Regulation of Egr2 expression in T cells and Egr2/3 function in tumour infiltrating T cells

Thesis submitted for the degree of Doctor of Philosophy by

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Declaration

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

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Abstract

The immune system is an organism's defence to protect the body against invading pathogens. T cells are one of the major components and essential for immune responses. The early growth response gene (Egr2) in T cells is important for maintaining immune functions of T cells by promoting adaptive immune responses, while controlling inflammation and preventing the development of autoimmune diseases. A recent study by our group demonstrated the function of Egr2 as a checkpoint regulator controlling the proliferation and differentiation of the T cells. In association, Egr2 and 3 play an indispensable role in the T cell immune response, but its function in tumour regression is less well known. Also, the mechanism regulating Egr2 expression in T cells is still unclear.

In this study, Egr2 was found to be significantly induced in tumour infiltrating lymphocytes (TILs) in a mouse melanoma tumour model. Deficiency of Egr2 and 3 resulted in rapid growth of tumour with impaired TIL expansion. The reduced expansion of TILs in Egr2/3 deficient mice were impaired in IL-2 production and expressed low levels of proliferation marker Ki-67, suggesting the positive role of Egr2 in $CD8^+$ TIL expansion and tumour regression. Furthermore, Egr2 expression was regulated by antigens and cytokines including, IL-2, IL-4 and IFNγ, IL-6. The latter regulatory function was mediated by IFNγ/STAT1 and IL-6/STAT3 signalling pathways.

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Last but surely not least, it gives me immense pleasure and joy to express my heartfelt gratitude to my brother Sina for all his unwavering love, efficient guidance and inspiration during my PhD. Thank you for being my first best friend and for creating unforgettable childhood memories and grown-up dreams.

Dedication

"The family is one of nature's masterpieces"
George Santayana

This thesis is dedicated to my parents Karim Taefehshokr and Nasrin Faraji, my uncle Ali Faraji, without whom none of my success would be possible.

I am eternally grateful for everything you've taught me

I love you so much

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List of Abbreviations

Ag	antigen
AP-1	activator protein-1
APC	antigen presenting cell
BCL-6	B cell lymphoma 6 protein
BCR	B cell receptor
BP	base pair
BSA	bovine serum albumin
BTLA	B and T lymphocyte attenuator
CCR	chemokine receptor
CD	cluster of differentiation
CTLA-4	cytotoxic T lymphocyte antigen- 4
DAG	diacylglycerol
DMEM	Dulbecco's modified Eagle medium
DN	double negative
DNA	deoxyribonucleic acid
DP	double positive
EGR	early growth response
FACS	fluorescence activated cell sorter
FBS	foetal bovine serum
EGFP	enhanced green fluorescence protein
FoxP3	fork head box P3
GATA-3	trans-acting T cell-specific transcription factor
GMCSF	granulocyte macrophage colony stimulating factor
H/E	haematoxylin and eosin staining
HEK	human embryonic kidney cells
HSC	haematopoietic stem cell
ICOS	inducible T cell co-stimulator
IL	interleukin
IFN	interferon
Ig	immunoglobulin

ITAMs	immunoreceptor tyrosine-based activation motif
iTreg	induced T regulatory cell
JAK	janus kinase
KO	knockout
LAG-3	lymphocyte activation gene 3
LPS	lipopolysaccharide
MACS	magnetic activated cell sorter
MAPK	mitogen activated protein kinase
MHC	major histocompatibility complex
MS	multiple sclerosis
NFAT	nuclear factor of activated T cells
NK	natural killer
PAMP	pathogen associated molecular patterns
PCR	polymerase chain reaction
PBS	phosphate buffered saline
PD-1	programmed cell death-1
PMA	phorbol 12-mysristate 1 acetate
PRR	pattern recognition receptors
PKC	protein kinase C
RA	rheumatoid arthritis
RNA	ribonucleic acid
SC	subcutaneous
SLE	systemic lupus erythematosus
SOCS	suppressor of cytokine signalling
SP	single positive
STAT	signal transducer and activator of transcription
TCR	T cell receptor
Tg	transgenic
TGF	transforming growth factor
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TILs	tumour infiltrating lymphocytes
TIM-3	T cell immunoglobulin and mucin domain containing-3
Th	T helper

TLR	toll-like receptor
Treg	T regulatory
UV	ultra violet
WT	wild type

CHAPTER 1 - INTRODUCTION

1.1 The function of the immune system

Immunity refers to the global ability of the host to resist against microbes predation. It has many facets and generally can be divided into adaptive immunity (acquired immunity) and innate immunity (natural immunity) (Hoebe *et al*., 2004), which work hand in hand. It is widely considered that any true immune system should be capable of doing three things including, recognition of various pathogens, killing these pathogens after their recognition and sparing self-tissues (self-tolerance) (Beutler, 2004).

1.2 Innate immunity

It is the first, rapid and non-antigen-specific acting line of defence against a broad range of exogenous and endogenous danger signals and can be divided into cellular and humoral components. Cellular innate immunity is largely dependent upon myeloid cells including mononuclear and polymorphonuclear phagocytes (Beutler, 2004). Mononuclear phagocytes are macrophages derived from monocytes in the blood, which are effective antigen presenting cells to T cells of adaptive immunity. They are capable of engulfing and killing microbes, while polymorphonuclear phagocytes, including neutrophils, basophils and eosinophils, are indispensable in containment of infections (Beutler and Rietschel, 2003). Also, natural killer (NK) cells are important features of cellular innate immunity, which have a host-protective role against viral infections and tumours via release of perforin and granzymes (Tosi, 2005). The humoral component of innate immunity includes complement, lysosome, lactoferrin and antimicrobial peptides, which can recognise and kill microbes (Beutler, 2004).

Antimicrobial peptides comprise polypeptides fewer than 100 amino acids that protect mucosal surfaces from infection and obstruct acute invasive infections. They include two large families of defensins and cathelicidins (Ganz, 2004, Singh *et al.,* 2002).

Innate immune receptors, pattern recognition receptors (PRR), recognise microbial infection through pathogen associated molecular patterns (PAMP), and among these, Toll-like receptors expressed on many epithelial cells, mononuclear phagocytes and leucocytes have a crucial role in recognition of microbes. They can induce activation of inflammation and antimicrobial immune responses (Medzhitov, 2001), for example, TLR2 and TLR4 binds mainly to gram-positive cell components and gram-negative lipopolysaccharide respectively (Tosi, 2005).

1.2.1 Complement system

The complement system consists of about 30 plasma proteins that are present as membrane-associated proteins or soluble proteins in the blood and its main function is recognition and elimination of microbes (Sarma and Ward, 2011). In addition, complement facilitates the clearance of apoptotic cells and cellular debris, with defects in its regulators causing autoimmune diseases (Zipfel and Skerka, 2009). Complement activation can be achieved through three different pathways including the classical, alternative and lectin pathways (Cho, 2015). The classical pathway is triggered by formation of immune complexes (target bound antibody), the lectin pathway by the recognition of microbial polysaccharide structures by binding of mannan-binding lectin (MBL) to mannose residues on microbial surfaces and the alternative pathway is initiated by carbohydrates, lipids and proteins found on foreign surfaces (Sarma and Ward, 2011, Cho, 2015). Finally, the terminal step leads to the formation of membrane

attack complex (MAC), which creates a pore in the membrane, leading to cell lysis (Zipfel and Skerka, 2009).

1.2.2 Phagocytosis

Is one of the initial steps in triggering host defence, inflammation and dying cell clearance, which is mediated by phagocytes including macrophages, dendritic cells and granulocytes (Murphy, 2012). Phagocytes are alerted via different chemotactic agents released by invaders and phagocytes through PRR binding to their targets (PAMPs) in a process known as particle recognition (Aderem, 2013). Phagocytes extend their plasma membrane to engulf and internalise pathogens in phagosomes. By fusing lysosomes, they mature into a phagolysosome where pathogens are killed and degraded by different microbicidal mechanisms (Owen *et al.,* 2013), including antimicrobial peptides, low pH, hydrolytic enzymes as well as reactive oxygen and nitrogen intermediates (Aderem, 2013). Efficiency of phagocytosis is increased by tagging of invading microbes via opsonins in a process known as opsonisation (Greenberg and Grinstein, 2002).

1.2.3 Inflammation

Inflammation is pervasive form of defence initiated due to the disrupted tissue homeostasis and different stimulants, including allergens, virulence factors, foreign bodies and necrotic cells, which leads to inflammatory responses (Ashley *et al.,* 2012). Protective inflammatory responses involve the recruitment of leukocytes and plasma proteins into the perturbed tissue and local sites of infection (Ashley *et al.,* 2012, Turvey and Broide, 2010). The main functions of inflammation is defined as the quick isolation of disturbance origin, separation of affected tissue and finally, restoring tissue

homeostasis (Medzhitov, 2008). The first step of the inflammatory cascade is achieved through the recognition of PAMPSs or damage-associated molecular patterns (DAMPs) by innate immune PRR receptors e.g. transmembrane Toll-like receptors (TLR) and intracellular nucleotide-binding oligomerisation-domain proteins (NOD)-like receptors (NLR) on tissue-resident macrophages (Turvey and Broide, 2010, Murphy, 2012, Tosi, 2005, Medzhitov, 2001). Then, this triggers mediators of inflammation: the cytokines (interleukins, interferons, and chemokines), eicosanoids and vasoactive amines (Turvey and Broide, 2010, Medzhitov, 2008). Inflammation alongside the cytokine milieu assists the maturation and activation of dendritic cells that provide the bridge between pathogen infection and autoimmunity (Ray *et al.,* 2013). In this mechanism, T lymphocytes that escape tolerance against the self-antigens in the thymus are activated when self-peptides are altered due to an external stimulus, leading to cellular infiltration and local inflammation (Turley, 2002). In recent years, it has been reported that cytokines were produced by keratinocytes in the skin that initiate active inflammation (Gröne, 2002). Inflammation is marked by:

- rubor (redness) due to the dilation of small blood vessels
- tumour (swelling) arising from increased permeability of the blood vessels and protein leakage
- calor (heat) results from increased blood flow and the metabolic activities of cytokines in peripheral parts of the body
- dolor (pain) associated with distortion of tissues caused by oedema and mediators of inflammation, such as prostaglandins and serotonin
- functiolaesa (loss of function) results from the pain and swelling that inhibits

mobility and disrupts the normal function of organ involved in the inflammatory process

Inflammation may be acute, lasting for a few days that can lead to the resolution of inflammation or chronic with longer duration of response and constant presence of inflammatory cells and molecules (Kumar *et al.,* 2007).

1.2.3.1 Acute Inflammation

This is a protective linear progression of response mounted by the immune system in response to stimulus to eliminate harmful pathogens and toxic substances (Ryan and Majno, 1977, Delgado *et al.,* 2003). The initiation step of the acute phase inflammatory response is marked by the dilation of blood vessels, vasodilation, to allow the exudation of polymorphonuclear neutrophils, plasma proteins and clotting factors into extravascular tissues spaces to prevent the spread of infectious agents (Figure 1.1) (Ashley *et al.,* 2012, Medzhitov, 2008). Activated neutrophils create a cytotoxic environment, removing their targets indiscriminately by the release of toxic substances from their granules. These noxious chemicals include reactive oxygen species (ROS), reactive nitrogen species, cathepsin G, proteinase 3 and elastase (Medzhitov, 2008). After pathogen elimination, pro-resolution molecules including lipoxin, an anti-inflammatory agent, reduce vascular permeability, promoting the recruitment of non-phlogistic monocytes respectively, which are required for wound healing (Serhan and Savill, 2005). These monocytes differentiate into phagocytic macrophages upon activation to clear apoptotic neutrophils and dying cells, leading to promotion of resolution and repair of damaged tissues (Figure 1.1) (Savill, 2001, Godson *et al.,* 2000). Also, other lipid mediators and anti-inflammatory cytokines, like resolvins (generated

from omega-3 unsaturated fatty acid), protectins and transforming growth factor-β (TGF-β), play an important role in the resolution of inflammation, in which they retard further neutrophil infiltration by initiating apoptosis and suppressing protein inflammatory signalling from TLR (Serhan and Savill, 2005, Medzhitov, 2008).

1.2.3.2 Chronic Inflammation

The regulation and control of inflammatory responses is important in maintaining homeostasis and avoiding pathological conditions, so pro-inflammatory and anti-inflammatory mediators should be tightly regulated and balanced (Hotamisligil, 2006). Repeated episodes of acute inflammation can also lead to chronic inflammation, while infectious organisms that are resistant to host defence and persist in tissues for extended periods can also cause chronic inflammation (Delgado *et al.,* 2003, Ashley *et al.,* 2012). Infiltration of circulating mononuclear macrophages, lymphocytes and plasma cells to the tissue sites is the hallmark of chronic inflammation often associated with tissue destruction, autoimmunity (Medzhitov, 2008). Due to the prolonged nature of the chronic inflammatory response, the host attempts to change from a tissue-damaging mode to a mode that promotes tissue repair by replacing them with either cells of the same type or fibrous connective tissues (Figure 1.1). Sometimes, the host is not capable of repairing the damaged tissue, thus leading to fatality (Nathan, 2002). Chronic inflammation has also given rise to the development of cancers, cardiovascular and neurodegenerative diseases, other severe systemic inflammatory diseases, as well as myocardial infarction and stroke (Delgado *et al.,* 2003, Ryan and Majno, 1997, Hotamisligil, 2006).

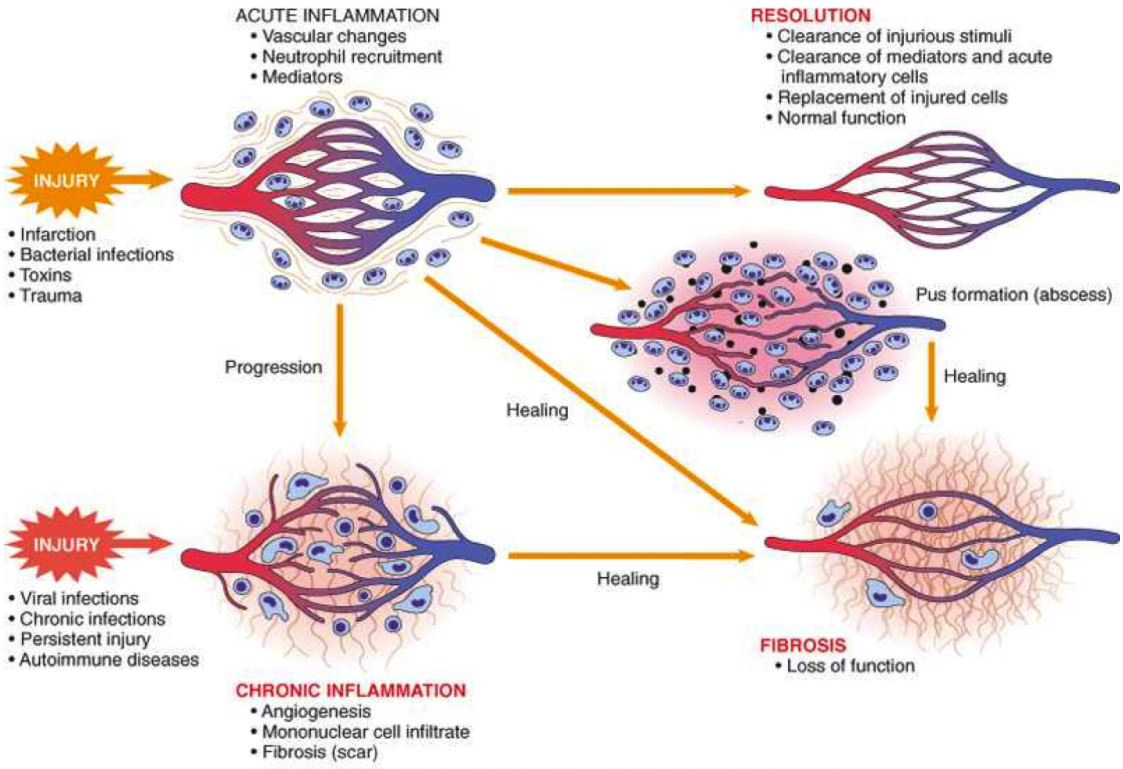


Figure 1.1 Schematic representing acute and chronic inflammation. In acute inflammation, inflammatory signals ends days later and neutrophils are recruited to the site of injury to resolve inflammation. On the other hand, chronic inflammation may last weeks and results in the infiltration of mononuclear macrophages and lymphocytes, which can culminate in the fibrosis of connective tissue (Kumar *et al.,* 2007).

1.2.4 Cytokines

In the immune system, cytokines form an integral part that have different roles in the immune cell development, immune-regulation, and modulation of immune effector functions. They play a crucial role in maintaining lymphocyte homeostasis under both steady state and inflammatory conditions, and aberrant cytokine expression can have noticeable implications to the host with regards to autoimmune disease and excessive tissue damage (O'Shea *et al.,* 2002). Numerous studies conducted on different cytokines have demonstrated their wide range of immune-modulatory effects on immune cells. IL-2, is historically recognised as a promoter factor that can enhance cell proliferation, whereas other cytokines, such as IL-10 and TNF-β primarily produced by Treg cells, have immune suppressive effects, inhibiting the generation of an autoimmune environment (Sanjabi *et al.,* 2009) (Table 1.1). These astonishing functions of cytokines on immune cells create a challenging aspect for researchers to explore the intricate and complicated mechanisms that govern their expression and function.

IL-2

As mentioned earlier, IL-2 contributes a pivotal role in promoting lymphoid proliferation, but a far more complex picture has been demonstrated in IL-2 and IL-2 receptor (IL-2R) knockout mouse models. Immune cells, including NK cells, natural killer T cells (NKT) and CD8 T cells produce IL-2 and CD4 T helper cells under homeostatic conditions mainly produce IL-2 in secondary lymphoid organs (Boyman and Sprent, 2012). During an immune response, the expression of IL-2 is induced in large amounts by activated antigen-specific $CD4^+$ and $CD8^+$ T cells, although IL-2 synthesis by CD8 cells is weak and to a lesser extent than CD4 cells (Malek, 2008). CD25, an IL-2 receptor subunit, is the third chain of the trimeric IL-2R and although it does

appear to contribute directly to signal transduction, it increases receptor affinity for IL-2. Under antigen-specific $CD4^+$ and $CD8^+$ T cell immune response, IL-2 is produced in large amounts, then consumed by CD25 effector T cells and Treg cells (Boyman and Sprent, 2012).

IL-2 plays a crucial role in the differentiation and survival of CD4 T helper subsets, including Th1, Th2 and Th17 along with homeostasis of regulatory T (Treg) cells (Sharma, 2007), (Figure 1.2). IL-2 promotes Th1 and Th2 differentiation mainly through the induction of T-bet and IL-4 expression respectively (Liao *et al.,* 2011, Cote-Sierra *et al.,* 2004). IL-2 signalling also downregulates IL-6-mediated STAT3 activation and favours the cells towards the development of $ROR\gamma t^+$ Th17 cells (Liao *et al.,* 2011). Due to diverse role of IL-2 under homeostatic and infection conditions in the immune system, it is regulated through different mechanisms.

One of the mechanisms involves IL-2 gene silencing by the transcription factor B lymphocyte-induced maturation protein 1 (Blimp1). In THE T cell-specific Blimp1 knockout mouse model, mice developed aberrant T cell homeostasis and fatal colitis. It was found that Blimp1 is crucial for directly repressing IL-2 transcription, upon T cell activation, IL-2 is induced, then induces Prdm1 expression (gene encoding Blimp1) which represses its own expression after T cell activation, ensuring that the magnitude of T cell effector immune response is appropriately controlled (Martins *et al.,* 2008).

Furthermore, IL-2 is demonstrated to have crucial impact on CD8 T cells during immune responses. IL-2 signalling affects different stages including primary expansion, contraction, memory generation and secondary expansion. The primary expansion of CD8 T cells in IL-2 deficient mice is threefold lower compared to WT counterparts in

response to viral infection, resulting in less-efficient viral clearance by virus-specific $CD8^+$ T cells (Boyman and Sprent, 2012). During a primary immune response, the strength and duration of IL-2 signals affects the differentiation of CD8 cells into short-lived effector T cells or long-lived memory T cells (Araki *et al.,* 2009, Pipkin *et al.,* 2010, Obar *et al.,* 2010). The affinity of IL-2 on cells with respect to CD25 expression is crucial for long-lived memory cells development and prolonged IL-2 signalling during priming promote effector differentiation of $CD8^+$ T cells (Kalia *et al.,* 2010). Kalia and colleagues (2010) demonstrated that a subset of virus-specific T cells sustain CD25 expression longer than the rest during an acute viral infection. In vivo examinations revealed that $CD25^{lo}$ cells, which are less sensitive to IL-2 upregulated CD127 and CD62L to give rise to functional long-lived memory cells, while $CD25^{hi}$ cells perceiving prolonged IL-2 signals appeared to be terminally differentiated, with a more pronounced effector phenotype.

Moreover, IL-2 is indispensable for the survival and homeostasis of naturally occurring T_{reg} ($CD25^+$ $CD4^+$) cells and consequently, the immunological self-tolerance sustained by Tregs (Setoguchi et al., 2005). IL2-deficient mice models represented severe autoimmune diseases such as type 1 diabetes and EAE, in which symptoms were prevented by IL-2 administration, leading to drastically prolonged survival and decreased autoantibodies (Gutierrez-Ramos *et al.,* 1990, Encinas *et al.,* 1999). The severe autoimmunity developed in IL-2RBeta-deficient mice and expression of IL-2Beta chains in these mice resulted in the production of a normal proportion of natural T_{reg} cells that inhibited the development of severe autoimmunity (Yu *et al.,* 2009). This highlights the use of IL-2 for strengthening natural self-tolerance and inhibiting autoimmune diseases. IL-2 is required for the promotion of Treg development and it

implements this function by upregulation of Foxp3, a master regulator of the T_{reg} lineage, and CD25 (Burchill *et al.,* 2008, Burchill *et al.,* 2007). IL-2 signalling was found to be important in both naturally occurring T cells (nTregs) and induced regulatory T cell (iTregs) by maintaining high expression levels of Foxp3 and induction of $Foxp3^+$ in the periphery respectively (Davidson *et al.,* 2007, Fontenot *et al.,* 2005).

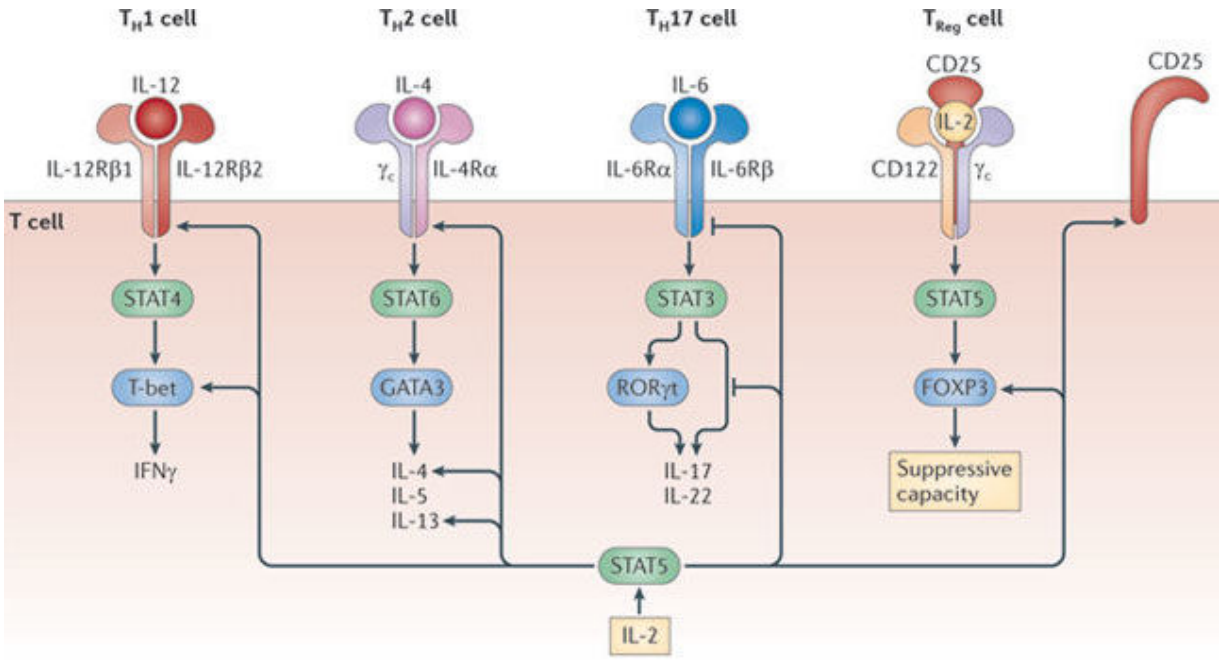


Figure 1.2 The role of IL-2 signals in controlling $CD4^+$ T cell subsets. Th0 can be activated to various Th subsets cells and the nature of the antigen drives the differentiation pathway, leading to an effector response. In this process, IL-2 signalling is activated via signal transducer and activator of transcription 5 (STAT5) and influences the polarisation of T helper subsets, including Th1, Th2 and Th17, alongside regulatory T cell homeostasis (Boyman and Sprent, 2012).

TGF-β

Transforming growth factor beta (TGF-β) is pleiotropic cytokine with potent inflammatory and regulatory functions in various cells of the immune system (Li and Flavell, 2008a). TGF-β signalling in the thymus is vital in the development of NKT, nTreg and $CD8^+$ T cells, when left unchecked cause rapidly fatal autoimmune diseases (Marie *et al.,* 2006). The abolition of TGF-β leads to severe multi-organ autoimmunity, tissue necrosis, organ failure and death (Li *et al.,* 2006, Shull *et al.,* 1992). TGF-β is indispensable in the survival of naïve T cells and its primary function is the maintenance of peripheral tolerance by inhibiting the proliferation and differentiation of CD4 and $CD8^+$ T cells (Li and Flavell, 2008b, Li *et al.,* 2006). Also, TGF-β with the requirement of IL-2 is essential for the induction of $Foxp3^+$ T regulatory cells (iTregs) that can inhibit the induction of autoimmunity (Davidson *et al.,* 2007). In contrast, TGF-β displays an opposing role towards antigen-experienced or memory $CD8^+$ T cells, enhancing their survival and increasing the production of IL-17 and IFNγ (Filippi *et al.,* 2008, Korn *et al.,* 2009).

IL-10

IL-10 is another key cytokine with immunological tolerance and suppressive function, mostly expressed by immune cells including dendritic cells, macrophages, NK cells, B cells, Th2, Th3 and Treg cells (Moore *et al.,* 2001). IL-27 and IL-6 independently promote the production of IL-10 producing T cells and TGF-β in conjunction with IL-27 or IL-6 can enhance this condition (Awasthi *et al.,* 2007, Stumhofer *et al.,* 2007). IL-10-expressing intestinal T cells are induced during systemic tolerance induction, highlighting the intestine as major site in the induction of immunological tolerance (Kamanaka *et al.,* 2006). IL-10 plays a pivotal role in survival and persistence of

intracellular pathogens, such as *Leishmania donovani* and *Mycobacterium tuberculosis*, suggesting a direct role of IL-10 in suppressing cell-mediated immune response and subverting this response upon its neutralisation (Chandra and Naik, 2008, De La Barrera *et al.,* 2004).

Table 1.1 Some common cytokines and their functions in the immune system

Cytokine	Biological Function	Signalling pathway	Reference
IL-2	• Promotes the differentiation of CD4 T helper subsets • Influences expansion and differentiation of $CD8^+$ T cells • Leads to development and survival $CD25^+$ $CD4^+$ Treg cells	STAT5-mediated	(Boyman and Sprent, 2012) (Sharma, 2007)
IL-4	• Promotes the differentiation of CD**4**$^+$ Th2 upon antigen stimulation • Regulates the proliferation and differentiation of mast, myeloid and dendritic cells	STAT6-mediated	(Zhu and Paul, 2008)
IL-6	• Activates $CD8^+$ T cells and encourages B cells to differentiate into plasma cells • Regulates bone homeostasis and mediates apoptosis inhibition • Promotes the production of acute phase protein from liver	STAT3-mediated	(Arango Duque and Descoteaux, 2014) (Scheller *et al.,* 2011)
IL-10	• Suppresses macrophage activation and production of inflammatory cytokines • Hinders the production of IFNγ by NK and Th1 cells • Favours the development of gastrointestinal pathologies, such as IBD	STAT3-mediated	(Stumhofer *et al.,* 2007)
IFNγ	• Elicits inflammatory response upon secretion by NK cells and macrophages • Stimulates Treg cell function • Induces anti-viral enzymes such as serine and threonine kinase	STAT1-mediated	(Kelchtermans *et al.,* 2008)

1.3 STAT proteins

The STAT proteins (signal transducers and activators of transcription) were identified as transcription factors, which are vital in mediating virtually all cytokine driven signalling. They can also be activated in response to growth factors, such as EGF and PDGF, through the intrinsic tyrosine kinases of the cognate receptors whose biological activities lead to many critical aspects of cell growth, survival differentiation, host resistance to pathogens and malignancies (Mitchell and John, 2005).

1.3.1 STAT domains

The three-dimensional structure of DNA bound dimers of the core STAT shows several obvious domains of the protein. Beginning around residue 130, coiled-coil domain, there are four-stranded long helical coils in which interaction with other proteins occur, with the DNA binding domain comprising ~300-500 residues containing several β-sheets, linker domain ~500-575 that are all alpha-helical, SH2 domain extending between residues 580-680, followed by the tyrosine residue ~700 that is phosphorylated (Bromberg and Jr, 2000) (Figure 1.3).

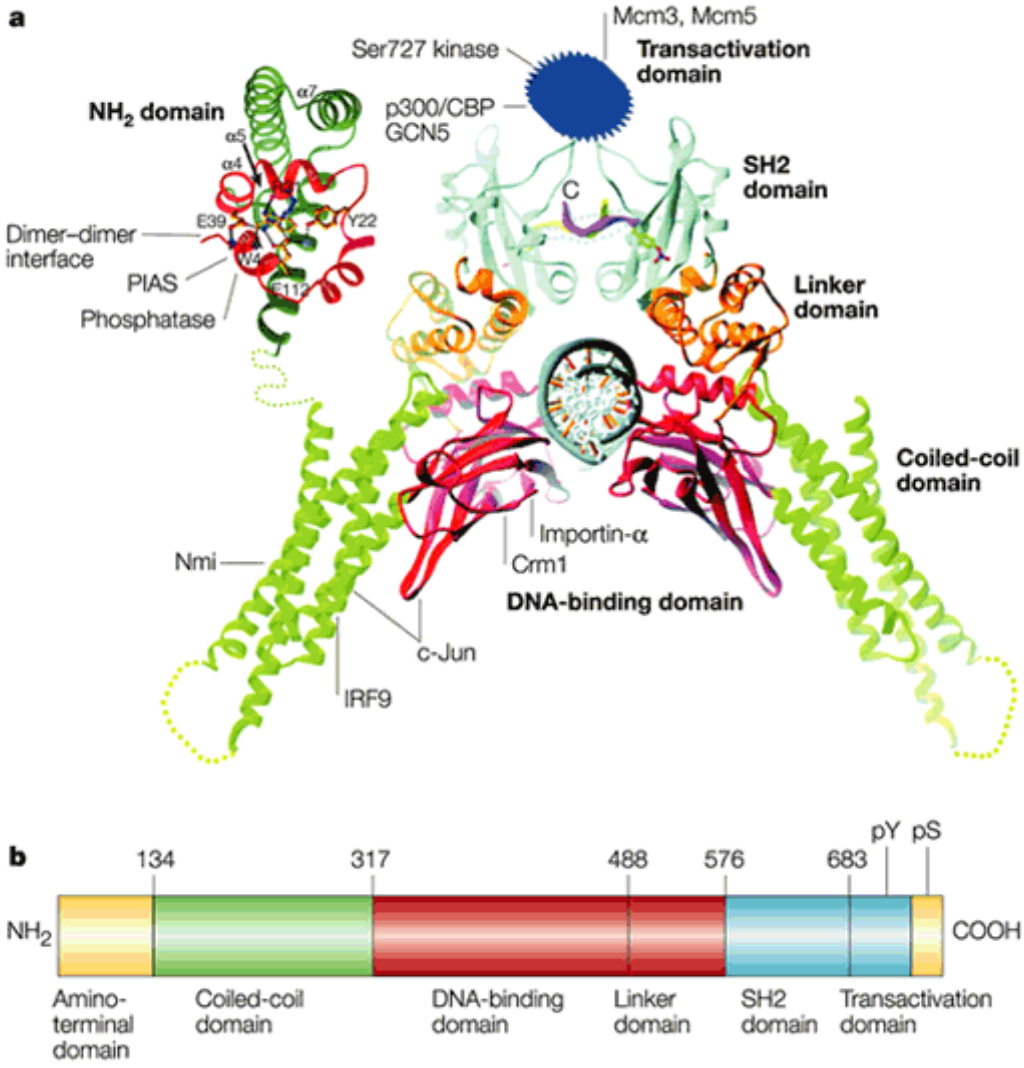


Figure 1.3 Crystal Structure of STAT domain and protein binding sites. a | Schematic showing the core structure (amino acids ~130-712) that reflects binding of STAT1 dimer to DNA and the location of binding sites of various proteins in various domains. b | STAT structure and related domains (Levy and Darnell, 2002).

