cognate Tfh cell activity to support GC B cells differentiation.

3.4.3 Egr2/3 expression is essential for Tfh differentiation

As seen in the previous section (section 3.4.2), a T cell defect in GC responses was very much likely. However, in order to confirm if the failure to generate GC and the consequences of that including impaired GC functions, was as a result of defective Egr2/3 expression in $CD4^+$ T cells or in B cells, WT $CD4^+$ cells or WT B220 cells were adoptively transferred into $Egr2^{-/-}$ $Egr3^{-/-}$ mice. This was followed by infection with VV_{WR} 24 hours after transfer. 14 days after infection, the spleens were harvested and analysed for the differentiation of Tfh, GC B cells and development of GCs (figure 3.4.6).

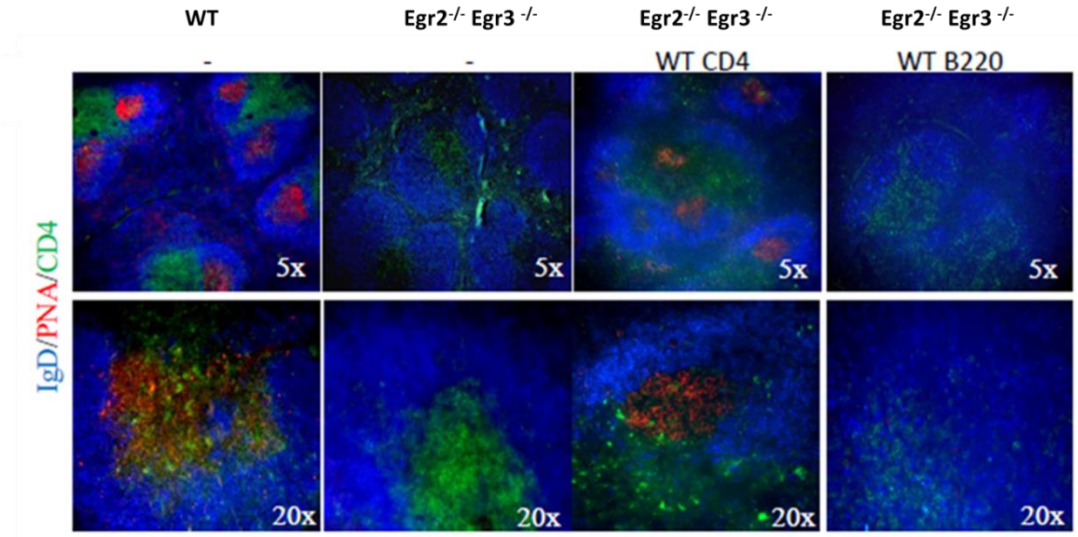

Figure 3.4.6: GC was induced in $Egr2^{-/-}$ $Egr3^{-/-}$ mice that received WT CD4 cells but not WT B220 cells. Immunofluorescence on spleen sections from WT and $Egr2^{-/-}$ $Egr3^{-/-}$ mice using GC marker PNA, CD4 and IgD on spleen. Magnification on images indicates objective used to acquire image.

Staining for GCs showed that GCs developed in $Egr2^{-/-}$ $Egr3^{-/-}$ mice that received the WT $CD4^+$ T cells and not in those that received WT B220 cells (figure 3.4.6). Next, $CD4^+$ T cells were isolated from the spleen and LN of these mice and stained for Tfh cell markers CXCR5 and PD-1. Using flow cytometry, Tfh cells were gated (figure 3.4.7A). The results obtained showed that $Egr2^{-/-}$ $Egr3^{-/-}$ mice receiving WT CD4 cells had significantly more Tfh cells and over half of them expressed Bcl6

similar to what was observed in WT mice (figure 3.4.7A and 3.4.3). Bcl6 and Egr2 expression was also increased in Egr2$^{-/-}$ Egr3$^{-/-}$ mice that received WT CD4 T cells compared to those that did not (figure 3.4.7B). Bcl6 was co-expressed with the Egr2 genes indicating that the Tfh cells were from the transferred WT CD4 cells (figure 3.4.8A and B). When the expression of GC B cells as indicated by GL7^{+} and B220^{+} cells was analysed, there was significantly higher numbers of GC B cells in Egr2$^{-/-}$ Egr3$^{-/-}$ mice receiving WT CD4 cells (figure 3.4.8). These results demonstrate that for the formation of GC, Egr2/3 expression is critical in Tfh cells.

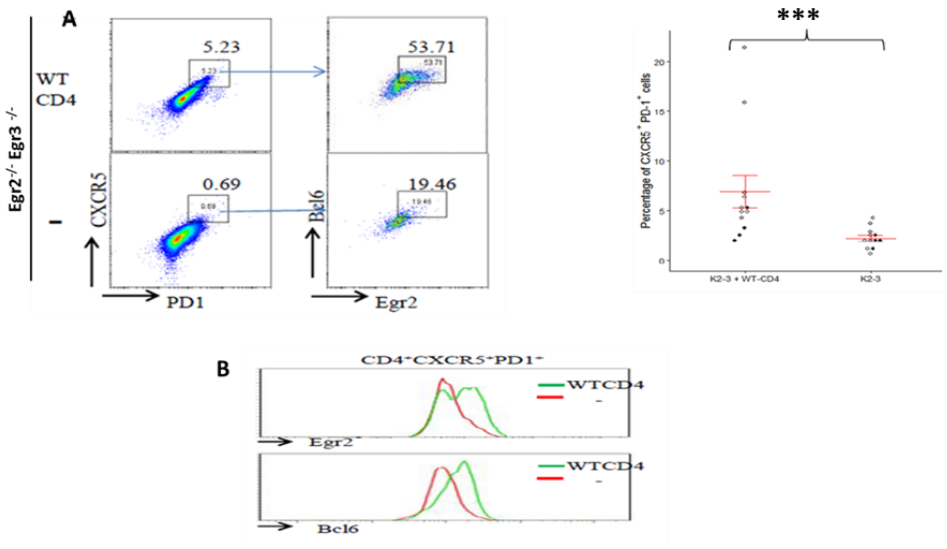

Figure 3.4.7: WT CD4 T cells transferred into Egr2$^{-/-}$ Egr3$^{-/-}$ mice increased Tfh cell numbers and Bcl6 expression. 2 X 10^{6} WT CD4 T cells or B220 cells were transferred into Egr2$^{-/-}$ Egr3$^{-/-}$ mice. 24 hours after transfer, mice were infected with VV$_{WR}$. 14 days after infection, spleens were harvested and splenocytes isolated and stained to evaluate CD4+, CXCR5+, PD-1+, Bcl6+ and Egr2+ T cells. Each dot presents an individual mouse from different experiments; Mann-Whitney's U test was used to evaluate statistical significance. P<0.001 = ***; P<0.01 = **; P<0.05 = *

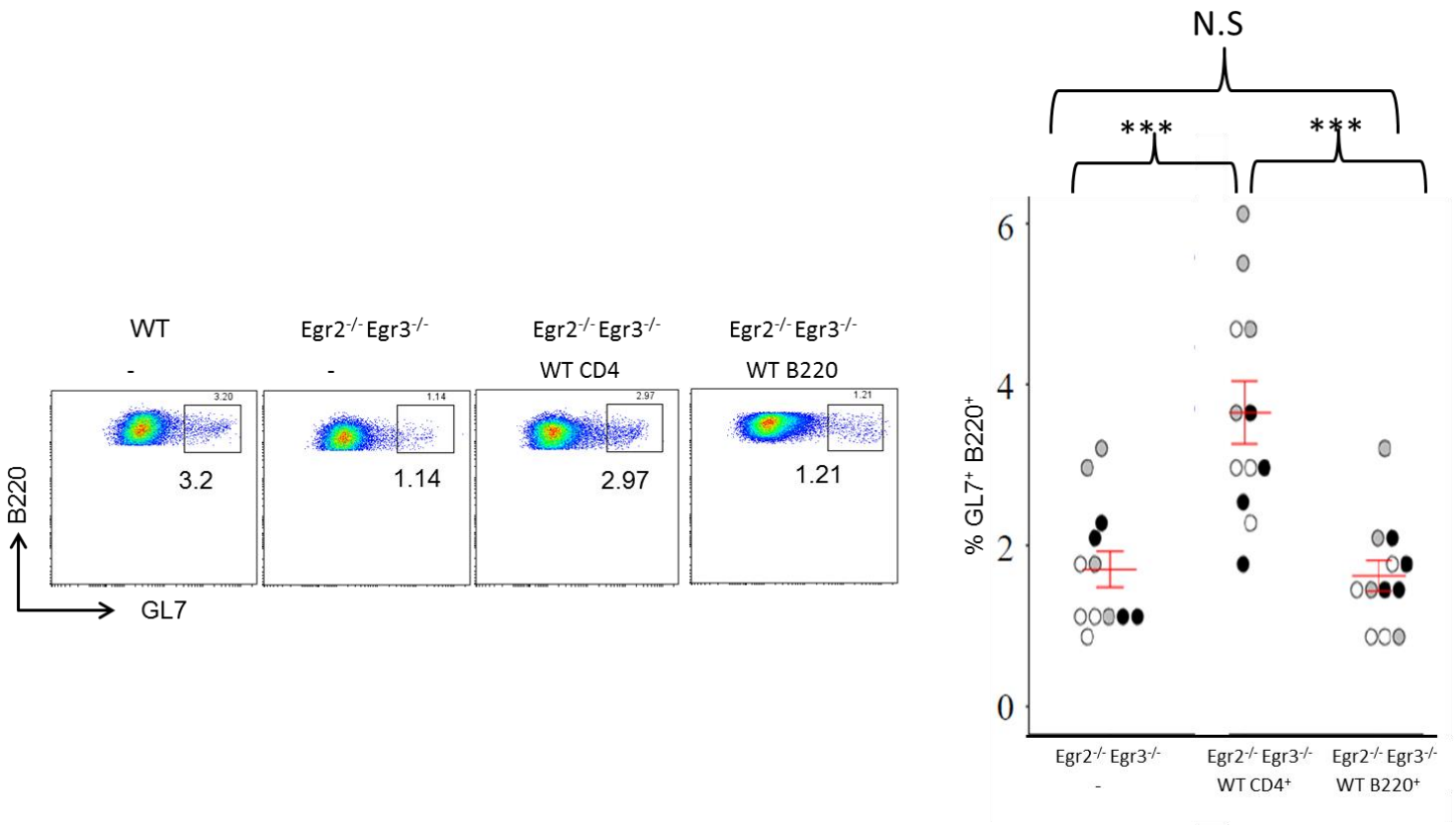

Figure 3.4.8: Transfer of WT CD4 cells into Egr2$^{-/-}$ Egr3$^{-/-}$ mice rescued the GC B cell phenotype. 2 X 10^6 WT CD4 T cells or B220 cells were transferred into Egr2$^{-/-}$ Egr3$^{-/-}$ mice. 24 hours after transfer, mice were infected with VV_{WR}. 14 days after infection, spleens were harvested and splenocytes isolated and stained for B220+ and GL7+ B cells**.** Each dot presents an individual mouse from different experiments; Mann-Whitney's U test was used to evaluate statistical significance. P<0.001 = ***; P<0.01 = **; P<0.05 = *; N.S = not significantly different

To verify that these findings were as a result of a $CD4^+$ T cell intrinsic mechanism rather than environmental influence including inflammatory environment found in $Egr2^{-/-}$ $Egr3^{-/-}$ mice, equal numbers of WT CD4/ $Egr2^{-/-}$ $Egr3^{-/-}$ B220 or $Egr2^{-/-}$ $Egr3^{-/-}$ CD4/WT B220 cells were adoptively transferred into $RAG^{-/-}$ mice. These cells were allowed to home for 10 weeks and thereafter infected with VV_{WR}. 14 days after infection, the spleens were harvested and analysed for GC development, Tfh cell and GC B cell differentiation in each of these mice. Mice that received WT CD4/ $Egr2^{-/-}$ $Egr3^{-/-}$ B220 were able to form germinal centres. This was in contrast to mice that received $Egr2^{-/-}$ $Egr3^{-/-}$ CD4/ WT B220 (figure 3.4.9).

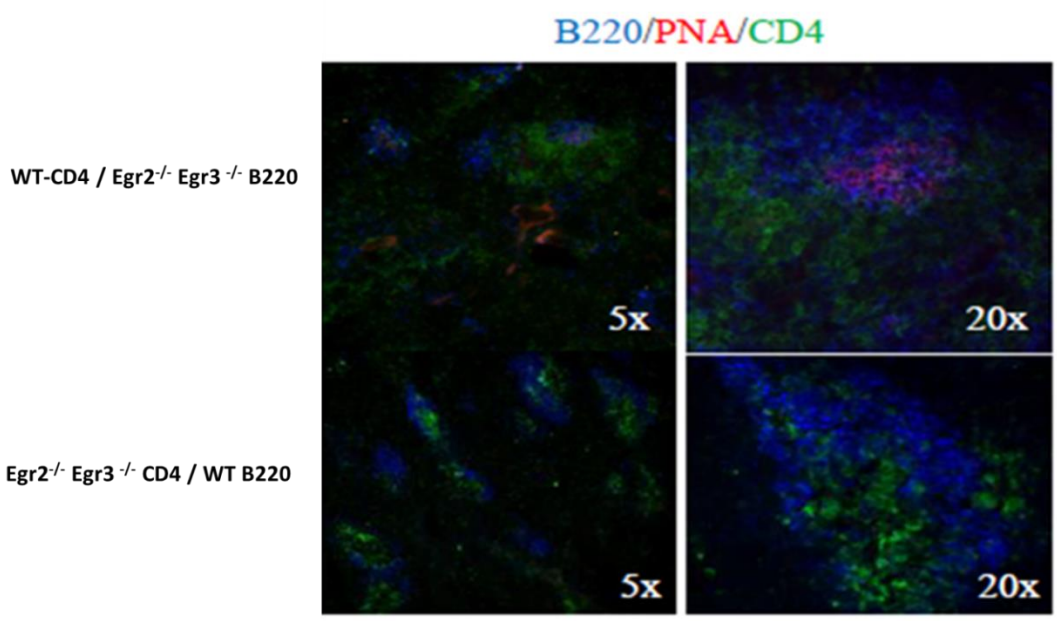

Figure 3.4.9: $RAG^{-/-}$ mice receiving WT CD4/ $Egr2^{-/-}$ $Egr3^{-/-}$ B220 were able to generate GC. 2.5 X 10^6 WT CD4 T cells/$Egr2^{-/-}$ $Egr3^{-/-}$ B220 cells or 2.5 X 10^6 $Egr2^{-/-}$ $Egr3^{-/-}$ CD4 T cells/WT B220 cells were transferred into Rag deficient mice. 10 weeks after transfer, mice were infected with VV_{WR}. 14 days after infection, spleens were harvested and analysed for the formation of germinal centres. Magnification on images indicates objective used to acquire image.