1.3.2 JAK-STAT signalling pathway

Cytokine receptors bind to a cytoplasmic tyrosine kinase known as janus kinase (JAK) and when it is activated via binding of the cytokine to its cognate receptor, regulatory molecule binds and brings two receptor molecules together to form a dimer. This dimerisation brings the two JAKs in close proximity, where they can phosphorylate each other, further activating JAK and allowing it to phosphorylate the receptor on target tyrosine residues (Rane and Reddy, 2002). Then, STAT proteins bind to phosphotyrosine residues on the receptor proteins and are phosphorylated by JAK on a single tyrosine residue around residue 700 of their 750850 amino-acid sequences. Once phosphorylated, STAT proteins, which are latent in the cytoplasm, become activated and a dimer is formed which is an active transcription factor. It travels to the nucleus where it binds to consensus DNA-recognition motifs, called gamma-activated sites (GAS), in the promoters of cytokine-inducible genes, resulting in gene transcription initiation (Mitchell and John, 2005). The degree and duration of gene activation are under strict regulation by a series of negatively acting proteins (Levy and Darnell, 2002) (Figure 1.4).

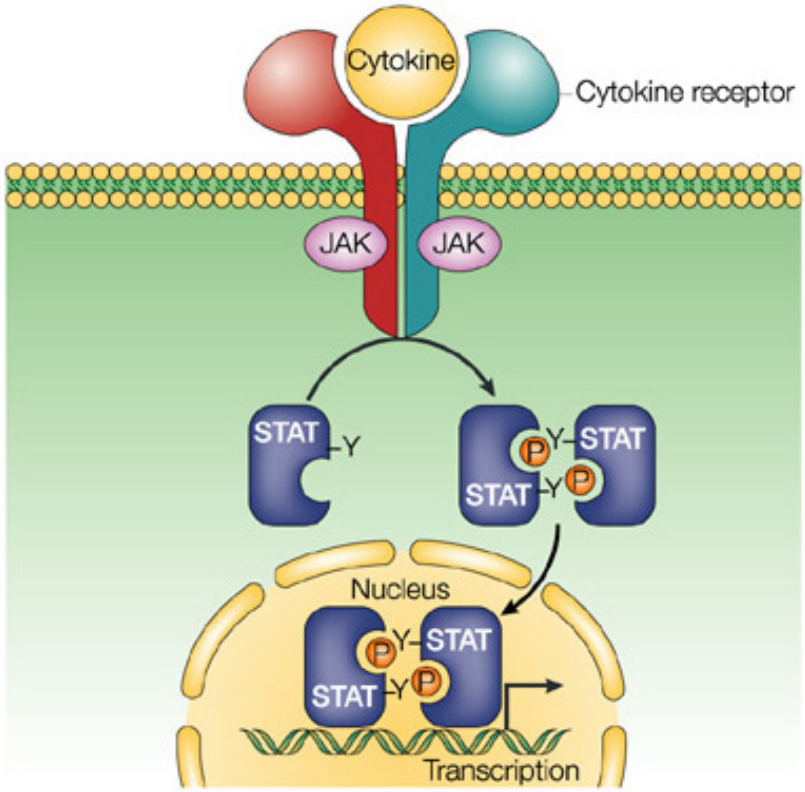


Figure 1.4 A schematic representation of canonical janus kinase - signal transducer of and activator of a transcription pathway. Cytokine-receptor interaction triggers sequential tyrosine phosphorylations. Receptor dimerisation allows Janus kinases (JAKs) phosphorylation, this is followed by the recruitment of the signal transducers and activators of transcription (STAT) and its tyrosine phosphorylation. Dimerisation of STAT is followed by nuclear entry (Shuai and Liu, 2003).

1.3.3 Janus kinases

They are characterised by a carboxy-terminal catalytic domain ranging from 250–300 amino acids, Src-homology 2 (SH2), SH3 domains, pleckstrin homology domain (PH), negative regulatory tyrosine in the carboxy terminus and myristylation or palmitoylation (lipid) modification sites at the N-terminus. In the absence of any SH2 or SH3 domains, these proteins encode a group of well-conserved domains termed as JAK homology (JH1-JH7) domains that follow a non-conserved amino terminus of about 30–50 amino acids (Rane and Reddy, 2002). There are four members of mammalian JAK

family including, Jak1, Jak2, Jak3 and tyrosine kinase 2 (TYK2) and their essential role is mediating the cytokine signalling pathway (George R. Stark *et al.,* 1998).

1.3.4 Biological functions of STATs

STAT1

Stat1 plays a critical role in growth arrest, promoting apoptosis and is also implicated as a tumour suppressor, thus proving that lack of STAT1 can promote tumour cell survival and establish tumours (Bromberg and Jr, 2000). IFNγ, IFNα, IFNβ and IL-27 activate STAT1 (Perona-Wright *et al.,* 2010). IL-21 preferentially activates STAT1 and STAT3, which is mediated by Y510 tyrosine in the cytoplasmic domain of IL-21R (Zeng *et al.,* 2007). STAT1 deficiency rather than the absence of both IFNγ and IFNα/β receptors results in pronounced impairment of NK cell function, demonstrating the IFN-independent roles of STAT1 for the NK cell response (Lee *et al.,* 2000) .

STAT2

The main function of STAT2 is its role in IFNα/β signalling, where it complexes with STAT1 and either heterodimerises by associating with DNA binding protein IRF-9 and forming ISGF-3 (IFN-stimulated gene factor 3), or they can form STAT1 homodimers (Darnell, 1997). STAT2 knockout mice are unresponsive to type I IFNs and exhibit enhanced susceptibility to viral infections and unique defects in macrophage and T cell responses (Park *et al.,* 2000).

STAT3

STAT3 plays an important role in the immune system, including regulation of steady state and emergency granulopoiesis, dendritic cell production and inhibition of macrophage inhibitory signalling (Panopoulos *et al.,* 2006, Yadav *et al.,* 2013). STAT3 is also involved in the activation of most genes in response to bacterial lipopolysaccharide (LPS) and its absence leads to significantly impaired responses to acute phase activators which is associated with bacterial infections (Alonzi *et al.,* 2001). It is an important signalling molecule in Th17 differentiation and when activated by both IL-6 and IL-23, promotes the Th17 programme, inhibiting Th1 and Th2 programmes by increasing expression of RORγt and reducing GATA3 and T-bet expression respectively (Yang *et al.,* 2007). IL-21 in combination with TGF-β independently induces Th17 differentiation in the absence of IL-6, suggesting an alternative pathway for Th17 differentiation *in vivo* in an IL-6 deficient environment (Korn *et al.,* 2007). Kortylewski and Yu showed for the first time that STAT3 deletion in the haematopoietic compartment improves tumour immune surveillance (Kortylewski *et al.,* 2005). STAT3 deletion in NK cells affect tumour cells, increasing the level of perforin and granzymes B, demonstrating an inhibitory role for STAT3 in NK cell dependent tumour surveillance by binding to the IFNγ promoter and interfering with STAT driven transcription (Gotthardt *et al.,* 2014). Furthermore, it has been shown that STAT3 is aberrantly activated in a type of inflammatory bowel disease, Crohn's disease, indicating a role of STAT3 in the pathogenesis of Crohn's disease in intestinal T cells (Lovato *et al.,* 2003).

STAT4

It is mainly phosphorylated by the IL-12 mediated signalling pathway in T cells, but STAT4 can also be activated by IFNγ stimulation, which has only been observed in human cells (Ho *et al.,* 1996). The phenotype of STAT4 deficient mice, in most respects, is similar to mice lacking the IL-12 or IL-12R subunit, having impaired Th1 differentiation, IFNγ production, and cell-mediated immunity (Wurster *et al.,* 2000).

STAT5

It has been demonstrated that STAT5 is a critical positive factor that promotes HSC fitness and multilineage haematopoiesis (Snow *et al.,* 2002). STAT5 is required in the earliest stages of differentiation from the lymphomyeloid repopulating HSCs (Bunting *et al.,* 2002). It also has a role in the regulation of T cell proliferation and its absence leads to severe autoimmunity (Moriggl *et al.,* 1999). The absence of NK cells in STAT5 knockout mice demonstrates its critical role in NK cell development (Lin and Leonard, 2000). IL-2 strongly induces STAT5 phosphorylation, and STAT5 proteins are activated by variety of cytokines and growth factors, including IL-3, IL-5, IL-7, IL-9, IL-15 and IL-21, with more sustained activation by IL-15 (Zeng *et al.,* 2007). STAT5a deficient mice are characterised by defects in responses to granulocyte macrophage colony stimulating factor (GM-CSF) and have loss of Prl-mediated mammary gland development, whereas STAT5b knockout mice have sexually dimorphic growth retardation (O'Shea *et al.,* 2002).

STAT6

IL-4 and IL-13 primarily activate STAT6 and coordinate the commitment and expansion of the Th2 response (Perona-Wright *et al.,* 2010). STAT6, recruited by IL-4, suppresses TGF-β-mediated Foxp3 induction by binding to the Foxp3 promoter, which can be reverted by retinoic acid and lead to iTregs increase (Takaki *et al.,* 2008). This highlights the therapeutic approaches facilitating tolerance induction in Th2-mediated diseases, such as allergy, using TGF-β1 with RA agonist.

1.3.5 Cross-talk between JAK-STAT and other pathways

It has been demonstrated that calcium-dependent pathways can regulate tyrosine and serine phosphorylation of STAT1 and JAK kinases that is mediated by calcium-activated calmodulin kinase II (CaMKII) and calcium-dependent tyrosine kinase (Pyk2). Inhibition of CaMK or Pyk2 suppresses STAT1-dependent IFNα-induced tyrosine phosphorylation of JAK1 and Tyk2 in macrophages and a mouse model of systemic lupus erythematosus *in vivo* (Wang *et al.,* 2008). The IFNγ induced increase in the expression of an anti-SMAD, Smad7, through the JAK1/STAT1 pathway effectively suppresses TGFβ-mediated Smad3 phosphorylation, preventing translocation of Smad3-Smad4 complex to the nucleus, nuclear accumulation of Smad3 and the activation of TGF-β responsive genes (Ulloa *et al.,* 1999). Integrins, such as transmembrane receptors that can mediate cell adhesion to matrix molecules, can control serine-threonine kinase protein kinase C ε (PKCε) phosphorylation and IFNγ-induced STAT1 phosphorylation is suppressed in PKCε$^{-/-}$ macrophages, indicating a mediatory role of PKCε between integrin and IFNγ-STAT1 signalling pathway (Ivaska *et al.,* 2003).

1.4 Adaptive immunity

It is long-lived, slowly developing and highly evolved antigen-specific protective response composed of cell-mediated and antibody production, which can show an extraordinary different range of specificity (Tosi, 2005). There is fundamental link between innate and adaptive immunity and the magnitude and quality of the adaptive immune response is hugely dependent on signals derived from innate immunity (Tough, 2002).

1.4.1 T cell development

T cells develop from progenitors derived from the pluripotent haematopoietic stem cells in the bone marrow. Lymphoid precursors migrate from the bone marrow and blood into the thymus, where they receive a signal, most probably from the stromal cells, that is transduced through the Notch 1 receptor to switch on specific genes. Notch 1 instructs the lymphoid precursor to become a T lymphocyte rather than B lymphocyte (Owen *et al.,* 2013). The mentioned progenitor cells undergo several stages of development to become mature T cells. These lymphoid precursors lack surface molecules (CD4/CD8) and their receptor genes are unarranged and this is called double negative stage, which can be divided into four stages based on the surface expression of CD44 and CD25 (Germain, 2002). During DN_1, CD44 is expressed, but CD25 is not ($CD44^+$, $CD25^-$) and in DN_2 ($CD44^+$, $CD25^+$), rearrangement of T cell receptor (TCR) β chain locus begins, cells with some D_β to J_β rearrangements and in DN_3 ($CD44^-$, $CD25^+$), it continues with $V_{\beta\ to}\ DJ_\beta$ rearrangement and in this step, pre-TCRα are expressed (Owen *et al.,* 2013). During this step, the β selection process starts as a productive β chain, then

thymocytes lose expression of CD25 once again and progress to DN_4 ($CD44^-$, $CD25^-$) in which pre-TCRs proliferate. Moreover, allelic exclusion occurs, in which T cells fully rearrange and express a TCRβ chain from only one of their two TCR alleles, so, β chain couples with pre-T α chain and rapid proliferation occurs in the sub-capsular cortex (Murphy, 2012).

Afterwards, thymocytes go through the development of $CD4^+$ and $C8^+$, known as double positive stage, which reside in the thymic cortex and are the most abundant subpopulation in the thymus. In this step, young DP thymocytes successfully rearrange and express a TCRα chain, which associates with the already produced TCRβ chain, taking the place of the surrogate TCRα chain, which is no longer actively expressed (Owen *et al.,* 2013).

Then, these lymphocytes receive a survival signal through undergoing positive or negative selection, but most lymphocytes (98%) never meet these criteria and die by apoptosis within the thymus. The majority fail positive selection and their receptor interacts so poorly with self-MHC molecules that they die by a process known as death by neglect. Positive selection is a process, which selects for those thymocytes bearing receptors capable of binding self-MHC molecules. These self-MHC molecules present self-peptides, which are typically arrived from intracellular or extracellular proteins that are degraded and interact with MHCI/II to receive the vital signal and differentiate (Murphy, 2012). Negative selection will remove thymocytes (5%) in medulla that bear TCRs capable of strongly binding with self-MHC peptides and errors in negative selection are responsible for autoimmune disorders (Murphy, 2012).

Afterwards, positively selected thymocytes must decide whether to join the $CD8^+$ cytotoxic T cell lineage or the $CD4^+$ helper T cell lineage. So, two models are proposed

for lineage commitment (Figure 1.1). First in the instructive model, TCR/CD4 and TCR/CD8 co-engagement generate unique signals that directly initiate distinct developmental programmes. If a thymocyte generated a TCR with an affinity for MHC class I, the TCR and CD8 would bind the MHC class I together, generating a signal that specifically initiates a programme to silence CD4 expression and induce expression of genes specific for cytotoxic T cell lineage function. Likewise, TCR/CD4 molecules generate distinct intracellular signals when they co-engage with MHC class II and makes CD8 to lose its expression (Germain, 2002).

By contrast, in the stochastic model, positively selected thymocytes randomly downregulate CD4 or CD8 and only those cells that express the correct co-receptor, the ones that can engage MHC with the TCR, generate a TCR signal strong enough to survive to mature, if the cell doesn't receive a signal through the correct co-receptor, it dies. Thus in this model, TCR/CD4 and TCR/CD8 co-engagement does not necessarily generate distinct signals (Germain, 2002) (Figure 1.5).

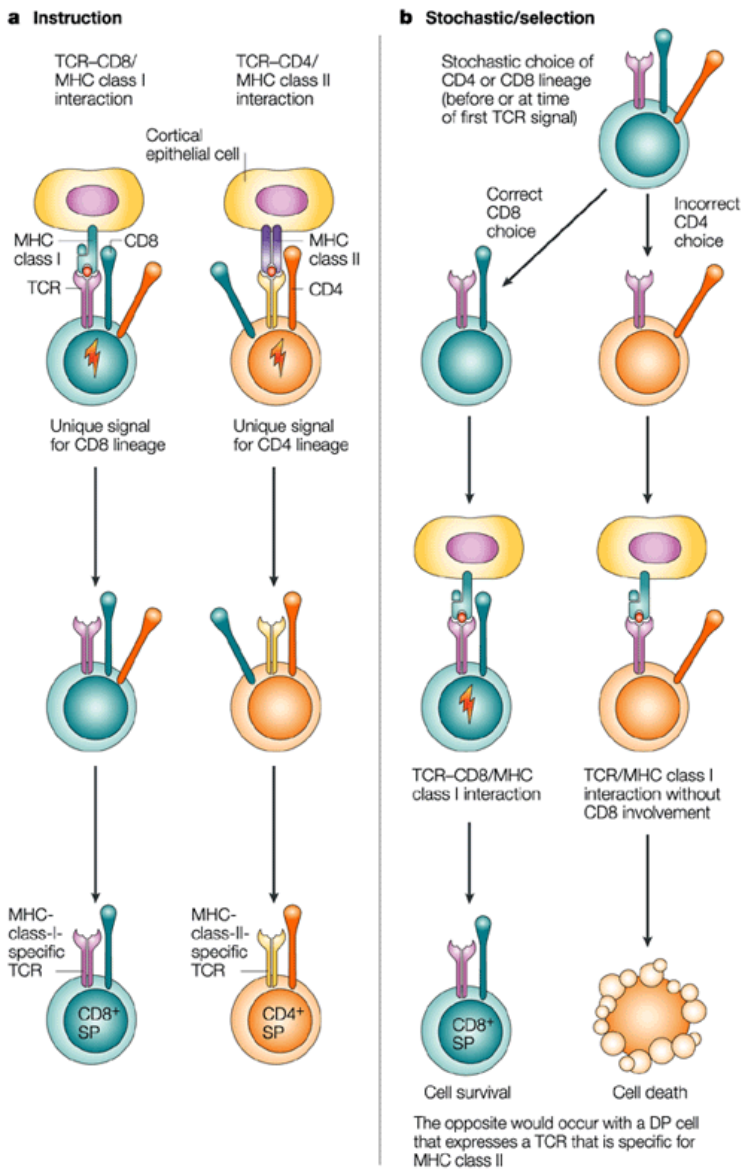


Figure 1.5 Instruction and stochastic model through engagement of TCR and CD4 and CD8 coreceptors. According to the one-step instructive model, coengagement of MHC class I–restricted TCR and CD8 or MHC class II–restricted TCR and CD4 induces alternative signals that promote specific CD8 or CD4 lineage programs of gene expression and lead directly to silencing of the inappropriate coreceptor. In contrast, the stochastic-selective model involves two steps: a random lineage choice, and selection of thymocytes whose coreceptor expression matches their TCR specificity, which requires signal mediated by coengagement of TCR and coreceptors (Germain, 2002).

Once a thymocyte successfully passes through the selection, it leaves the thymus and those mature T cells that exit the thymus are referred to as recent thymic emigrants (RTEs), which are self-restricted, self-tolerant and single positive. Final maturation is influenced by their interactions with both MHC and non-MHC ligands in secondary lymphoid organs (Figure 1.6) (Murphy, 2012).

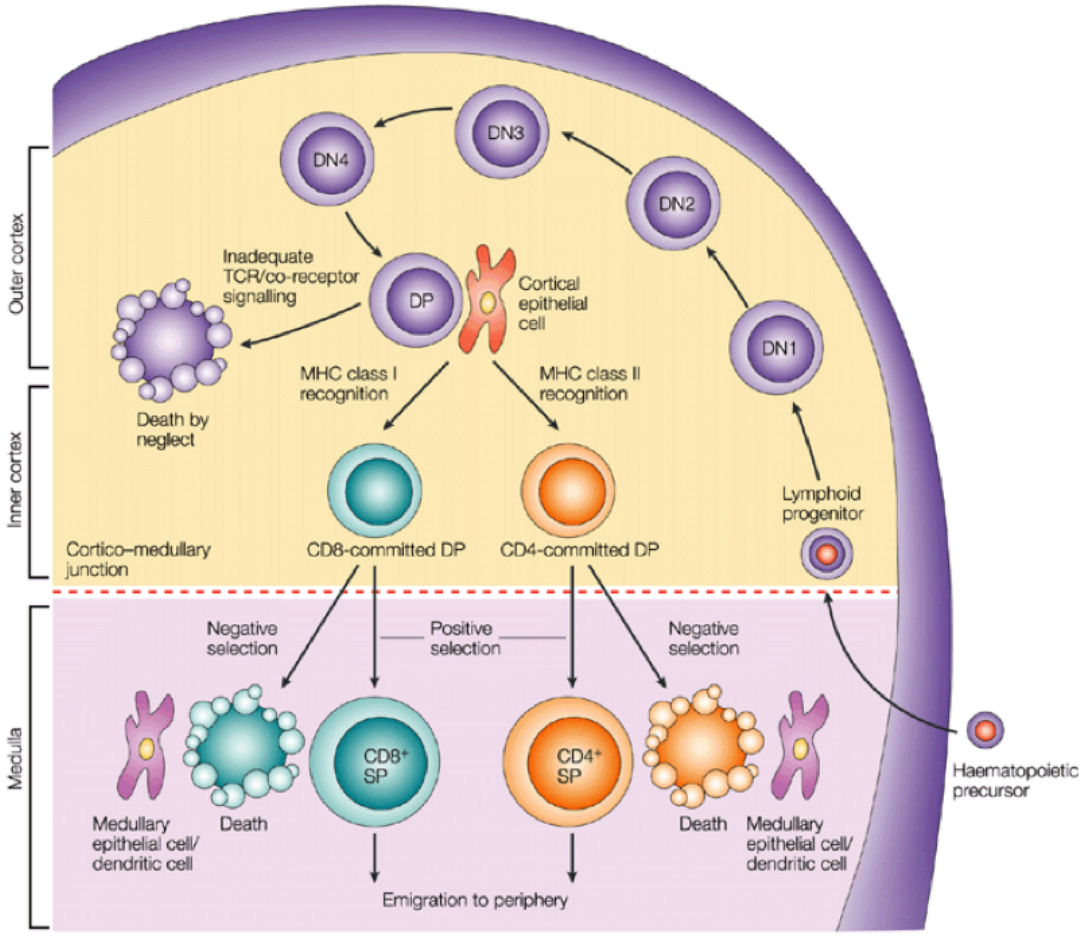


Figure 1.6 Schematic representing T cell development. Progenitor (PG) cells from the bone marrow colonise the thymus where they commit to natural killer cells (NK) or T cell lineage. They undergo progressive differentiation from double-negative (DN) to double positive (DP) to single-positive CD4 or CD8 thymocytes. The immature DN thymocytes can be further divided into DN1 to DN4 based on their CD44 and CD25 expression. Progression from DP stage to SP can occure through immature CD8+ single positive (ISP) cell intermdiate (Germain, 2002).

1.4.2 T cell activation and differentiation

T cells can be activated by engagement with three signals. Firstly, TCR binds with T cell co-receptor protein complex, CD3. The second essential signal for T cell activation and proliferation, CD28, is a glycoprotein presented on T lymphocytes, which as a co-stimulatory molecule binds to B7-1 (CD80) and B7-2 (CD86) ligands on antigen presenting cells, finally enhancing transcription and stability of IL-2 mRNA, thereby T cells can proliferate and produce IL-2, as a T cell growth factor (Linsong Li *et al.,* 1999). In contrast, interruption in the CD28 pathway not only suppresses the immune response but also induces antigen-specific tolerance (J. *et al.,* 1996).

CD4 can play a central role in immune protection via its ability to help B cells make antibody, recruit polymorphonuclear phagocytes to the site of infection, induce macrophages to develop increased microbicidal activity and producing chemokines and cytokines to orchestrate the full immune responses (Zhu and Paul, 2008). During their first interaction with antigens and pattern of signals they receive, CD4 T cells can be differentiated into four populations including, Th1, Th2, Th17 and regulatory T cells (Zhu *et al.,* 2010).

Th1 cells mediate immune responses against intracellular microorganisms, with T-bet being the master transcriptional factor and Th1 principal cytokine production being IFNγ and IL-2. By contrast, Th2 cells are critical for immunity to many extracellular pathogens with GATA3 as main transcriptional factor and Th2 cells can produce mainly IL-4, IL-5 and IL-13 (Zhu and Paul, 2008).

Th17 mediates immune responses, particularly against extracellular bacteria and fungi, and its master regulator gene is RORγt and it produces IL-17, IL-21 and IL-22, whereas,

Treg cells play central role in maintaining self-tolerance, with Foxp3 as their main transcriptional factor and can produce TGF-β, IL-10 and IL-35 (Figure 1.7) (Zhu *et al.,* 2010).

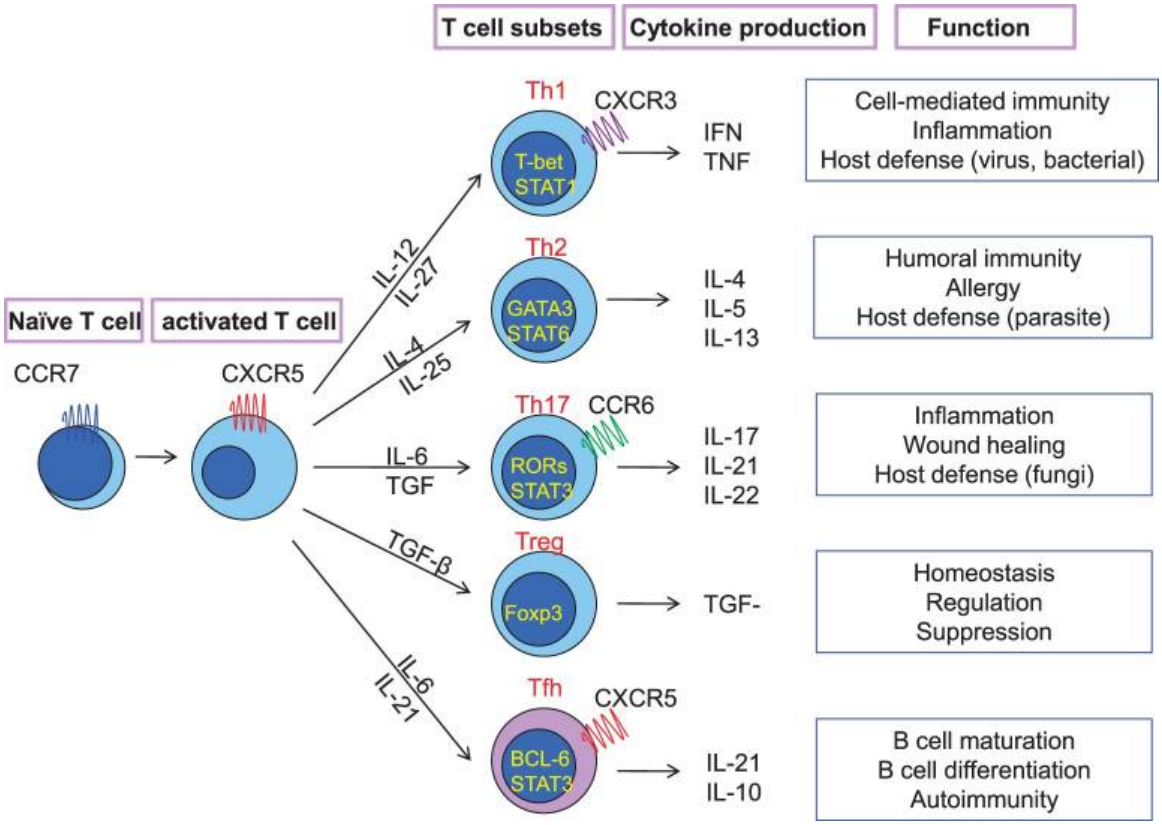


Figure 1.7 CD4 T helper cell lineage fate and function. It summarises major set of CD4 T cells, including their unique products, specific transcriptional factors, critical cytokines for their fate determination and their function. The molecules secreted by these subsets, e.g., IFNγ for Th1 cells, are finely tuned to control the pathogen that mediated the release of the specific molecules by the APC during activation of the Th0 cells into the various subsets (Zhang *et al.,* 2013).

1.4.3 B cell development and differentiation

B lineage cells develop from haematopoietic stem cells (HSCs) in bone marrow through several stages and in these stages, multipotent progenitors (MPP) differentiate into lymphoid progenitors and B cell-restricted progenitors originate from early lymphoid progenitors, followed by common lymphoid progenitors (CLP), pro-B cells, pre-B cells and immature B cells (Figure 1.8) (Ichii *et al.,* 2014). Immunoglobulin gene rearrangements are required for the process of B lymphopoiesis and mature B cells as well as T cells. The activation of recombination enzymes, such as recombination-activating gene (RAG-1 and RAG-2) and terminal deoxynucleotidyl transferase, promotes the D to J and V to DJ rearrangements in the Ig heavy (IgH) chain locus during the differentiation from CLP to pro-B stage. Bone marrow stromal cell-derived IL-7 is a non-redundant cytokine for B cell development that promotes V to DJ rearrangement and transmits survival and proliferation signals (Ichii *et al.,* 2014).

On the completion of heavy chain rearrangement, the cell is classified as a pre-B cell that expresses the first Ig expression in B cell development. The membrane bound complex of μ heavy chain and surrogate light chain appears on the pre-B cell associated with the αIg-B heterodimer to form the pre-B cell receptor, only pre-B cells that are able to express membrane bound μ heavy chain in association with surrogate light chain are able to proceed along the maturation pathway (Owen *et al.,* 2013).

In the next stage, pre-B cells develop into mature B cells, which show complete membrane of IgM on the cell and the κ and λ chain is also attached to μ. Furthermore, immature B cells react with antigens with high avidity, such as polyvalent antigens in bone marrow, leading to apoptosis rather than activation; this property is important for

negative selection of B cells that are specific for innate antigens available in bone marrow. By contrast, immature B cells that do not bind self-antigens express D chain and membrane IgD with their IgM remain in bone marrow and become mature naïve (resting) B cells (Murphy, 2012).

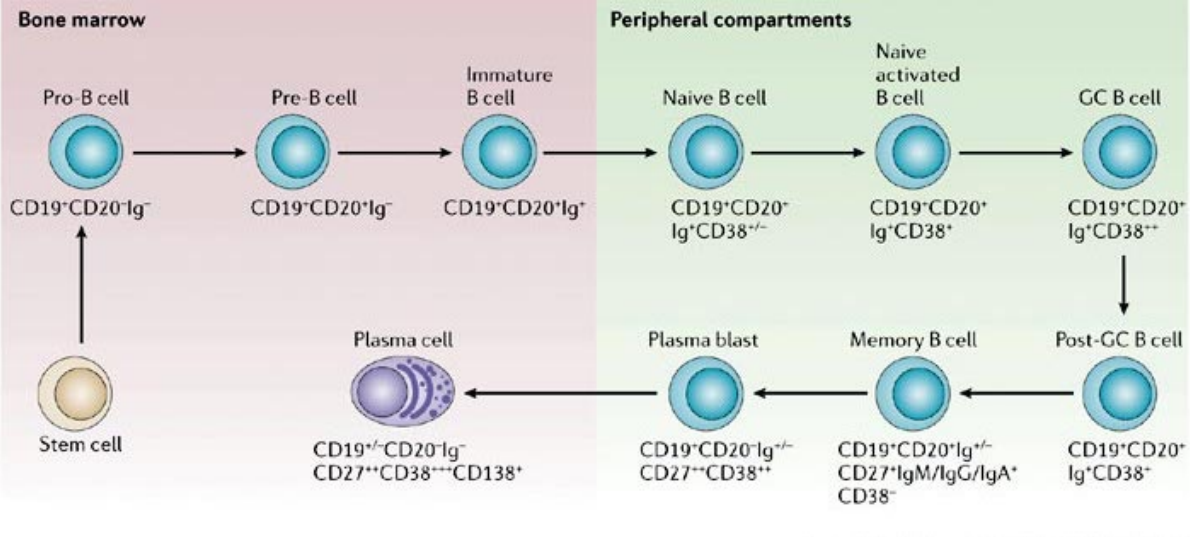


Figure 1.8 B cell maturation stages and their cell surface markers. B cell lymphocytes arise from progenitors derived from hematopoietic stem cells (HSCs). B cell membrane receptors evolve and change throughout the B cell life span. CD20 is expressed on all stages of Bc ell development except the first and last stage (W *et al.,* 2006).

In the other words, to complete development into mature B cells, immature B cells leave the bone marrow and complete their maturation in the spleen before migration to lymphatic organs. After mature B cells migrate to secondary lymphoid organs, they proliferate and differentiate in the presence of antigen. There are two main procedures at this level, somatic hypermutation and immunoglobulin isotype switching. Somatic hypermutation occurs in variable antibody genes, in both heavy and light chain genes,

leading to affinity maturation (the affinity of binding the antibody to antigen), while changing the constant heavy chain occurs in isotype switching (Murphy, 2012).

B cell activation is enhanced through the activity of CD21, a surface receptor in a complex with surface proteins CD19 and CD81 (all three are collectively known as the B cell co-receptor complex). Once a B cell encounters its cognate antigen and receives an additional signal from a T helper cell, it can further differentiate into either plasma B cells or memory B cells (Figure 1.9) (Owen *et al.,* 2013).

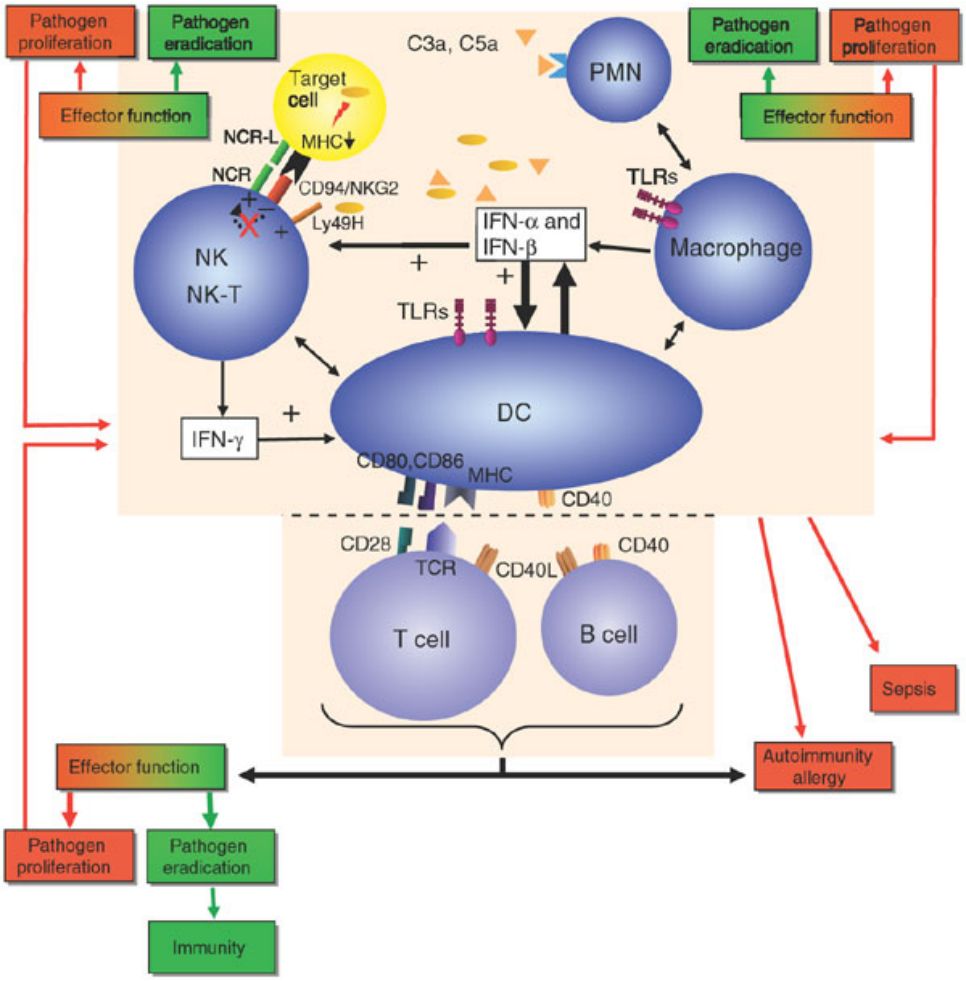


Figure 1.9 The interface between innate and adaptive immunity. Innate immune system components recognise the microbes and help with the successful removal of pathogens. These include specialised receptors on NK cells, complement system, TLRs that are expressed on myeloid and lymphoid cells, which recognise specific microbially derived molecular structures. A well orchestrated innate and adaptive immunity lead to eradication of pathogen and host immunity (Hoebe *et al.,* 2004).

1.5 Autoimmunity

One of the key features of the immune system is immune tolerance that is designed to preserve self-tissues while allowing effective responses against infections. In autoimmunity, dysregulated immune responses of the body occur against its own cells and tissues. Typically, autoimmune diseases are multi-etiological entities, where environmental, genetic abnormalities, hormonal defects along with derailed immunoregulatory processes lead to the development of the disease (Nagy *et al.,* 2015). Therefore, faulty tolerance mechanism, imbalance of pro and anti-inflammatory cytokines, abnormal auto-antigen scavenging machinery and antigen presentation can contribute to the development and continuation of autoimmune diseases (Ermann and Fathman, 2001). Altered survival of T cells and defects in the inhibitory signalling pathways of T cells is one of the major factors that can contribute to autoimmunity and they are referred as T cell-mediated autoimmune disease, such as rheumatoid arthritis (RA), multiple sclerosis, type 1 diabetes, inflammatory bowel disease (IBD), systemic lupus erythematosus, Sjogren's syndrome and psoriasis (Wu *et al.,* 2012).

Death receptor Fas (CD95) is vital in T cell deletion and if self-reactive T cell deletion is disrupted, autoimmunity might also arise, whereas if self-antigens are presented on activated mature APCs, this can lead to activation of self-reactive T cells resulting in

autoimmunity (Ohashi, 2002). Lack of the receptor tyrosine kinases, such as Tyro3, Axl and Mer, that are regarded as negative regulators of APC activation can contribute to APC activation and development of broad-spectrum autoimmunity (Lu and Lemke, 2001).

Enhanced activation of the PI3K-PKB pathway and in particular, changes in the activity of the PI3K subunit $p65^{PI3K}$, PTEN and PKB alter Fas-mediated apoptosis, thereby contributing to the loss of lymphocyte homeostasis and prolonged survival signals, resulting in autoimmunity (Jones *et al.,* 2000, Cristofano *et al.,* 1999). Activation of PI3K, an important molecule that regulates cell growth, proliferation and survival, leads to the activation of $p65^{PI3K}$ to T cells that will result in the activation of downstream effectors including protein kinase B (PKB/Akt) and Rac. This leads to predominant expansion of $CD4^+$ T cells, splenomegaly, increased level of serum immunoglobulin and autoimmune kidney disease (R.-Borlado *et al.,* 2000).

In the absence of Cbl-b, a cytoplasmic protein that works as a negative regulator of T cell signalling, IL-2 production is enhanced after TCR stimulation, indicating predisposition towards autoimmunity, with symptoms such as multi-organ inflammation and production of autoantibodies, demonstrating the role of Cbl-b as negative regulator of mature T cell activation (Ohashi, 2002).

Abnormal activation of effector subsets of $CD4^+$ T helper cells, including Th1 and Th17, play a key role in mediating many aspects of organ-specific autoimmune diseases. Furthermore, Th1 and Th17 can induce production of inflammatory chemokines and cytokines, including IFN-γ, IL-1β, IL-6, IL-12, IL-17, IL23 and TNF-α, thus promoting inflammatory infiltration leading to the onset of autoimmune diseases (Wu *et al.,* 2012).

Moreover, sometimes pathogens express antigens that are similar to host self-antigens and pathogen-activated responding T cells might cross-react with self-tissue to initiate organ-specific autoimmune disease, a mechanism known as molecular mimicry (Oldstone, 1987, Oldstone *et al.,* 1991).

In addition, Tregs are important and play a crucial role in immune homeostasis, maintaining peripheral tolerance for self-tissues to prevent autoimmunity. In autoimmune diseases, their number is selectively decreased or there is a diminished suppressor function of Tregs (Koonpaew *et al.,* 2006). Similarly, $CD4^{+}CD25^{high}$ Tregs are significantly decreased in SLE patients, which is associated with the reduced expression level of FoxP3 mRNA and protein (Valencia *et al.,* 2007). Periphery Tregs (pTregs) arise extrathymically upon antigen exposure, which can complement tTregs to establish tolerance by participating locally at the site of inflammation to control immunity. One of their prominent functions is maintenance of foetal tolerance during pregnancy (Yadav *et al.,* 2013).

1.6 Early growth response (Egr) gene family

The early growth response genes are a family of transcription factors comprising four members: Egr1 (Krox-24/NGFI-A), Egr2 (Krox-20), Egr3 (NGFI-3/Pilot) and Egr4 (NGFI-C). All members share three cyc2-Hys2 zinc fingers that bind to the cognate GC-rich consensus DNA binding motif of 28-30 amino acids (Figure 1.10) (Poirier *et al.,* 2008, Warner *et al.,* 1998). Egr genes are expressed in many different cell types including neurons, B and T cells and their expression is rapidly induced in response to mitogens, apoptotic signals and differentiation (Decker *et al.,* 1998).

Egr1 has a regulatory role in T cell proliferation and is rapidly induced after IL-2 stimulation (Perez-Castillo *et al.,* 1993). It binds to the regulatory ZIP elements and acts as a potent coactivator for the nuclear factor of activated T cells (NFATc) within the IL-2 promoter leading to IL-2 transcription (Decker *et al.,* 1998). Egr1 also plays a role in thymocyte development and by enhancing expression of anti-apoptosis molecule bcl-2 and Id3 inhibitor, it promotes positive selection of both CD4 and CD8 single positive cells (Bettini *et al.,* 2002). Id3 promotes thymocyte maturation and during positive selection, Id3 expression is upregulated in response to TCR signalling (Rivera *et al.,* 2000, G *et al.,* 2001). Egr1 expression is essential to sustain macrophage lineage differentiation (Krishnaraju *et al.,* 1995).

Egr2 expression plays a vital role in the onset of myelination in the peripheral nervous system and hindbrain development (Warner *et al.,* 1998), while Egr3 is associated with muscle-spindle development (O'Donovan *et al.,* 1999). Egr4 was initially identified in the central nervous system, significantly expressed in the forebrain, and it plays a crucial role in male murine fertility (Tourtellotte *et al.,* 1999). Egr4 is also regarded as a novel regulator of posterior hindbrain development (Bae *et al.,* 2015).

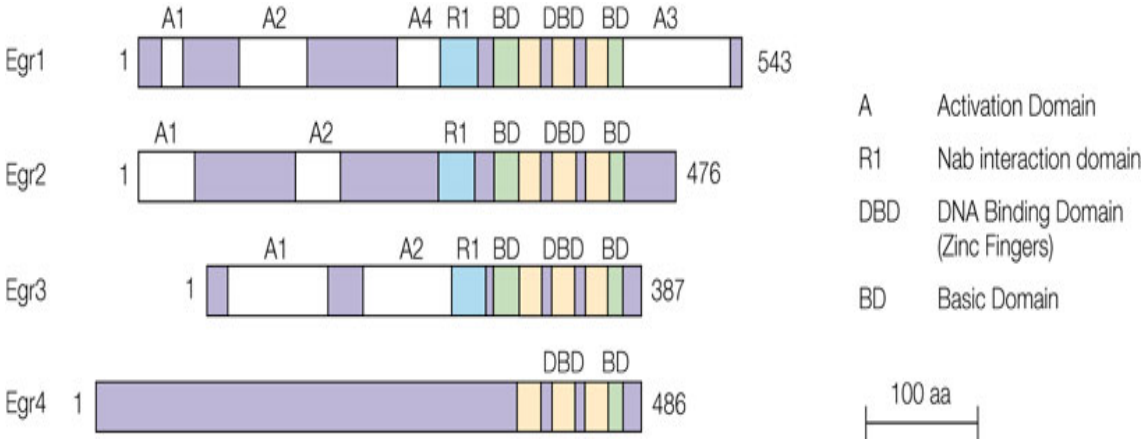


Figure 1.10 Schematic representing the structure of four members of the Egr family. Between the members Egr2, Egr3, followed by Egr1 are closely related. Nab interaction domain is present in Egr1, Egr2 and Egr3. The zinc fingers are conserved between all memebers and two basic regions flanking the zinc fingers are conserved between Egr1, Egr2 and Egr3. (Poirier *et al.,* 2008).

1.6.1 The role of Egr2 and Egr3 in the immune system

Egr2 and Egr3 are expressed in selected stages of B and T cell development and are repressed in ISP thymocytes and pro-B cells, which suggests an indispensable role of Egr molecules in the development of DN cells following β selection and withdrawal of Egr2 expression after β selection for the differentiation of ISP cells to DP cells (Li *et al.,* 2011).

Egr2 and Egr3 are negative regulators of T cell activation and inhibit T cell function by suppressing NAB2 and Egr1 expression (Collins *et al.,* 2008). NAB2 as a coactivator collaborates with Egr1, promoting IL-2 production and T cell function (Collins *et al.,* 2006). Cells overexpressing Egr2 and Egr3 showed increased Cbl-b, and T cells from $Egr3^{-/-}$ had much less Cbl-b in comparison to controls from wild type mice, supporting the hypothesis that upregulation of inhibitory genes involved in T cell activation can be promoted by Egr2 and Egr3 genes (Safford *et al.,* 2005). Silencing Egr2 gene expression prevents full induction of anergy and enhance responsiveness of $CD3^{+}CD28^{-}$ stimulated cells to stimulation, resulting in restored state proliferation and IL-2 production in anergic cells, indicating the role of Egr2 role in conferring the anergic state (Harris *et al.,* 2004).

Egr2 and Egr3 are crucial for the control of inflammation and antigen-induced proliferation of B and T cells. In this regard, Egr2 and 3 deficient mice led to the diminished activation of SOCS1 and SOCS3, resulting in enhanced activation levels of

IFN-γ and Th17 polarisation respectively, suggesting a fundamental role of Egr2 and Egr3 in limiting immunopathology during productive adaptive immune responses and preventing the development of autoimmune disease (Figure 1.11) (Li *et al.,* 2012). SOCS1 and SOCS3 deficiency leads to activation of peripheral T cells, resulting in the development of inflammatory disease (Chong *et al.,* 2005, Croker *et al.,* 2004). The suppressor of cytokine signalling (SOCS) 1 and SOCS3 negatively regulate STAT1 and STAT3, the major signal transducers pathways, respectively, which control the differentiation and development of Th1 and Th17 respectively (Ramgolam and Markovic-Plese, 2011). A recent study identified that Egr2 and 3 are potent antagonists of T-bet function in CD4 and CD8 effector T cells, indicating a distinct mechanism in controlling effector T cell differentiation that can limit immunopathology (Singh *et al.,* 2017).

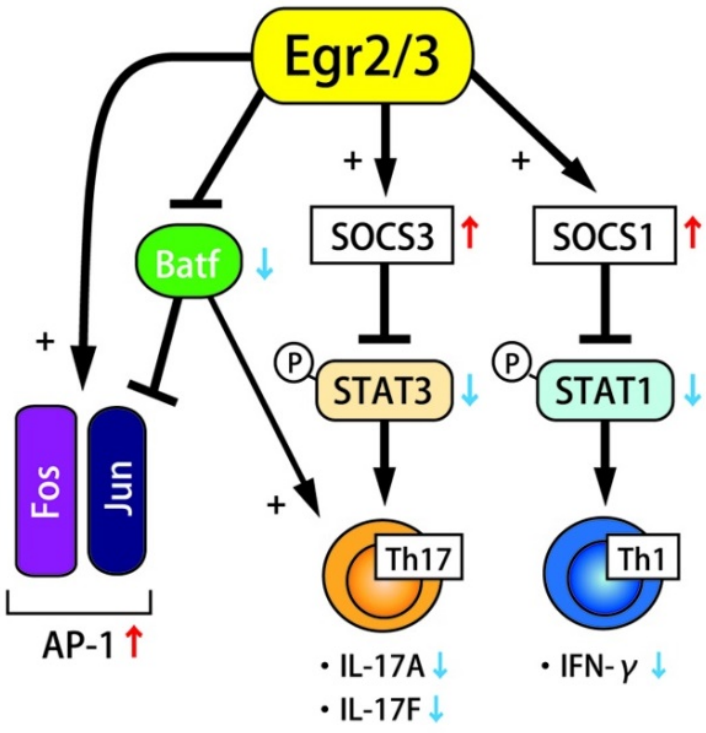


Figure 1.11 Overview of the role of Egr2 and Egr3 functions under inflammatory conditions. Egr2 and Egr3 genes are able to control the inflammation by suppressing STAT1/3 phosphorylation indirectly, preventing the development of autoimmune disease (Sumitomo *et al.,* 2013).

Egr2 has a role in the control of the expansion of effector T cells and TCR signalling is required for the induction of Egr2 (Zhu *et al.,* 2008). Zhu et al. (2008) demonstrated that $CD44^{high}$ T cells are hyperactive *in vivo* in Egr2/3 deficient mice, resulting in development of a late onset lupus-like autoimmune disease characterised by severe glomerulonephritis, skin lesions, hair loss, significantly increased infiltration of T cells into multiple organs, enhanced total serum Ig and accumulation of IFNγ and IL-17 producing $CD4^{+}$ T cells in comparison to age-matched WT mice counterparts. Thus, this reflected a vital intrinsic role of Egr2, not only in inflammation and proliferation of effector T cells, but also in prevention of developing a lupus-like autoimmune disease. $CD44^{high}$ is regarded as a marker for memory and effector T cells (Schumann *et al.,* 2015). The pathogenesis of systemic lupus erythematosus is closely related to the significant production of pro-inflammatory chemokines and cytokines IL-17 and IFN-γ (Crispin *et al.,* 2008). Moreover, another study demonstrated that in treatment for SLE through downregulation of Akt phosphorylation, diminished expression of Egr2 and Egr3 was ameliorated by hCDR1 peptide, which led to down-regulated IL-2 mRNA expression and IFNγ secretion, but enhanced expression levels of E3 ligase Cbl-b and TGFβ, inducing anergy, thereby demonstrating the crucial role of Egr2 and Egr3 as negative regulators for the maintenance of SLE (Sela *et al.,* 2008). Akt is an important kinase that plays a vital role in survival, activation and proliferation of T cell, providing co-stimulatory signals that can be activated either by CD28 or following cross-linking of

TCR (Kane and Weiss, 2003). Egr2 and 3 through regulation of BCL-6 have been found to play essential role in Tfh development (Ogbe *et al.,* 2015).

Furthermore, Egr2 and 3 are potent activators of FasL expression and can bind to the Fas ligand (Fas L) regulatory element in the FasL promoter and its transcription is induced after TCR activation via the NFAT dependent induction of Egr2 and Egr3 (Rengarajan *et al.,* 2000). FasL is a transmembrane protein belonging to the tumour necrosis factor family, which is expressed on cytotoxic T lymphocytes and crosslinks with Fas, cell surface receptors, initiating apoptotic signal leading to cell death (Huang *et al.,* 1999). Egr3 expression alone is sufficient to induce FasL mRNA in nonlymphoid cells HeLa, suggesting a direct role in activation of FasL (Mittelstadt and Ashwell, 1998), whereas Egr 2 does not affect apoptosis induction of thymocytes in the thymus and FasL expression is not altered in T cells (Li *et al.,* 2011, Zhu *et al.,* 2008).

1.6.2 Regulation of Egr2

Egr2 expression is activated during the G0/G1 transition in cultured cells (Chavrier *et al.,* 1988) and is expressed in the selected stages of B and T cell development (Li *et al.,* 2011). Thus far, it has been demonstrated that Egr2 is induced by the TCR signalling pathway (Shao *et al.,* 1997, Carter *et al.,* 2007, Seiler *et al.,* 2012, Zhu *et al.,* 2008). Rengarajan *et al.* (2000) reported that Egr2 is a NFAT-regulated target gene, the expression of which was severely impaired in $NFATp^{-/-}$ and $NFAT4^{-/-}$ mice, whereas Egr2 was transactivated by NFATp overexpression. Other reports also confirmed the regulatory role of NFAT in Egr2 expression in T cells (Lazarevic *et al.,* 2009). Although Egr2 is required for AP-1 activation after antigen receptor signalling in naïve T and B cells (Li *et al.,* 2012), it was demonstrated that Egr2 is induced by NFAT in the absence

of Ap-1, suggesting that NFAT target genes do not require AP-1 activity (Collins *et al.,* 2008). Moreover, Ly 108, a SLAM family receptor, increased co-stimulation and sustained CD3-induced Egr2 expression in developing thymocytes (Dutta *et al.,* 2013).

A recent study showed that Egr2 can be induced through CD26 mediated co-stimulation along with the preferential production of IL-10 in $CD4^+$ T cells. It has been suggested that not only NFAT but also Raf-MEK-ERK signalling is involved in the induction of Egr2 expression (Hatano *et al.,* 2015). MAPK/ERK signalling association with the Egr2 induction has been reported in osteoprogenitors through the stimulatory effect of epidermal growth factor receptor (EGFR) (Chandra *et al.,* 2013), suggesting that the ERK signalling pathway is also associated with the induction of Egr2 expression in T cells.

1.7 Tumour immunosurveillance

Cancer is defined as the accumulation of numerous genetic alterations and the loss of normal cellular regulatory processes, with the initiation of several events proceeding and expanding effective anti-cancer immune responses against cancer cells. In the first step, after oncogenesis, neoantigens are released and captured by dendritic cells (Tian *et al.,* 2011). In the processing step, immunologic signals such as pro-inflammatory cytokines and factors are released, then captured antigens are presented by dendritic cells to T cells, leading to priming and activation of effector T cells against tumour specific antigens (Pardoll, 2012). Next, activated effector T cells traffic and infiltrate the tumour cells, recognise them and kill their target cancer cells. After killing cancer cells, additional tumour-associated antigens are released to enhance the depth and breadth of the immune response in the subsequent revolutions of the cycle (Chen and Mellman,

2013). The cancer cycle in cancer patients does not perform optimally, in this process tumour antigens may not be recognised, dendritic cells and T cells may not detect antigens as foreign thereby creating T regulatory cell responses, or T cells may not properly infiltrate the tumours (Motz and Coukos, 2013).

1.7.1 Immune therapy

Understanding the vital aspect of immune-regulatory pathways in tumours is important in cancer immunotherapy and different mechanisms, including conserved or acquired somatic mutations and T cell exhaustion are involved in making dysfunctional T cells (Speiser *et al.,* 2016). Adoptive cell therapy (ACT) using autologous TILs with anti-tumour activity has emerged to be the most effective therapy in metastatic melanoma in experimental animals as well as cancer patients (Rosenberg *et al.,* 2008). The generation of a tumour specific T cells by autologous lymphocytes from peripheral blood using a retrovirus that encodes TCR can mediate metastatic melanoma regression, so genetically engineered lymphocytes have the potential to be used therapeutically for cancer (Morgan *et al.,* 2006).

Furthermore, fundamental T cell unresponsiveness in advanced tumours is driven by TGF-β signalling through Smad2/3 transcriptional factors, which in turn upregulate Foxp1. This suppresses $CD8^+$ T cell proliferation and upregulation of Granzymes B and IFNγ in response to tumour antigens (Stephen *et al.,* 2014, Delisle *et al.,* 2013). Stephen *et al.* (2014) showed that tumour-reactive Foxp1 deficient $CD8^+$ T cells have superior anti-tumour effector functions, which can induce the regression of aggressive established tumours. Among the various approaches to therapeutic anti-tumour immunity, blockade of immune checkpoints has shown promising results and different

studies have highlighted immune checkpoint pathways as a prominent mechanism of immune resistance, in particular against T cells, that are specific for tumour antigens (Pardoll, 2012).

1.7.2 Immune checkpoints

Immune checkpoints are a plethora of pathways incorporated into the immune system that are vital for maintaining self-tolerance and modulating the amplitude and duration of immune responses against pathogenic infection in the peripheral tissues to minimise collateral tissue damage (Figure 1.12) (Pardoll, 2012).

CTLA-4

CTLA4, the first clinically targeted immune checkpoint receptor, is expressed exclusively on T cells where it mainly regulates the amplitude of the early stages of T cell activation. Primarily, CTLA4 has a counteractive influence on the activity of the CD28, a T cell co-stimulatory receptor. It was demonstrated that CTLA4 has a much higher overall affinity for both CD80 and CD86, hence with CTLA4 expression on the surface of T cells and by outcompeting CD28 in binding CD80 and CD86, it diminishes the activation of T cells. It also actively delivers inhibitory signals to the T cell (Rudd *et al.,* 2009). After tumour implantation, an endogenous anti-tumour immune response was generated in the animals and CTLA4 blockade could boost endogenous response, which ultimately led to induction of tumour regression. The endogenous immune responses are not substantially induced when the tumours are poorly immunogenic. In such cases, the combination of a vaccine and a CTLA4 antibody could induce a strong

enough immune response to decrease tumour growth and in some cases, eliminate established tumours completely (van Elsas *et al.,* 1999, Leach *et al.,* 1996).

PD-1

The immune responses should be tightly regulated and during the inflammatory immune response to infection, PD-1 plays a major role in limiting the activity of T cells in peripheral tissues and restrict autoimmunity (Keir *et al.,* 2006). PD-1 is expressed when T cells become activated and binds to one of its ligands, it suppresses T cells activation through inhibiting kinases that are involved in T cell activation via the phosphatase SHP2. In a similar fashion to CTLA4, PD-1 expression is highly induced on Treg cells, where it can increase their proliferation in the presence of ligand (Keir *et al.,* 2008). T_{Reg} cells are highly infiltrated into tumours that can further suppress effector immune responses, so blockade of the PD-1 pathway can revert this function, enhancing anti-tumour immune responses by diminishing the number and suppressive activity of intra-tumoural Treg cells (Francisco *et al.,* 2009). PD-1 expression is also induced in other activated non-T lymphocyte subsets, including B cells and NK cells, which suppress their lytic activity (Fanoni *et al.,* 2011).

Hence, although PD1 blockade is regarded as a fundamental approach in enhancing the effector T cell activity in tissues and in the tumour microenvironment, it also increases the activity of NK cell in tumours and tissues, probably either indirectly or through direct effects on $PD1^+$ B cells amplifying antibody production (Velu *et al.,* 2009). A recent study showed that T cell invigoration of the tumour burden ratio is associated with an anti-PD-1 response (Huang *et al.,* 2017). Huang *et al.* (2017) studied patients with stage IV melanoma and found that exhausted T cells were the major targets for the

PD-1 blockade, which could be detected in the peripheral blood, with Tex in the blood sharing the same TCR clonotypes with tumour infiltrating T cells.

PD-1 is highly expressed on TILs from multiple cancers and PD-1 ligands are usually increased on the tumour cell surface in many different human tumours (Dong *et al.,* 2002). Indeed, taken together, these findings provide underlying support for enhancing anti-tumour effector functions in the tumour microenvironment by the blockade of the PD-1 pathway (Iwai *et al.,* 2002, Konishi *et al.,* 2004).

LAG-3

Lymphocyte activation gene-3 (Lag-3) is highly expressed on Treg cells, where they are vital for increasing the immunosuppressive activity of Treg cells (Huang *et al.,* 2004, Goldberg and Drake, 2011). PD-1 and LAG3 in particular, are commonly co-expressed on exhausted or anergic T cells (Grosso *et al.,* 2009, Blackburn *et al.,* 2009).

TIM3

T cell immunoglobulin and mucin domain containing-3 (TIM3) is encoded by the HAVCR2 gene. It inhibits responses of T helper 1 (T_H1) cell and TIM3 antibodies enhance anti-tumour immune responses (Zhu *et al.,* 2005). In animal models, coordinated blockade of PD-1 and TIM3 amplifies anti-tumour immunity and blockade of each individual molecule has led to modest effect tumour rejection (Sakuishi *et al.,* 2010, Fourcade *et al.,* 2010).

BTLA

The expression of B and T lymphocyte attenuator (BTLA) on activated virus-specific $CD8^+$ T cells is relatively low, but it can be much higher on TILs from melanoma patients. $BTLA^{hi}$ T cells are suppressed in the presence of its ligand, HVEM. So, BTLA may be a pertinent inhibitory receptor for T cells in the tumour microenvironment (Lasaro *et al.,* 2011).

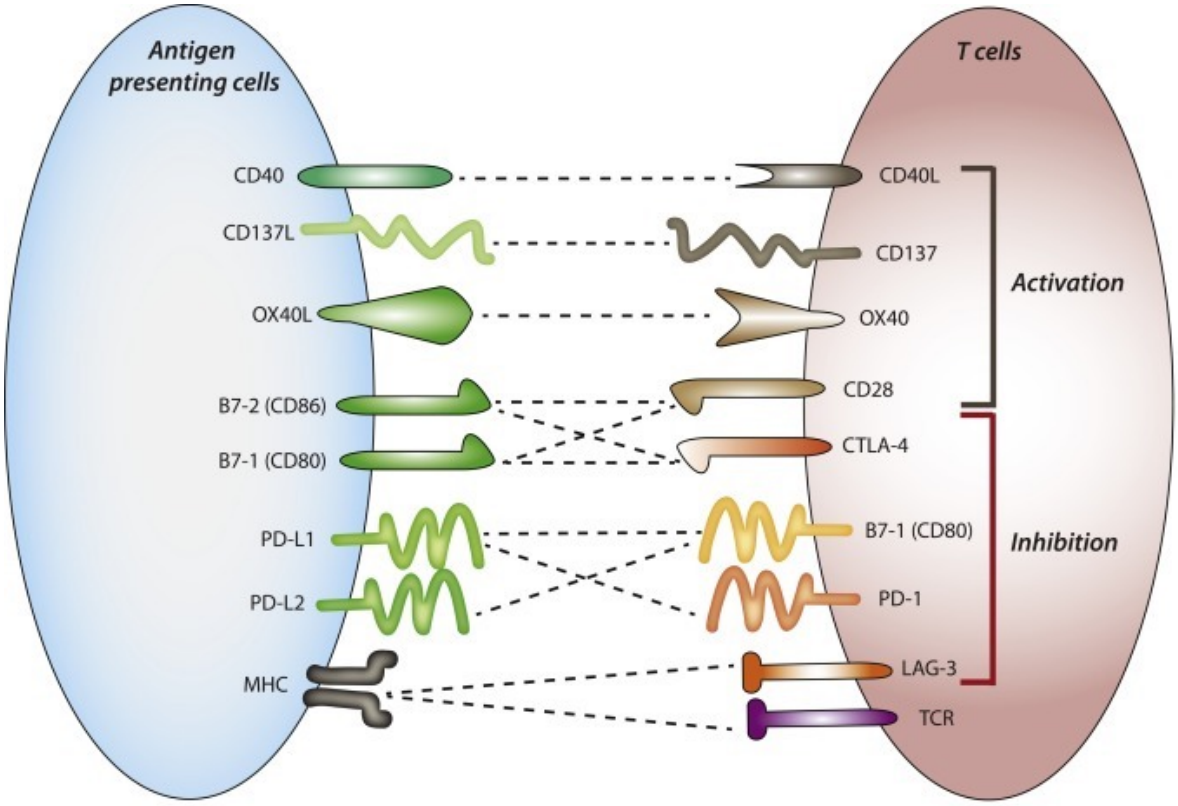


Figure 1.12 Co-stimulatory and co-inhibitory interactions regulate T cell immune responses. The interaction between antigen presenting cells (APCs) and TCR regulate T cell responses to antigen in lymph nodes, peripheral tissues or tumours. Upon T cell activation, multiple inhibitory immune checkpoint receptors are expressed on T cells and suppress immune responses by engagement of their respective ligands on antigen presenting cells (APC) or tumour cells. (Di Giacomo *et al.,* 2017).

1.8 Aims of the study

Previous studies have demonstrated that Egr2 and Egr3 are vital for antigen-induced proliferation of B and T cells and the control of inflammation. Furthermore, investigation of lymphocyte specific Ege2 and Egr3 double knockout mice demonstrated the indispensable role of Egr2 and Egr3 in preventing the development of autoimmune disease and limiting immunopathology during productive adaptive immune responses (Li *et al.,* 2012). Moreover, a recent study by our group demonstrated an important role of Egr2, that it serves as a checkpoint controller in adaptive immunity, regulating clonal proliferation and differentiation in both adaptive and homeostatic responses (Miao *et al.,* 2017). Based on the previous findings, it was hypothesised that Egr2/3 function is important for the potent anti-tumour immune response. Therefore, this study investigated the mechanism involved in the function of Egr2/3 in TILs, analysed Egr2 expression in association with immune checkpoint blockers, and addressed the effects of cytokines including IL-2, IL-4 and IL-10 on Egr2 expression in T cells. The aims were as follows:

- Define Egr2/3 functions in TILs and assess tumour growth in Egr2/3 KO and GFP-Egr2 mice models for effective anti-tumour immunity
- Evaluate the role of Egr2/3 in the regulation of immune checkpoint molecules in the tumour microenvironment
- Study the regulation of Egr2 expression under different cytokine stimulation
- Investigate molecular mechanisms that are involved in the regulation of Egr2 expression

CHAPTER 2 - Methods and Materials

2.1 Mice

2.1.1 Experimental model

All mice used in this study, including hCD2-Cre-Egr2$^{-/-}$Egr3$^{-/-}$ mice and GFP Egr2 knock-in mice, were approved under the authority of a UK Home Office project licence. All the mice, both male and female, were used at 7–8 weeks of age and were maintained in the biological services unit, Brunel University, in accordance with the guidelines of the committee on the operation of animals, Scientific Procedures Act 1986.

2.1.2 Egr2 and Egr3 knockout mouse model

Systemic deletion of Egr2 can cause defects in brain development, leading to lethal death phenotypes of mice in the birth (Gabet *et al.,* 2010), so the conditional knockout model in lymphocytes using the CD2 promoter via Cre/LoxP system (Zhu *et al.,* 2008) was used in this study.

In this model, the target genes were floxed with the LoxP gene at both sides of Egr2 and Cre, a recombinase enzyme initially found in the P1 bacteriophage virus, which is attached to the CD2 promoter in lymphocytes and recognises and cuts at these LoxP sites (Figure 2.1). LoxP sites are 34 base pairs long and attract Cre to recombine the DNA surrounding them, so hCD2-Cre mice and Egr2$^{Loxp/LoxP}$ were crossed to generate CD2-specific Egr2$^{-/-}$ mice. Then, the mice were backcrossed with a C57BL/6 background to purify the line. Polymerase chain reaction (PCR) verified Cre and LoxP gene expression. Egr3 knockout mice were generated using a neomycin cassette, with the entire Egr3 gene cloned into a pBluescript vector flanking LoxP sites and 1.5 kb neomycin-resistant positive selection cassette added to create a sequence of homology.

This fragment disrupted the transcription of the Egr3 gene, removing the DNA binding domain. This vector was transferred into embryonic stem cells, the cells were cloned and injected into blastocytes, then inserted into C57BL/6 pseudopregnant females. The offspring were backcrossed to C57BL/6 mice for 13 generations until a stable germline was established (Tourtellotte WG and J, 1998).