RAG$^{-/-}$ mice lack a natural lymphocyte population, and so gating for transferred lymphocytes, a distinct population of CD4^{+} T cells and B220 was established for further analysis (figure 3.4.10). Following sorting for CD4^{+} cells, Tfh cells were gated in the 2 groups of mice. It was observed that there was significantly higher amount of Tfh cells in mice that received WT CD4/ Egr2$^{-/-}$ Egr3$^{-/-}$ B220 compared with those that received Egr2$^{-/-}$ Egr3$^{-/-}$ CD4/ WT B220 (figure 3.4.11). This suggests that the increased number of Tfh cells in these mice that received WT CD4/ Egr2-/- Egr3-/- B220 cells restored GC formation. When GC B cell differentiation was analysed, it was found that there were significantly more GC B cells in RAG$^{-/-}$ mice that received WT CD4/ Egr2$^{-/-}$ Egr3$^{-/-}$ B220 (figure 3.4.12). This indicated that B cells from Egr2$^{-/-}$ Egr3$^{-/-}$ mice were capable of differentiating into GC B cells in response viral challenge provided adequate T cell help is present.

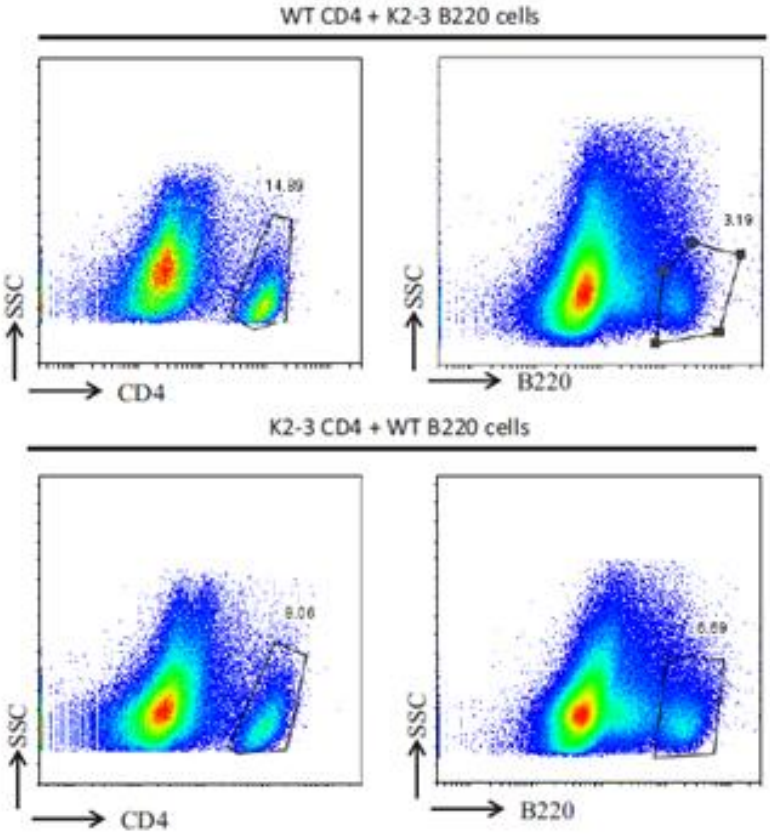

Figure 3.4.10: Staining for CD4 T cells and B220 in splenocytes isolated from RAG-/- mice receiving identified distinct population of lymphocytes in RAG$^{-/-}$ mice following adoptive transfer.

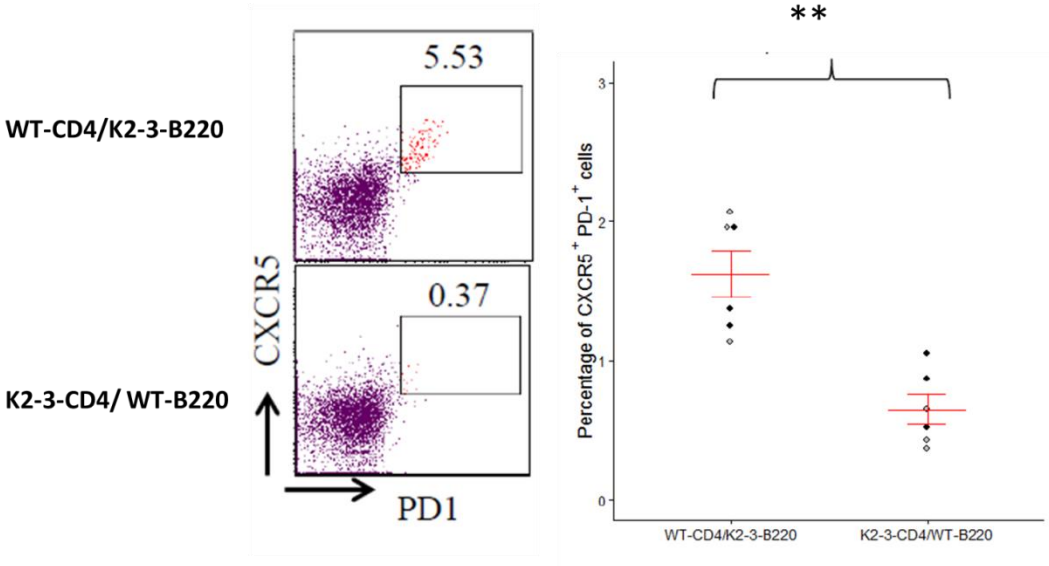

Figure 3.4.11: Adoptive transfer of WT CD4/Egr2$^{-/-}$ Egr3$^{-/-}$ B220 into RAG$^{-/-}$ mice increased CD4^{+} CXCR5^{+} PD-1^{+} Tfh cells. Each dot presents an individual mouse from different experiments; Mann-Whitney's U test was used to evaluate statistical significance. P<0.001 = ***; P<0.01 = **; P<0.05 = *

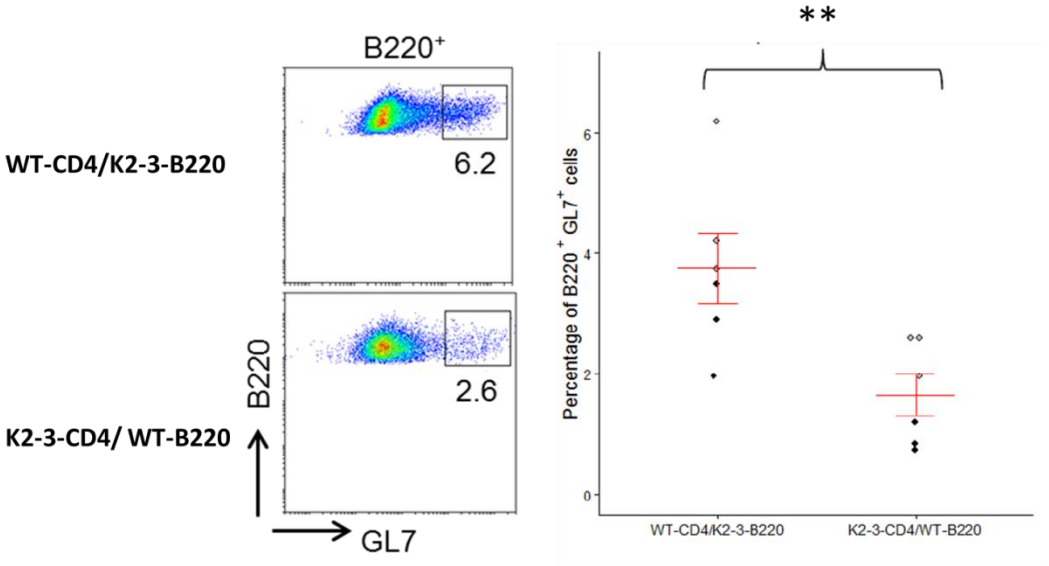

Figure 3.4.12: RAG-/- mice with adoptive transfer of WT CD4/Egr2$^{-/-}$ Egr3$^{-/-}$ B220 had significantly higher percentage of GC B cells. Each dot presents an individual mouse from different experiments; Mann-Whitney's U test was used to evaluate statistical significance <0.001 = ***; P<0.01 = **; P<0.05 = *

In order to further confirm that GC B cells from Egr2$^{-/-}$ Egr3$^{-/-}$ were intrinsically normal and the absence of GC formation and function was due to a Tfh cell defect, B cells from WT and Egr2$^{-/-}$ Egr3$^{-/-}$ mice were isolated 8 days after VV_{WR} infection and gated for GC B cells (Figure 3.4.13). These GC B cells were then analysed for the expression of Bcl6 and Egr2. Almost all GC B cells from WT and Egr2$^{-/-}$ Egr3$^{-/-}$ mice expressed Bcl6 however, in the WT GC B cells; there was no Egr2 expression (figure 3.4.13). This indicates that Egr2/3 may not be responsible for the regulation of GC B cell differentiation and confirms that defective GC formation and function was as a result of Tfh cell defect.

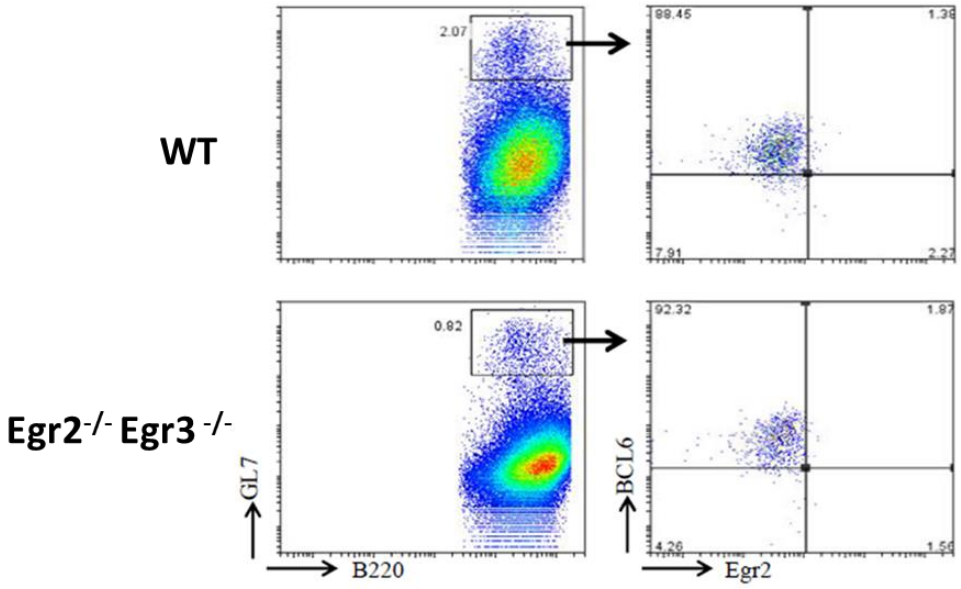

Figure 3.4.13: GC B cells from WT mice do not express Egr2 protein. B cells from VV_{WR} infected mice were isolated 8 days following infection and analysed for GC B cell differentiation and the expression of Egr2 and Bcl6 transcription factors. We found no expression of Egr2 in these B cells from WT and Egr2$^{-/-}$ Egr3$^{-/-}$ mice.

Anti-CD40 and IL-4 stimulation has been reported to initiate B cell differentiation into antibody-secreting cells (ASC) by mimicking T cell help *in vitro* (Kallies *et al.*, 2007). Lastly, we assessed the ability of CD19+ B cells isolated from Egr2$^{-/-}$ Egr3$^{-/-}$ mice to differentiate into ASC using anti-CD40 and IL-4 stimulation. The results obtained from that experiment showed significantly higher levels of secreted immunoglobulin isotypes IgG1, IgG2a, IgG2b, IgG3, IgM and IgA in Egr2$^{-/-}$

Egr3$^{-/-}$ B220 cells after *in vitro* stimulation compared to the *in vivo* stimulated Egr2$^{-/-}$ Egr3$^{-/-}$ B220 cells (Figure 3.4.14). This suggests that the class switch machinery in B cells from Egr2$^{-/-}$ Egr3$^{-/-}$ mice is functional and would undergo isotype switching in the presence of appropriate helper cells.

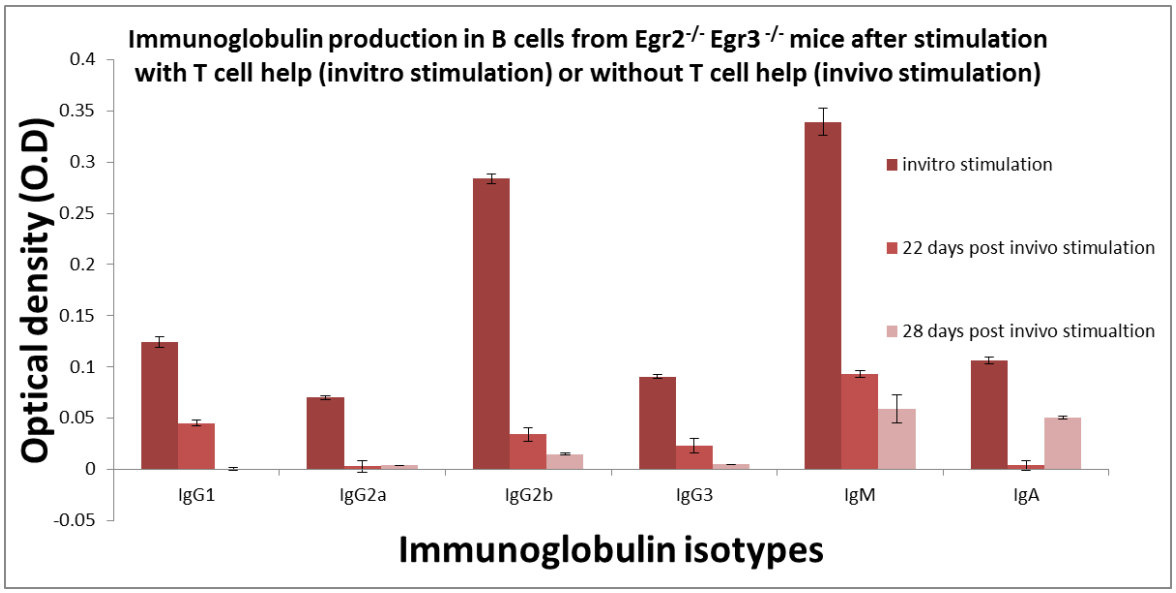

Figure 3.4.14: B cells from Egr2$^{-/-}$ Egr3$^{-/-}$ mice show significantly higher antibody titres in the presence of T cell help. *In vitro* stimulation of Egr2$^{-/-}$ Egr3$^{-/-}$ B220 cells was done using anti-CD40 and IL-4. Antibody titre for *in vivo* stimulated B cells were obtained from VV_{WR} infected sera. WT control shows comparable Ig levels in both *in vitro* and *in vivo* stimulations (Appendix figure 2, page 198)

Taken together these results illustrate that after antigenic challenge, Egr2/3 genes appear to be essential for Tfh cell differentiation and in the absence of these genes, GC formation and function fails. It further suggests that Bcl6 regulation of GC B cells may be regulated through an Egr2/3 independent mechanism. Thus GC B cells in Egr2$^{-/-}$ Egr3$^{-/-}$ mice are in essence, intrinsically normal and will respond to Tfh cells normally. This indicates that Egr2/3 function in a Tfh cell-intrinsic manner to regulate the differentiation of Tfh cells as well as the formation of GC and its functions.

3.4.4 Adoptive transfer of Egr2 or Bcl6 expressing CD4 cells rescue GC formation and Tfh phenotype in Egr2$^{-/-}$ Egr3 $^{-/-}$ mice

We have demonstrated that Egr2/3 expression was necessary for the maintenance of basal numbers of Tfh cells and its subsequent differentiation after antigenic stimulation. We next assessed if adoptive transfer of Egr2 into Egr2$^{-/-}$ Egr3$^{-/-}$ CD4^{+} T cells would rescue GC formation and Tfh differentiation *in vivo*. Expression construct for Egr2 were transfected into Egr2$^{-/-}$ Egr3$^{-/-}$ CD4^{+} T cells. These were then transferred into Egr2$^{-/-}$ Egr3 $^{-/-}$ mice. 24 hours after adoptive transfer, the mice were then infected with VV_{WR} and 14 days post infection, spleens were collected for analysis. Constitutive expression of the Egr2 gene in Egr2$^{-/-}$ Egr3$^{-/-}$ CD4^{+} T cells was able to mitigate the absence of germinal centres in the Egr2$^{-/-}$ Egr3$^{-/-}$ mice (figure 3.4.15).

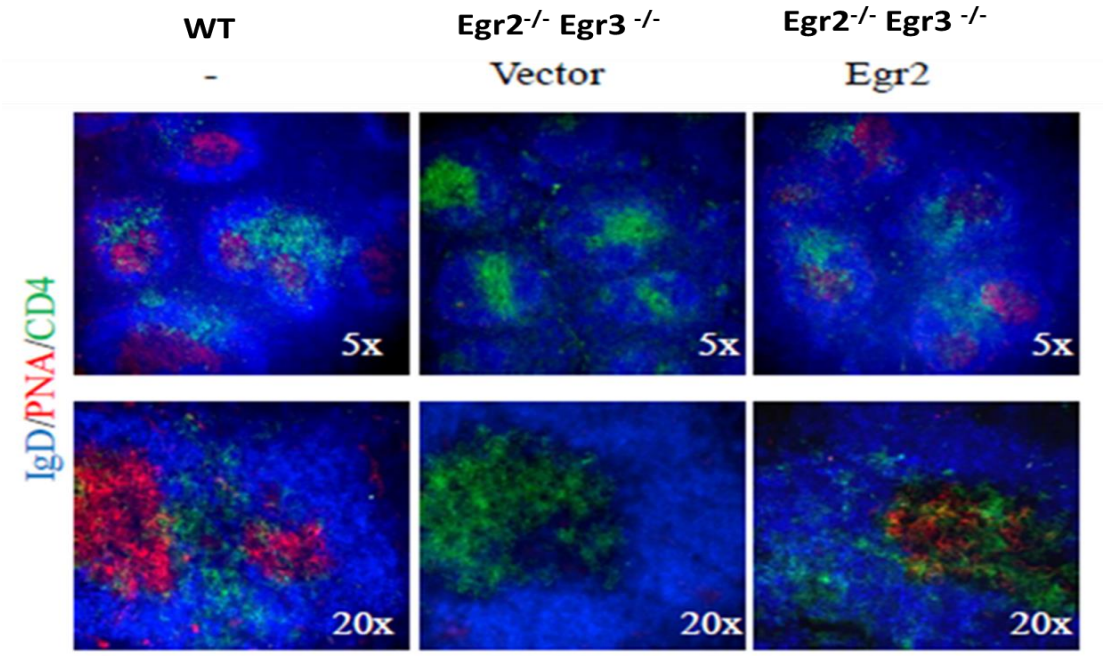

Figure 3.4.15: The development of GC was rescued by the forced expression of Egr2 in CD4 T cells from Egr2$^{-/-}$ Egr3$^{-/-}$ mice. 2 x 10^6 Egr2 expressing lentivirus were transfected into CD4 T cells from Egr2$^{-/-}$ Egr3$^{-/-}$ mice which were then adoptively transferred into Egr2$^{-/-}$ Egr3$^{-/-}$ mice, next day, mice were infected and 14 days after infection, spleens were collected for analysis of GC formation Magnification on images indicates objective used to acquire image.

Forced expression of the Egr2 gene in $CD4^+$ T cells of $Egr2^{-/-}$ $Egr3^{-/-}$ mice was also able to rescue the phenotype of the $CD4^+$ $CXCR5^+$ $PD\text{-}1^+$ Tfh cells to levels very similar to WT counterpart (figure 3.4.16). Indeed this was also the case for GC B cells differentiation. GC B cell numbers as assessed by $GL7^+$ B220 cells were elevated in $Egr2^{-/-}$ $Egr3^{-/-}$ mice receiving Egr2- expressing $CD4^+$ T cells (figure 3.4.16).

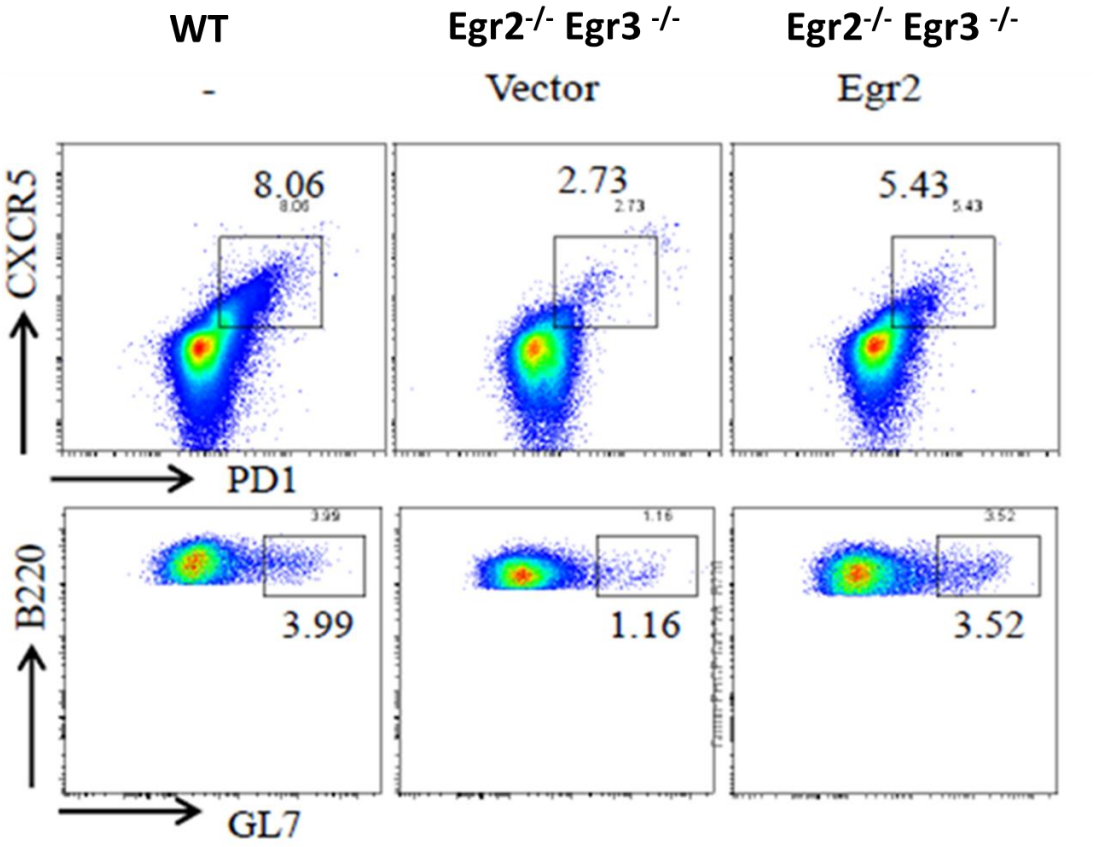

Figure 3.4.16: Constitutive expression of Egr2 in $Egr2^{-/-}$ $Egr3^{-/-}$ CD4 cells restored Tfh cell differentiation and GC B cell differentiation. 2 x 10^6 Egr2 expressing lentivirus were transfected into CD4 T cells from $Egr2^{-/-}$ $Egr3^{-/-}$ mice which were then adoptively transferred into $Egr2^{-/-}$ $Egr3^{-/-}$ mice, next day, mice were infected and 14 days after infection, spleens were collected for analysis of Tfh and GC B cell differentiation.

To investigate the possibility that Egr2 controls Tfh differentiation by regulating Bcl6 expression, Bcl6 expression construct were transfected into $CD4^+$ T cells from $Egr2^{-/-}$ $Egr3^{-/-}$ mice. This was then subsequently adoptively transferred into $Egr2^{-/-}$ $Egr3^{-/-}$ mice. Following this, the mice were infected. Similar to Egr2-expressing CD4 cells transferred into $Egr2^{-/-}$ $Egr3^{-/-}$ mice, forced expression of Bcl6 in $Egr2^{-/-}$ $Egr3^{-/-}$ CD4 T cells permitted the formation of GCs in these $Egr2^{-/-}$ $Egr3^{-/-}$ mice (figure 3.4.17). Analysis of the expression of $CD4^+$ $CXCR5^+$ $PD\text{-}1^+$ Tfh cells showed $Egr2^{-/-}$ $Egr3^{-/-}$ mice that received $Egr2^{-/-}$ $Egr3^{-/-}$ CD4 T cells with constitutively expressed Bcl6 were able to restore the Tfh cell phenotype as well as rescue the differentiation of GC B cell in response to viral challenge (figure 3.4.18). The results presented here demonstrate that Egr2 may control Tfh differentiation by the regulation of the Bcl6 gene.

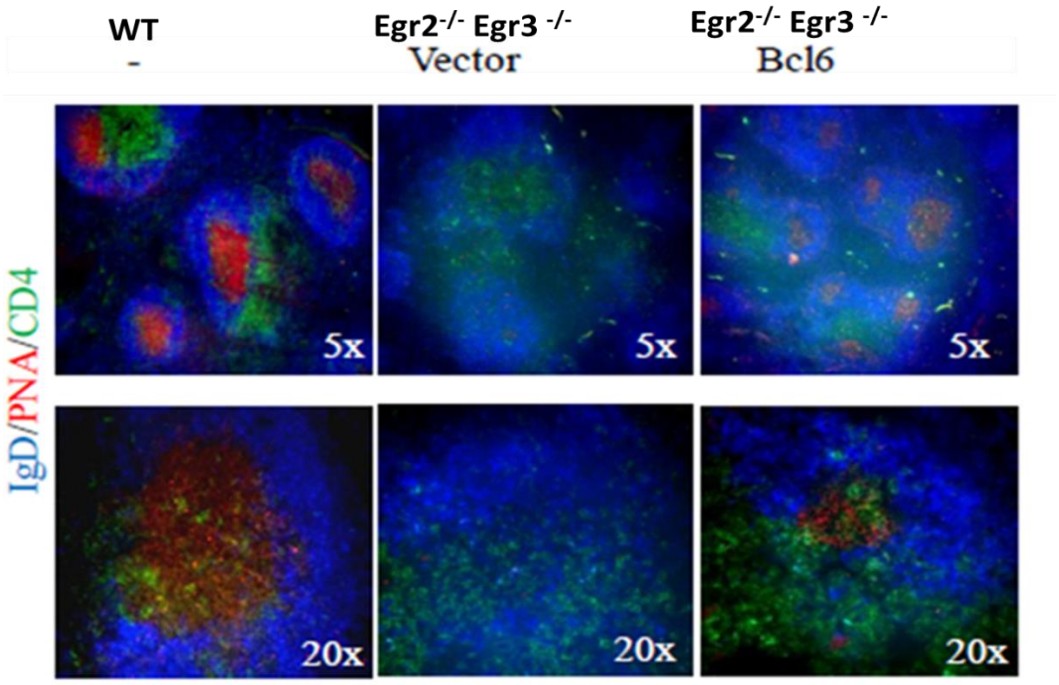

Figure 3.4.17: GC development is rescued in $Egr2^{-/-}$ $Egr3^{-/-}$ mice receiving Bcl6 transfected $Egr2^{-/-}$ $Egr3^{-/-}$ CD4 T cells. 2 x 10^6 Bcl6 expressing lentivirus were transfected into CD4 T cells from $Egr2^{-/-}$ $Egr3^{-/-}$ mice which were then adoptively transferred into $Egr2^{-/-}$ $Egr3^{-/-}$ mice, next day, the mice were challenged using VV_{WR}. 14 days after challenge, mice were sacrificed and samples analysed for GC formation. Magnification on images indicates objective used to acquire image.

Chapter 3 - Results

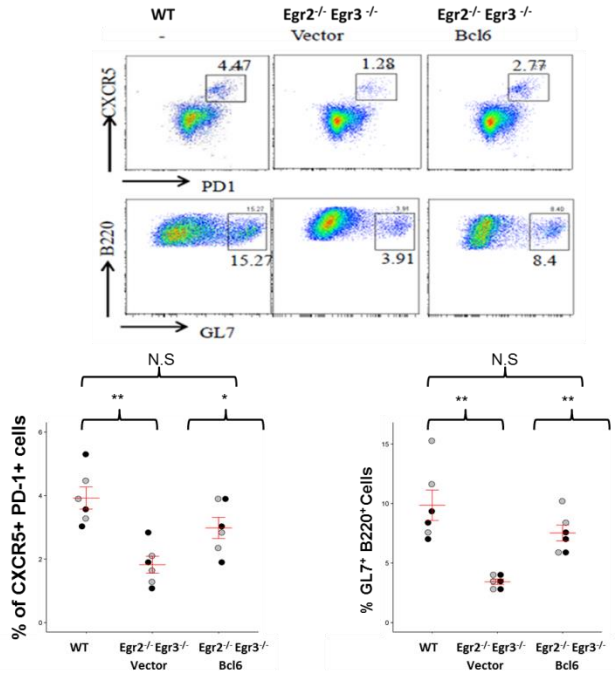

Figure 3.4.18: Forced expression of Bcl6 in CD4 T cells from Egr2$^{-/-}$ Egr3$^{-/-}$ mice is able to restore Tfh differentiation and GC B cell development in Egr2$^{-/-}$ Egr3 $^{-/-}$ mice. 2 x 10^6 Bcl6 expressing lentivirus were transfected into CD4 T cells from Egr2$^{-/-}$ Egr3$^{-/-}$ mice which were then adoptively transferred into Egr2$^{-/-}$ Egr3$^{-/-}$ mice, next day, the mice were challenged using VV_{WR}. 14 days after challenge, mice were sacrificed and spleens were collected for analysis of Tfh and GC B cell differentiation.Each dot presents an individual mouse from different experiments; Mann-Whitney's U test was used to evaluate statistical significance. P<0.001 = ***; P<0.01 = **; P<0.05 = *; N.S = not significantly different

3.4.5 Differentiation of Tfh-like cells in *in vitro* culture is impaired in Egr2$^{-/-}$ Egr3 $^{-/-}$ mice

I had earlier reviewed how IL-21, IL-6 and Bcl6 amongst other molecules are necessary for Tfh differentiation *in vivo*. Nurieva et al, 2008 has also reported that in *in vitro* culture, IL-21 recombinant protein in the presence of Th1, Th2 and Th17 cytokine antagonists would drive the differentiation of Tfh-like cells (Nurieva *et al.*, 2008). We sought to determine the role of the Egr2/3 genes in the *in vitro* differentiation of Tfh-like cells in the presence of IL-21. For this, Isolated CD4^{+} T cells were stimulated and cultured under Th0 or Tfh-like inducible cultures. For cells treated under Tfh conditions, over 60% of the WT cells differentiated into Tfh-like cells and majority of these cells co-expressed Egr2 and Bcl6 (Figure 3.4.19A) again, highlighting the importance of Egr2 expression in these Tfh cells. In the Egr2$^{-/-}$ Egr3$^{-/-}$ CD4^{+} cells, culturing under Tfh conditions resulted in only about 20% of CD4^{+} cells differentiating into Tfh-like cells (Figure 3.4.19A). WT CD4^{+} T cells cultured under Tfh-like conditions show high Bcl6 mRNA expression while contrastingly low levels of Bcl6 were observed in Tfh-like CD4^{+} T cells from Egr2$^{-/-}$ Egr3 $^{-/-}$ mice suggesting a role for Egr2/3 genes in the upregulation of Bcl6 in Tfh-like cells (figure 3.4.19B). This is consistent with *in vivo* data.