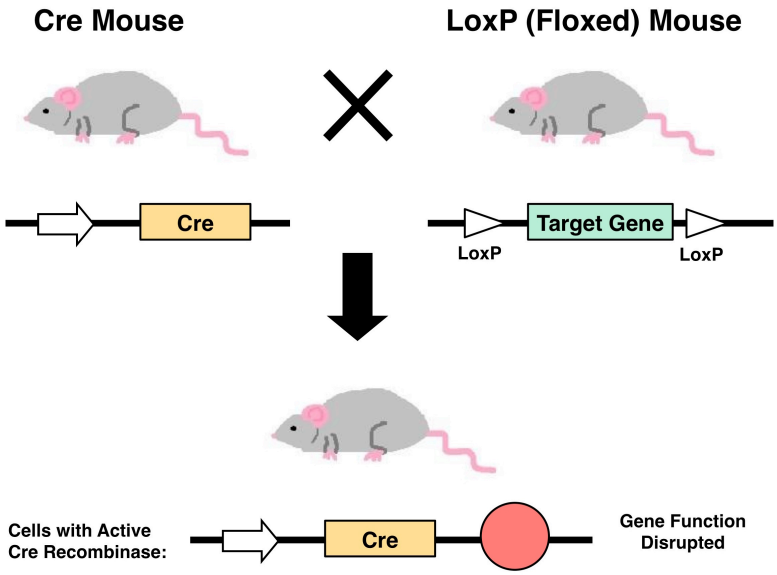


Figure 2.1 **Cre/LoxP system:** conditional knockout via Cre/LoxP system in which target gene is flanked by two LoxP sites and hCD2 Cre-mediated recombination results in the excision of the Egr2 in the lymphocytes (Sauer, 1998).

2.1.3 GFP-Egr2 knock-in mouse model

Knock-in is the replacement of the gene by the same version of gene using homologous recombination, in which a gene for a green fluorescent protein (GFP) can be engineered downstream from the promoter of the gene of interest, so every time that promoter is activated, the cells that the promoter turns on will glow green (Owen *et al.,* 2013).

In this study, the GFP-Egr2 knock-in mouse model was used as a wild type model. The GFP-Egr2 gene was cloned into a pBabe vector to generate a construct in which the target gene was disrupted with the neoR gene and the thymidine kinase tk^{HSV} gene was located outside the target gene. This construct was introduced into the cultured embryonic stem cells (ES) isolated from the inner mass of a C57BL/6N JM8 mouse blastocyst. Afterwards, incorporated homologous recombinant cells were selected. For this purpose, 24 hours after transfection, cells were positively selected through a neoR gene that confers neomycin resistance, so that when a neomycin-like drug is added to the culture, non-recombinant ES cells are killed because they lack the neoR gene. Then, cells were treated with gancyclovir and negatively selected so that non-homologous recombinants carrying the tk^{HSV} gene were killed. Those cells in which the construct was inserted at random retained the expression of the tk^{HSV} gene, whereas those cells in which the construct inserted by homologous recombination lost the tk^{HSV} gene. The thymidine kinase gene from herpes simplex virus confers sensitivity to gancyclovir. Finally, homologous ES recombinants survive this selection scheme. Afterwards, positive cells were injected into a mice blastocyst, which was then implanted into a pseudopregnant female C57BL/6 to yield chimeric offspring composed of cells derived from the genetically altered ES cells and the cells derived from normal cells of the host blastocyst. Chimeric mice were mated with C57BL/6N to generate homozygous lines.

2.1.4 Genotyping of GFP-Egr2 and Egr2/Egr3 knockout mice models

Mice were genotyped prior to use to confirm excision of Egr2 and Egr3 genes and to validate the infusion of GFP to the Egr2 gene in Egr2/3 knockout and GFP-Egr2 mice models respectively.

In this procedure, DNA was extracted from the mouse ear punch using REDExtract-N-Amp Tissue PCR Kit (Sigma) according to the manufacturer's protocol. Mice ear tissues were punched, collected and placed in Eppendorf tubes. Then, 20 µl of extraction buffer was added and vortexed for 10 seconds, before the addition of 5 µl tissue preparation buffer. The samples were vortexed and centrifuged, then incubated in a heating block for 30 minutes at 55°C to allow the protease enzyme present in the buffers to break down proteins. This was followed by incubation for 3 minutes at 95°C to denature the enzyme. Afterwards, 20 µl neutralisation buffer was added to stop the reaction and the samples were vortexed and centrifuged, before the addition of 150 µl, 200 µl and 300 µl of autoclaved water to Egr2 and Egr3 knockout, GFP-Egr2 knock-in and Egr2 transgenic mice respectively to dilute them as necessary. PCR was performed to amplify the genomic DNA from mice samples using gene-specific sense and antisense primers. This technique utilises *Thermus aquaticus* (Taq) polymerase, which is thermostable, to produce new copies of the gene of interest using dNTP, which adds nucleotides into newly synthesised single-stranded DNA. PCR mixtures were prepared as follows: 0.25 µl sense and antisense primer, 5 µl mastermix for Egr2 KO mice or 0.5 µl sense and antisense primer, 0.5 µl Neo primer and 5 µl mastermix for Egr3 KO mice. Then, the 1.5 µl ear sample was added into each PCR tube. For GFP-Egr2 knock-in mice, PCR reactions were prepared using 10 µl mastermix, 0.5 µl mixed-primer and 0.5 µl ear DNA samples. The details of the PCR for each primer is shown in Table 2.1.

Table 2.1 PCR cycles and product sizes for primers in the genotyping of $Egr2^{-/-}$ $Egr3^{-/-}$, Tg and knock-in mice.

GENOTYPING SAMPLE	PRIMER	PCR PROTOCOL	BAND SIZE (bp)
EGR-2 KO - LoxP	Sense - GTG TCG CGC GTC AGC ATG CGT Antisense - GGG AGC GAA GCT ACT CGG ATA CGG	95°C for 5 min94°C for 40sec} 65°C for 40sec } 30 Cycles 72°C for 40sec} 72°C for 5min 4°C for 10min	Homo = 200bp Hetero = 150bp and 200bp
EGR-2 KO - Cre	Sense - CCA ACA ACT ACC TGT TCT GCC G Antisense - TCA TCC TTG GCA CCA TAG ATC AGG	95°C for 5min 94°C for 40sec} 56°C for 40sec } 40 Cycles 72°C for 40sec} 72°C for 5min 4°C for 10min	150bp
Egr2 Transgenic	Sense - CCA CCA GTC TCA CTT CAG TTC C Antisense - CAG CTG CTG CAG AAA ACC ACT G	95°C for 5min 94°C for 40sec} 55°C for 40sec } 30 Cycles 72°C for 40sec} 72°C for 5min 4°C for 10min	350bp
Egr3 KO	Sense - CTA TTC CCC CCA GGA TTA CC Antisense - TCT GAG CGG GCT GAA ACG Neo - GAT TGT CTG TTG TGC CCA GTC	95°C for 5min 94°C for 40sec} 57°C for 40sec } 35 Cycles 72°C for 40sec} 72°C for 5min 4°C for 10min	Homo = 700bp Hetero = 350bp and 700bp
GFPEgr2	Sense - GCTCAGTTCAAC CCC TCT CC Antisense - GGATTTTGTCTACGGCCTTG	95°C for 3min 95°C for 20sec} 60°C for 20sec } 30 Cycles 72°C for 30sec} 72°C for 5min 4°C for 10min	WT = 119bp Homo = 854bp

Then, PCR products obtained from the amplification were loaded on 2% agarose gels, prepared using 3 g of agarose powder (Invitrogen, cat. Number-15510-027) dissolved in 150 ml of 0.5% TAE buffer (Sigma) and cooled down before addition of 15 µl (1 µl for every 10 ml) of gel red for fluorescence tagging. The agarose solution was poured into moulding blocks and left to solidify for 20 minutes. Then, samples were added to the

wells with 100 bp DNA ladder and run in an electrophoresis tank containing 1X TAE Buffer at 100 V. Gels were visualised under ultraviolet (UV) light using a BioRad Imager.

2.2 Cell culture

2.2.1 Lymphocyte collection from spleen

Spleens were harvested from mice and homogenised in PBS using a mesh. The cells were collected and centrifuged at 1300 rpm for 5 minutes at room temperature. The supernatant was removed, and cells were resuspended in 0.8% ammonium chloride solution and incubated at 37°C for 5 minutes. The ammonium chloride creates an osmotic potential causing the erythrocytes to burst, thereby depleting red blood cells, while maintaining the integrity of white blood cell populations. Then, cells were washed up with PBS and centrifuged at 1300 rpm for 5 minutes and the supernatant was discarded immediately.

2.2.2 CD4+ T cell isolation

Naïve CD4 T cells were isolated using a Magnetic Activated Cell Sorting (MACS) kit according to the manufacturer's instructions. The cells were resuspended in bead buffer (50 ml PBS+ 3 ml RPMI) and 25 µl CD4 bead buffer was added to both KO and Tg mice samples. The mixture was incubated on ice for 20 minutes with periodic agitation every 5 minutes. Then, beads buffer was added to 10 ml and centrifuged for 5 minutes at 1300 rpm. The supernatant was removed, and the pellet was resuspended in 500 µl–1 ml bead buffer. Magnetic columns were calibrated using 3 ml bead buffer and 200 µl of the cells were added to the centre of the column and was left to pass through the matrix.

The column was then washed twice using 2–4 ml of bead buffer. T cells were then extracted from the samples into newly labelled 15 ml tubes.

2.2.3 Cell culture and stimulation

Two different methods were used to stimulate the cells. In the first approach, purified $CD4^+$ and $CD8^+$ T cells were stimulated with plate-bound anti-CD3 at 2.5 μg/ml and anti-CD28 at 1.25 μg/ml overnight unless otherwise stated. In the second method, the cells were stimulated with a combination of phorbol 12-myristate 13-acetate (PMA) and inomycin at 200 ng/ml for 5–6 hours unless otherwise indicated. For analysis of cytokine expression by flow cytometry, cells were stimulated with PMA and inomycin in the presence of Golgi Stop (BD Biosciences) overnight according to the manufacturer's instructions. The cells were maintained in complete RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS), 300 μg/L L-glutamine, 50 μM β-mercaptoethanol (2-ME) and 50 μg/ml gentamicin (All from Invitrogen). Cells were incubated at 37°C in 5% CO_2.

Table 2.2 Cytokine information

Cytokine	Company
IFNγ	eBioscience
IL-2	
IL-4	
IL-6	BD Bioscience
IL-10	

2.3 Flow cytometry

Flow cytometry is a technique used to investigate physical and chemical parameters of a cell, including its size, density and complexity, at the single cell level based on the intensity of signals. In this respect, cells were labelled with antibodies bearing fluorochromes that attach to specific epitopes on the cells of interest enabling their detection. To analyse intracellular proteins, such as cytokines, and nuclear proteins, such as transcription factors, firstly cells were stained for cell surface markers, then fixed with formaldehyde to allow for the stabilisation of the cell membrane and permeabilised to facilitate the entry of antibodies into the cell. Finally, the cells are passed through a laser that sends the signal to appropriate detectors. The signals are translated electronically so that the user can interpret the data. In this study, the cells were resuspended in FACS solution and were analysed with a HF15 ACEA Novocyte flow cytometer.

2.3.1 Flow cytometry antibodies

The antibodies used in flow cytometry analysis are shown below in Table 2.3.

Table 2.3 Antibody information

Fluorescence	Antibody specific for	Company
Phycoerythrin (PE)	CD8	eBioscience
	CD44	
	CD27	
	PD-1	Milteni Biotech
	CTLA-4	
	TIM-3	
	4-1BB	
	ICOS	
	TIGIT	
	BTLA	
	OX-40	
PerCP	CD4	eBioscience
	Granzyme B	

2.3.2 Nuclear and intracellular cytokine staining

The cells were stimulated with 200 ng/ml inomycine and 200 ng/ml PMA in the presence of 1 µl/ml Golgi Stop according to the manufacturer's protocol. The following day, cells were collected and washed with 1X PBS. For nuclear or intracellular staining, cells were resuspended in 1X fixation/permeabilisation buffer (EBioscience) and incubated at room temperature for 20 minutes. Cells were centrifuged and resuspended in 1X permeabilisation buffer and incubated for 20 minutes at room temperature. Following centrifugation, the cell pellet was resuspended in fluorescently labelled antibodies diluted in perm/wash buffer and incubated for 20 minutes at room temperature. Then, cells were washed to removed unbound antibodies and resuspended in 1X PBS and passed through the flow cytometer.

2.4 Calcium phosphate transfection

EL4 cells were plated in DMEM medium with glutamine, 10% serum and 1X gentamicin with 40–60% confluency. On the second day, to achieve high transfection efficiency, the medium was changed two hours before transfection and 1 ml of DNA mixture was prepared according to the following protocol: water, GFP-Egr2 plasmid (1 μg/μl) and 1X TE buffer were vortexed and 2.5 M $CaCl_2$ was added to the mixture, while vortexing, 2X HBS was added in a dropwise manner to the mixture. After mixing completely, the DNA mixture was immediately added to the cells and they were incubated overnight. The cells were then harvested, resuspended in FACS solution and sorted by a FACSAria sorter.

2.5 Luciferase assay

The luciferase assay system is extremely rapid, simple and sensitive technique for the quantification of luciferase activity. The principle is based on the measurement of the light generated when the enzyme and substrates are combined. Firefly luciferase, a monomeric protein, encodes an enzyme that oxidises D-luciferin using ATP-Mg^{2+} as a co-substrate, yielding a fluorescent product molecule, oxyluciferin that can be quantified by measuring the released light.

In a 24-well plate, EL4 were seeded the day before transfection to reach the appropriate confluency. The next day, the medium was changed two hours before transfection and the calcium phosphate method was used to introduce DNA into cells as described in section 2.5. The cells were transfected with 2 μg of total DNA per well with the vectors specified for each individual experiment.

Luciferase assay reagent was prepared according to the manufacturer's instructions prior to the experiment. EL4 cells were washed gently with 1X PBS, before the addition of 100 µl 1X Passive Lysis Buffer (Promega), prepared according to the manufacturer's instruction. The cells were agitated for 15 minutes at room temperature, then collected and further vortexed for 60 seconds to aid the lysis process. At this stage, protein contents were released by the lysate and Bradford assay was performed to determine the protein concentration to normalise luciferase readings to the protein concentration. Then, 10 µl of the lysate was added into a 96-well plate, followed by 50–100 µl of luciferase assay reagent. Luminescence was measured on a Glomax luminometer (Promega). The experiments were performed in triplicate.

2.6 Bradford assay

The Bradford assay was used to determine the concentration of protein in solution. In this procedure, the Bradford reagent contains a dye named Brilliant Blue G, which complexes with proteins in the lysate. The protein-dye complex shifts the absorbance from 495 nm to 595 nm, with the amount of absorption corresponding to the protein content. The absorbance was measured at 595 nm on Glomax luminometer (Promega). A standard curve was prepared using a serial dilution of bovine serum albumin (BSA) from 0 mg/ml to 10mg/ml. The protein concentration of unknown samples was then determined by plotting the standard curve of absorbance values against BSA concentration. The samples were diluted by 1:250 in H_2O and placed in a 96-well plate, to which an equal amount of Bradford reagent was added. This was briefly incubated at room temperature for 5 minutes and absorbance was measured at 595 nm wavelength. Each treatment was performed in triplicate.

2.7 Tumour studies

2.7.1 Tumour inoculation

Egr2/3 KO and WT mice ranging from 8–10 weeks of age were used in this study. For experiments, mice received 1×10^6 B16 melanoma tumour cells via subcutaneous injection in both left and right flank. All mice were maintained for 14 days and monitored for tumour growth on a regular basis.

2.7.2 TIL isolation

Tumours were harvested from mice at the indicated time points and cut into small (<3 mm) pieces, then incubated in dissociation buffer [RPMI medium supplemented with 5% FBS, collagenase type I (Sigma-Aldrich, 1 μg/ml) and DNAse I (Sigma-Aldrich, 100μg/ml)] for 40 minutes, with vortexing every 10 minutes. Then, cells were passed through a 50-μm cell strainer and washed three times with DMEM by spinning at 1300 rpm for 5 minutes. TILs were further purified for $CD4^+$ and $CD8^+$ counterparts using a MACS positive selection kit (see section 2.3.2) according to the manufacturer's instructions.

2.8 Statistical analysis

Student's unpaired t-tests were performed to assess the differences between two groups. Error bars represent the variability of the data and indicate the standard error of the mean (SEM). A P value ≤ 0.05 was considered as statistically significant.

CHAPTER 3 - Egr2/3 Mice Models

3.1 Aim

Selection of genetic modified mice for experiments by genotyping.

3.2 Introduction

Two genetic modified mouse lines were used in this study, CD2-Egr2/3-/- and GFP-Egr2 knock-in, both of which had been previously established by our group (Zhu *et al.,* 2008, Miao *et al.,* 2017). However, due to difficulties in breeding homozygous CD2-Egr2/3-/- mice, CD2-Egr2/3-/- mice were obtained by breeding CD2-Egr2-/- with Egr3-/+ parental mice, so the experimental mice were selected by genotyping. The GFP-Egr2 knock-in mice were also genotyped for this project.

3.3 Identification of the CD-2 specific $Egr2^{-/-}$ and $Egr3^{-/-}$ mice from mixed homo/hetro breeding

In this model, conditional KO using the lymphocyte specific CD2 promoter was used to generate $Egr2^{-/-}$ mice. For this purpose, firstly, the Cre-LoxP system was used and mice with floxed Egr2 gene were crossbred with hCD2-Cre mice. The PCR genotyping of mice showed homozygous Egr2 LoxP, with an upper band size of 210 bp and Cre positive, with a band size of 150 bp (Figure 3.1, A). Egr2 can be heterozygous if loxp results show both upper and lower bands at 210 bp and 195 bp respectively, however to have an Egr2 KO mouse, it is important that it has the Cre genes. Secondly, the Egr3 neo product that disrupts the Egr3 gene was used to generate $Egr3^{-/-}$ mice. This product has the possibility of 2 bands, with the homozygous completely knocking out Egr3 at 800 bp and the heterozygous being only party knocked out at 800 bp and 400 bp (Figure 3.1, B). If Egr3 is not knocked out, then only a 400 bp product is expected; these are wild type. Lastly, Egr2 and Egr3 KO mice were crossbred to generate Egr2 and Egr3 deficient lymphocytes.

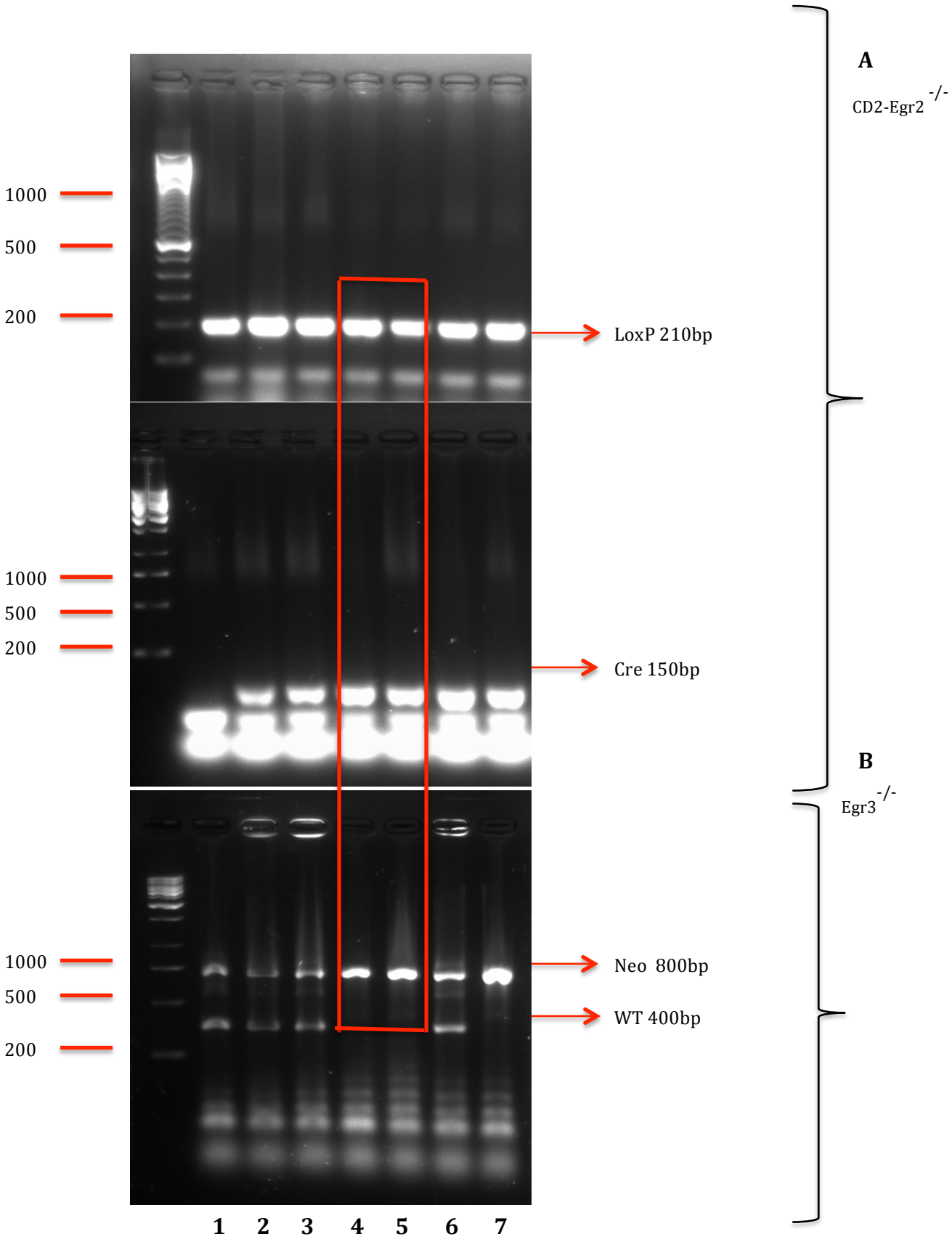

A
CD2-Egr2 -/-
1000
500
200
LoxP 210bp
1000
500
200
Cre 150bp
B
Egr3 -/-
1000
500
200
Neo 800bp
WT 400bp
1 2 3 4 5 6 7

Figure 3.1 G**enotyping of CD-2 specific Egr2-/- and Egr3-/- mice.** (A) CD-2 specific **h**omozygous deletion of Egr2 in mice expressing only LoxP homozygous bands and Cre bands (B) Egr3 homozygous deletion were those that expressed neomycin-resistant upper bands. Lane 1 **is** Egr2 and Egr3 heterozygous**;** lane 2, 3, 6 **are** Egr2 homozygous and Egr3 heterozygous**; la**ne 4, 5 **are** Egr2 and Egr3 homozygou**s; l**ane 7 **is** wild **type** for LoxP as control and **Cre** positive and Egr3 homozygous. Mice with homozygous deletion for both Egr2 and Egr3 allele (lane 4 and 5, highlighted in red) were considered for the study.

3.3.1 Identification of GFP-Egr2 mice

In addition to the phenotyping of the CD2-Egr2/3 knockout mice, the genotypes and phenotype of GFP-Egr2 knock-in mice were analysed according to our reports (Miao *et al.,* 2017). PCR genotyping results showed an upper band of 854 bp in the GFP-Egr2 homozygous mouse and lower bands at 119 bp indicated that GFP-Egr2 in these mice had not been inherited, so they were wild type. However, heterogeneity was shown with the presence of both upper and lower bands (Figure 3.2). In addition, the expression of GFP was measured by FACS (Figure 3.3). As confirmed previously, the GFP was stably fused with Egr2 (Miao *et al.,* 2017), therefore, the positivity of GFP represents expression of Egr2. Figure 3.3 showed that Egr2 was not expressed in naïve T cells and stimulation with anti-CD3 rapidly induced expression of Egr2 in about 18% of naïve T cells.

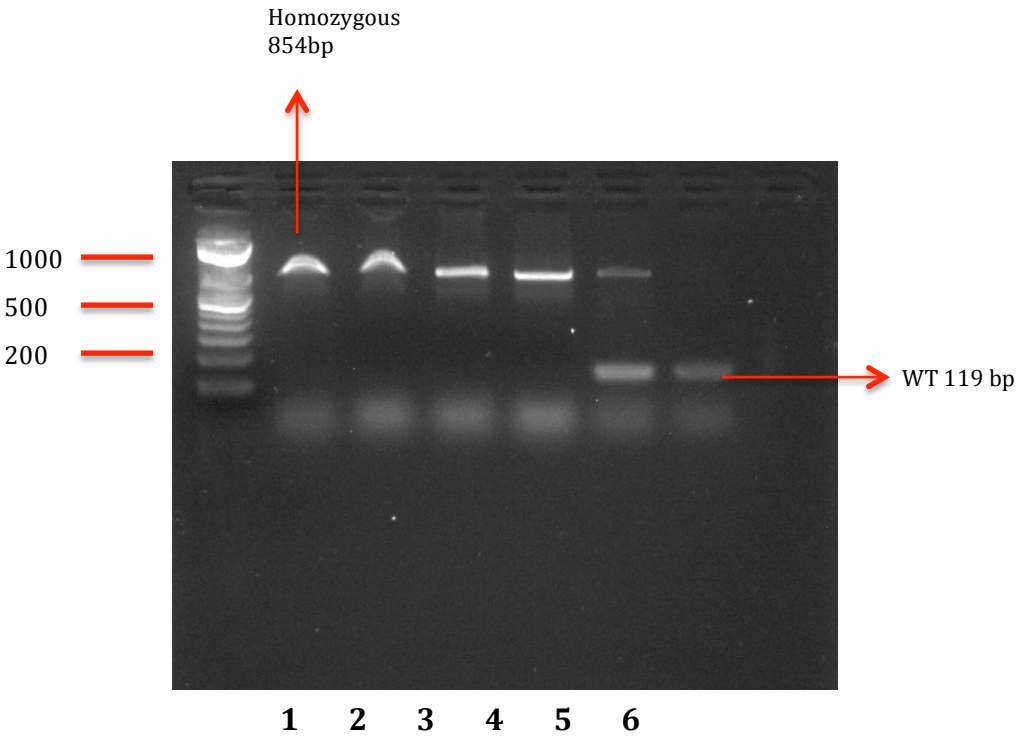


Figure 3.2 PCR product obtained from genotyping of GFP-Egr2. It represents bands for homozygous expression of GFP-Egr2 gene. Lane 1, 2, 3, and 4 show homozygous GFP-Egr2, while lane 5 shows heterozygous GFP-Egr2, lane 6 shows a wild type lower band. Homozygous mice (lane 1, 2, 3 and 4) were considered for the study.

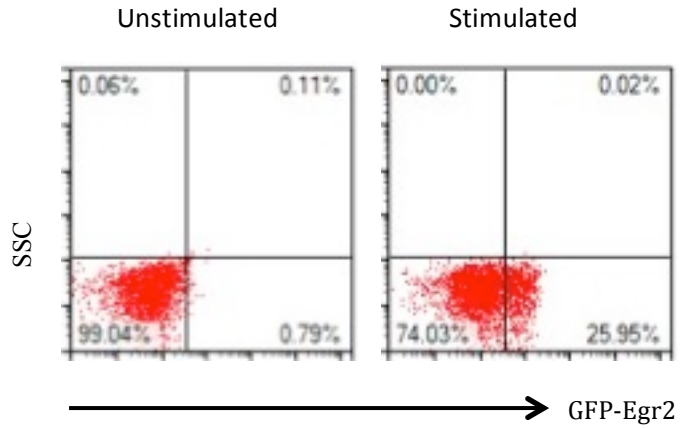


Figure 3.3 Dot plots of cultured GFP-Egr2 CD4+ T under stimulation after 24 hours, analysed for the induction of Egr2 expression. Splenic $CD4^+$ T cells were isolated and cultured in vitro with anti-CD3 (2.5 μg/ml) and anti-CD28 (1.25 μg/ml) for 24 hours and analysed by flow cytometry.

3.4 Discussion

The indispensable roles of Egr2 and Egr3 have been demonstrated in controlling inflammation and preventing the development of autoimmune disease (Zhu *et al.,* 2008, Li *et al.,* 2012). Also, Egr2 and Egr3 have been regarded as negative regulators of T cell activation that can inhibit T cell function (Harris *et al.,* 2004, Safford *et al.,* 2005, Collins *et al.,* 2008). This chapter focuses on the selection of genetic modified mice (Egr2/3 KO and GFP-Egr2) for the experiments.

Systemic knockout of the Egr2 was involved with neonatal fatalities involving hindbrain development and preventing myelination in the peripheral nervous system in mice (Topilko *et al.,* 1994, Poirier *et al.,* 2008). Transgenic mice contain multiple copies of a transgene in a random locus which can lead to a different expression level in every cell generated. However, in the knock-in model, the gene of interest ubiquitously integrated in the locus of a mouse leads to a comparable expression level for every transgene introduced, in which the endogenous murine promoter controls its level of expression (Menalled, 2005).

In this study, the knock-in mice model was established using replacement of Egr2 tagged with a GFP reporter gene using homologous recombination. The mice model was confirmed using genotyping and flow cytometry, which finally showed GFP-Egr2 homologous and protein level expression. This model was then used to monitor in vivo expression of Egr2 in physiologic conditions.

We already have shown enhanced proliferative responses in T and B cells in Egr2 cTg mouse and conversely, impaired proliferation was observed in Egr2 and 3cKO mice. So, a high level of IL-2 with decreased level of IFNγ and low level of IL-2 with high IFNγ

expression was observed in our transgenic and knockout models respectively (Li *et al.,* 2012). Our recent study showed a similar expression profile of GFP-Egr2^{+} and GFP-Egr2^{-} to our transgenic and knockout models respectively (Roberta *et al.,* 2003, Peterson *et al.,* 2003). This showed the further advantages of using the GFP-Egr2 model to profile both knockout and transgenic models.

Chapter 4 - Egr2/3 are important for T cell mediated responses against tumours

4.1 Aim

To investigate the potential function of Egr2/3 in tumour infiltrating T cells.

4.2 Introduction

The nascent tumour cells can be identified and destroyed by the immune system in a process called immunosurveillance that functions as the main defence against cancer (Vesely *et al.,* 2011). T cell mediated immune response, including CD8 and T helper cells, counteracts cancer progression by establishing antigen-specific T cells with memory and effector properties (Karwacz *et al.,* 2011). TILs are deployed as part of the host immune response to cancer and infiltrate tumour nests (Lee *et al.,* 2016). Several studies have demonstrated that more brisk T cell infiltrate lymph node metastases and adoptive transfer of autologous TILs with IL-2 has led to tumour regression in patients with metastatic melanoma (CR *et al.,* 2015).

Cancer cells express antigens that elicit T cell-mediated responses, but these responses are limited during malignant progression by the development of immunosuppressive mechanisms including T cell deletion, insufficient T cell activation and peripheral hypo-responsiveness of anti-cancer T cells in the tumour microenvironment that drive immune evasion (Baitsch *et al.,* 2012). T cell hypo-responsiveness can be caused by clonal anergy or T cell exhaustion (Abe *et al.,* 2012). Dendritic cells are central regulators of adaptive immunity and their maturation is vital to induce co-stimulatory signals to T cells, but while dendritic cells maturation occurs in tumours and considering suppressive mechanisms within the tumours, inducing potent immunity is insufficient (Gardner and Ruffell, 2016). Several studies in metastatic melanoma patients have demonstrated that $CD8^+$ T cells can be directed to melanocyte

differentiation antigens or tumour-restricted antigens, including MAGE-3, NY-ESO-1 and Melan-A/Mart-1, but they are not terminally differentiated (Roberta *et al.,* 2003, Peterson *et al.,* 2003). This partial maturation of anti-tumour $CD8^+$ T cells can happen due to the lack of CD4 cells in the tumour tissues and inadequate production of IL-15 and IL-7 that can prevent efficient infiltrated $CD8^+$ T cell maturation (Champagne *et al.,* 2001, Prlic *et al.,* 2002). Additionally, lack of key chemokines in a subset of melanoma metastases prevents the migration of effective $CD8^+$ T cells, which can lead to reduced anti-tumour immunity (Harlin *et al.,* 2009). Chemokines, including CXCR4 and CCR5, provide co-stimulatory signals for T cells, ensuring sustained T cell-APC interactions (Molon *et al.,* 2005).

Egr2 and Egr3 are highly induced in naïve T cells in response to antigen stimulation and infection in vivo, suggesting a vital role in regulating T cell-mediated immune responses (Anderson *et al.,* 2006, Best *et al.,* 2013). Egr2 is an essential regulator for the T cell immune response against viral infection by promoting T cell differentiation through binding to Tbx21 and fostering the expression of T-bet (Du *et al.,* 2014). Moreover, in our study, Egr2 and 3 have been demonstrated as essential regulators for the efficient proliferation of naïve B and T cells in response to mitogenic antigen receptor stimulation, in which severe defects in B and T cell activation was evidenced by impaired proliferation and production of IL-2 in Egr2 and Egr3 deficient mice model (Li *et al.,* 2012). Later, Egr2 has been suggested as a checkpoint that regulates the transition between the clonal expansion and differentiation of effector T cells (Miao *et al.,* 2017).

In contrast, a study by Zheng and colleagues (2012) examined the anergy characteristic of Egr2 in T cells and showed that melanoma tumour growth is markedly slowed in Egr2-deleted mice, indicating that cell-intrinsic expression of Egr2 can contribute to tumour escape from immune destruction in vivo. To further examine the function of Egr2 in tumour growth and its role in efficient anti-tumour therapy, the Egr2/3 KO and WT mice models were used in our study. This chapter aimed to investigate the role of Egr2 as one of the key genes that might have an effective role in T cell immune responses against melanoma tumour models.

4.3 B16 mouse melanoma tumour model to assess tumour growth in mice with lymphocyte specific deletion of Egr2/3

B16 melanoma is regarded as low-immunogenic and there is difficulty in inducing therapeutic immune responses to B16 (Overwijk and Restifo, 2001). To assess tumour growth in Egr2/3 KO mice, 1×10^6 B16 melanoma cells were injected subcutaneously into both the left and right flank of the mice. Then, after 14 days, average tumour size was 1 mm. Mice were culled, and spleen and tumour tissues were harvested. After measurement of the size and weight of tumour tissues, tumours from wild type mice were ±0.2 g, while those from Egr2/3 KO mice were ±0.8 g (Figure 4.1), suggesting that possible expression of Egr2/3 in lymphocytes in TILs can promote anti-tumour immune responses.

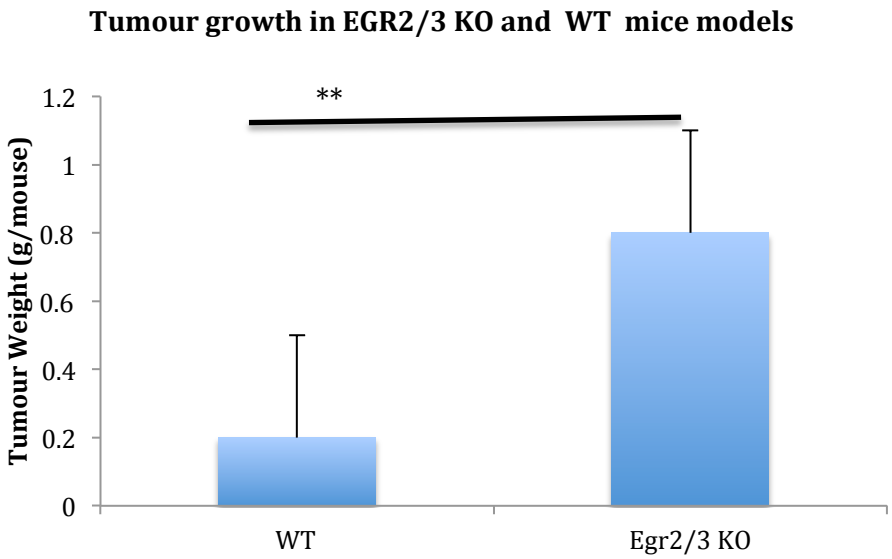


Figure 4.1 Tumour growth in Egr2/3 KO and WT mice models. Tumours developed in subcutaneously injected B16 melanoma mouse 14 days after inoculation. Tumour burden ratio was measured according to the tumour mass per mouse (n = 5 mice). This is representative of three independent experiments.

4.3.1 CD4 and CD8 T cells from TILs are significantly reduced in tumours from Egr2/3 KO mice

Next, $CD4^+$/ $CD8^+$ TILs were measured as Egr2/3 are important mostly in effector T cells in response to viral infection. In wild type mice, 11×10^6 $CD4^+$ cells and 20×10^6 $CD8^+$ cells were recovered from each gram of tumour, while in tumours from knockout mice only 5.5×10^6 $CD4^+$ cells and 4×10^6 $CD8^+$ cells were recovered (Figure 4.2).

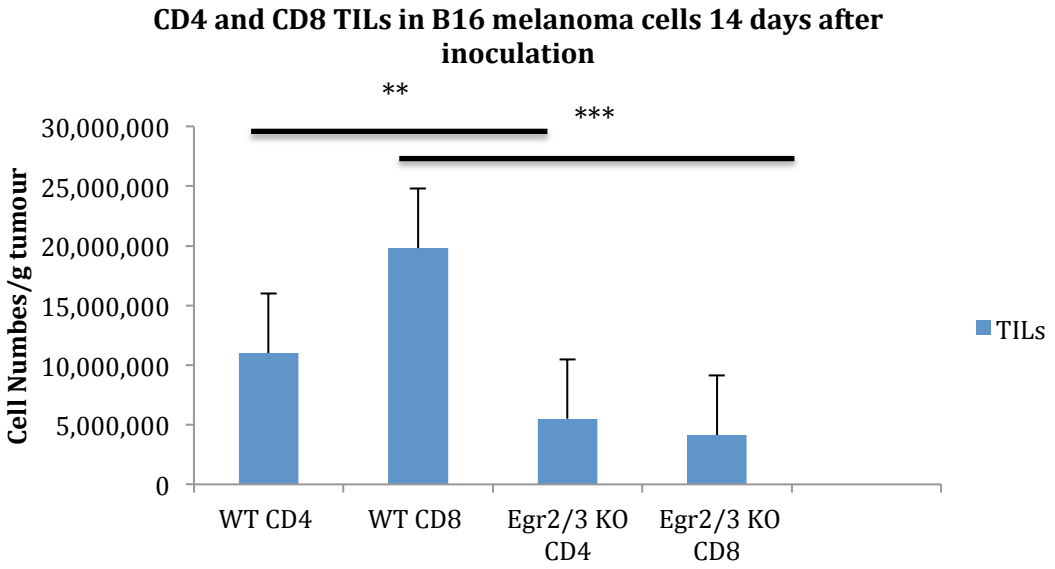
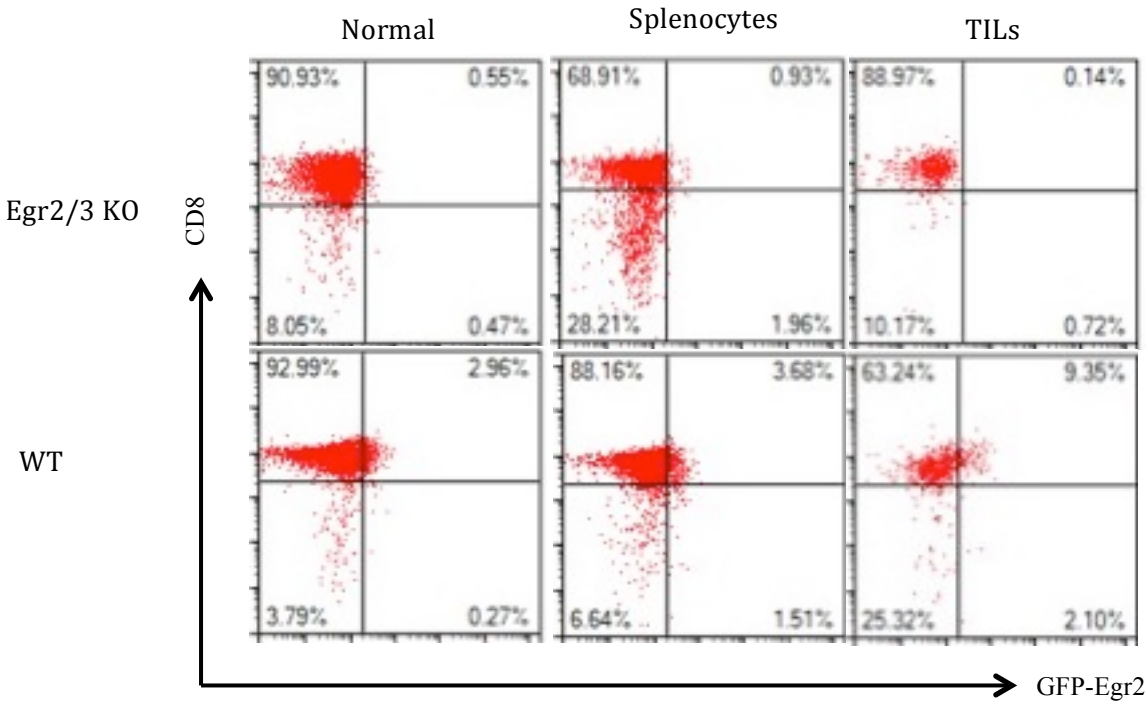


Figure 4.2 $CD8^+$ /$CD4^+$ TILs in Egr2/3 KO and WT mice models. Tumours were harvested from subcutaneous injected B16 melanoma mice 14 days after inoculation. **P ≤ 0.01, ***P≤ 0.001, total number of the TILs were counted per gram, n= 5 mice. This is representative of three independent experiments.

4.3.2 Egr2 expression is significantly increased in CD8 TILs

We have shown that Egr2 and 3 are only induced in antigen stimulated T cells and play a major role in promoting adaptive immune responses against virus infection (Miao *et al.,* 2017). To assess Egr2 expression in T cells under tumour conditions, $CD8^+$ TILs were isolated and analysed for Egr2 expression. Results showed that 10% WT $CD8^+$ TILs expressed Egr2, while in $CD8^+$ cells from splenocytes, only 5% expressed Egr2 (Figure 4.3 & 4.4). Egr2 positive CD8 WT TILs also expressed higher levels of CD44, indicating that these cells have an effector function (Figure 4.3).



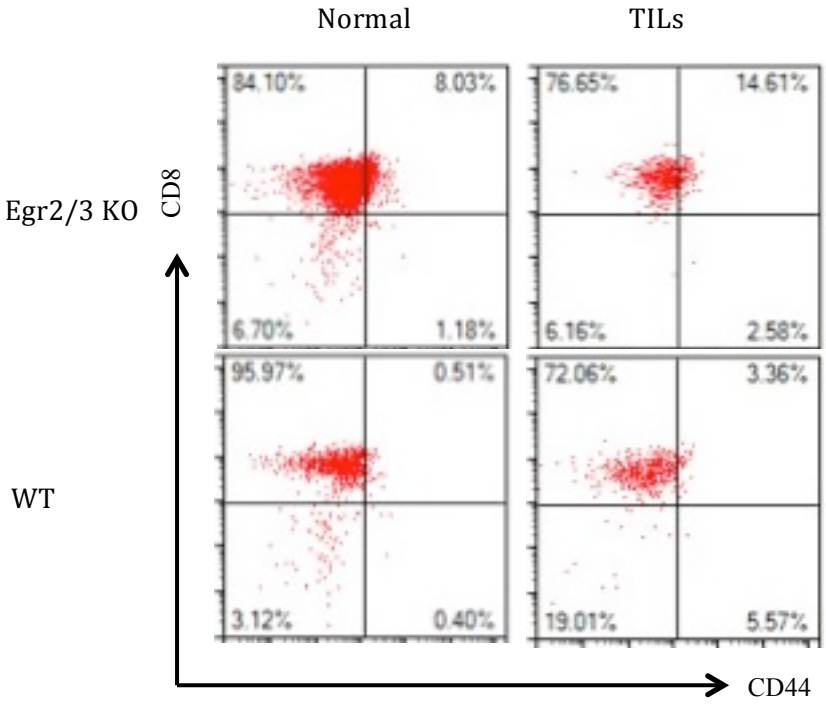


Figure 4.3 Dot plots of Egr2/3 KO and WT CD8 TILs/splenocytes analysed for the expression of Egr2 and CD44. Egr2/3 KO $CD8^+$ TILs/ splenocytes and WT $CD8^+$ TILs/ splenocytes were obtained from subcutaneously injected B16 melanoma mice 14 days after inoculation. The cells were isolated and stained with the CD8 antibody and analysed for Egr2 and CD44 expression by flow cytometry. Results represent n=3-5 mice in three independent experiments.

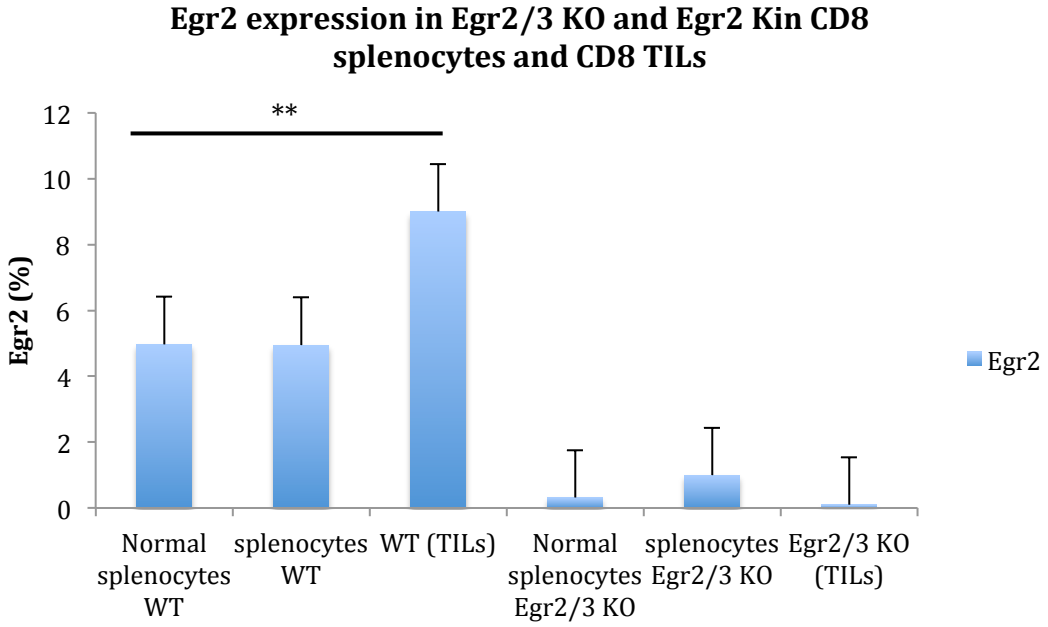


Figure 4.4 Expression of Egr2 in Egr2/3 KO and WT CD8 TILs/splenocytes. Egr2/3 KO $CD8^+$ TILs/splenocytes and WT $CD8^+$ TILs/ splenocytes were isolated from B-16 melanoma cells and analysed for the expression of Egr2. **P ≤ 0.01 versus control.

4.3.3 Production of effector cytokines in CD8 TILs

Egr2/3 are important for clonal expansion, but suppress inflammatory cytokine production in T cells (Miao *et al.,* 2017). To explore the mechanism for the role of Egr2 in tumour growth, effector cytokines and cytotoxic molecules including IFNγ and Granzyme B were assessed. Results showed that Granzyme B expression in Egr2/3 KO CD8 TILs was not significantly different to their wild type counterparts and IFNγ was relatively high in Egr2/3 KO CD8 TILs (Figure 4.5 & 4.6), indicating that Egr2/3 have less effect on the expression of IFNγ and Granzyme B under tumour conditions.

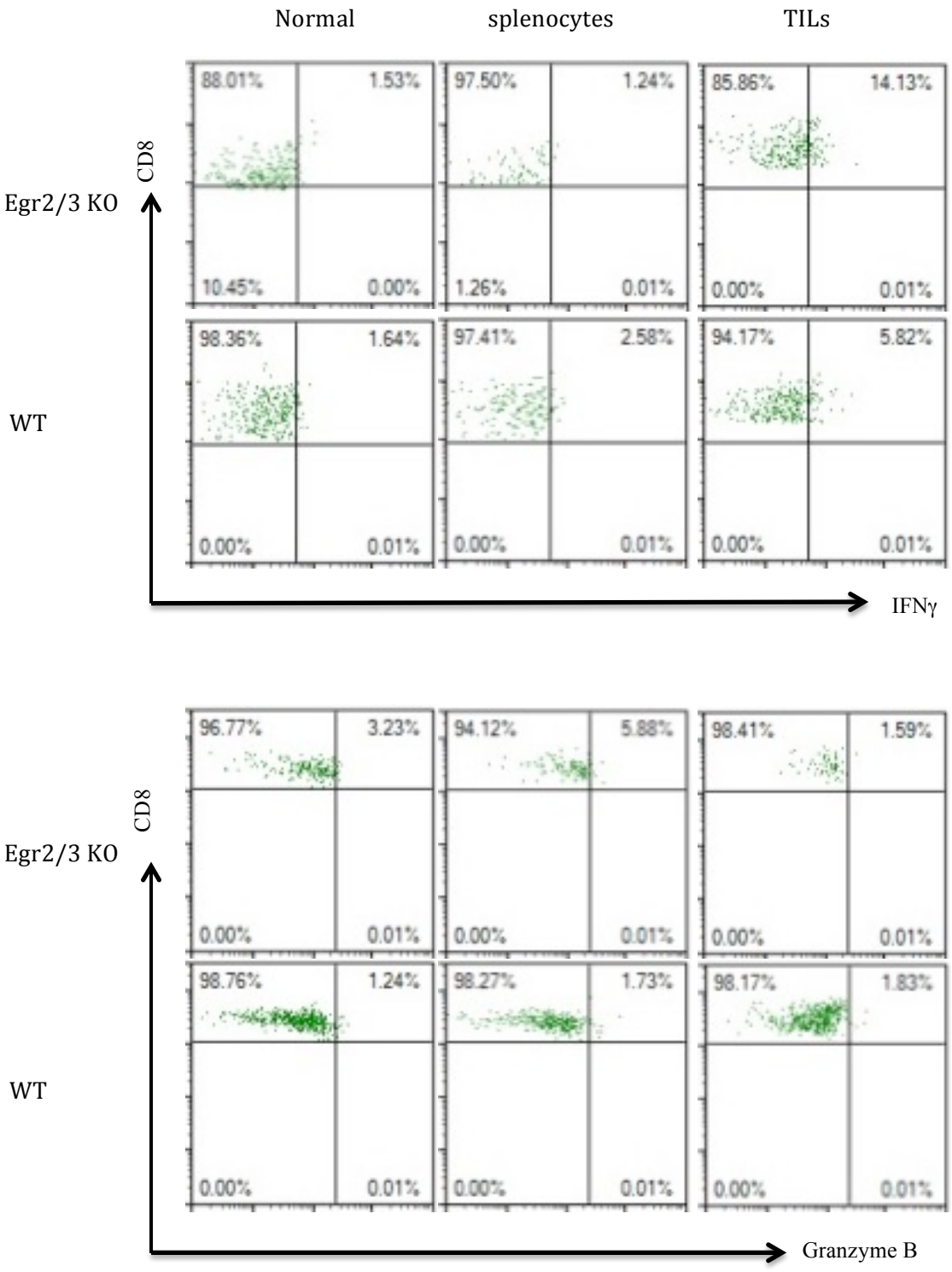


Figure 4.5 Dot plots of Egr2/3 KO TILs and WT CD8 TILs/splenocytes analysed for the expression of IFNγ and Granzyme B. Egr2/3 KO CD8+ TILs and WT CD8+ TILs were obtained from subcutaneously injected B16 melanoma mice 14 days after inoculation. The cells were isolated and stained with the CD8 antibody and analysed for IFNγ and Granzyme B expression by flow cytometry. Results represent n = 3–5 mice in three independent experiments.

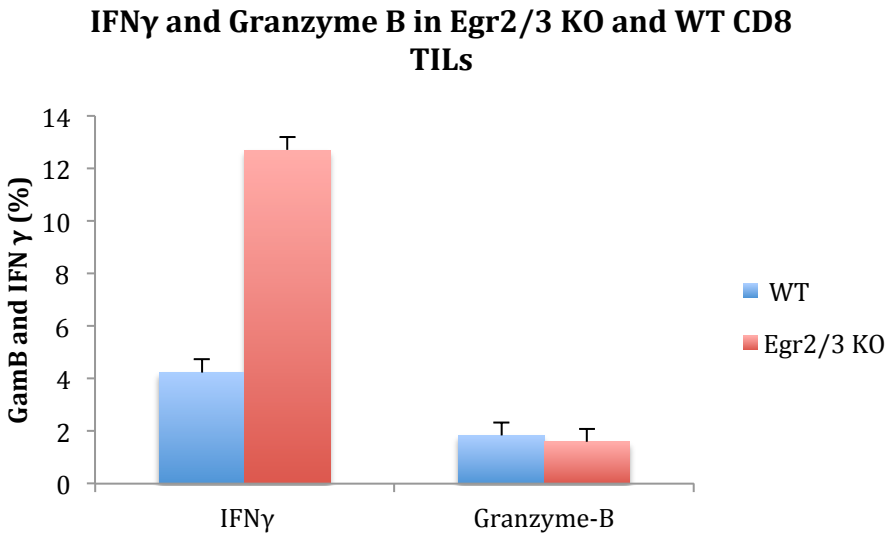


Figure 4.6 Expression of IFNγ and Granzyme B in Egr2/3 KO and WT CD8 TILs. Egr2/3 KO $CD8^+$ TILs and WT $CD8^+$ TILs were isolated from B-16 melanoma cells and analysed for the expression of IFNγ and Granzyme B.

4.3.4 CD8 TILs produce more IL-2 and are more proliferative than their counterparts from Egr2/3 KO mice

To further investigate the mechanism for the function of Egr2/3 in TIL T cells, the expression of IL-2 and Ki-67 was assessed. Previously, we found that Egr2/3 are important for antigen-specific T cells to produce IL-2 and express Ki67, a marker for T cell proliferation (Li *et al.,* 2012). TILs were stained with antibodies for CD8 plus anti-IL-2 and anti-Ki67 antibodies, showing that there were 7% IL2 positive CD8 cells in TILs from wild type mice, while only 2% from Egr2/3 KO mice. Regarding Ki67 positive CD8 T cells, there were 16% and 9% from wild type and Egr2/3 KO respectively (Figure 4.7 & 4.8). These results indicate that CD8 T cells in TILs from Egr2/3 KO mice experienced impaired expansion.

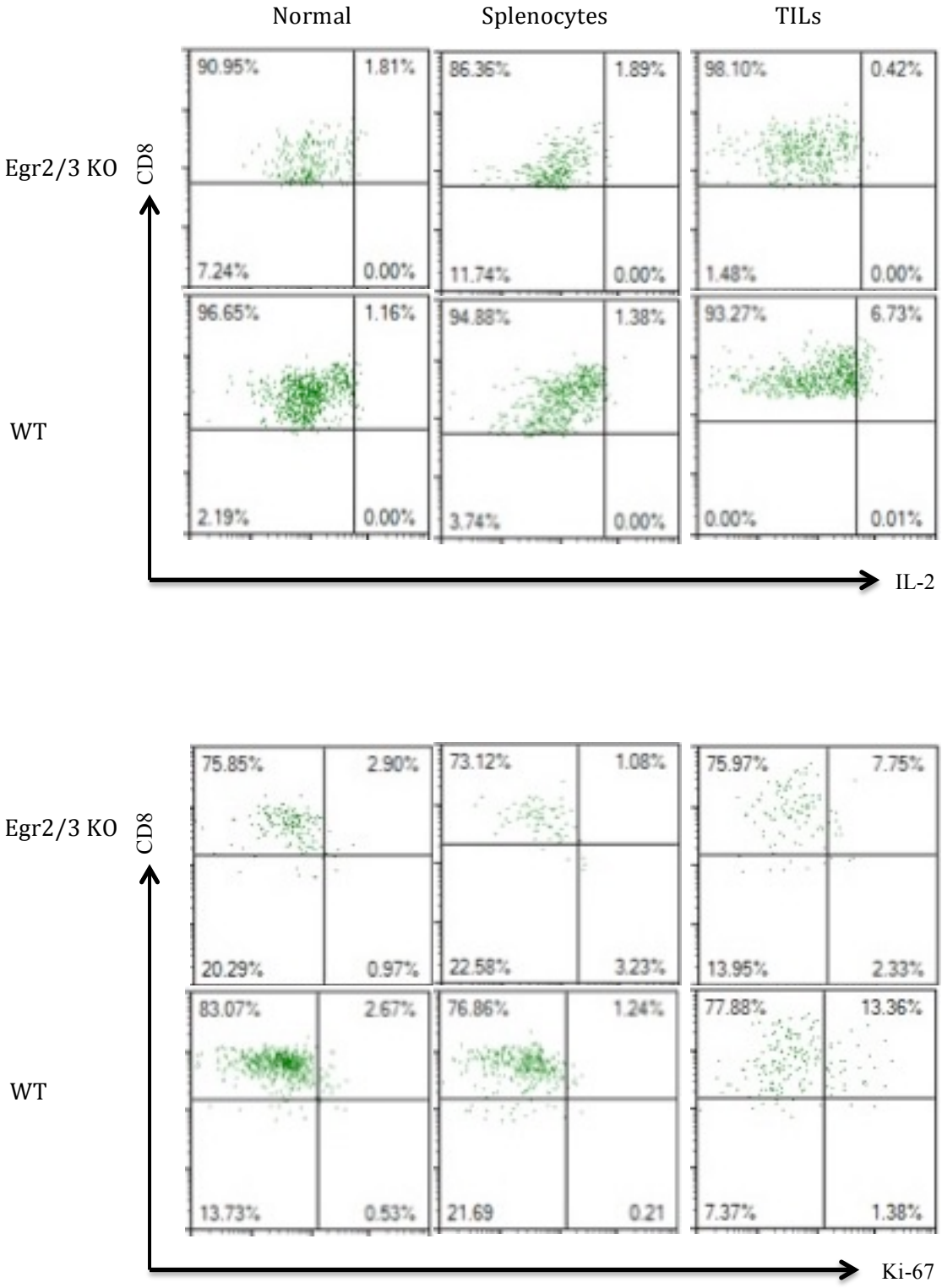


Figure 4.7 Dot plots of Egr2/3 KO TILs and WT CD8 TILs/splenocytes analysed for the expression of IL-2 and Ki-67.

Egr2/3 KO $CD8^+$ TILs and WT $CD8^+$ TILs obtained from SC-injected B16 melanoma mice 14 days after inoculation. The cells were isolated and stained with the CD8 antibody and analysed for IL-

2 and Ki-67 expression by flow cytometry. Results represent n = 3–5 mice in three independent experiments.

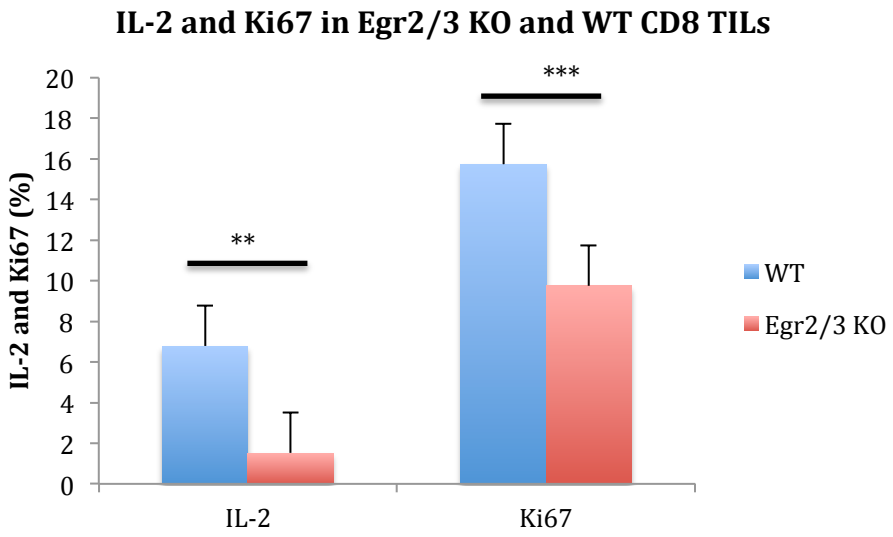


Figure 4.8 Expression of IL-2, Ki67 in Egr2/3 KO and WT CD8 TILs. Egr2/3 KO $CD8^+$ TILs and WT $CD8^+$ TILs were isolated from B-16 melanoma cells and analysed for the expression of IL-2 and Ki67. **P ≤ 0.01, ***P ≤ 0.001 versus control.

4.4 Discussion

Tolerance to tumour antigens is one of the mechanisms for poor tumour immune responses (Rabinovich *et al.,* 2007). However, the mechanisms are unknown. Recently, it has been reported that tumour infiltrating $CD8^+$ T cells become anergic, with increased expression of Egr2, which drives the expression of anergic molecules like lymphocyte activation gene-3 (LAG-3) and cytotoxic and regulatory T cell molecule (CRTAM), which may play role in tumour escape from immune destruction in vivo (Zheng *et al.,* 2012). In this study, we have now found that deficiency of Egr2/3 results in poor immune response against B16 tumour models.

Egr2 and Egr3 control the expression of Myc, a key transcriptional factor for T cell proliferation, in both CD4 and CD8 T cells in response to viral infection (Wang *et al.,* 2011, Nie *et al.,* 2012). Egr2 and 3 deficient B and T cells have shown impaired proliferation as evidenced by defects in IL-2 production. It was also demonstrated that Egr2 is required for AP-1 activity by directly blocking the function of Batf, a suppressor of AP-1, highlighting the indispensable role of Egr2 as a positive regulator for antigen receptor induced proliferation in adaptive immune responses (Li *et al.,* 2012). Increased Ki67 expression in $CD8^+$ TILs, representing TILs expansion, accelerate the rate of memory $CD8^+$ TILs that can be highly enriched for melanoma antigen specificity leading to critical tumour therapy (Chacon *et al.,* 2015).

Recently, studies from checkpoint blockages showed that expansion of tumour specific T cells in TILs is more important than their ability to produce effector cytokines (Abiko *et al.,* 2015). Abiko et al. demonstrated that IFNγ accelerates tumour growth by inducing PD-L1 in the tumour in a subcutaneous model. Also, absolute numbers of

infiltration of $CD8^+$ TILs are reduced by the expression of PD-L1. This suggested that IFNγ status and lymphocyte infiltration might be key for effective anti-tumour immunotherapy.

We found a distinct mechanism for Egr2/3 in promoting anti-tumour responses of CD8 TILs. The effector cytokines, including IFNγ and Granzyme B, that are important for anti-tumour responses are not reduced in CD8 TILs from Egr2/3 KO mice. However, the major difference is the expansion of CD8 TILs, that are significantly defect in Egr2/3 KO mice. Also, CD44 expression was higher in Egr2 positive CD8 WT TILs, reflecting that these cells might have an effector function. Thus, the induction of Egr2/3 in TIL T cells plays an important function in promoting expansion of tumour specific T cells, which promote an efficient $CD8^+$ T cell immune response in the tumour tissue.

CHAPTER 5 - Analysing expression of immune checkpoint molecules in the tumour microenvironment

5.1 Aim

To assess the expression of checkpoint molecules including PD-1, Lag-3 and CTLA-4 in T cells in the presence or absence of Egr2/3 in tumour conditions.

5.2 Introduction

Co-stimulatory and co-inhibitory molecules on T cells are key players in the activation step of the adaptive immune system, regulating the expansion and effector functions of antigen-specific T cells. During T cell activation, co-stimulatory molecules, CD80 and CD86, on APC bind to CD28 enhance T cell proliferation and activate Th1 and Th2 immune responses. However, co-inhibitory molecules such as PD-1, CTLA-4 and Lag-3 serve as checkpoint regulators to control the activation of T cells (Chen, 2004). These molecules are highly induced in T cells in the tumour microenvironment and play a major role in immune tolerance towards tumour (Vinay and Kwon, 2012, Hernandez-Chacon *et al.,* 2011).

The central role of CTLA4 for keeping T cell activation in check was dramatically demonstrated by the lethal systemic immune hyperactivation phenotype of *Ctla4*-knockout mice (Tivol *et al.,* 1995, Waterhouse *et al.,* 1995). The increased PD1 expression on $CD8^+$ TILs may either reflect an anergic or exhausted state, as has been suggested by decreased cytokine production by $PD1^+$ compared with $PD1^-$ TILs from melanomas (Ahmadzadeh *et al.,* 2009). Lag-3 co-expression on T cells is sustained during a tumour and chronic viral infection, that frequently co-express additional inhibitory receptors such as PD-1, TIGIT, TIM3, leading to a T cell dysfunctional state exemplified by lack of proliferation, cytokine expression and catalytic activity (Andrews *et al.,* 2017). Dual Blockade of PD-1 and Lag-3 synergises to enhance $CD8^+$ T cell

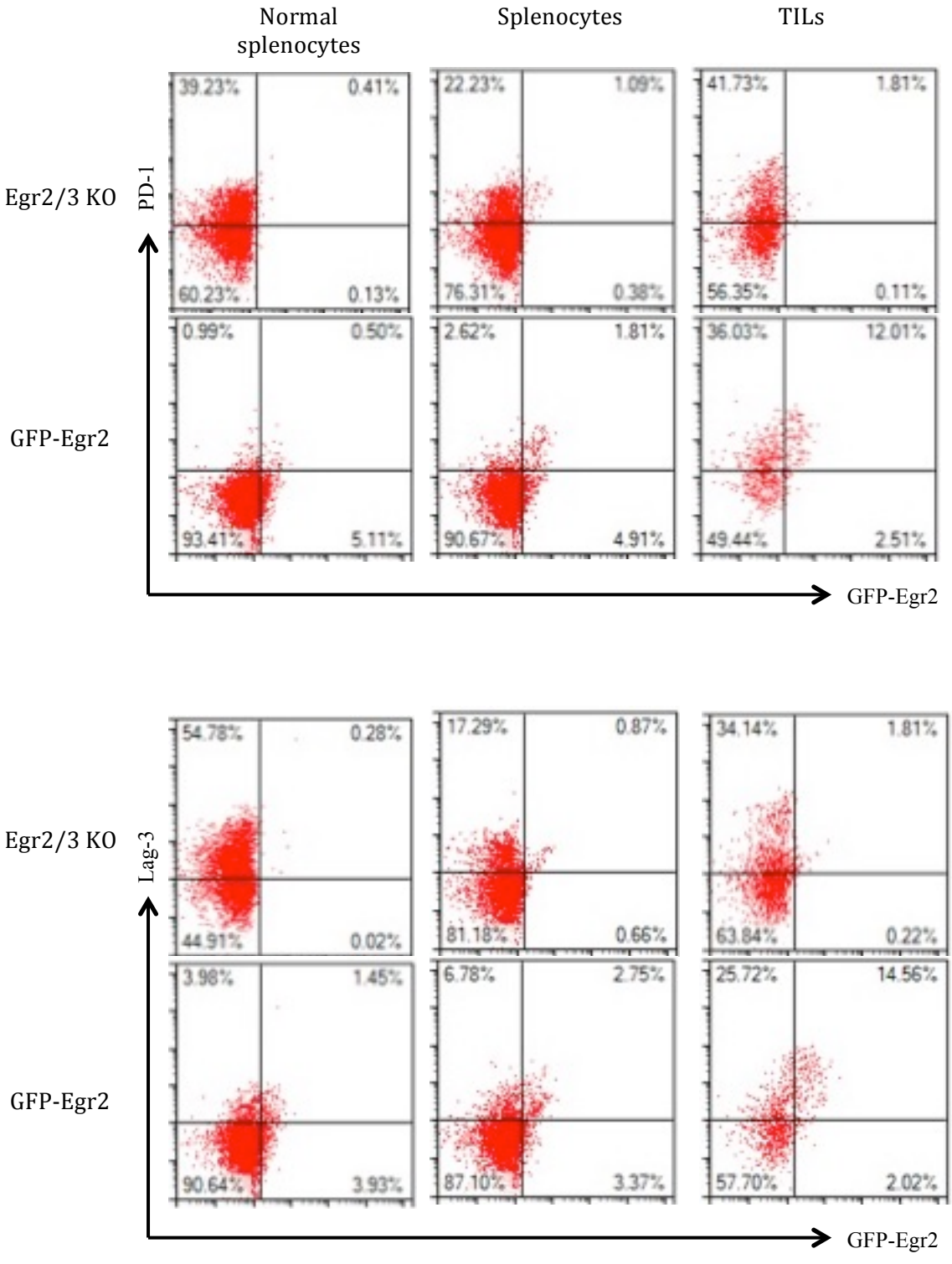
responses during chronic viral infection, suggesting targeting multiple co-inhibitory receptor pathways to adjust T cell immune responses against persisting antigens (Blackburn *et al.,* 2009).