When IL-21 expression levels were analysed by RT-PCR, it was found to be down regulated in Egr2$^{-/-}$ Egr3$^{-/-}$ Tfh-like cells (Figure 3.4.19B). IL-21 is an important cytokine that supports Tfh cell differentiation, GC B cell differentiation and function (Bollig *et al.*, 2012; Linterman *et al.*, 2010; Zotos *et al.*, 2010; Johnston *et al.*, 2009; Bryant *et al.*, 2007). Absence of this cytokine indicates deregulation of normal Tfh activity in the Egr2$^{-/-}$ Egr3$^{-/-}$ mice. This supports earlier data showing a failure to generate GC B cells due to deregulated Tfh help in the Egr2$^{-/-}$ Egr3$^{-/-}$ mice. These results indicate a possible role for Egr2/3 in the upregulation of IL-21 production during Tfh differentiation.

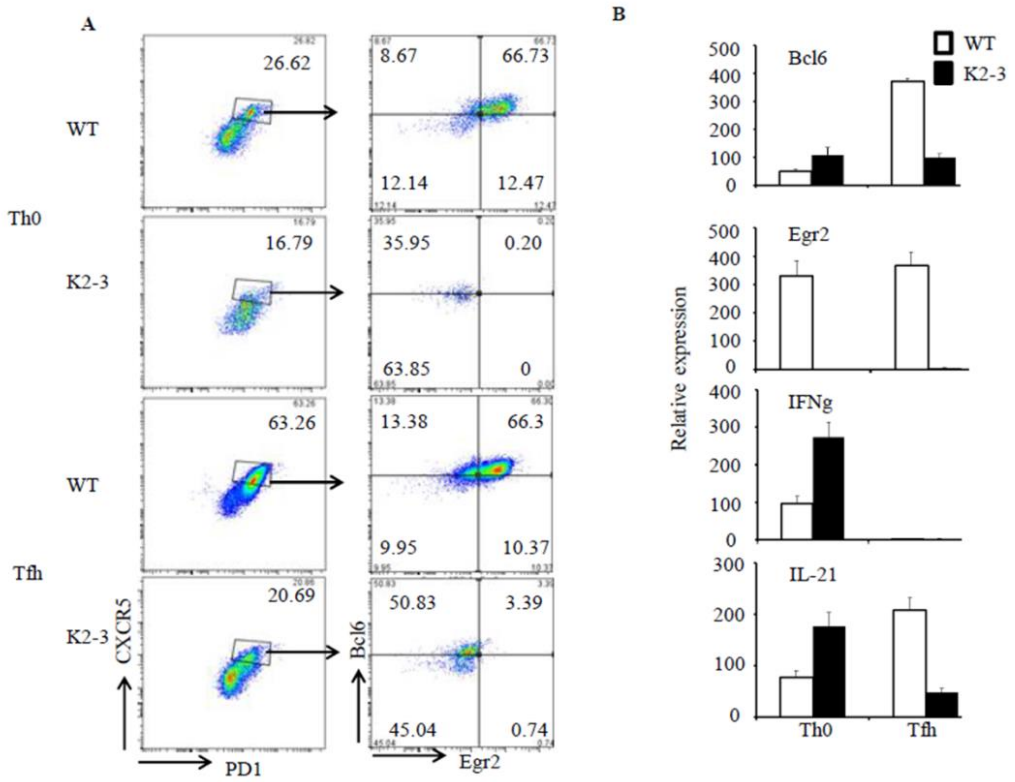

Figure 3.4.19: Lack of the Egr2/3 genes impairs the differentiation of CD4 T cells into Tfh-like cells in *in vitro* cultures. A. Isolated CD4 T cells were cultured for 5 days in the presence (Tfh-like conditions) or absence (Th0 conditions) of recombinant IL-21, anti-IFN-γ and anti-IL4 for 5 days after which they were stained and analysed for the differentiation of Tfh cells. **B.** RT-PCR was also performed on these cells to analyse the expression of effector cytokines.

3.5 Molecular mechanism of Bcl6 regulation by Egr2

3.5.1 $CD4^+$ Th cell are activated after antigenic stimulation in $Egr2^{-/-}$ $Egr3^{-/-}$ mice

In the previous sub-chapters, I had elaborated on the absence of GC in $Egr2^{-/-}$ $Egr3^{-/-}$ mice and the defective differentiation of Tfh cells within these mice. This may have been responsible for the reduced number of GC B cells and defective GC formation and function in $Egr2^{-/-}$ $Egr3^{-/-}$ mice. It became imperative, therefore, to understand the molecular mechanism that governs Egr2/3 gene regulation of Tfh cell differentiation. For this 2 possibilities were assessed. The first was if impaired $CD4^+$ Th cell activation after antigenic stimulation was responsible for the defective Tfh differentiation in $Egr2^{-/-}$ $Egr3^{-/-}$ mice. The second was if Egr2 directly interacted with Bcl6, the signature transcription factor for Tfh cells, to promote Tfh cell differentiation.

In order to investigate Th differentiation in $Egr2^{-/-}$ $Egr3^{-/-}$ mice *in vivo*, the activation of CD4 T cells and the production of effector cytokines before and after VV_{WR} infection were analysed. Our results show that following VV_{WR} infection, CD4 T cells in $Egr2^{-/-}$ $Egr3^{-/-}$ mice differentiated into $CD44^{high}$ effector phenotype and upregulated effector cytokine levels (figure 3.5.1 and 3.5.2). These suggest that activation and differentiation into effector subsets was not defective in $Egr2^{-/-}$ $Egr3^{-/-}$ mice and thus could be excluded as the cause for defective Tfh cell differentiation in $Egr2^{-/-}$ $Egr3^{-/-}$ mice.

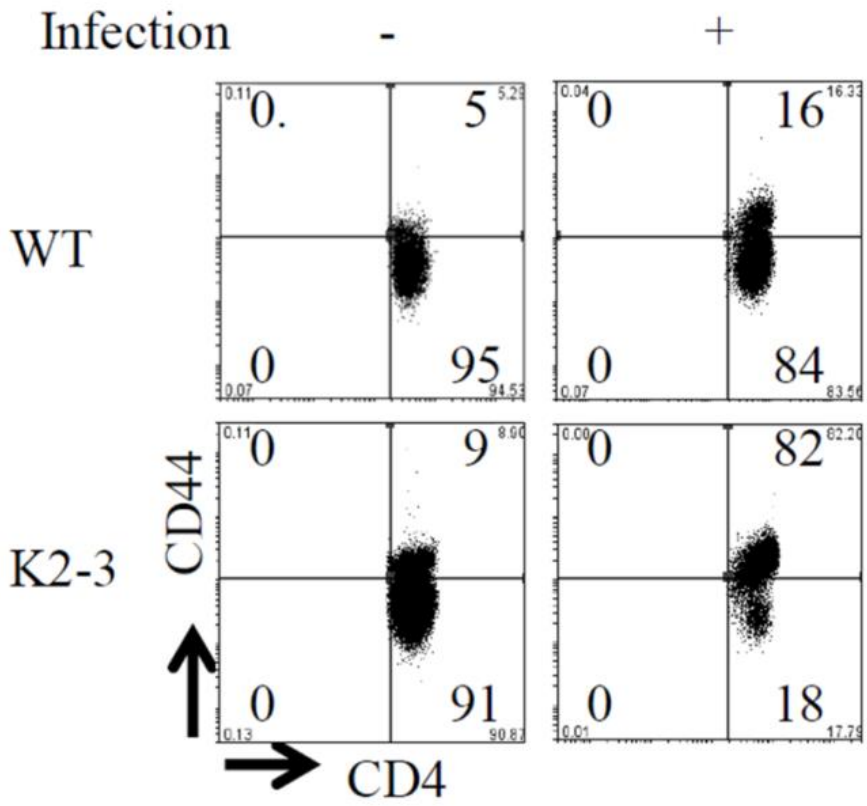

Figure 3.5.1: High expression of CD44+ CD4 T cells in Egr2$^{-/-}$ Egr3$^{-/-}$ mice following VV$_{WR}$ infection. CD4 T cells from WT and Egr2$^{-/-}$ Egr3$^{-/-}$ mice were analysed for activation marker CD44 before and after VV$_{WR}$ infection. Egr2$^{-/-}$ Egr3$^{-/-}$ mice show high expression of activation marker CD44.

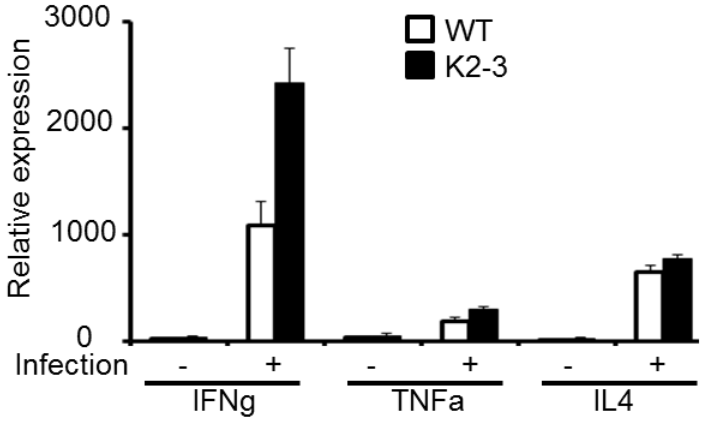

Figure 3.5.2: Effector cytokines are upregulated in Egr2$^{-/-}$ Egr3$^{-/-}$ mice following VV$_{WR}$ infection. mRNA expression levels for effector cytokines from CD4 T cells before and after infection show increased production of effector cytokines after VV$_{WR}$ infection. Data is presented relative to the expression of β-actin mRNA.

3.5.2 Egr2 interaction with the Bcl6 gene

We have shown that the Egr2/3 genes are capable of increasing the expression of Bcl6 in WT Tfh cells after infection and that $Egr2^{-/-}$ $Egr3^{-/-}$ mice had reduced Bcl6 expression levels. The impaired expression of Bcl6 in $Egr2^{-/-}$ $Egr3^{-/-}$ mice points towards a possible role of the Egr2 or Egr3 genes in the regulation of Bcl6 in Tfh cells. Therefore, the possibility of Egr2 interaction with the Bcl6 gene was assessed. Using the multiTF utility software, we investigated the potential role of Egr2 in Bcl6 regulation of Tfh cells. Analysis of the Bcl6 gene locus highlighted 3 potential Egr2 binding sites on the 1st intron (site 1), distal promoter region (site 2) and downstream of the Bcl6 gene (site 3) on the Bcl6 gene (figure 3.5.3).

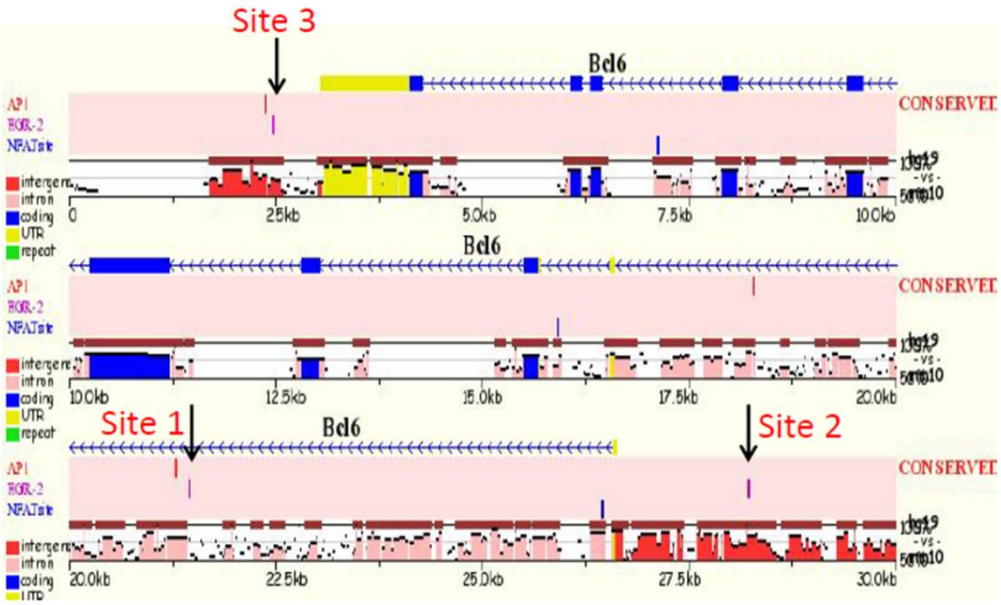

Figure 3.5.3: Analysis of Bcl6 gene locus shows it has 3 potential Egr2 binding sites.

Using EMSA (performed by Dr. Tizong Miao at Queen Mary University, London), we next analysed the interaction between the Egr2 protein and these potential Egr2 binding sites on Bcl6 gene by probing the ability of oligos obtained from these binding sites to competitively bind to Egr2 protein. We found that the 3 different binding sites were able to effectively compete for Egr2 binding against a probe containing the Egr2 consensus sequence (5'-TGTAGGGGCGGGGGCGGGGTTA -3') (Figure 3.5.4). In order for us to verify gene interaction obtained via EMSA, we used chromatin Immunoprecipitation (ChIP) assay (performed by Dr. Tizong Miao at Queen Mary University, London), on WT CD4 T cells and found that Egr2 was able to bind to 2 of the 3 potential binding sites (Figure 3.5.4). This demonstrates that Egr2 protein does indeed interact with these binding sites on the Bcl6 gene locus and can therefore regulate the expression of Bcl6 in Tfh cells.