Egr2 and Egr3 are critical for maintaining immune homeostasis and their crucial function as a checkpoint, controlling the transition between clonal expansion and differentiation in effector T cells was recently demonstrated (Miao *et al.,* 2017). Some tumour infiltrating $CD8^+$ T cells become anergic, with increased expression of Egr2 driving the expression of anergic markers like LAG-3 and CRTAM, which can contribute to tumour escape from immune destruction in vivo (Zheng *et al.,* 2012). Egr2-driven cell surface proteins Lag-3, PD-1 and 4-1BB can identify a subpopulation of dysfunctional tumour antigen-specific $CD8^+$ TIL, as reflected by defective IL-2 production, contributing to the immune suppressive tumour microenvironment (Williams *et al.,* 2014, Williams *et al.,* 2017). Therefore, the role of Egr2 in the regulation of these immune checkpoint molecules was studied in the tumour microenvironment in this chapter.

5.3 Expression of immune checkpoint molecules

Studies have shown that Egr2 plays a role in driving the expression of immune checkpoint molecules such as Lag-3 and PD-1 (Zheng *et al.,* 2012, Williams *et al.,* 2014). To pinpoint the role of Egr2 in regulating immune checkpoint markers under indirect and direct tumour conditions, $CD8^+$ splenocytes and $CD8^+$ TILs were isolated and stained with different immune checkpoint molecules and analysed by flow cytometry (Figure 5.1 & 5.2). Egr2/3 KO $CD8^+$ splenic cells reflected the significant increase in the expression of immune checkpoint markers in comparison with GFP-Egr2 $CD8^+$ T cells.

Also, Egr2/3 KO CD8⁺ TILs were not significantly different to GFP-Egr2 CD8⁺ TILs in terms of expression of immune checkpoint markers (Figure 5.1 & 5.2). Collectively, the results indicated that Egr2/3 function in anti-tumour immune responses is not due to their regulatory role in immune checkpoint repressions.



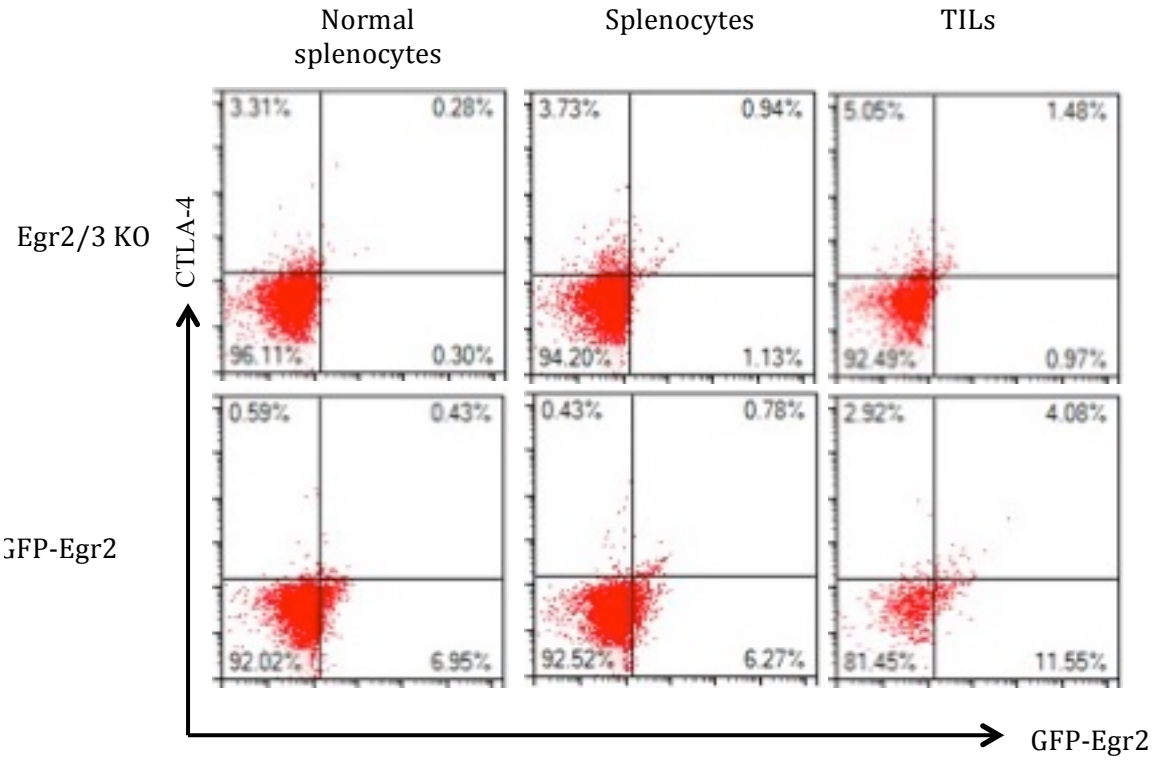


Figure 5.1 Dot plots of Egr2/3 KO and GFP-Egr2 CD8 TILs/splenocytes analysed for the expression of immune checkpoint molecules. Egr2/3 KO $CD8^+$ splenocytes/TILs and GFP-Egr2 $CD8^+$ splenocytes/TILs were obtained from subcutaneously injected B16 melanoma mice 14 days after inoculation. The cells were isolated and stained with the checkpoint markers for flow cytometry. Results represent n = 3–5 mice in three independent experiments.

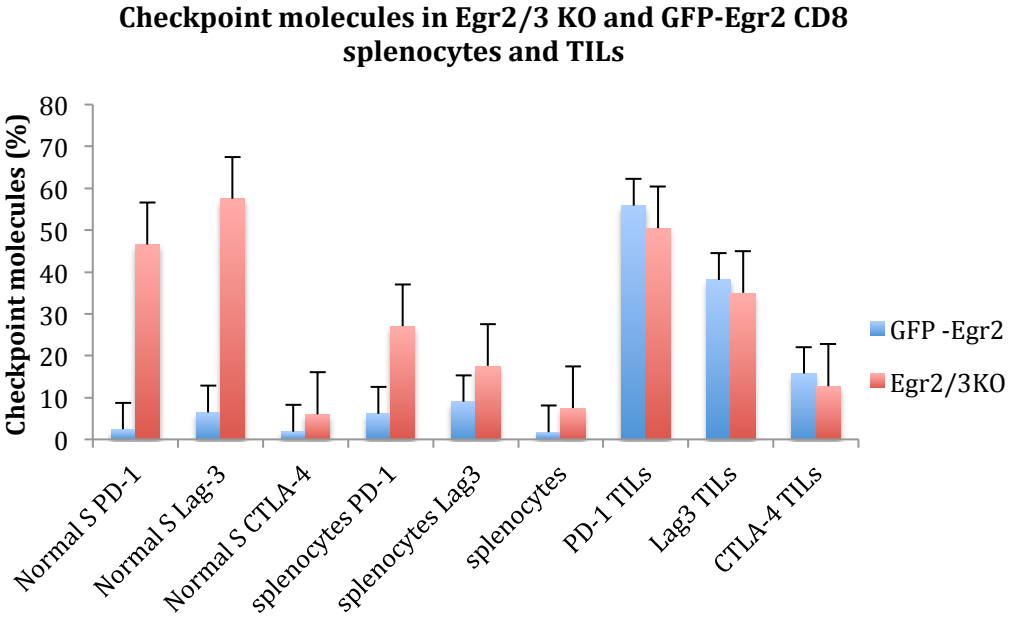


Figure 5.2 Expression of immune checkpoint molecules in Egr2/3 KO and GFP-Egr2 CD8 TILs/splenocytes. Egr2/3 KO $CD8^+$ T and GFP-Egr2 $CD8^+$ T were isolated and stained with the immune checkpoint molecules. Results represent three independent experiments.

5.4 Discussion

Immune checkpoint molecules play the non-redundant role in modulating immune responses, providing a prognostic role and promising therapeutic approaches in cancer and autoimmunity conditions (Kyi and Postow, 2014, Pardoll, 2012). PD-1, CTLA-4, Lag-3 are the most successful targets for anti-tumour immune therapy (Flemming, 2012). These molecules are mostly induced in the tumour microenvironment (Seidel *et al.,* 2018). Dual targeting of PD-1 and Lag-3 synergistically enhances the anti-tumour immune response in various tumour models by increasing $CD4^+$ and $CD8^+$ T cell infiltration as well as increased IFNγ and TNFα production (Andrews *et al.,* 2017).

We established that $CD8^+$ TILs from wild type mice have similar or low expression of these molecules, indicating Egr2/3 function in TIL T cells is not due to counter-regulation of checkpoint repression. Thus, Egr2/3 function in promoting anti-tumour responses is distinct to checkpoint blockages.

CHAPTER 6- Effect of cytokines on Egr2 expression in T cells

6.1 Aim

We have now shown that Egr2/3 are important for expansion of TILs which affect the anti-tumour responses. However, Egr2 is a de novo transcription factor that is only expressed in pre-activated T cells by antigen stimulation (Zheng *et al.,* 2012). Previously, our group reported that different cytokines have different effects on Egr2 expression in T cells (Miao *et al.,* 2017). Therefore, analysis of the potential function of different cytokines in induction of Egr2 in T cells may explain the importance of tumour microenvironment factors to control the Egr2 expression in TILs.

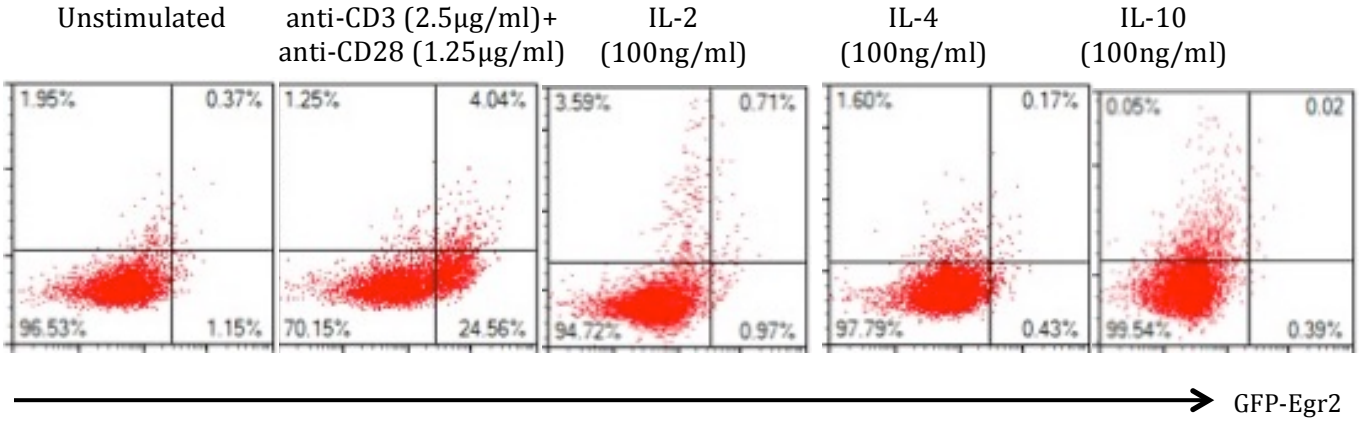
6.2 Introduction

Previously, our group showed that Egr2 and Egr3 are rapidly induced by antigens in naïve T cells and promptly suppressed by IFNγ via inhibitory feedback mechanisms, allowing optimal clonal expansion and coupling of expansion with effector differentiation of viral responding T cells (Miao *et al.,* 2017). However, the regulation of Egr2 through IFNγ and Jak-STAT signalling pathway was not elucidated, so it was hypothesised that Egr2 expression is mediated through IFNγ/STAT1 and IL-6/STAT3 signalling pathway.

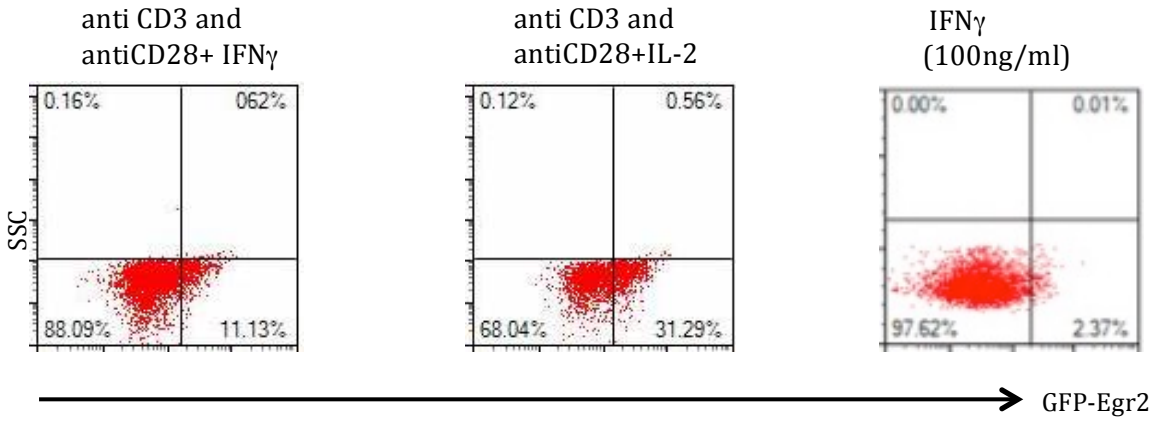
6.3 Egr2 expression is regulated by IL-2, IL-4 and IL-10 cytokines

The cytokines are associated with T cell activation and produced in the tumour microenvironment are IL-2, IL-4, IL-6, IL-10, IL-12, INFγ and TGF-β (Landskron *et al.,* 2014). Among them, IL-2, IL-4 and IL-10 share an important promising function in TILs, T cells and cancer immunotherapy (Seo *et al.,* 2001, Jiang *et al.,* 2016). Therefore, the possibility that these cytokines induce Egr2 expression in T cells was investigated. IL-2 and INFγ increased and decreased Egr2 expression respectively in $CD8^+$ T cells (Figure

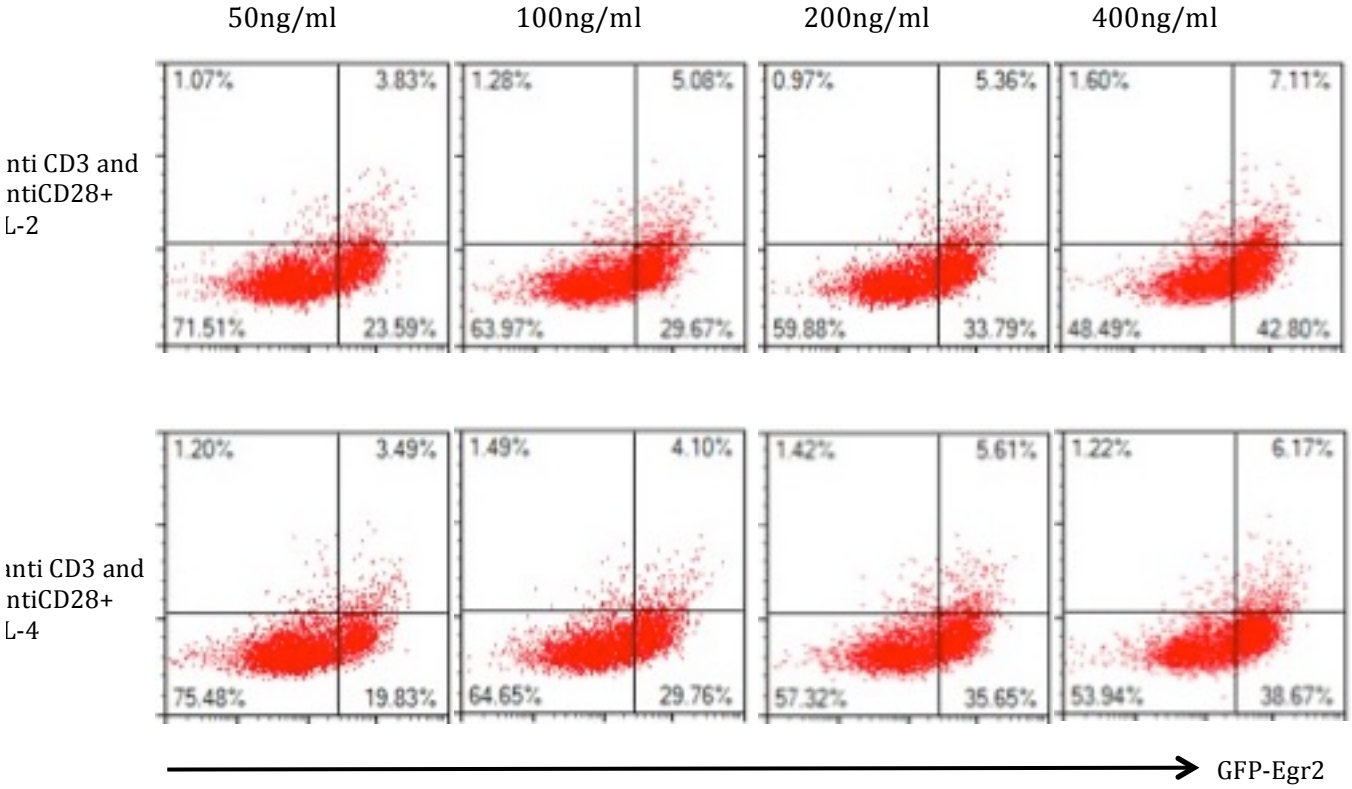
6.1, A). To further study the regulation of Egr2 expression under cytokine stimulation, GFP-Egr2 $CD4^+$ T cells were stimulated with anti-CD3 and anti-CD28 in the presence of IL-2, IL-4 and IL-10 in a serial titration (Figure 6.1 & 6.2). It was observed that cytokines including IL-2, IL-4 and IL-10 increased Egr2 induction and regulated its expression. However, IL-2 and IL-4 used to promote TIL expansion and function, and IL-10 produced by the tumour microenvironment can repress the function of TILs (Seo *et al.,* 2001, Rosenberg, 2014). In this study, Egr2 was only associated with expansion of TILs but had less of an effect on the function of TILs. Therefore, IL-10 may be important to balance the adaptive immune responses to tumours, while limiting the inflammatory effects.


Unstimulated
anti-CD3 (2.5µg/ml)+ anti-CD28 (1.25µg/ml)
IL-2 (100ng/ml)
IL-4 (100ng/ml)
IL-10 (100ng/ml)
1.95%
0.37%
96.53%
1.15%
1.25%
4.04%
70.15%
24.56%
3.59%
0.71%
94.72%
0.97%
1.60%
0.17%
97.79%
0.43%
0.05%
0.02
99.54%
0.39%
GFP-Egr2

A)


anti CD3 and antiCD28+ IFNγ
anti CD3 and antiCD28+IL-2
IFNγ (100ng/ml)
SSC
0.16%
062%
88.09%
11.13%
0.12%
0.56%
68.04%
31.29%
0.00%
0.01%
97.62%
2.37%
GFP-Egr2

B)


50ng/ml
100ng/ml
200ng/ml
400ng/ml
nti CD3 and
ntiCD28+
L-2
1.07%
3.83%
71.51%
23.59%
1.28%
5.08%
63.97%
29.67%
0.97%
5.36%
59.88%
33.79%
1.60%
7.11%
48.49%
42.80%
ınti CD3 and
ntiCD28+
L-4
1.20%
3.49%
75.48%
19.83%
1.49%
4.10%
64.65%
29.76%
1.42%
5.61%
57.32%
35.65%
1.22%
6.17%
53.94%
38.67%
GFP-Egr2

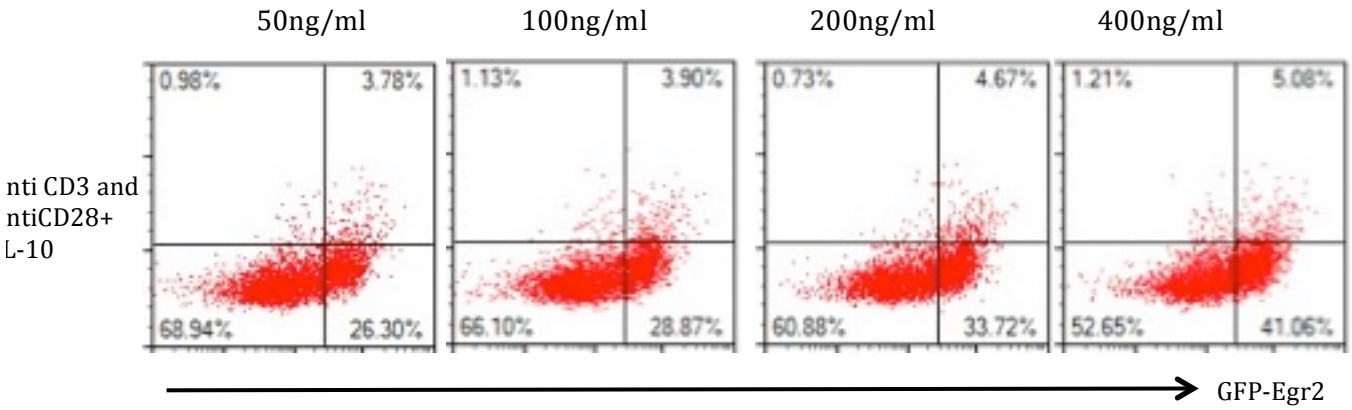


Figure 6.1 Dot plots of cultured GFP-Egr2 CD4+ and CD8+ T cells in the presence of IL-2, IL-4 and IL-10 after 24 hours analysed for the induction of Egr2 expression. A) Splenic $CD8^+$ T cells were isolated and cultured in vitro with anti-CD3 (2.5 μg/ml) and anti-CD28 (1.25 μg/ml) in the presence of IL-2 and IFNγ (100 ng/ml) for 24 hours and analysed by flow cytometry. B) Splenic $CD4^+$ T cells were isolated and cultured in vitro with anti-CD3 (2.5 μg/ml) and anti-CD28 (1.25 μg/ml) in the presence of IL-2, IL-4 and IL-10 (50-400 ng/ml) in a serial titration for 24 hours and analysed by flow cytometry.

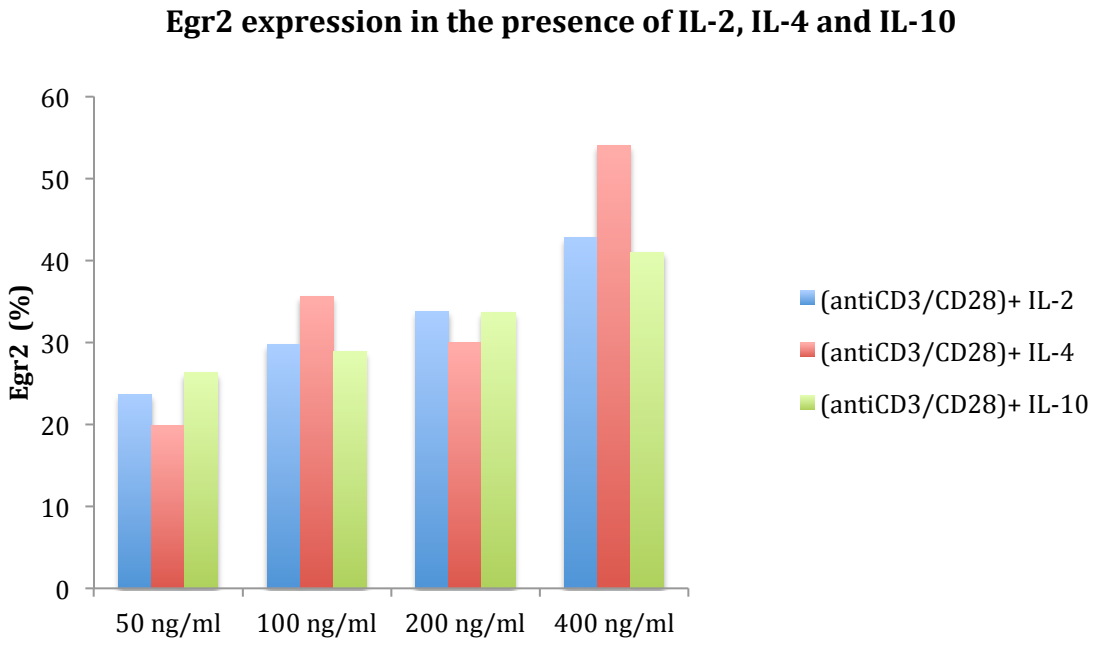


Figure 6.2 GFP-Egr2 CD4+ T cells with antiCD3 and anti-CD28 in the presence of IL-2, IL-4 and IL-10 after 24 hours analysed for the expression of Egr2 expression. Splenic $CD4^+$ T cells were isolated and cultured in vitro with anti-CD3 (2.5 μg/ml) and anti-CD28 (1.25 μg/ml) in the presence of IL-2, IL-4 and IL-10 (50-400 ng/ml) in a serial titration for 24 hours and analysed by flow cytometry.

6.3.1 Egr2 expression is inhibited in the presence of IFNγ and IL-6 in activated T cells

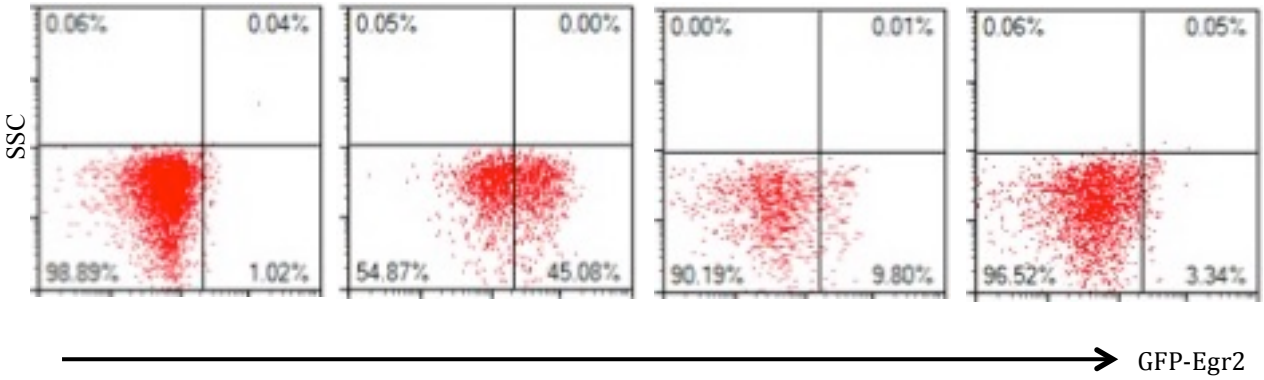
To investigate the regulation of Egr2 expression in T cells by these cytokines, GFP-Egr2 $CD4^+$ T cells were stimulated with anti-CD3 and anti-CD28 in the presence and absence of INFγ and IL-6. Anti-CD3 and anti-CD28 induced Egr2 expression in GFP-Egr2 $CD4^+$ T cells by 45% and 60.06%, this expression was suppressed to 10% and 17.76 in the presence of INFγ and IL-6 respectively (Figure 6.3 A & B). As shown in Figure 6.4, inhibition of Egr2 expression in the presence of IFNγ was very significant (P=0.01) compared with only anti-CD3 and anti-CD28 treated cells.

Moreover, cells with only INFγ and IL-6 stimulation did not show activation in GFP-Egr2 cells similar to the unstimulated controls (Figure 6.3 A & B). The inhibited expression of

Egr2 in the presence of IL-6 was significant (P= 0.05) compared with the anti-CD3 and anti-CD28 treated cells (Figure 6.5). These results demonstrate that INFγ and IL6, the major inflammatory cytokines in tumour microenvironment, are repressors for Egr2 expression in T cells. Our results may indicate the suppression of Egr2 expression in T cells by these cytokines is one the mechanisms for tolerogenic effects of tumour microenvironment.

A)

		anti-CD3 (2.5 µg/ml)+ anti-CD28 (1.25 µg/ml)	anti-CD3(2.5 µg/ml)+ anti-CD28 (1.25 µg/ml)	-
IFNγ	-	-	200ng/ml	200ng/ml



B)

		anti-CD3 (2.5 µg/ml)+anti-CD28 (1.25 µg/ml)	anti-CD3 (2.5 µg/ml)+ anti-CD28 (1.25 µg/ml)	-
IL-6	-	-	200ng/ml	200ng/ml

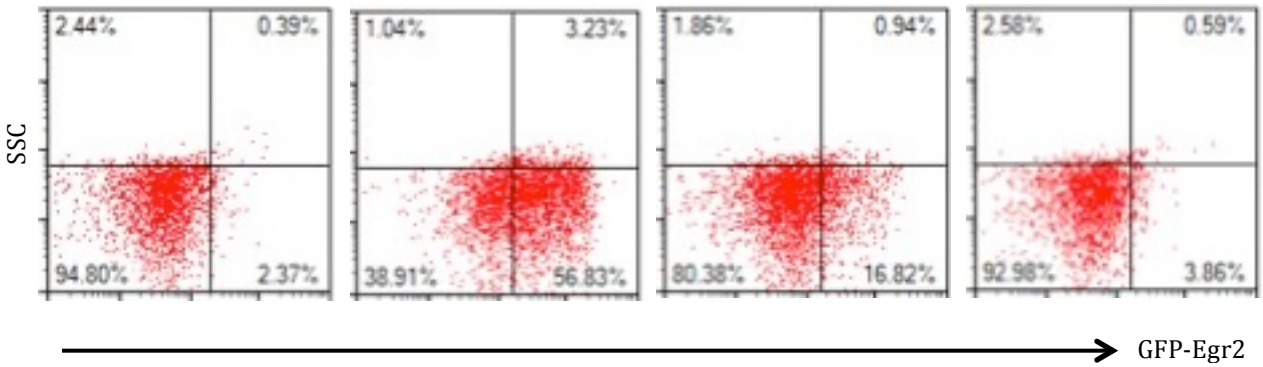


Figure 6.3 Dot plots of cultured GFP-Egr2 CD4+ T cells in the presence of IFNγ and IL-6 after 48 hours analysed for the induction of Egr2 expression. A) Splenic $CD4^+$ T cells were isolated and cultured in vitro with anti-CD3 (2.5 μg/ml) and anti-CD28 (1.25 μg/ml) in the presence of IFNγ (200ng/ml) for 48 hours and analysed by flow cytometry. B) Splenic $CD4^+$ T cells were isolated and cultured in vitro with anti-CD3 (2.5 μg/ml) and anti-CD28 (1.25 μg/ml) in the presence of IL-6 (200ng/ml) for 48 hours and analysed by flow cytometry. Flow cytometric results are representative of three experiments, with two or more mice per experiment.