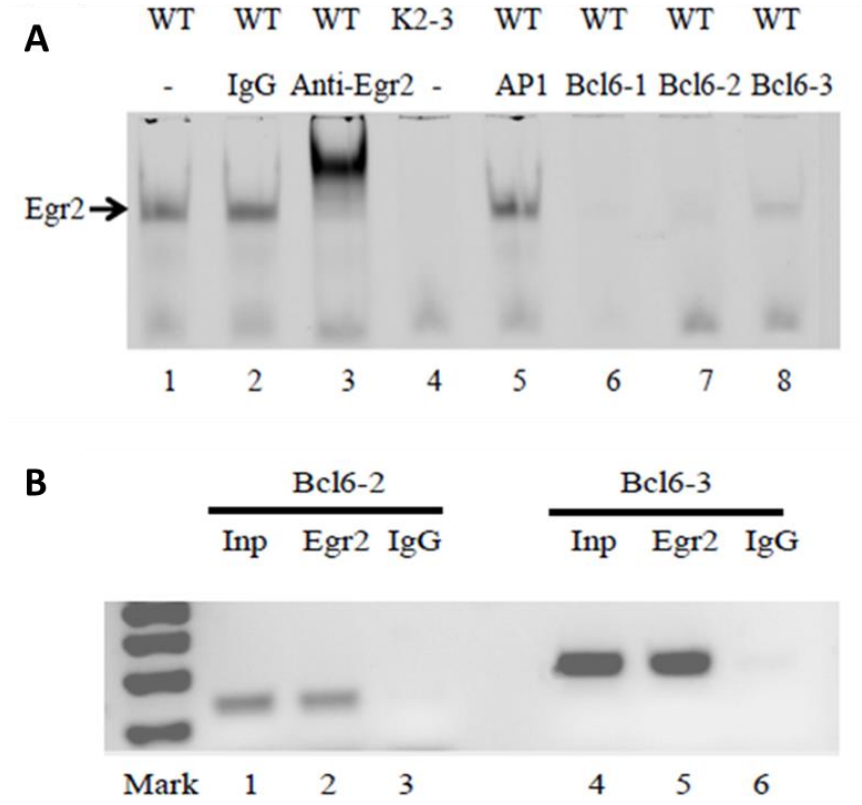

Figure 3.5.4: EMSA and ChIP assay confirms that Egr2 interacts with the Bcl6 gene. **A**. EMSA shows the interaction between Egr2 protein and the 3 binding sites on the Bcl6 gene locus **B.** Chromatin immunoprecipitation confirms Egr2 protein interacts with the 2 of the potential Egr2 binding sites on the Bcl6 gene.

3.5.3 Forced expression of Bcl6 or Egr2 restored the normal expression of Tfh regulatory genes Bcl6 and Blimp1

To confirm the functionality of Egr2 regulation of the Bcl6 gene, CD4 T cells from $Egr2^{-/-}$ $Egr3^{-/-}$ mice were transfected with either Egr2 or Bcl6 expression constructs and then analysed for the expression of Tfh regulatory genes Bcl6 and Blimp1. Expression of Egr2 drove the expression of Bcl6 while reducing the relative expression of Bcl6 antagonist, Blimp-1. Similarly, Bcl6 lentivirus increased the expression of Bcl6 in these $Egr2^{-/-}$ $Egr3^{-/-}$ CD4 cells while downregulating the Blimp1 mRNA level (figure 3.5.5). This indicates that Egr2 interaction with Bcl6 may be necessary for its physiological function.

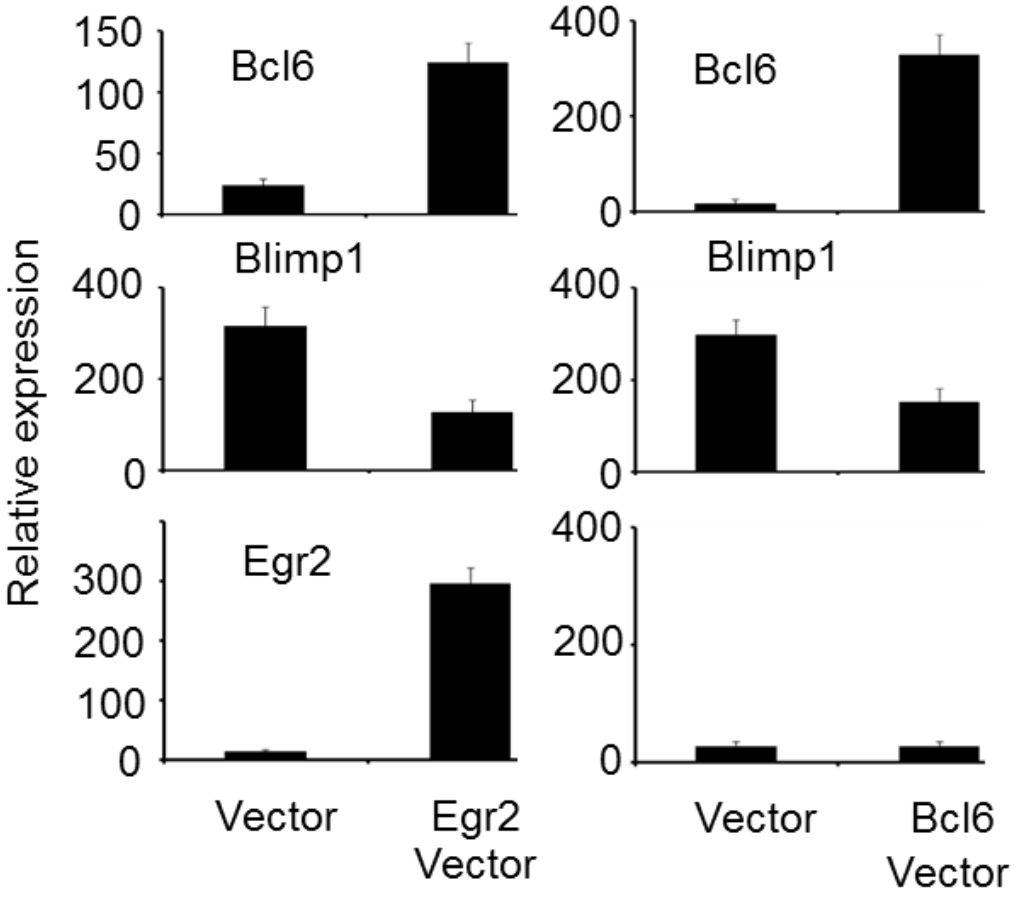

Figure 3.5.5: Constitutive expression of Egr2 or Bcl6 in vectors transferred into $Egr2^{-/-}$ $Egr3^{-/-}$ mice restored Bcl6 expression in CD4 T cells from these $Egr2^{-/-}$ $Egr3^{-/-}$ mice. mRNA was extracted and RT-PCR was performed on $Egr2^{-/-}$ $Egr3^{-/-}$ CD4 T cells transfected with either an empty lentivirus or an Egr2 or Bcl6 expressing lentivirus

Finally, when the expression of some of the other genes involved in Tfh cell regulation were analysed**,** IRF4, cMaf, CXCR5 and SH2D1A were found to remain relatively similar in WT and Egr2$^{-/-}$ Egr3$^{-/-}$ mice (figure 3.5.6). All of these data taken together demonstrates that Egr2 control of Tfh cells differentiation is by direct regulation of the Tfh signature transcription factor Bcl6.

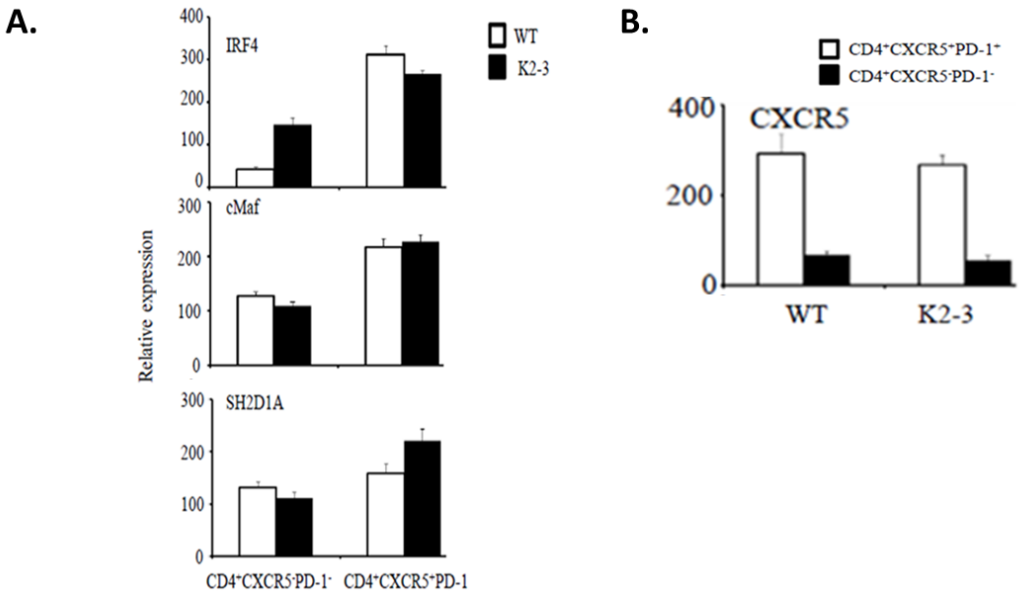

Figure 3.5.6: Relative expression of other Tfh related genes were similar in WT and Egr2$^{-/-}$ Egr3$^{-/-}$ mice. RT-PCR performed on sorted CXCR5+ PD1+ and CXCR5- PD1- CD4 T cells from WT and Egr2$^{-/-}$ Egr3$^{-/-}$ mice show that the expression of IRF4, cMAF, SH2D1A, CXCR5 were relatively similar between both mice groups

Chapter 4 General Discussion

Studies into the early growth response genes 2 and/or 3 have achieved significant strides in recent years (Du *et al.*, 2014; Miao *et al.*, 2013; Li *et al.*, 2012). These transcription factors are gaining grounds as regulators of the immune system. They are reported to control development, activation and effector function of cellular mediators of the innate and adaptive immune system including NK cells (Lazarevic *et al.*, 2009a; Bassiri and Nichols, 2009), macrophages (Syed *et al.*,) and lymphocytes (Li *et al.*, 2012; Li *et al.*, 2011). Limitations in Egr2 *in vivo* studies include neonatal fatalities arising from the systemic knockout (KO) of the Egr2 gene (Baloh *et al.*, 2009). While some researchers have used siRNA to study Egr2 functions (Barbeau *et al.*, 2014; Fang *et al.*, 2011), this can be limiting in the sense that *in vitro* studies do not necessary correlate with *in vivo* studies as it is an arduous task replicating the cellular microenvironment found within an organism. This necessitated the creation of conditional KO of the Egr2 gene in order to study its role in various systems. For this study, I have used the lymphocyte-specific CD2-promoter to knockout the Egr2 gene in order to investigate the role of Egr2/3 in the control of inflammation pathology and regulation of follicular helper T cell differentiation.

4.1 Loss of Egr2/3 in lymphocytes leads to organ pathology due to chronic inflammation

It has been previously reported that in the absence of the Egr2/3 genes, mice show high levels of pro-inflammatory cytokines such as IFNγ, IL-17A, IL-17F and GM-CSF as well as develop autoimmune diseases (Li *et al.*, 2012; Zhu *et al.*, 2008). Li et al, found that Egr2/3 genes directly regulated the expression of the SOCS1 and SOCS3 genes (Li et al., 2012). These genes control the Th1 and Th17 lineage specific STATS (STAT1 and STAT3) (Li et al., 2012). They proposed that Egr2 and 3 might be necessary for reducing immunopathology during immune responses (Li *et al.*, 2012). Data from that study is supported by other researcher's findings showing increased levels of IFNγ and IL-17 in Egr2 single KO mice (Miao *et al.*, 2013; Iwasaki and Medzhitov, 2010; Zhu *et al.*, 2008)

From this study, it has now been shown that the onset of immunopathology within these $Egr2^{-/-}$ $Egr3^{-/-}$ mice coincides with the age from which high levels of

pro-inflammatory cytokines are visible in the serum of Egr2$^{-/-}$ Egr3$^{-/-}$ mice as reported by Li et al, 2012. This information could further understanding of the molecular basis of organ pathology arising from inflammation.

Egr2 and Egr3 genes have previously been associated with the development of autoimmune diseases (Li et al., 2012, Zhu et al., 2008). The results presented by this thesis identified severe organ damage in these Egr2$^{-/-}$ Egr3$^{-/-}$ mice. The link between organ damage and autoimmunity is supported by reports associating the presence of autoantibodies, pro-inflammatory cytokines and activated self-reactive CD4 T cells in the observed pathology in SLE and MS autoimmune disease patients as well as in mouse models of autoimmunity (Ichinose *et al.*, 2011; Blair *et al.*, 1994). Other reports also show that organ pathology arising from axon damage is closely associated with the presence of chronic inflammation within the nervous tissues in MS patients (Brück, 2005). A publication by Miao et al., 2013 also showed defective Egr2 expression in MS patients (Miao *et al.*, 2013). From these publications, organ pathology in Egr2$^{-/-}$ Egr3$^{-/-}$ mice can be linked with inflammation and autoimmune diseases development. This indicates that our model of Egr2$^{-/-}$ Egr3$^{-/-}$ mice can serve as an appropriate model for the study of autoimmune diseases.

The Th1 cytokine IFNγ has long been known to be capable of activating macrophages (Schroder *et al.*, 2004). As high IFNγ levels are a feature of Egr2$^{-/-}$ Egr3$^{-/-}$ mice, IFNγ could contribute to organ pathology by activating macrophages as observed by CD11b staining in the spleens of Egr2$^{-/-}$ Egr3$^{-/-}$ mice. The presence of APCs like macrophages at inflammatory sites has been implicated in the presentation of auto-antigens to T lymphocytes in some studies (Bailey *et al.*, 2007; Gutcher and Becher, 2007). The role of macrophages within the spleens of Egr2$^{-/-}$ Egr3$^{-/-}$ mice in promoting autoimmunity is yet to be investigated however; macrophages produce IL-1 and TNF which can further drive chronic inflammation and the ensuing organ pathology (Gutcher and Becher, 2007). In essence, Egr2/3 genes are necessary to prevent organ pathology that may contribute to the development of autoimmune diseases.

4.2 Transcriptional programme involved in Tfh cell differentiation

As mentioned earlier in the literature review, antigen presentation and co-stimulation by dendritic cells along with cytokine production induces the differentiation of naïve CD4 T cells into one of a number of effector subsets. Egr2/3 have been identified to play a role in effector T cell differentiation (Du *et al.*, 2014). In naïve $CD4^+$ T cells, upregulation of Tbx21 (the gene encoding Tbet) skews the differentiation of Th cells into the Th1 pathway. Th1 cells mediate the activation of cytotoxic $CD8^+$ T cells. Researchers have shown increased numbers of Th1 cells after prolonged stimulation of $CD4^+$ T cells in Egr2cKO (Zhu *et al.*, 2008). IFNγ produced by Th1 cells was increased in our mouse model as well and was implicated in the development of autoimmunity (Li *et al.*, 2012; Zhu *et al.*, 2008). Th17 cells are induced by the expression of the Rorc (gene encoding RORγt) transcription factor in the presence of Tgfβ, IL-6 and IL-21. Th17 levels in Egr2cKO mice were increased following TCR ligation leading to enhanced production of IL-17 (Miao *et al.*, 2013; Li *et al.*, 2012; Zhu *et al.*, 2008). High IL-17 levels have been linked with organ pathology in CD2-$Egr2^{-/-}$ mice (Miao *et al.*, 2013).

T Follicular helper cells, a newer member of the effector T cell subset develops when Bcl6 expression is elevated in newly activated $CD4^+$ T cells in the presence of IL-6 and IL-12 in mouse and IL-6, IL-12, IL-21 and IL-23 in humans. Differentiated Tfh cells provide help to follicular B cells inducing their differentiation into germinal centre (GC) B cells. GC B cells then migrate back into follicles to form GC that mediates T cell dependent humoral immune responses. In the absence of Tfh cells, GC responses are defective and an immunodeficient phenotype arises (Pratama and Vinuesa, 2014; Yusuf *et al.*, 2010). As the signature transcription factor for this lineage, the expression of Bcl6 is paramount for Tfh differentiation and consequently for optimal T cell dependent humoral immunity. $Bcl6^{-/-}$ T cells were unable to differentiate into Tfh cells (Hollister *et al.*, 2013; Yu and Vinuesa, 2010; Yu *et al.*, 2009). Lack of Tfh cells in these $Bcl6^{-/-}$ mice meant they were unable to provide help to GC B cells and consequently these mice failed to develop GC and had defective GC responses in response to antigens (Yu *et al.*,

2009). In this study, using Egr2$^{-/-}$ Egr3$^{-/-}$ mice model, array analysis measured Bcl6 expression levels in total CD4^{+} T cells from Egr2$^{-/-}$ Egr3$^{-/-}$ to be at least 2 fold downregulated in stimulated and unstimulated conditions compared to WT mice. Bcl6 expression levels was also found to be defective in stimulated and unstimulated total CD4^{+} T cells and Tfh cells isolated from Egr2$^{-/-}$ Egr3$^{-/-}$ mice *invitro*. *In vivo* analysis of Tfh differentiation in Egr2$^{-/-}$ Egr3$^{-/-}$ mice before and after acute viral infection showed reduced Bcl6 protein levels and significantly lower numbers of Tfh cells in these mice. These findings are in line with those of Robert Johnson et al and Yu et al both in 2009. They demonstrated higher levels of Bcl6 mRNA in WT Tfh cells, lower levels in non-Tfh cells as well as a lack of Tfh cells in Bcl6 null mice (Johnston *et al.*, 2009; Yu *et al.*, 2009). When the Bcl6 gene was overexpressed in lentiviral constructs, and then adoptively transferred into Egr2$^{-/-}$ Egr3$^{-/-}$ mice before viral challenge, it rescue of the differentiation of Tfh cells and GC B cells in Egr2$^{-/-}$ Egr3$^{-/-}$ mice. This is in agreement with previous studies showing overexpression of Bcl6 induced Tfh differentiation (Johnston *et al.*, 2009; Yu *et al.*, 2009) and pointing to a role of Egr2 in driving Tfh cells differentiation by Bcl6 regulation. This role of Egr2 in Bcl6 regulation was confirmed by the discovery that Egr2 directly interacted with the Bcl6 gene.

Although Bcl6 is regarded as the master transcription factor for this lineage, a number of other transcription factors work alongside Bcl6 to promote or inhibit the Tfh cell program and thus regulate T cell dependent humoral immune responses. Blimp1 is a direct repressor of Bcl6 (Hollister *et al.*, 2013; Johnston *et al.*, 2009). Bcl6 was shown to directly inhibit Blimp1 expression in Tfh specifically (Hollister *et al.*, 2013). Blimp1 levels are elevated in non-Tfh CD4^{+} T cells and mice overexpressing the Blimp1 protein had severely impaired Tfh differentiation (Johnston *et al.*, 2009). This correlates with the findings of this study showing increased Prdm1 (gene coding for Blimp1) levels in array analysis of total CD4^{+} T cells from Egr2$^{-/-}$ Egr3$^{-/-}$ mice. This was confirmed by RT-PCR on Tfh cells from the same mice. Consequently, these mice had reduced differentiation of Tfh cells evident by staining for Tfh markers CXCR5, PD-1. More so, overexpression of Egr2

or Bcl6 in Egr2$^{-/-}$ Egr3$^{-/-}$ CD4 T cells reduced the expression of Blimp1 in these cells suggesting a role for Egr2 in Bcl6 physiological function in Tfh cells.

Ascl2, a transcription factor active in Tfh cells is reported to be critical for CXCR5 expression and is upregulated early to initiate Tfh migration to T-B cell border (Liu *et al.*, 2014b). There was no significant difference in the expression of CXCR5 mRNA levels in Egr2$^{-/-}$ Egr3$^{-/-}$ mice inferring that Egr2/3 genes were not involved in upregulation of CXCR5 expression during Tfh cell differentiation. This is supported by the works of Liu et al., 2014. They found that in Bcl6^{lo} T cells, CXCR5 expression was upregulated after NP-OVA immunisation (Liu *et al.*, 2014b).

cMaf and IRF4 induce IL-21 production in Tfh cells and is expressed at high levels in human Tfh cells (Kroenke *et al.*, 2012). Constitutive expression of cMaf was able to significantly increase IL-21 and CXCR5 expression to drive Tfh differentiation (Kroenke *et al.*, 2012). Interferon regulatory factor 4 (IRF4) is reported to be involved in Th2, Th17, Th9 and Tfh differentiation (Bollig *et al.*, 2012). IRF4$^{-/-}$ mice failed to differentiate into Tfh cells and lacked GC (Bollig *et al.*, 2012). Although a comprehensive study has not been carried out on the mechanism for IRF4 regulation of Tfh cell differentiation, Bollig et al., 2012, suggests that it may be through interaction with Bcl6 as they found Bcl6 levels to be significantly altered in their model (Bollig *et al.*, 2012). It is therefore important to mention there was no difference in the expression levels of cMaf and IRF4 between WT and Egr2$^{-/-}$ Egr3$^{-/-}$ mice.

Batf is reported to directly regulate Bcl6 and cMaf expression in CD4^{+} T cells and Batf$^{-/-}$ CD4^{+} T cells showed reduced expression of both Bcl6 and cMaf (Ise *et al.*, 2011). We previously reported enhanced Batf activity in Egr2$^{-/-}$ Egr3$^{-/-}$ mice (Li et al., 2012). High Batf levels and low Tfh levels in our model suggest that Egr2 inhibition of Batf activity in AP-1 signalling does not hinder Batf and Bcl6 interaction.

In this study, a novel transcriptional regulator of Tfh differentiation in the form of the Egr2/3 genes has now been identified. Our model of CD2-Egr2$^{-/-}$ Egr3$^{-/-}$ is very similar in phenotype to the Bcl6$^{-/-}$ model showing upregulation of Blimp1 in CD4^{+} T cells, aberrant Tfh differentiation, impaired GC B cell differentiation and aberrant GC formation and function (Yu *et al.*, 2009; Johnston *et al.*, 2009; Fukuda

et al., 1997). Normal expression of Blimp1 was only restored in $Egr2^{-/-}$ $Egr3^{-/-}$ mice by the expression of Egr2 or Bcl6 in lentiviral constructs. This is in agreement with experiments conducted by other researchers showing down regulation of Blimp1 expression after forced Bcl6 expression (Johnston *et al.*, 2009). As expected, adoptive transfers of WT $CD4^{+}$ T cells with endogenous expression of Egr2 and Bcl6 into $Egr2^{-/-}$ $Egr3^{-/-}$ mice, restored Tfh and GC B cell differentiation as well as GC formation. Indeed this was the case with transfer of Egr2 or Bcl6 expression construct into $Egr2^{-/-}$ $Egr3^{-/-}$ mice. Although GC B cell numbers were significantly reduced in $Egr2^{-/-}$ $Egr3^{-/-}$ mice, it was proposed that Egr2 regulated Bcl6 in a Tfh-specific manner because transfer of WT B220 cells into $Egr2^{-/-}$ $Egr3^{-/-}$ mice did not have a similar effect in restoration of GC B cell differentiation and GC responses. Certainly other studies have shown how a reduction in Tfh numbers can severely abrogate GC B cell differentiation (Liu *et al.*, 2014b; Bollig *et al.*, 2012; Yu *et al.*, 2009; Johnston *et al.*, 2009; Fukuda *et al.*, 1997). Finally, the interaction between Egr2 – Bcl6 was confirmed in $CD4^{+}$ T cells by EMSA and ChIP assay thereby identifying Egr2 as a novel regulator of the all-important Tfh cell differentiation.

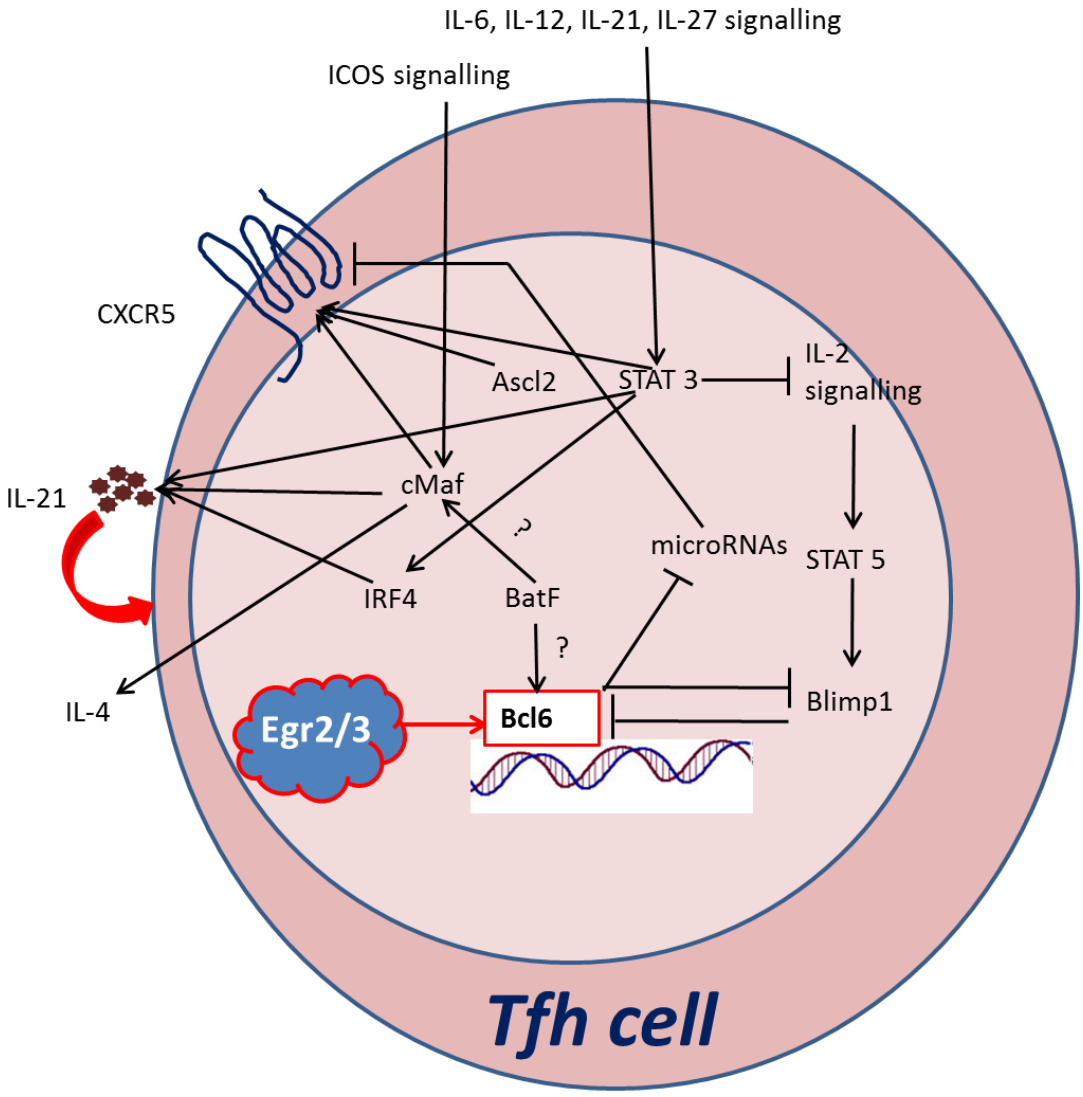

Figure 4.1: Proposed model showing molecular interactions involved in the differentiation of T follicular helper (Tfh) cells including Egr2/3 genes.

4.3 Tfh cells are indispensable for induction of GC function

GC B cells require help from Tfh cells to undergo clonal proliferation, somatic hypermutation, class switch recombination and the development of B cell immunological memory in the GCs (Nutt and Tarlinton, 2011). GC B cells first initiate contact with Tfh cells at the T-B cell border to induce the genetic program required for their differentiation. As mentioned earlier, in the absence of Tfh cells, GC B cell differentiation, GC formation and T dependent antibody immune response is defective in our model and other models (Liu *et al.*, 2014b; Bollig *et al.*, 2012; Yu *et al.*, 2009; Johnston *et al.*, 2009; Fukuda *et al.*, 1997). Although we found that Egr2/3 did not seem to play a direct role in the regulation of GC B cell differentiation in our model, GC functions like class switching and high affinity antibody production were severely diminished presumably due to lack of help from Tfh cells. This is supported by the findings of researchers where severely impaired GC functions were observed in their models in the absence of Tfh cells (Liu *et al.*, 2014b; Ise *et al.*, 2011; Johnston *et al.*, 2009; Yu *et al.*, 2009).

During somatic hypermutation (SHM), point mutation occurs at high frequency on the V region of the immunoglobulin heavy chain. This changes the immunoglobulin receptor affinity which results in the production of high affinity antibodies (Muramatsu *et al.*, 2000). In $Egr2^{-/-}$ $Egr3^{-/-}$ mice, in line with defective Tfh differentiation and absence of GCs, there was impaired production of neutralising antibodies and OVA-specific antibodies. This is consistent with reports from Bcl6 conditional knockout mice model where absence of Tfh cells lead to severely abrogated levels of antigen-specific antibody production following sheep red blood cell (SRBC) immunisation (Hollister *et al.*, 2013)

Class switch recombination (CSR) involves the change in the constant region of immunoglobulin heavy chain (C_H) from Cμ coding for IgM, to the C_H of other immunoglobulin isotypes like IgG, IgE or IgA depending on nature and location of antigen (Muramatsu *et al.*, 2000). Class switching is necessary in order to improve and expand antibody effector functions. T cell intrinsic defect in Tfh differentiation in $Batf^{-/-}$ mice led to a loss of IgG1 and IgG3 production in response to T cell–dependent hapten nitrophenol–chicken γ-globulin (Ise *et al.*, 2011). Similarly, in

Egr2$^{-/-}$ Egr3$^{-/-}$ mice, there was defective productive of antibodies marked by low serum levels of IgG1 in response to viral challenge and OVA protein immunisation. Consequently, our mice fared much worse than WT mice showing more severe clinical pathology and an inability to clear virus as efficiently as WT after VV_{WR} challenge. IL-21 levels were found to be significantly less in Tfh-like cells in Egr2$^{-/-}$ Egr3$^{-/-}$ mice. As IL-21 is reported to be essential for B cell activation and antibody secretion (Hollister *et al.*, 2013), lack of IL-21 may contribute to defective antibody production.