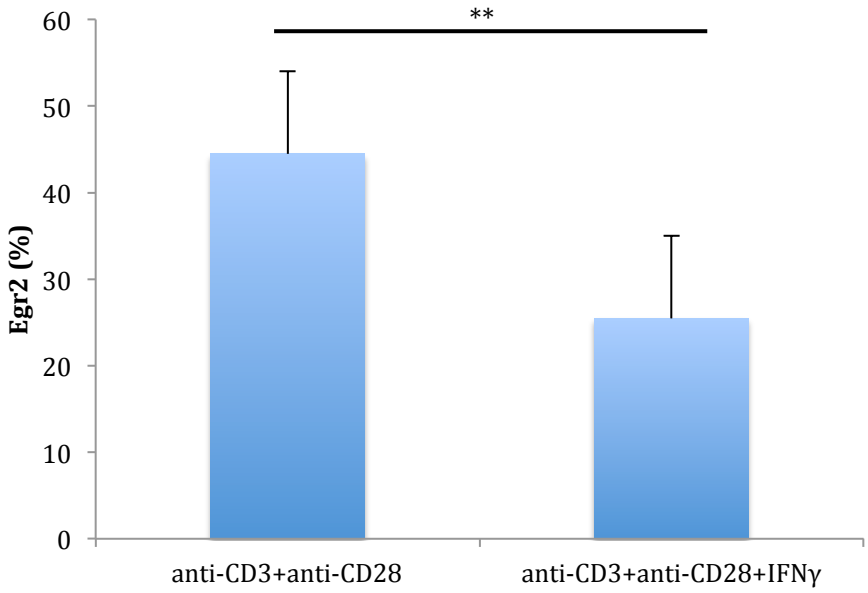


Figure 6.4 GFP-Egr2 $CD4^+$ T cells in the presence of IFNγ after 48 hours exhibiting Egr2 expression. Splenic CD4+ T cells were stimulated using soluble anti-CD3 (2.5 μg/ml) and anti-CD28 (1.25 μg/ml) and co-cultured in the presence of 200ng/ml IFNγ for 48 hours. **P ≤ 0.01 versus stimulated control.

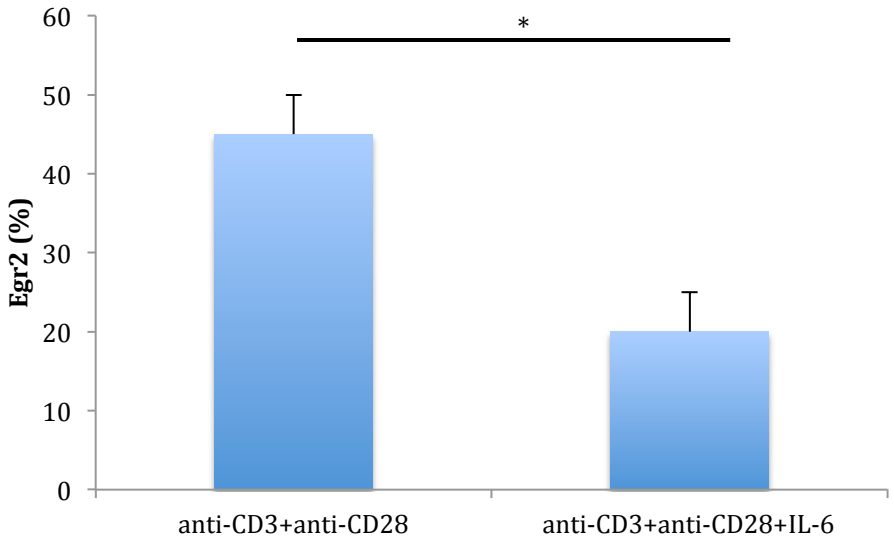


Figure 6.5 GFP-Egr2 CD4+ T cells in the presence of IL-6 after 48 hours, exhibiting Egr2 expression. Splenic $CD4^+$ T cells were stimulated using soluble anti-CD3 (2.5 μg/ml) and anti-CD28 (1.25 μg/ml) and co-cultured in the presence of 200ng/ml IL-6 for 48 hours. *P ≤ 0.05 versus stimulated control.

6.3.2 Analysis of the proximal regions of the Egr2 locus revealed STAT1 and STAT3 binding sites

To investigate the potential binding sites of STAT1 and STAT3 in the Egr2 promoter region, we analysed 3 kb upstream of Egr2 locus by functional regulatory elements multiple-sequence alignment analysis (Ovcharenko *et al.,* 2005). There were four binding sites for STAT1 and three sites for STAT3: 5'- cGGAAgtg-3', 5'-tttaatTTTCCGGAAtggctc-3', 5'-cGGAAttc-3' for STAT1 and 5'-tttaatTTTCCGGAAtggctc-3'- and 5'-cGGAAttc-3' for STAT3 (Timofeeva *et al.,* 2013). The STAT1 binding sites partially overlapped with STAT3, cGGAAttc and tttaatTTTCCGGAAtggctc, suggesting that Egr2 transcription is regulated by these two transcription factors in response to IFNγ and IL6 stimulation (Figure 6.6).

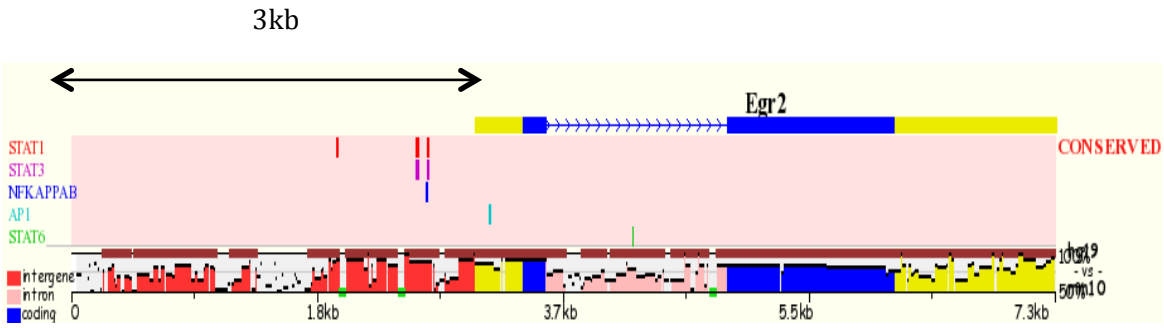


Figure 6.6 Egr2 promoter with STAT-1 and STAT-3 binding sites using Mulan multi-sequence analysis.

6.3.3 Suppressed Egr2 expression is reverted in the presence of STAT1 and STAT3 inhibitor

To investigate the effect of INFγ and IL-6 on STAT1 and STAT3 mediated Egr2 induction, the STAT1 inhibitor (Fludarabine) and STAT3 inhibitor (Stattic) were used. Fludarabine inhibits the cytokine-induced activation of STAT1 and STAT1-dependent gene transcription by substantially decreasing phosphorylation of STA1 at Tyr701, preventing IFNγ induced STAT1 activation and leading to a loss of STAT1 from lymphocytes through diminished protein synthesis (Frank *et al.,* 1999). Stattic is a non-peptidic small molecule that exerts its effect by preventing the activating kinases from binding to the STAT3 SH2 domain, thereby inhibiting dimerisation and DNA binding of STAT3 (Schust *et al.,* 2006).

GFP-Egr2 $CD4^+$ T cells were stimulated with anti-CD3 (2.5 μg/ml) anti-CD28 (1.25 μg/ml) in the presence of INFγ (200ng/ml) and STAT1 inhibitor (10μM), increased Egr2 expression to 66.48% (Figure 6.7 A). The induced Egr2 expression in the presence of INFγ and STAT1 inhibitor was highly significant (P=0.001) compared with anti-CD3/anti-CD28 and INFγ treated samples (Figure 6.8).

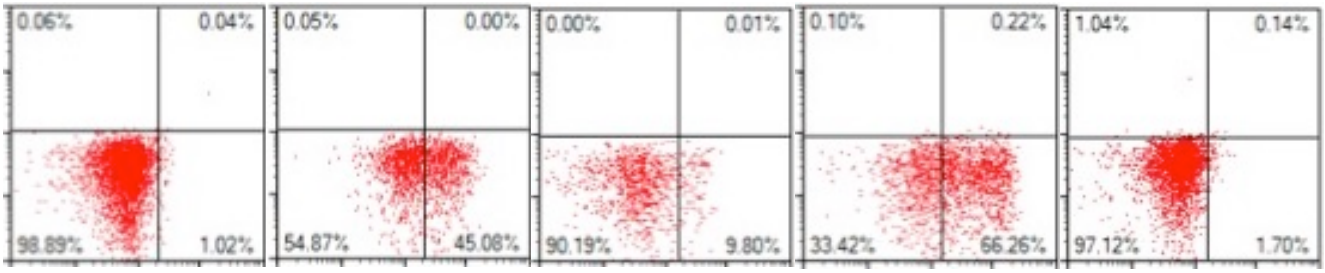
Likewise, cells in the presence of IL-6 (200 ng/ml) and STAT3 inhibitor (20 μM) showed an increase in Egr2 expression to 74.95% (Figure 6.7 B). The induced Egr2 expression in the presence of IL-6 and STAT3 inhibitor was highly significant (P=0.001) compared with anti-CD3/anti-CD28 and IL-6 treated samples (Figure 6.9). Moreover, the cells stimulated only with the Fludarabine and Stattic did not express Er2.

Thus, these results showed that Egr2 expression is mediated by INFγ/STAT1 and IL6/STAT3 signalling pathway. The increased Egr2 expression in the presence of STAT1

and STAT3 inhibitors indicated the negative regulatory role of cytokine STAT in Egr2 expression.

A)

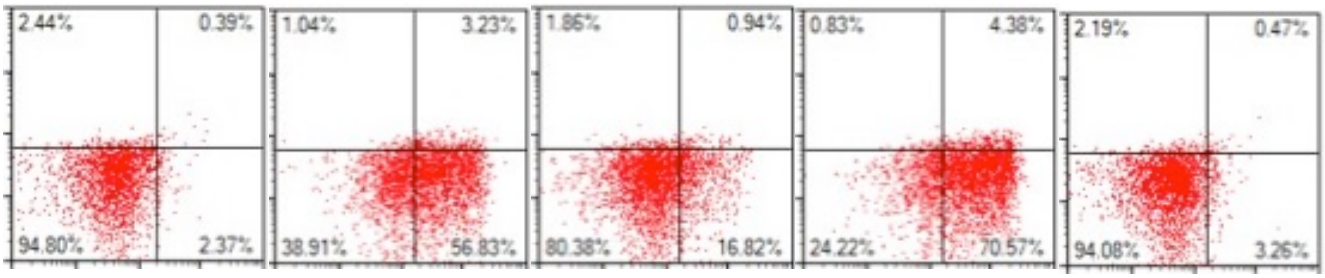
	-	anti-CD3 (2.5 µg/ml)+ anti-CD28 (1.25 µg/ml)	anti-CD3 (2.5 µg/ml)+ anti-CD28 (1.25 µg/ml)	anti-CD3 (2.5 µg/ml)+ anti-CD28 (1.25 µg/ml)	-
FNγ	-	-	200ng/ml	200ng/ml	-
ludarabine	-	-	-	10µM	10µM



GFP-Egr2

B)

	-	anti-CD3 (2.5 µg/ml)+ anti-CD28 (1.25 µg/ml)	anti-CD3 (2.5 µg/ml)+ anti-CD28 (1.25 µg/ml)	anti-CD3 (2.5 µg/ml)+ anti-CD28 (1.25 µg/ml)	-
L-6	-	-	200ng/ml	200ng/ml	-
tattic	-	-	-	20µM	20µM



GFP-Egr2

Figure 6.7 Dot plots **of cultured GFP-Egr2 CD4$^+$ T cells stimulated with IFNγ and IL-6 in the presence of Fludarabine and Stattic after 48 hours assessed for Egr2 expression.** A) Splenic CD4$^+$ T cells were isolated and cultured in vitro with anti-CD3 (2.5 μg/ml) and anti-CD28 (1.25 μg/ml) in the presence of IFNγ (200 ng/ml) for 24 hours. The following day, Fludarabine (10 μM) was added to the cells, incubated for 24 hours and analysed by flow cytometry. B) Splenic CD4$^+$ T cells were isolated and cultured in vitro with anti-CD3 (2.5 μg/ml) and anti-CD28 (1.25 μg/ml) in the presence of IL-6 (200ng/ml) for 24 hours. The following day, Stattic (20 μM) was added to the cells, incubated for 24 hours and analysed by flow cytometry. Flow cytometric results are representative of three experiments.

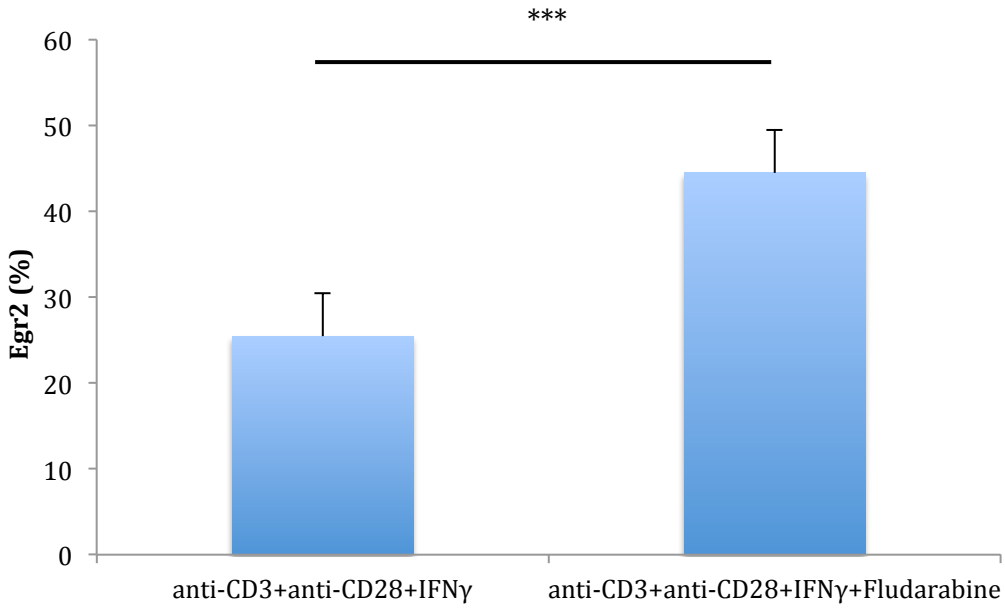


Figure 6.8 GFP-Egr2 CD4$^+$ T cells in the presence of IFNγ and fludarabine after 48 hours exhibiting Egr2 expression. Splenic CD4$^+$ T cells were stimulated using soluble anti-CD3 (2.5 μg/ml) and anti-CD28 (1.25 μg/ml) and co-cultured in the presence of IFNγ (200 ng/ml) and fludarabine (10 μM) for 48 hours. T-test ***P ≤ 0.001.

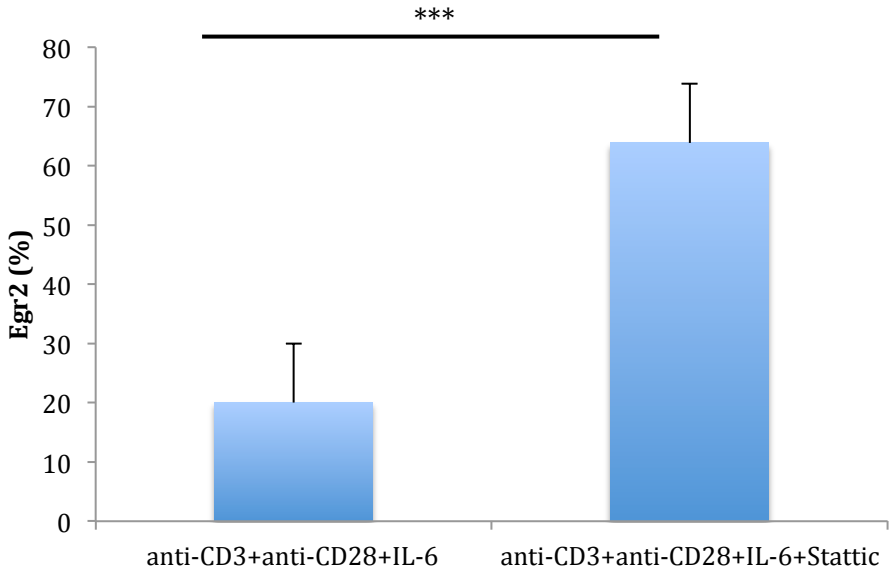


Figure 6.9 GFP-Egr2 CD4$^+$ T cells in the presence of IL-6 and Stattic after 48 hours exhibiting Egr2 expression. Splenic CD4$^+$ T cells were stimulated using soluble anti-CD3 (2.5 μg/ml) and anti-CD28 (1.25 μg/ml) co-cultured in the presence of IL-6 (200 ng/ml) and fludarabine (10 μM) for 48 hours. T-test ***P ≤ 0.001.

6.3.4 Egr2 promoter activity is decreased in the presence of IFNγ in which it was reverted by the addition of Fludarabine

Although IFNγ and IL-6 through STAT1 and STAT3 suggested their regulatory role for Egr2 expression, it still poses the question as to whether this mediatory role can be directly related to Egr2 promoter activity. To test if STAT1 is involved in IFNγ mediated Egr2 expression, a luciferase reporter assay was performed with an Egr2-pGL2-b reporter gene containing the Egr2 promoter. Analogous to previous experiments, IFNγ inhibited Egr2 expression as shown in Figure 6.4 by the very significant reduction in luciferase activity, while Egr2 promoter activity was increased when transfected Egr2-pGL2-b was treated with both STAT1 inhibitor (Fludarabine) and IFNγ under the same conditions (Figure 6.10).

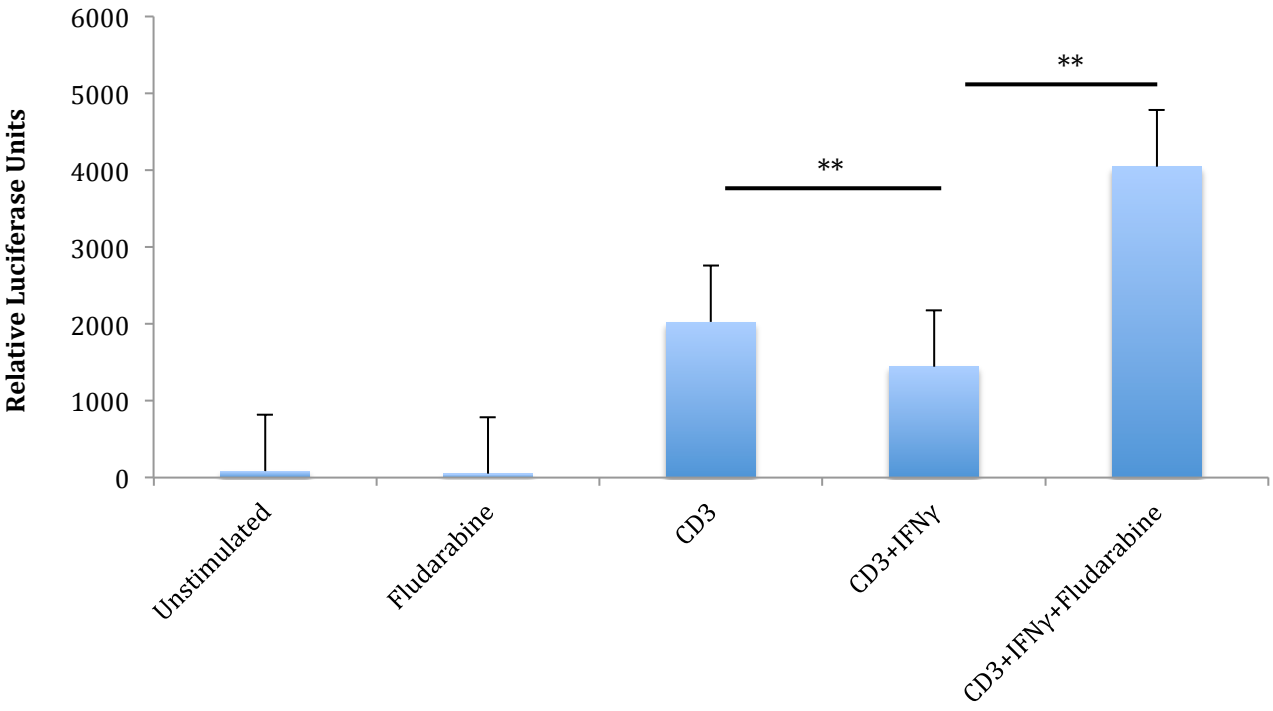


Figure 6.10 Egr2 promoter activity is regulated through IFNγ and STAT-1 pathway. Firefly luciferase vector (Egr2-pGL2-b) was transfected into EL4 cells, then 24 hours after transfection, cells were treated with soluble anti-CD3 (2.5 μg/ml) in the presence and absence of IFNγ (200 ng/ml) and fludarabine (10 μM) for 16–18 hours. Cells were harvested for protein extraction followed by measurement of luciferase activity. The experiment was performed in triplicate. **P ≤ 0.01 versus stimulated control.

6.4 Discussion

Several studies have highlighted the essential role of cytokines in inhibition and progression of tumours, but regulatory mechanisms in TIL proliferation and function in immunosuppressive tumours are not well understood (Zhou *et al.,* 2014). Egr2$^{-/-}$ and Egr3$^{-/-}$ mice have demonstrated that Egr2 and Egr3 are important for anti-tumour responses in B16 tumour models. Their function is associated largely with the expansion of TIL T cells, but less with the function of TIL T cells.

We have now investigated the effects of cytokines that have been reported in tumour microenvironment on the regulation of Egr2 expression in T cells. The results demonstrated that the cytokines, IL-2 and IL-4, that promote T cell expansion increased expression of Egr2 in activated T cells, while pro-inflammatory cytokines, including IFNγ and IL-6, suppress Egr2 expression. As TIL T cells from wild type mice are expanded significantly in comparison to TILs from Egr2/3 KO mice, the mechanisms reported from TIL T cells after IL-2 and IL-4 treatment may result partly from induction of Egr2 (Rosenberg, 2014). However, the pro-inflammatory cytokines have effects on both the function of TIL T cells and tumour metastasis (Landskron *et al.,* 2014). These cytokines have been reported to have a negative impact on TIL expansion (Abiko *et al.,* 2015), therefore, excessive production of these cytokines by the tumour microenvironment may limit the expansion of TIL T cells through inhibition of Egr2 expression.

CHAPTER 7 - General Discussion

7.1 Introduction

Egr2 and Egr3 are expressed in different stages of effector T cells in adaptive immune responses and they play crucial role in controlling the inflammation and antigen-induced proliferation of B and T cells (Li *et al.,* 2012). A recent study demonstrated that Egr2 and Egr3 are rapidly induced by the antigens in naïve T cells, which are required for clonal expansion of antigen-specific T cells. Also, the expression of Egr2/3 is suppressed by inflammatory cytokines, such as IFNγ, through inhibitory feedback mechanisms. This mechanism allows optimal clonal expansion and coupling of expansion with effector differentiation of responding T cells against viral infection, which proves their indispensable role in limiting immunopathology during productive adaptive immune responses (Miao *et al.,* 2017). Egr2, through promoting T cell differentiation and fostering the expression of T-bet, is a vital regulator for the T cell immune response against viral infection (Du *et al.,* 2014).

The findings from this study showed that Egr2/3 are important for T cell mediated immune responses in tumours by promoting the expansion of tumour infiltrating T cells. In the absence of Egr2/3, tumour infiltrating T cells were significantly reduced, with a low expression of Ki-67, a proliferating marker and IL-2. These results were associated with over growth of B16 melanoma tumours. However, the effector function, such as production of IFNγ and Granzyme B, was not affected or increased in Egr2/3 deficient T cells, indicating the importance of the expansion of tumour specific T cells in anti-tumour responses. Moreover, Egr2 expression is regulated by different cytokines, including IL-2 and IL-4 that increased Egr2 induction in activated T cells. However, inflammatory cytokines, including INFγ and IL-6, suppressed Egr2 expression through STAT1 and STAT3 signalling pathway respectively, highlighting a mechanism that can

affect Egr2 function, either by favouring TIL expansion or applying tolergenic effects of the tumour microenvironment. Egr2 has been reported to induce checkpoint regulators, such as Lag3 in T cells from TILs (Williams *et al.,* 2014). However, we provide results from Egr2/3 deficient T cells from TILs that Egr2/3 are not directly involved in the regulation of immune checkpoint molecules in the tumour microenvironment.

7.2 Egr2/3 are important for efficient T cell immune responses against tumours

Immune therapies with anti-checkpoint regulators, such as anti-PD-1 and anti-CTLA4, have outstanding efficacy for multiple solid tumours (Seidel *et al.,* 2018), which demonstrate that tumour specific immune responses of T cells are important for controlling tumours and the tumour microenvironment induces checkpoint regulators to escape immune surveillance (Pardoll, 2012). However, it is not known whether the tumour specific T cells are suppressed for their proliferation or effector function or both (Chen and Mellman, 2013). Consistent observations demonstrated that the number of T cells in TILs is associated with the survival rate of cancer patients in multiple solid tumours (Abiko *et al.,* 2015), indicating the expansion of TIL T cells may be fundamentally important, while inflammatory effectors without proliferation will negatively impact on anti-tumour activity. Lack of both TIL proliferation and sensitivity in T cell expansion in combination with CTLA-4 activation hampers the treatment of metastatic melanoma patients (Hodi *et al.,* 2010).

Egr2, by promoting T cell differentiation and boosting T-bet expression, has been found as a key regulator in T cell response (Du *et al.,* 2014). It was demonstrated that Egr2 and 3 are crucial regulators in the efficient proliferation of naïve B and T cells in response to mitogenic antigen receptor stimulation. Egr2 also plays an essential role in the

regulation of the transition between the clonal expansion and differentiation of effector T cells during infection (Li *et al.,* 2012, Miao *et al.,* 2017). In this study, considerably high tumour growth was observed in Egr2/3 KO mice model in comparison with WT mice. Moreover, $CD8^+$ TIL expansion was significantly defected in Egr2/3 KO mice, however, effector cytokine induction was not impaired. This provided a mechanism by which Egr2/3 can promote TIL expansion, resulting in tumour regression. Collectively, it was demonstrated that Egr2/3, a component of the tumour microenvironment, efficiently promotes anti-tumour $CD8^+$ T cell-mediated immune response in vivo, significantly contributing to tumour regression.

7.3 Role of Egr2 and Egr3 in the regulation of Immune checkpoint molecules

Immune checkpoints are incorporated into the immune system that play an important role in maintaining self-tolerance and modulating the amplitude and duration of immune responses against infection and cancer (Pardoll, 2012). The cell surface proteins Lag-3, PD-1 and 4-1BB induced by Egr2 can identify a subpopulation of dysfunctional tumour antigen-specific $CD8^+$ TIL, as shown by defective IL-2 production, leading to immune suppressive tumour microenvironment (Williams *et al.,* 2014, Williams *et al.,* 2017) (Table 7.1). PD-1 and Lag-3 targeting through increased $CD4^+$ and $CD8^+$ T results in a coordinated immune activation in the large-tumour setting and a significantly higher survival rate (Andrews *et al.,* 2017). This emphasises the importance of the regulation and mechanisms of immune checkpoint molecules in terms of T cell-mediated immune response, potential therapeutic approaches to prevent autoimmunity and cancer.

We have shown that PD-1, Lag-3 and CTLA-4 expression in T cells is significantly higher in Egr2/3 KO mice in comparison with GFP-Egr2 mice. Previously, our group demonstrated that the loss of self-tolerance and development of lupus-like autoimmune disease in Egr2/3 KO mice (Zhu *et al.,* 2008). In line with our model, this enhanced expression of immune checkpoint molecules could be related to the tangible effect of inflammation that could be exerted on the expression of immune checkpoint molecules. However, checkpoint molecules including PD-1, Lag-3 and CTLA-4 did not show any difference between Egr2/3 KO and GFP-Egr2 TILs, indicating that anti-tumour function of Egr2/3 in TILs T cells is not due to their regulatory role in the repression of immune checkpoint molecules.

7.4 Egr2 expression is regulated reciprocally through cytokines

The pivotal role of the cytokines in the progression and suppression of tumours has been studied in several studies (Zhou *et al.,* 2014). It has been shown that IL-2, IL-4 and IFNγ play fundamental roles in TIL T cells and cancer immunotherapy (Jiang *et al.,* 2016). IL-2 and IL-4 encourage T cell proliferation and infusion of these cytokines in patients with metastatic melanoma and renal cell carcinoma has led to significant eradication of tumour cells (Sim and Radvanyi, 2014). The intrinsic role of Egr2/3 has been highlighted in the regulation of effector T cells that can control the development of the autoimmune disease (Li *et al.,* 2012). Furthermore, Egr2 expression is essential for T cell clonal expansion and a negative feedback mechanism exerted by effector cytokines, such as IFNγ, is important to boost T cell differentiation (Miao *et al.,* 2017). With these key findings in mind, we set out to establish the mechanism by which cytokines control the expression of Egr2 in T cells within the tumour microenvironment.

According to our results, IL-2 and IL-4, important cytokines involved in T cell activation, increased Egr2 expression, whereas pro-inflammatory cytokines, including IFNγ and IL-6, suppressed the induction of Egr2 in T cells. This suggests that IL-2 and IL-4 promote T cell expansion in activated T cells, while excessive production of IFNγ and IL-6 may inhibit T cell expansion through the suppression of Egr2 in activated T cells, with the latter providing a distinct mechanism for tolergenic effects of the tumour microenvironment. Furthermore, it was, shown that Egr2 suppressed expression via IFNγ and IL-6, mediated through the IFNγ/STAT1 and IL-6/STAT3 pathway (Table 7.1).

Table 7.1 Summary of the regulators of Egr2 expression.
The contributions of this thesis regarding the understanding of the regulation of Egr2 gene within adaptive immunity are highlighted in blue.

Molecule/Signalling pathway	Mouse model/Cell line	Effects on Egr2 expression	Reference
NFAT	NFATp$^{-/-}$ and NFAT4$^{-/-}$ T cells	**Positive**	**Rengarajan et al., 2000; Rivera et al., 2000; Lazarevic et al., 2009**
Ly 108	**T cells**	**Positive**	(Dutta et al., 2013)
MPK/ERK	**T cells**	**Positive**	(Chandra et al., 2013)
CD26/Raf-MEK-ERK	**CD4^{+} T cells**	**Positive**	(Hatano et al., 2015)
IFN-γ	**T cells**	**Negative**	(Miao et al., 2017)
IFN-γ/STAT1 and IL-6/STAT3	**T cells**	**Negative**	**From this thesis**

7.5 Conclusion

Our previous studies have demonstrated that Egr2 and Egr3 have a crucial role in B and T cell development and prevention of autoimmunity. Also, the fundamental function of Egr2 as a checkpoint molecule in controlling the proliferation and differentiation of T cells has been demonstrated. Based on the findings reported, we investigated the function of Egr2 in adaptive immunity using a B16 melanoma tumour model. The results showed that significant Egr2 expression in T cells under tumour microenvironment can result in enhanced $CD8^+$ TIL expansion, whereas Egr2/3 KO counterparts showed higher tumour growth followed by impaired $CD8^+$ TIL expansion. This highlighted the fundamental function of Egr2 that can exert efficient T cell mediated immune response, boosting tumour immune therapy.

Secondly, functions of cytokines, as tumour microenvironment factors, in terms of Egr2 expression in T cells were investigated. It was shown that IL-2 and IL-4 increase Egr2 expression, which can lead to expansion of TILs T cells, whereas IFNγ and IL-6 suppress Egr2 expression in activated T cells, suggesting a distinct tolerant mechanism of the tumour microenvironment to the immune response. Moreover, Egr2 expression was mediated through IFNγ/STAT1 and IL-6/STAT3 signalling pathways (Figure 7.1). Lastly, our study reflected that anti-tumour function of Egr2/3 is not due to their role in the inhibition of immune checkpoint molecules including PD-1, Lag-3 and CTLA-4.

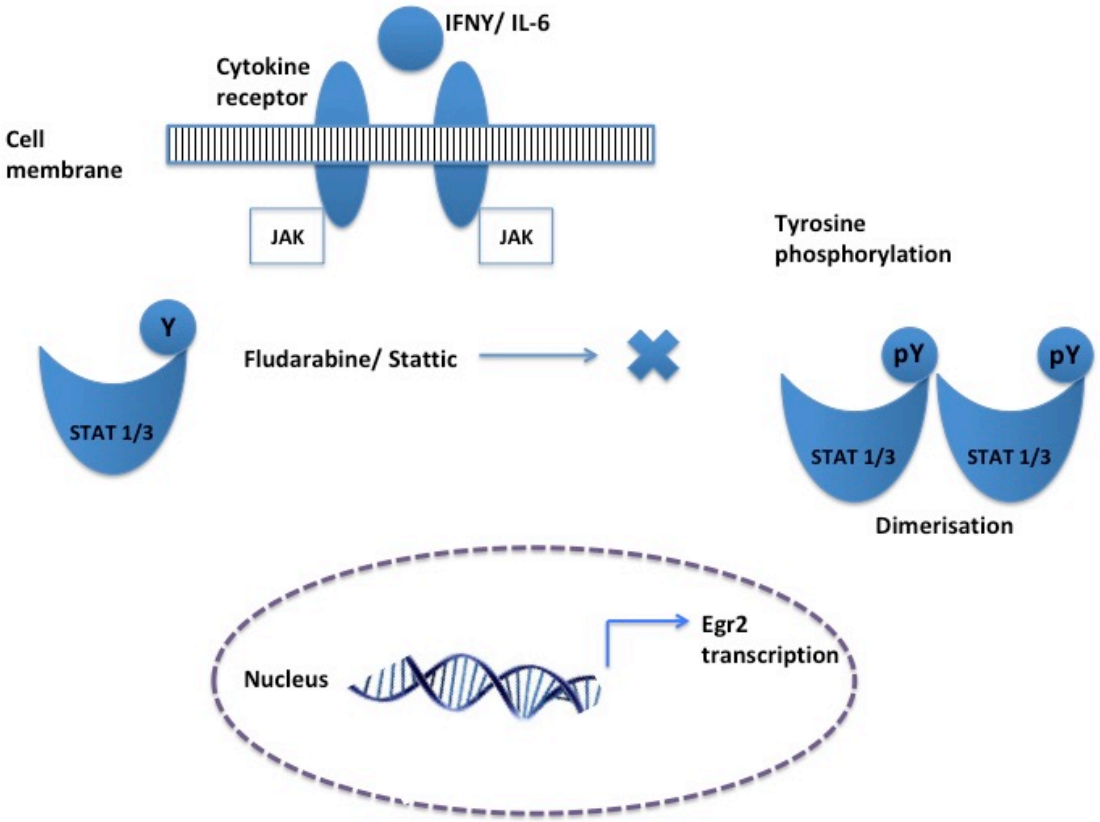


Figure 7.1 Schematic model representing Egr2 regulation via INFγ/STAT1 and IL6/STAT3 mediated signalling pathways in the adaptive immune response. Upon antigen (positive for Egr2 expression) and cytokine stimulation (including INFγ and IL-6 and negative for Egr2 expression), STAT1 inhibitor (Fludarabine) and STAT3 inhibitor (Stattic) block tyrosine phosphorylation and dimerisation, thereby preventing translocation of STATs to the nucleus and binding to the Egr2 promoter region, that finally block the inhibitory effects of cytokines on Egr2 expression.