In line with the absence of GC, antibody production during secondary exposure to antigens was defective in Egr2$^{-/-}$ Egr3$^{-/-}$ mice. As B cell immunological memory is still very much unclear (Berkowska *et al.*, 2011; Toyama *et al.*, 2002). The Egr2/3 mouse model could be used to study the development of GC dependent B cell immunological memory.

Mouse model	Similarities with Egr2$^{-/-}$ Egr3$^{-/-}$ mice model	Reference
Bcl6$^{-/-}$ bone marrow cells in Rag-/- mice and Bcl6$^{fl/fl}$/CD4-Cre mice	Defective differentiation of Tfh cells and GC B cells leading to diminished numbers of these cells. Absence of GC, defective SHM and CSR, defective antibody immune responses, low affinity memory B cell formation	(Hollister *et al.*, 2013; Yu *et al.*, 2009; Johnston *et al.*, 2009; Toyama *et al.*, 2002; Fukuda *et al.*, 1997)
Blimp1 overexpression	Lack of Tfh phenotype, severely reduced T cell dependent antibody responses	(Johnston *et al.*, 2009)
IRF4$^{-/-}$ mice	Impaired Tfh cell differentiation, Absence of GC, lack of GC B cells, GC function is defective.	(Ochiai *et al.*, 2013; Bollig *et al.*, 2012)
Ascl2$^{fl/fl}$/CD4-Cre	Defective humoral immune response, impaired Tfh and GC B cell differentiation	(Liu *et al.*, 2014b)
c-Maf$^{-/-}$ fetal liver cells in Rag-/- mice	Low Tfh numbers, Dysregulated. IL-21 and CXCR5 production.	(Kroenke *et al.*, 2012; Bauquet *et al.*, 2009)
Batf$^{-/-}$ mice	Impaired Tfh differentiation, Absence of GC, defective GC B cell differentiation and aberrant GC function including class switching, failure to induce AID	(Ise *et al.*, 2011)

Table 4.1: Summary of mouse models used in the study of transcription factors reported to be involved in Tfh differentiation, GC B cell differentiation, GC formation and function presented along with similarities shared between these models and our Egr2$^{-/-}$ Egr3$^{-/-}$ mouse model.

4.4 Relevance of this study in human diseases and the design of therapeutic strategies

As elaborated earlier, studies looking into Tfh cell biology are actively gaining grounds due to the role these cells play in diseases and the potentials of manipulating them in rational therapeutic strategies. A lot of knowledge of the molecular signatures of Tfh cells only became clear in the last 10 years with the discovery of regulators of its biology (Liu *et al.*, 2014; Johnston *et al.*, 2009; Yu *et al.*, 2009; Fukuda *et al.*, 1997; Ye *et al.*, 1997; Dent *et al.*, 1997). Studies using mouse models of diseases like X-linked lymphoproliferative disorders (Crotty *et al.*, 2003; Czar *et al.*, 2001), common variable immunodeficiency diseases (CVID) (Warnatz and Voll, 2012) and autoimmune diseases (Ma and Deenick, 2014) have found deregulated expression of Tfh-related molecules and consequently dysregulated Tfh cell numbers (Fahey *et al.*, 2011; Simpson *et al.*, 2010). Indeed, these were similar with the disease phenotype observed in human sufferers of these diseases (Ma and Deenick, 2014).

25% of patients suffering from CVID develop early onset autoimmunity with altered T and B cell homeostasis (Warnatz and Voll, 2012). CVID patients also show dysregulated expression pattern for IL-2 and IFN-γ although researchers are yet to uncover what roles these cytokines may play in the development of autoimmunity within these patients (Warnatz and Voll, 2012). Interestingly, these deregulated immune functions are linked with defective antigen receptor signaling similar to what is observed in $Egr2^{-/-}$ $Egr3^{-/-}$ mice (Warnatz and Voll, 2012; Fischer *et al.*, 1996). As the phenotype of $Egr2^{-/-}$ $Egr3^{-/-}$ mice strongly correlates with that of CVID with low Tfh numbers, deregulated cytokine expression, autoimmunity and TCR defects, there is a possibility of a role of the Egr2/3 genes in CVID and its associated pathologies. Thus Egr2/3 studies may highlight potential immunotherapeutic targets for the treatment of these diseases.

In this study, severe reduction in Tfh cell numbers was observed during primary infection with VV_{WR}. This resulted in adverse clinical pathology, high viral retention and failure to produce neutralising antibodies. Our findings correlate with those of other researchers showing that reduced Tfh cell numbers severely

abrogates anti-viral immune response to LCMV (Crotty, 2014; Fahey *et al.*, 2011; Harker *et al.*, 2011). This indicates that Tfh cell levels are crucial in mediating antigen specific humoral immune responses. Adoptive transfer of Egr2 was able to rescue Tfh cell numbers and the GC phenotype. Egr2 interaction with the Tfh signature transcription factor Bcl6 to generate Tfh cells was also aptly validated. As Tfh cells are paramount in providing B cell help for the generation of long lasting affinity-matured isotype-switched antibodies, our discovery of a novel regulator of this all important Th subset, Tfh cells, sheds more light on the programming of Tfh cell differentiation. Such knowledge of Tfh biology is imperative in the design or improvement of therapeutic strategies for diseases like malaria, hepatitis B virus (HBV), HCV and influenza where increasing antigen specific Tfh cell levels would enhance humoral immune responses (Ma and Deenick, 2014; Moon *et al.*, 2012; XING, XU and YU, 2012).

Knowledge of Tfh cell biology can also be utilised in the studies of secondary immunodeficiencies like Human Immunodeficiency Virus (HIV). HIV may result in acquired immunodeficiency syndrome (AIDS) which had a remarkable death toll of 1.5 million adult and children in 2013 (World Health Organisation, 2015). In HIV patients, there is a reported accumulation of Tfh cells in circulation and within secondary lymphoid organs leading to the development of hypergammaglobulinimea with high IgG levels against GAG proteins (Lindqvist *et al.*, 2012). Due to the high mutation rates of the HIV virus, anti-GAG antibodies are ineffective in controlling disease progression (Ma and Deenick, 2014). The challenge therefore is to generate broadly neutralising antibodies (bNAB) that target highly conserved sequences such as those against the envelop proteins gp120 (Cubas *et al.*, 2013). This way, antibodies will recognise a large range of different mutated strains of the virus (Ma and Deenick, 2014). Studies in Tfh cell biology are currently being manipulated for the generation of these bNAB in HIV (Pissani and Streeck, 2014). Our study elucidating how Egr2/3 genes regulate Tfh cell levels and GC dependent immune responses could certainly contribute positively to anti-HIV therapy design as it throws more light into the regulation of Tfh biology and antibody immune responses mediated by Tfh cells.

Finally, in the absence of the Egr2/3 genes, mice are reported to develop autoimmune diseases with high pro-inflammatory cytokine levels (Li *et al.*, 2012; Zhu *et al.*, 2008). Results from this study have demonstrated that increased levels of inflammation-inducing cytokines coincide with organ pathology in the $Egr2^{-/-}$ $Egr3^{-/-}$ mice. In diseases like multiple sclerosis (MS), the cause of organ pathology that drives the disease is still very much unknown (Ma and Deenick, 2014). Recently, Egr2 was found to be downregulated in T lymphocytes of patients suffering from multiple sclerosis (MS) (Miao *et al.*, 2013). The study by Miao et al, 2013 along with the link between Egr2/3 and organ pathology highlighted by this study could provide better understanding of MS and potentially other autoimmune diseases. These studies could highlight potential therapeutic targets for the treatment of MS. Immunotherapy involving Egr2/3 could possibly be utilised to abrogate disease progression or severity. The $Egr2^{-/-}$ $Egr3^{-/-}$ model could thus be ideal; for the development of novel therapeutic strategies that can be manipulated for the treatment of autoimmune diseases.

4.5 Egr2/3 are essential regulators of optimal humoral immune responses and inflammation pathology

The immune system is tightly regulated to facilitate optimal immune response while averting adverse immune pathology. Studies on the phenotype of $Egr2^{-/-}$ $Egr3^{-/-}$ mice suggest a model of aberrant immune function and deregulated homeostasis (Miao *et al.*, 2013; Li *et al.*, 2012; Zhu *et al.*, 2008). To add to this, our study has now identified Egr2/3 as novel transcription factors that regulates Tfh differentiation and plays a role in immunopathology arising due to chronic inflammation.

Egr2/3 genes are reported to be upregulated after *in vitro* antigenic stimulation where they interact with Batf. Egr2 interaction with Batf prevents Batf mediated blocking of AP-1 signalling and thus drives optimal T cell antigen receptor induced proliferation (Li *et al.*, 2012). In the study undertaken here, I have been able to demonstrate that the differentiation of T follicular helper cells is defective in $Egr2^{-/-}$ $Egr3^{-/-}$ mice. This led to severely impaired GC B cell differentiation and a lack of GC, which contributed to severe pathology, high viral load, and an absence of neutralising antibodies in these mice. WT $CD4^{+}$ T cells containing endogenous Egr2 or Egr2 expressing lentivirus was however able to reverse these defects. It is can be said, therefore, that in the absence of Egr2/3 genes, Tfh differentiation and humoral immune responses to T cell dependent antigens are impaired thus identifying a crucial role for these molecules in T cell dependent antibody responses.

Based on these findings, a schematic model is presented below to illustrate the novel role of the Egr2/3 genes in the differentiation of Tfh cells and promotion of humoral immune responses in the GC (figure 4.2).

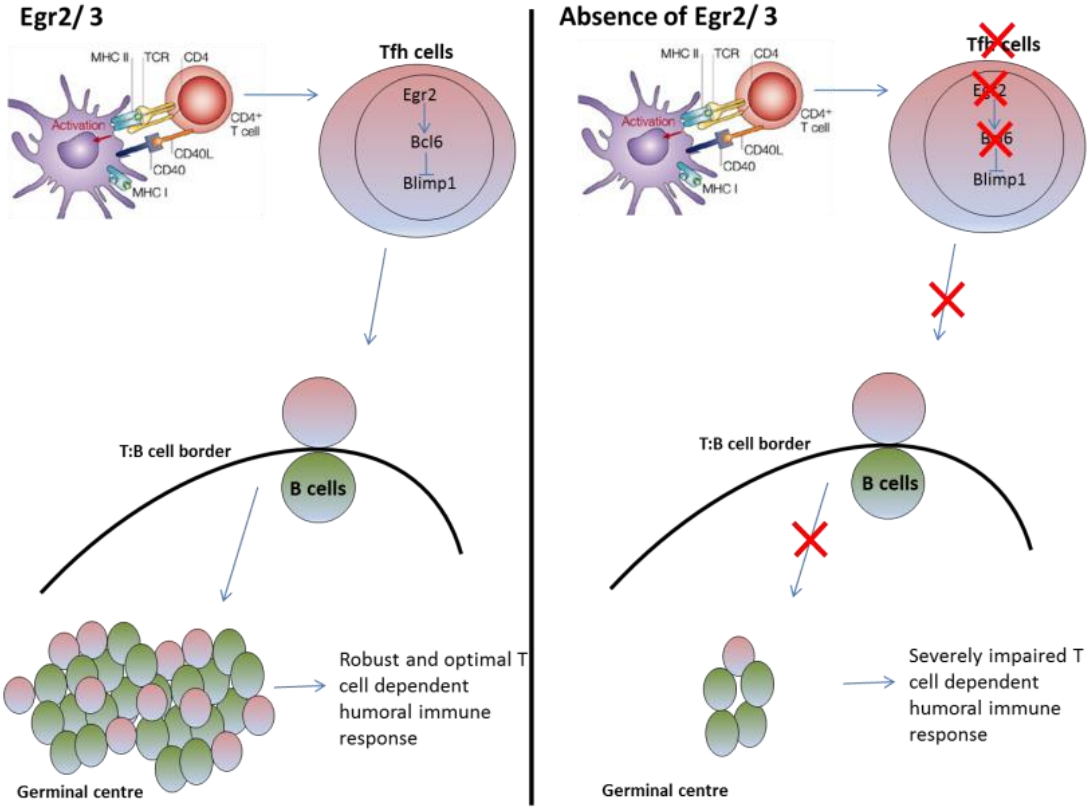

Figure 4.2: Schematic model showing how Egr2 interacts with Bcl6 to promote Tfh cell differentiation. In the presence of all other mediators of Tfh cell differentiation, Egr2 expression will promote the differentiation of Tfh cells, GC B cells and the formation of GC leading to optimal T cell dependent immune responses

Egr2/3 genes have also been reported to regulate immune homeostasis (Du *et al.*, 2014; Li *et al.*, 2012; Zhu *et al.*, 2008). In the absence of Egr2/3 genes, homeostatic proliferation in T and B lymphocytes led to the appearance of highly activated phenotype with associated high levels of inflammatory cytokines. These mice also developed autoimmune disease and became moribund after about 8 months (Li et al., 2012). Our study has linked organ pathology to the ensuing chronic inflammatory phenotype observed in $Egr2^{-/-}$ $Egr3^{-/-}$ mice. It is therefore clear from our research and those of others that the Egr2/3 genes are fundamental in the regulation of vital antibody responses while limiting chronic inflammation pathology. These newly elucidated roles of the Egr2/3 genes, along with findings of other researchers are hence summarised in figure 4.3.

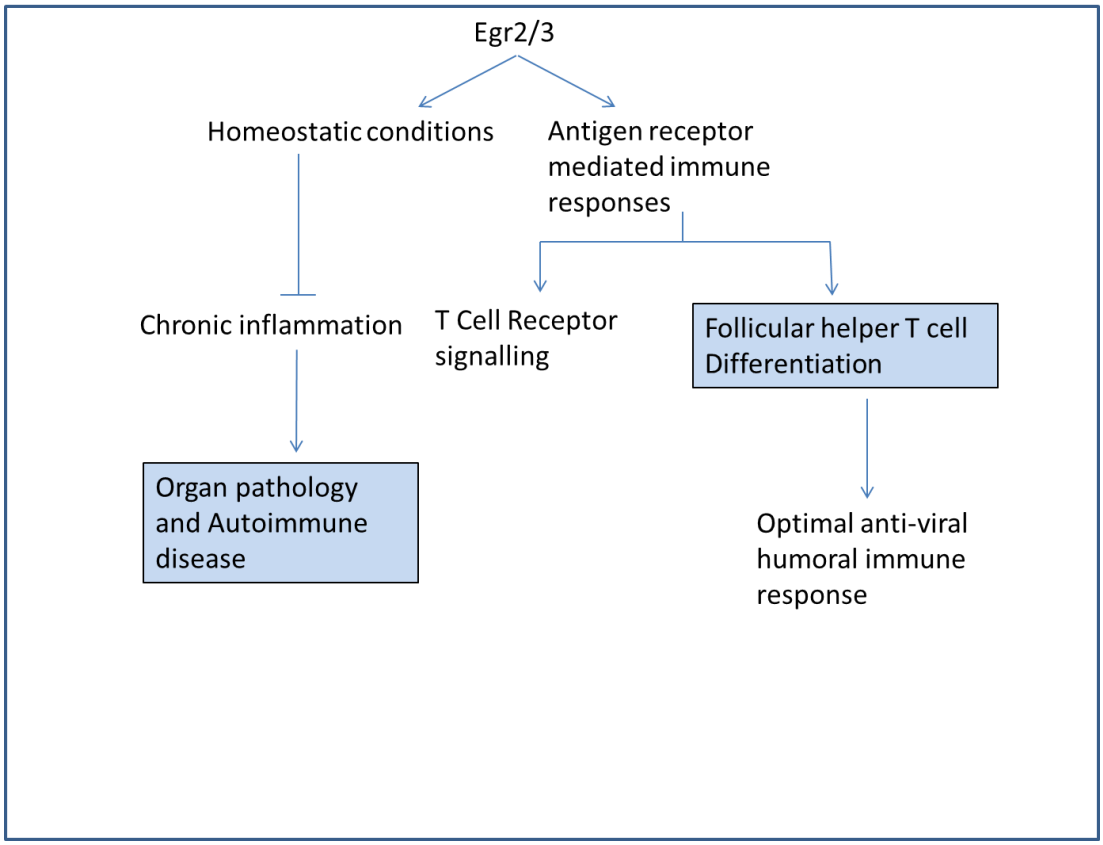

Figure 4.3: A propose model showing the updated functions of the Egr2 and 3 genes within the immune system. Contributions made by this study into understanding the role of the Egr2/3 genes within the immune systems are highlighted in blue

Chapter 5 Conclusion and Future work

The work presented by this thesis places emphasis on the essential role of the early growth response genes 2 and 3 (Egr2/3) in the regulation of systemic inflammation pathology and effector T cell differentiation. The most significant finding of this thesis is the novel role for the Egr2/3 genes in the regulation of Tfh cell differentiation, which, by extension, leads to the generation of optimal T cell dependent antibody immune responses. These roles of the Egr2/3 genes illustrate the paramount functions they play in the regulation of the immune system where they avert inflammation pathology while promoting potent humoral immune responses. The findings of this thesis can thus be summarised as follows:

- Increased levels of pro-inflammatory cytokines in the $Egr2^{-/-}$ $Egr3^{-/-}$ mice may contribute to systemic organ pathology. This may play a pathogenic role in the development of systemic inflammatory autoimmune disease observed in the $Egr2^{-/-}$ $Egr3^{-/-}$ mice. This therefore makes the Egr2 and 3 genes essential for the prevention of organ pathology arising due to chronic inflammation.
- In the absence of the Egr2 and 3 genes, GCs are absent and GC functions including the production of high affinity antibodies and class switching are severely impaired.
- The Egr2/3 genes control GC formation and functions by regulating the differentiation of Tfh cells, which provide cognate help to GC B cells that populate the GC.
- The molecular mechanism of Egr2 regulation of Tfh cell differentiation is through its interaction with Bcl6, the Tfh signature transcription factor. This promotes the physiological function of Bcl6.
- This study has contributed greatly to the knowledge of Tfh biology and can be used in the development of therapeutic strategy.
- The Egr2/3 mouse model can therefore serve as an appropriate model for further studies into autoimmune disease development, Tfh biology and therapy design.

This study did not however go without limitations including validating the roles of the Egr2/3 genes in the generation of immunological memory. Current

concepts in GC responses indicate that the GC mediates class switch recombination, somatic hyper mutation and the development of immunological memory. Very little is known about memory B cell once they are generated and researchers have proposed that memory B cells may be generated in the absence of GCs (Berkowska *et al.*, 2011; Toyama *et al.*, 2002). The $Egr2^{-/-}$ $Egr3^{-/-}$ mice showed protection from re-challenge during secondary infection. As this study was unable to delineate the mechanism of immunological protection in these KO mice following re-challenge, an area of future research could be investigations into the development of immunological memory in our mouse model. Studies looking into the development of immune memory during second exposure to antigens in $Egr2^{-/-}$ $Egr3^{-/-}$ mice would increase the understanding of immunological memory during T cell dependent immune responses. As immunological memory is the bedrock of vaccinations, findings from that study could also be manipulated in vaccine discovery. Other potential areas of future research are highlighted below.

It has been reported that although Tfh and Tfr cells arise from distinct precursor cells, they both still require similar cues including Bcl6, CD28, SAP expression and B cell interaction for their formation and maintenance (Linterman *et al.*, 2011; Chung *et al.*, 2011). As $Egr2^{-/-}$ $Egr3^{-/-}$ mice had impaired Bcl6 expression, further investigation into Tfr cells in the $Egr2^{-/-}$ $Egr3^{-/-}$ mice model could highlight more regulatory functions of these genes and holds potentials in the therapy for auto-antibody mediated diseases.

The interferons are very prominent anti-viral mediators inducing non-specific cytolytic effects in affected organs. IFNγ levels in our model are elevated however we consistently observe very severe clinical pathology in these mice after primary infection. It would be interesting to examine what functions IFNγ may play in the context of viral infection within $Egr2^{-/-}$ $Egr3^{-/-}$ mice.

In line with high IFNγ, another important area for future exploration would be to understand the mechanism of how Egr2 and 3 regulate IFNγ during inflammation and thus control organ pathology. This would further highlight the roles of these genes in immune function and can aid in targeted therapeutics in the future.

IL-21 is an important cytokine necessary for Tfh development and also implicated in the pathogenesis of autoimmune diseases (Liu and King, 2013). Microarray data indicated high levels of IL-21 in total CD4 T cells from Egr2/3 deficient mice. However when these cells were polarized to induce Tfh-like cells in vitro, IL-21 production was severely compromised. The increased IL-21 levels in total CD4 cells can be attributed to the highly activated Th17 cell phenotype which is reported to be present in Egr2/3 deficient mice (Li et al., 2012). These pathological levels of IL-21 may drive autoimmunity in these mice (Liu and King, 2013). In order to understand the differential expression of IL-21 in Egr2/3 deficient mice, further investigation is necessary in order to elucidate the mechanism by which IL-21 is regulated specifically in Tfh cells by the Egr2 and/or Egr3 genes.

The GC is reported to play a role in the development of autoantibodies in autoimmunity (Ma and Deenick, 2014; Ma *et al.*, 2012; Deenick *et al.*, 2010). In our mouse model, we observe the contrary; an absence of GC yet, a lupus-like autoimmune phenotype. There is a growing body of work linking other B cell populations with autoantibody production. Some reports have shown that circulating B cells in the blood and red pulp B cells are able to respond to antigens and switch immunoglobulin classes especially under inflammatory conditions (Giltiay, Chappell and Clark, 2012). Another study by William J et al., (2002), used a mouse model of autoimmunity, MPR.Fas^{LPR}, and found that autoreactive B cells producing hypermutated anti-IgG rheumatoid factor autoantibodies were found in the T cell zone and red pulp area of the spleen and these antibodies were produced independent of the GC and did not require CD4 T cell help (Giltiay, Chappell and Clark, 2012; William *et al.*, 2002). As the levels of pro-inflammatory cytokines are profound in Egr2/3 deficient mice, this may contribute to the development of autoreactive B cells outside of the GC in our mice model however this is yet to be investigated. The findings from this study has identified a mouse model that can be used for the study of GC independent autoantibody production and may provide insights into some of the key cellular and molecular players in GC independent autoantibody production which could be targeted for therapeutic purposes.

A number of cytokines and STATs have been reported to be required for Tfh differentiation. IL-6, IL-12 and IL-21 are reported to be essential for Tfh

differentiation in vivo and in vitro (Crotty, 2011; Eto et al., 2011; Linterman et al., 2010) and STAT 1 is important for the IL-6 dependent induction of Bcl6 in Tfh cells and their subsequent differentiation (Choi et al., 2013). In another review by Choi et al, 2013, IL-21 signalling via STAT3 was found to be partially involved in the upregulation of Bcl6 and thus promote Tfh cell differentiation (Choi, Yang and Crotty, 2013). Egr2/3 deficient mice are reported to have high expression of STAT 1 and 3 due to an absence of Egr2 induced regulation of SOCS1 and 3 (Li et al., 2012). As IL-6 together with Tgf-β have been shown to induce Egr2 expression in CD4 cells (Miao et al., 2013), The role of STAT 1 and 3 in Tfh cell differentiation in Egr2/3 deficient mice may depend upon factors such as the cytokine milieu present during activation and differentiation, when these cytokines are produced, whether or not their receptors are expressed, and also the interplay between these and other signalling pathways including STAT 5 which regulates IL-2 production that dampens Tfh differentiation.

It is clear from this thesis and publications from our group that the Egr2/3 genes are essential in maintaining homeostasis and inducing optimal immune responses after antigenic stimulation (Miao et al., 2013 Li et al, 2012, Li et al., 2011). Egr2 has been found to interact with Bcl6 (Ogbe *et al.*, 2015), Batf (Miao et al., 2013), SOCS1 and 3 (Li et al., 2012), T bet and Notch (Du et al, 2014). These genes regulate important aspects and components of the immune system. It is therefore important to investigate other potential genes that may be regulated by these transcription factors. This holds the potentials of discovering novel regulators of the immune system or novel functions of these Egr2/3 genes. A possible method of approaching this question can be through ChIP-Seq analysis which will enable the identification of genes that interact with Egr2 and/or Egr3.

In conclusion, this thesis highlights that the Egr2 and 3 genes are imperative for preventing the development of systemic inflammation pathology and also critical for the differentiation of Tfh cells. These genes can therefore be targeted in the design of therapeutic strategies that can be used for the benefit of humanity.

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Conference presentation

The findings from this thesis were presented in the following conference:

Ogbe A.T., Miao T., Symonds A.L.J., Li S., and Wang P., (2014), Early growth response gene -2 and -3 are required for the differentiation of T follicular helper cells by regulation of Bcl6 expression – ***Poster presentation at "The British Society of Immunologist Annual Congress", Brighton, United Kingdom.***

Publications

Conference publications

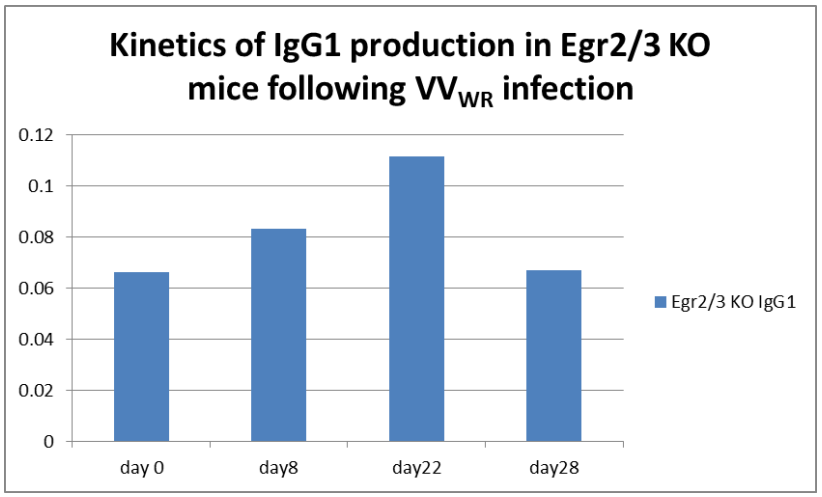
Ogbe A.T., Miao T., Symonds A.L.J., Li S., and Wang P., (2014), Early growth response genes-2 and-3 are required for the differentiation of T follicular helper cells by regulation of Bcl6 expression**.** *IMMUNOLOGY* vol. 143, 78-79

Journal Publications

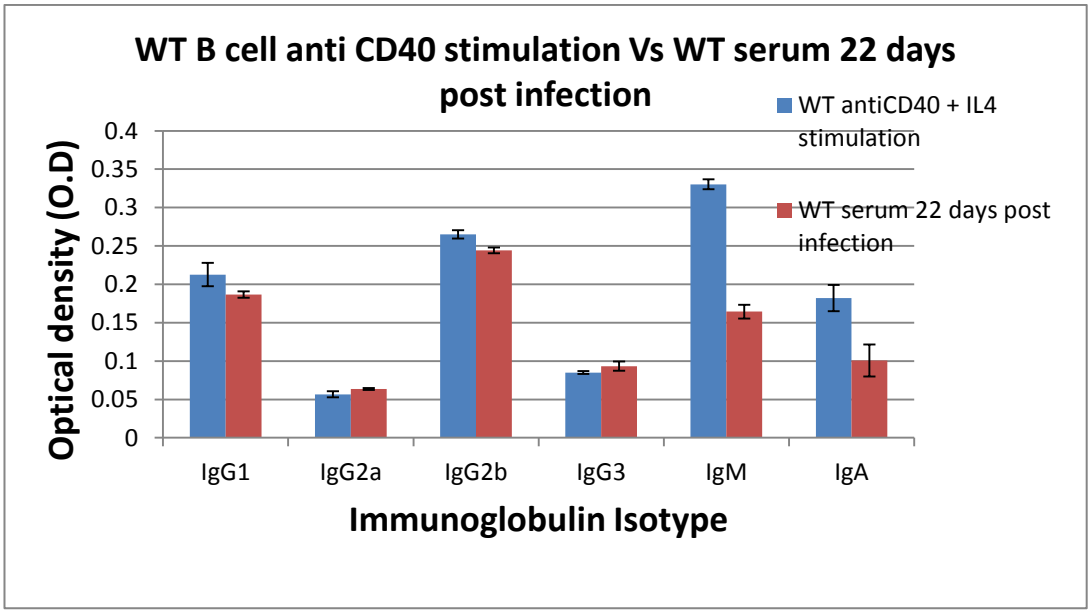
Ogbe A., Miao T., Symonds A.L.J., Omodho B., Singh R., Bhullar P., Li S., and Wang P., Early Growth Response Gene 2 and 3 Regulate the Expression of Bcl6 and Differentiation of T Follicular Helper Cells – **Manuscript in press at Journal of Biological Chemistry (JBC)**

http://www.jbc.org/content/early/2015/05/15/jbc.M114.634816.abstract

Appendix



Appendix figure 1: Graphical representation of IgG1 production in $Egr2^{-/-}$ $Egr3^{-/-}$ mice over the course of VV_{WR} infection. Data presented as an appendix to figure 3.3.8 on page 103.



Appendix figure 2: Immunoglobulin isotypes produced by WT control after *in vitro* stimulation using anti-CD40 and rIL-4 compared with serum from 22 days post VV_{WR} infection in WT mice. Data shows comparable Ig levels in the in vitro stimulated VS in vivo stimulated WT B cells. Data is presented as an appendix to figure 3.4.14 on page 120