7.6 Future work

The findings from this study demonstrate a novel function of Egr2/3 in an effective anti-tumour immune response through promoting the expansion of TIL T cells and provoked a discussion regarding Egr2 for efficient anti-tumour therapy. It would be interesting to use RNA-seq for TILs to determine the gene expression regulated by Egr2 in the tumour condition. It would be beneficial to investigate antigen-specific tumour models and other potential effector mechanisms within this mice model for developing a strong strategy against cancers. For the regulation of Egr2 through the cytokine STAT (IFN-γ/STAT1 and IL-6/STAT3) signalling pathway, it would be interesting to quantify the expression of Egr2 and Egr3 during T cell signalling and investigate the mechanisms of other cytokines; as we have found an enhancing effect of IL-2, IL-4 and IL-10 in terms of Egr2 expression. This will help to find out mechanisms in boosting T cell proliferation and functions for efficient tumour immune therapy. Last but not least, it would be interesting to further identify additional immune checkpoints and delineate the mechanism for any interaction of Egr2 and checkpoint molecules expressed on target cells for therapeutic applications in terms of infection, cancer and autoimmunity.

List of References

ABE, B. T., SHIN, D. S., MOCHOLI, E. & MACIAN, F. 2012. NFAT1 supports tumor-induced anergy of CD4+ T cells. *Cancer research,* 72**,** 4642-4651.

ABIKO, K., MATSUMURA, N., HAMANISHI, J., HORIKAWA, N., MURAKAMI, R., YAMAGUCHI, K., YOSHIOKA, Y., BABA, T., KONISHI, I. & MANDAI, M. 2015. IFN-γ from lymphocytes induces PD-L1 expression and promotes progression of ovarian cancer. *British Journal Of Cancer,* 112**,** 1501.

ADEREM, A. 2013. Phagocytosis and the Inflammatory Response. *The journal of infectious diseases***,** s340-5.

AHMADZADEH, M., JOHNSON, L. A., HEEMSKERK, B., WUNDERLICH, J. R., DUDLEY, M. E., WHITE, D. E. & ROSENBERG, S. A. 2009. Tumor antigen–specific CD8 T cells infiltrating the tumor express high levels of PD-1 and are functionally impaired. *Blood,* 114**,** 1537.

ALONZI, T., MARITANO, D., GORGONI, B., RIZZUTO, G., LIBERT, C. & POLI, V. 2001. Essential Role of STAT3 in the Control of the Acute-Phase Response as Revealed by Inducible Gene Activation in the Liver. *Molecular and Cellular Biology,* 21**,** 1621-1632.

ANDERSON, P. O., B.A. MANZO, A. SUNDSTEDT, S. MINAEE, A. SYMONDS, S. KHALID, M.E. RODRIGUEZ-CABEZAS, K. NICOLSON, S. L., D.C.WRAITH, A. & P.WANG 2006. Persistent antigenic stimulation alters the transcription program in T cells, resulting in antigen-specific tolerance. *Eur J Immunol,* 36**,** 1360-3.

ANDREWS, L. P., MARCISCANO, A. E., DRAKE, C. G. & VIGNALI, D. A. A. 2017. LAG3 (CD223) as a Cancer Immunotherapy Target. *Immunological reviews,* 276**,** 80-96.

ARAKI, K., TURNER, A. P., SHAFFER, V. O., GANGAPPA, S., KELLER, S. A., BACHMANN, M. F., LARSEN, C. P. & AHMED, R. 2009. mTOR regulates memory CD8 T-cell differentiation. *Nature,* 460**,** 108.

ARANGO DUQUE, G. & DESCOTEAUX, A. 2014. Macrophage Cytokines: Involvement in Immunity and Infectious Diseases. *Frontiers in Immunology,* 5**,** 491.

ASHLEY, N. T., WEIL, Z. M. & NELSON, R. J. 2012. Inflammation: Mechanisms, Costs, and Natural Variation. *Annual Review of Ecology, Evolution, and Systematics,* 43**,** 385-406.

AWASTHI, A., CARRIER, Y., PERON, J. P. S., BETTELLI, E., KAMANAKA, M., FLAVELL, R. A., KUCHROO, V. K., OUKKA, M. & WEINER, H. L. 2007. A dominant function for interleukin 27 in generating interleukin 10–producing anti-inflammatory T cells. *Nature Immunology,* 8**,** 1380.

BAE, C. J., JEONG, J. & SAINT-JEANNET, J. P. 2015. A novel function for Egr4 in posterior hindbrain development. *Sci Rep,* 5**,** 7750.

BAITSCH, L., FUERTES-MARRACO, S. A., LEGAT, A., MEYER, C. & SPEISER, D. E. 2012. The three main stumbling blocks for anticancer T cells. *Trends in Immunology,* 33**,** 364-372.

BEST, J. A., BLAIR, D. A., KNELL, J., YANG, E., MAYYA, V., DOEDENS, A., DUSTIN, M. L., GOLDRATH, A. W. & IMMUNOLOGICAL GENOME PROJECT, C. 2013. Transcriptional insights into the CD8(+) T cell response to infection and memory T cell formation. *Nat Immunol,* 14**,** 404-12.

BETTINI, M., XI, H., MILBRANDT, J. & KERSH, G. J. 2002. Thymocyte Development in Early Growth Response Gene 1-Deficient Mice. *The Journal of Immunology,* 169**,** 1713-1720.

BEUTLER, B. 2004. Innate immunity: an overview. *Molecular Immunology,* 40**,** 845-859.

BEUTLER, B. & RIETSCHEL, E. T. 2003. Innate immune sensing and its roots: the story of endotoxin. *Nat Rev Immunol,* 3**,** 169-176.

BLACKBURN, S. D., SHIN, H., HAINING, W. N., ZOU, T., WORKMAN, C. J., POLLEY, A., BETTS, M. R., FREEMAN, G. J., VIGNALI, D. A. A. & WHERRY, E. J. 2009. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nat Immunol,* 10**,** 29-37.

BOYMAN, O. & SPRENT, J. 2012. The role of interleukin-2 during homeostasis and activation of the immune system. *Nature Reviews Immunology,* 12**,** 180.

BROMBERG, J. & JR, J. E. D. 2000. The role of STATs in transcriptional control and their impact on cellular function. *Oncogene,* 19**,** 2468-2473.

BUNTING, K. D., BRADLEY, H. L., HAWLEY, T. S., MORIGGL, R., SORRENTINO, B. P. & IHLE, J. N. 2002. Reduced lymphomyeloid repopulating activity from adult bone marrow and fetal liver of mice lacking expression of STAT5. *Blood,* 99**,** 479.

BURCHILL, M. A., YANG, J., VANG, K. B., MOON, J. J., CHU, H. H., LIO, C.-W. J., VEGOE, A. L., HSIEH, C.-S., JENKINS, M. K. & FARRAR, M. A. 2008. Linked T Cell Receptor and Cytokine Signaling Govern the Development of the Regulatory T cell Repertoire. *Immunity,* 28**,** 112-121.

BURCHILL, M. A., YANG, J., VOGTENHUBER, C., BLAZAR, B. R. & FARRAR, M. A. 2007. IL-2 Receptor β-Dependent STAT5 Activation Is Required for the Development of Foxp3⁺ Regulatory T Cells. *The Journal of Immunology,* 178**,** 280.

CARTER, J. H., LEFEBVRE, J. M., WIEST, D. L. & TOURTELLOTTE, W. G. 2007. Redundant role for early growth response transcriptional regulators in thymocyte differntiation and survival *The Journal of Immunology* 6796-6805.

CHACON, J. A., SARNAIK, A. A., CHEN, J. Q., CREASY, C., KALE, C., ROBINSON, J., WEBER, J., HWU, P., PILON-THOMAS, S. & RADVANYI, L. 2015. Manipulating the tumor microenvironment ex vivo for enhanced expansion of tumor-infiltrating

lymphocytes for adoptive cell therapy. *Clinical cancer research : an official journal of the American Association for Cancer Research,* 21**,** 611-621.

CHAMPAGNE, P., OGG, G. S., KING, A. S., KNABENHANS, C., ELLEFSEN, K., NOBILE, M., APPAY, V., RIZZARDI, G. P., FLEURY, S., LIPP, M., FÖRSTER, R., ROWLAND-JONES, S., SÉKALY, R.-P., MCMICHAEL, A. J. & PANTALEO, G. 2001. Skewed maturation of memory HIV-specific CD8 T lymphocytes. *Nature,* 410**,** 106.

CHANDRA, A., LAN, S., ZHU, J., SICLARI, V. A. & QIN, L. 2013. Epidermal growth factor receptor (EGFR) signaling promotes proliferation and survival in osteoprogenitors by increasing early growth response 2 (EGR2) expression. *J Biol Chem,* 288**,** 20488-98.

CHANDRA, D. & NAIK, S. 2008. Leishmania donovani infection down-regulates TLR2-stimulated IL-12p40 and activates IL-10 in cells of macrophage/monocytic lineage by modulating MAPK pathways through a contact-dependent mechanism. *Clinical and Experimental Immunology,* 154**,** 224-234.

CHAVRIER, P., ZERIAL, M., LEMAIRE, P., ALMENDRAL, J., BRAVO, R. & CHARNAY, P. 1988. A gene encoding a protein with zinc fingers is activated during G0/G1 transition in cultured cells. *EMBO,* 7**,** 29-35.

CHEN, D. S. & MELLMAN, I. 2013. Oncology meets immunology: the cancer-immunity cycle. *Immunity,* 39**,** 1-10.

CHEN, L. 2004. Co-inhibitory molecules of the B7-CD28 family in the control of T-cell immunity. *Nature Rev. Immunol.,* 4**,** 336-347.

CHO, H. 2015. Complement regulation: physiology and disease relevance. *Korean J Pediatr,* 58**,** 239-44.

CHONG, M. M., METCALF, D., JAMIESON, E., ALEXANDER, W. S. & KAY, T. W. 2005. Suppressor of cytokine signaling-1 in T cells and macrophages is critical for preventing lethal inflammation. *Blood,* 106**,** 1668-75.

COLLINS, S., LUTZ, M. A., ZAREK, P. E., ANDERS, R. A., KERSH, G. J. & POWELL, J. D. 2008. Opposing regulation of T cell function by Egr-1/NAB2 and Egr-2/Egr-3. *Eur J Immunol,* 38**,** 528-36.

COLLINS, S., WOLFRAIM, L. A., DRAKE, C. G., HORTON, M. R. & POWELL, J. D. 2006. Cutting Edge: TCR-Induced NAB2 Enhances T Cell Function by Coactivating IL-2 Transcription. *The Journal of Immunology,* 177**,** 8301-8305.

COTE-SIERRA, J., FOUCRAS, G., GUO, L., CHIODETTI, L., YOUNG, H. A., HU-LI, J., ZHU, J. & PAUL, W. E. 2004. Interleukin 2 plays a central role in Th2 differentiation. *Proceedings of the National Academy of Sciences of the United States of America,* 101**,** 3880-3885.

CR, B., A, M., S, R. & SS, A. A. 2015. Trends in Adoptve Tumor Infltratng Lymphocytes (TILs) Therapy. *BAOJ Cancer Research & Therapy,* 1.

CRISPIN, J. C., OUKKA, M., BAYLISS, G., COHEN, R. A., VAN BEEK, C. A., STILLMAN, I. E., KYTTARIS, V. C., JUANG, Y. T. & TSOKOS, G. C. 2008. Expanded Double Negative T Cells in Patients with Systemic Lupus Erythematosus Produce IL-17 and Infiltrate the Kidneys. *The Journal of Immunology,* 181**,** 8761-8766.

CRISTOFANO, A. D., KOTSI, P., PENG, Y. F., CORDON-CARDO, C., ELKON, K. B. & PANDOLFI, P. P. 1999. Impaired Fas Response and Autoimmunity in Pten^{+/−} Mice. *Science,* 285**,** 2122.

CROKER, B. A., METCALF, D., ROBB, L., WEI, W., MIFSUD, S., DIRAGO, L., CLUSE, L. A., SUTHERLAND, K. D., HARTLEY, L., WILLIAMS, E., ZHANG, J.-G., HILTON, D. J., NICOLA, N. A., ALEXANDER, W. S. & ROBERTS, A. W. 2004. SOCS3 Is a Critical Physiological Negative Regulator of G-CSF Signaling and Emergency Granulopoiesis. *Immunity,* 20**,** 153-165.

DARNELL, J. E. 1997. STATs and Gene Regulation. *Science,* 277**,** 1630.

DAVIDSON, T. S., DIPAOLO, R. J., ANDERSSON, J. & SHEVACH, E. M. 2007. Cutting Edge: IL-2 Is Essential for TGF-β-Mediated Induction of Foxp3⁺ T Regulatory Cells. *The Journal of Immunology,* 178**,** 4022.

DE LA BARRERA, S., ALEMÁN, M., MUSELLA, R., SCHIERLOH, P., PASQUINELLI, V., GARCÍA, V., ABBATE, E. & SASIAIN, M. D. C. 2004. IL-10 down-regulates costimulatory molecules on Mycobacterium tuberculosis-pulsed macrophages

and impairs the lytic activity of CD4 and CD8 CTL in tuberculosis patients. *Clinical and Experimental Immunology,* 138**,** 128-138.

DECKER, E. L., SKERKA, C. & ZIPFEL, P. F. 1998. The Early Growth Response Protein (EGR-1) Regulates Interleukin-2 Transcription by Synergistic Interaction with the Nuclear Factor of Activated T Cells. *Journal of Biological Chemistry,* 273**,** 26923-26930.

DELGADO, M., ABAD, C., MARTINEZ, C., JUARRANZ, M. G., LECETA, J., GANEA, D. & GOMARIZ, R. P. 2003. PACAP in Immunity and Inflammation. *Annals of the New York Academy of Sciences,* 992(1)**,** 141-157.

DELISLE, J. S., GIROUX, M., BOUCHER, G., LANDRY, J. R., HARDY, M. P., LEMIEUX, S., JONES, R. G., WILHELM, B. T. & PERREAULT, C. 2013. The TGF-β-Smad3 pathway inhibits CD28-dependent cell growth and proliferation of CD4 T cells. *Genes And Immunity,* 14**,** 115.

DI GIACOMO, A. M., VALENTE, M., COVRE, A., DANIELLI, R. & MAIO, M. 2017. Immunotherapy targeting immune check-point(s) in brain metastases. *Cytokine & Growth Factor Reviews,* 36**,** 33-38.

DONG, H., STROME, S. E., SALOMAO, D. R., TAMURA, H., HIRANO, F., FLIES, D. B., ROCHE, P. C., LU, J., ZHU, G., TAMADA, K., LENNON, V. A., CELIS, E. & CHEN, L. 2002. Tumor-associated B7-H1 promotes T-cell apoptosis: A potential mechanism of immune evasion. *Nat Med,* 8**,** 793-800.

DU, N., KWON, H., LI, P., WEST, E. E., OH, J., LIAO, W., YU, Z., REN, M. & LEONARD, W. J. 2014. EGR2 is critical for peripheral naïve T-cell differentiation and the T-cell response to influenza. *Proceedings of the National Academy of Sciences of the United States of America,* 111**,** 16484-16489.

DUTTA, M., KRAUS, Z. J., GOMEZ-RODRIGUEZ, J., HWANG, S. H., CANNONS, J. L., CHENG, J., LEE, S. Y., WIEST, D. L., WAKELAND, E. K. & SCHWARTZBERG, P. L. 2013. A role for Ly108 in the induction of promyelocytic zinc finger transcription factor in developing thymocytes. *J Immunol,* 190**,** 2121-8.

ENCINAS, J. A., WICKER, L. S., PETERSON, L. B., MUKASA, A., TEUSCHER, C., SOBEL, R., WEINER, H. L., SEIDMAN, C. E., SEIDMAN, J. G. & KUCHROO, V. K. 1999. QTL influencing autoimmune diabetes and encephalomyelitis map to a 0.15-cM region containing Il2. *Nature Genetics,* 21**,** 158.

ERMANN, J. & FATHMAN, C. G. 2001. Autoimmune diseases: genes, bugs and failed regulation. *Nat Immunol,* 2**,** 759-761.

FANONI, D., TAVECCHIO, S., RECALCATI, S., BALICE, Y., VENEGONI, L., FIORANI, R., CROSTI, C. & BERTI, E. 2011. New monoclonal antibodies against B-cell antigens: Possible new strategies for diagnosis of primary cutaneous B-cell lymphomas. *Immunology Letters,* 134**,** 157-160.

FILIPPI, C. M., JUEDES, A. E., OLDHAM, J. E., LING, E., TOGHER, L., PENG, Y., FLAVELL, R. A. & VON HERRATH, M. G. 2008. Transforming Growth Factor-β Suppresses the

Activation of CD8⁺ T-Cells When Naïve but Promotes Their Survival and Function Once Antigen Experienced. *Diabetes,* 57**,** 2684.

FLEMMING, A. 2012. PD1 makes waves in anticancer immunotherapy. *Nature Reviews Drug Discovery,* 11**,** 601.

FONTENOT, J. D., RASMUSSEN, J. P., GAVIN, M. A. & RUDENSKY, A. Y. 2005. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nature Immunology,* 6**,** 1142.

FOURCADE, J., SUN, Z., BENALLAOUA, M., GUILLAUME, P., LUESCHER, I. F., SANDER, C., KIRKWOOD, J. M., KUCHROO, V. & ZAROUR, H. M. 2010. Upregulation of Tim-3 and PD-1 expression is associated with tumor antigen–specific CD8⁺ T cell dysfunction in melanoma patients. *The Journal of Experimental Medicine,* 207**,** 2175.

FRANCISCO, L. M., SALINAS, V. H., BROWN, K. E., VANGURI, V. K., FREEMAN, G. J., KUCHROO, V. K. & SHARPE, A. H. 2009. PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. *The Journal of Experimental Medicine,* 206**,** 3015.

FRANK, D. A., MAHAJAN, S. & RITZ, J. 1999. Fludarabine-induced immunosuppression is associated with inhibition of STAT1 signaling. *Nature Medicine,* 5**,** 444-447.

G, B., CB, C., C, L., J, A.-L., SM, H. & C, M. 2001. Regulation of the helix-loop-helix proteins, E2A and Id3, by the Ras-ERK MAPK cascade. *Nat Immunol,* 2**,** 165-71.

GABET, Y., BANIWAL, S. K., LECLERC, N., SHI, Y., KOHN-GABET, A. E., COGAN, J., DIXON, A., BACHAR, M., GUO, L., TURMAN, J. E., JR. & FRENKEL, B. 2010. Krox20/EGR2 deficiency accelerates cell growth and differentiation in the monocytic lineage and decreases bone mass. *Blood,* 116**,** 3964-71.

GANZ, T. 2004. Defensins: antimicrobial peptides of vertebrates. *Comptes Rendus Biologies,* 327**,** 539-549.

GARDNER, A. & RUFFELL, B. 2016. Dendritic Cells and Cancer Immunity. *Trends in Immunology,* 37**,** 855-865.

GEORGE R. STARK, IAN M. KERR, BRYAN R. G. WILLIAMS, ROBERT H. SILVERMAN & SCHREIBER, R. D. 1998. HOW CELLS RESPOND TO INTERFERONS

. *Annu. Rev. Biochem,* 67**,** 227–64.

GERMAIN, R. N. 2002. T-cell development and the CD4-CD8 lineage decision. *Nat Rev Immunol,* 2**,** 309-22.

GODSON, C., MITCHELL, S., HARVEY, K., PETASIS, N. A., HOGG, N. & BRADY, H. R. 2000. Cutting Edge: Lipoxins Rapidly Stimulate Nonphlogistic Phagocytosis of Apoptotic Neutrophils by Monocyte-Derived Macrophages. *The Journal of Immunology,* 164**,** 1663.

GOLDBERG, M. V. & DRAKE, C. G. 2011. LAG-3 in Cancer Immunotherapy. *In:* DRANOFF, G. (ed.) *Cancer Immunology and Immunotherapy.* Berlin, Heidelberg: Springer Berlin Heidelberg.

GOTTHARDT, D., PUTZ, E. M., STRAKA, E., KUDWEIS, P., BIAGGIO, M., POLI, V., STROBL, B., MÜLLER, M. & SEXL, V. 2014. Loss of STAT3 in murine NK cells enhances NK cell–dependent tumor surveillance. *Blood,* 124**,** 2370.

GREENBERG, S. & GRINSTEIN, S. 2002. Phagocytosis and innate immunity. *Current Opinion in Immunology,* 14**,** 136-145.

GRÖNE, A. 2002. Keratinocytes and cytokines. *Veterinary Immunology and Immunopathology,* 88**,** 1-12.

GROSSO, J. F., GOLDBERG, M. V., GETNET, D., BRUNO, T. C., YEN, H.-R., PYLE, K. J., HIPKISS, E., VIGNALI, D. A. A., PARDOLL, D. M. & DRAKE, C. G. 2009. Functionally Distinct LAG-3 and PD-1 Subsets on Activated and Chronically Stimulated CD8 T Cells. *The Journal of Immunology,* 182**,** 6659.

GUTIERREZ-RAMOS, J. C., ANDREU, J. L., REVILLA, Y., VIÑUELA, E. & MARTINEZ-A, C. 1990. Recovery from autoimmunity of MRL/lpr mice after infection with an interleukin-2/vaccinia recombinant virus. *Nature,* 346**,** 271.

HARLIN, H., MENG, Y., PETERSON, A. C., ZHA, Y., TRETIAKOVA, M., SLINGLUFF, C., MCKEE, M. & GAJEWSKI, T. F. 2009. Chemokine Expression in Melanoma

Metastases Associated with CD8⁺ T-Cell Recruitment. *Cancer Research,* 69**,** 3077.

HARRIS, J. E., BISHOP, K. D., PHILLIPS, N. E., MORDES, J. P., GREINER, D. L., ROSSINI, A. A. & CZECH, M. P. 2004. Early Growth Response Gene-2, a Zinc-Finger Transcription Factor, Is Required for Full Induction of Clonal Anergy in CD4+ T Cells. *The Journal of Immunology,* 173**,** 7331-7338.

HATANO, R., OHNUMA, K., OTSUKA, H., KOMIYA, E., TAKI, I., IWATA, S., DANG, N. H., OKUMURA, K. & MORIMOTO, C. 2015. CD26-mediated induction of EGR2 and IL-10 as potential regulatory mechanism for CD26 costimulatory pathway. *J Immunol,* 194**,** 960-72.

HERNANDEZ-CHACON, J. A., LI, Y., WU, R. C., BERNATCHEZ, C., WANG, Y., WEBER, J., HWU, P. & RADVANYI, L. 2011. Co-stimulation through the CD137/4-1BB pathway protects human melanoma tumor-infiltrating lymphocytes from activation-induced cell death and enhances anti-tumor effector function. *Journal of immunotherapy (Hagerstown, Md. : 1997),* 34**,** 236-250.

HO, I. C., HODGE, M. R., ROONEY, J. W. & GLIMCHER, L. H. 1996. The Proto-Oncogene c-maf Is Responsible for Tissue-Specific Expression of Interleukin-4. *Cell,* 85**,** 973-983.

HODI, F. S., O'DAY, S. J., MCDERMOTT, D. F., WEBER, R. W., SOSMAN, J. A., HAANEN, J. B., GONZALEZ, R., ROBERT, C., SCHADENDORF, D., HASSEL, J. C., AKERLEY, W., VAN

DEN EERTWEGH, A. J. M., LUTZKY, J., LORIGAN, P., VAUBEL, J. M., LINETTE, G. P., HOGG, D., OTTENSMEIER, C. H., LEBBÉ, C., PESCHEL, C., QUIRT, I., CLARK, J. I., WOLCHOK, J. D., WEBER, J. S., TIAN, J., YELLIN, M. J., NICHOL, G. M., HOOS, A. & URBA, W. J. 2010. Improved Survival with Ipilimumab in Patients with Metastatic Melanoma. *The New England journal of medicine,* 363**,** 711-723.

HOEBE, K., JANSSEN, E. & BEUTLER, B. 2004. The interface between innate and adaptive immunity. *Nature Immunology,* 5**,** 971-74.

HOTAMISLIGIL, G. S. 2006. Inflammation and metabolic disorders. *Nature,* 444**,** 860-867.

HUANG, A. C., POSTOW, M. A., ORLOWSKI, R. J., MICK, R., BENGSCH, B., MANNE, S., XU, W., HARMON, S., GILES, J. R., WENZ, B., ADAMOW, M., KUK, D., PANAGEAS, K. S., CARRERA, C., WONG, P., QUAGLIARELLO, F., WUBBENHORST, B., D'ANDREA, K., PAUKEN, K. E., HERATI, R. S., STAUPE, R. P., SCHENKEL, J. M., MCGETTIGAN, S., KOTHARI, S., GEORGE, S. M., VONDERHEIDE, R. H., AMARAVADI, R. K., KARAKOUSIS, G. C., SCHUCHTER, L. M., XU, X., NATHANSON, K. L., WOLCHOK, J. D., GANGADHAR, T. C. & WHERRY, E. J. 2017. T-cell invigoration to tumour burden ratio associated with anti-PD-1 response. *Nature,* 545**,** 60.

HUANG, C.-T., WORKMAN, C. J., FLIES, D., PAN, X., MARSON, A. L., ZHOU, G., HIPKISS, E. L., RAVI, S., KOWALSKI, J., LEVITSKY, H. I., POWELL, J. D., PARDOLL, D. M., DRAKE, C. G. & VIGNALI, D. A. A. 2004. Role of LAG-3 in Regulatory T Cells. *Immunity,* 21**,** 503-513.

HUANG, D. C. S., HAHNE, M., SCHROETER, M., FREI, K., FONTANA, A., VILLUNGER, A., NEWTON, K., TSCHOPP, J. & STRASSER, A. 1999. Activation of Fas by FasL induces apoptosis by a mechanism that cannot be blocked by Bcl-2 or Bcl-XL. *PNAS,* 96**,** 14871-14876.

ICHII, M., ORITANI, K. & KANAKURA, Y. 2014. Early B lymphocyte development: Similarities and differences in human and mouse. *World J Stem Cells,* 6**,** 421-31.

IVASKA, J., BOSCA, L. & PARKER, P. J. 2003. PKC[epsi] is a permissive link in integrin-dependent IFN-[gamma] signalling that facilitates JAK phosphorylation of STAT1. *Nat Cell Biol,* 5**,** 363-369.

IWAI, Y., ISHIDA, M., TANAKA, Y., OKAZAKI, T., HONJO, T. & MINATO, N. 2002. Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. *Proceedings of the National Academy of Sciences,* 99**,** 12293-12297.

J., D., L, L. T., WALUNAS & BLUESTONE, J. A. 1996. CD28/B7 SYSTEM OF T CELL COSTIMULATION. *Annual Review of Immunology,* 14**,** 233-258.

JIANG, T., ZHOU, C. & REN, S. 2016. Role of IL-2 in cancer immunotherapy. *Oncoimmunology,* 5**,** e1163462.

JONES, R. G., PARSONS, M., BONNARD, M., CHAN, V. S. F., YEH, W.-C., WOODGETT, J. R. & OHASHI, P. S. 2000. Protein Kinase B Regulates T Lymphocyte Survival, Nuclear

Factor κb Activation, and Bcl-X_L Levels in Vivo. *The Journal of Experimental Medicine,* 191**,** 1721.

KALIA, V., SARKAR, S., SUBRAMANIAM, S., HAINING, W. N., SMITH, K. A. & AHMED, R. 2010. Prolonged Interleukin-2Rα Expression on Virus-Specific CD8+ T Cells Favors Terminal-Effector Differentiation In Vivo. *Immunity,* 32**,** 91-103.

KAMANAKA, M., KIM, S. T., WAN, Y. Y., SUTTERWALA, F. S., LARA-TEJERO, M., GALÁN, J. E., HARHAJ, E. & FLAVELL, R. A. 2006. Expression of Interleukin-10 in Intestinal Lymphocytes Detected by an Interleukin-10 Reporter Knockin tiger Mouse. *Immunity,* 25**,** 941-952.

KANE, L. P. & WEISS, A. 2003. The PI-3 Kinase/Akt pathway and T cell activation: Pleiotropic pathways downstream of PIP3. *Immunological Reviews,* 12**,** 7-20.

KARWACZ, K., BRICOGNE, C., MACDONALD, D., ARCE, F., BENNETT, C. L., COLLINS, M. & ESCORS, D. 2011. PD‐L1 co‐stimulation contributes to ligand‐induced T cell receptor down‐modulation on CD8⁺ T cells. *EMBO Molecular Medicine,* 3**,** 581.

KEIR, M. E., BUTTE, M. J., FREEMAN, G. J. & SHARPE, A. H. 2008. PD-1 and Its Ligands in Tolerance and Immunity. *Annual Review of Immunology,* 26**,** 677-704.

KEIR, M. E., LIANG, S. C., GULERIA, I., LATCHMAN, Y. E., QIPO, A., ALBACKER, L. A., KOULMANDA, M., FREEMAN, G. J., SAYEGH, M. H. & SHARPE, A. H. 2006. Tissue

expression of PD-L1 mediates peripheral T cell tolerance. *The Journal of Experimental Medicine,* 203**,** 883.

KELCHTERMANS, H., BILLIAU, A. & MATTHYS, P. 2008. How interferon-γ keeps autoimmune diseases in check. *Trends in Immunology,* 29**,** 479-486.

KONISHI, J., YAMAZAKI, K., AZUMA, M., KINOSHITA, I., DOSAKA-AKITA, H. & NISHIMURA, M. 2004. B7-H1 Expression on Non-Small Cell Lung Cancer Cells and Its Relationship with Tumor-Infiltrating Lymphocytes and Their PD-1 Expression. *Clinical Cancer Research,* 10**,** 5094.

KOONPAEW, S., SHEN, S., FLOWERS, L. & ZHANG, W. 2006. LAT-mediated signaling in CD4⁺CD25⁺ regulatory T cell development. *The Journal of Experimental Medicine,* 203**,** 119.

KORN, T., BETTELLI, E., GAO, W., AWASTHI, A., JAGER, A., STROM, T. B., OUKKA, M. & KUCHROO, V. K. 2007. IL-21 initiates an alternative pathway to induce proinflammatory Th17 cells. *Nature genetics,* 448**,** 484-488.

KORN, T., BETTELLI, E., OUKKA, M. & KUCHROO, V. K. 2009. IL-17 and Th17 Cells. *Annual Review of Immunology,* 27**,** 485-517.

KORTYLEWSKI, M., KUJAWSKI, M., WANG, T., WEI, S., ZHANG, S., PILON-THOMAS, S., NIU, G., KAY, H., MULE, J., KERR, W. G., JOVE, R., PARDOLL, D. & YU, H. 2005.

Inhibiting Stat3 signaling in the hematopoietic system elicits multicomponent antitumor immunity. *Nat Med,* 11**,** 1314-1321.

KRISHNARAJU, K., NGUYEN, H. Q., LIEBERMANN, D. A. & HOFFMAN, B. 1995. The Zinc Finger Transcription Factor Egr-1 Potentiates Macrophage Differentiation of Hematopoietic cells. *Molecular and Cellular Biology,* 15**,** 5499-5507.

KUMAR, V., MITCHELL, R., ABBAS, A. K. & FAUSTO, N. 2007. Robbins Basic Pathology 8th Edition *Elsevier.*

KYI, C. & POSTOW, M. A. 2014. Checkpoint blocking antibodies in cancer immunotherapy. *FEBS Letters,* 588**,** 368-376.

LANDSKRON, G., DE LA FUENTE, M., THUWAJIT, P., THUWAJIT, C. & HERMOSO, M. A. 2014. Chronic inflammation and cytokines in the tumor microenvironment. *J Immunol Res,* 2014**,** 149185.

LASARO, M. O., SAZANOVICH, M., GILES-DAVIS, W., MRASS, P., BUNTE, R. M., SEWELL, D. A., HUSSAIN, S. F., FU, Y.-X., WENINGER, W., PATERSON, Y. & ERTL, H. C. J. 2011. Active Immunotherapy Combined With Blockade of a Coinhibitory Pathway Achieves Regression of Large Tumor Masses in Cancer-prone Mice. *Molecular Therapy,* 19**,** 1727-1736.

LAZAREVIC, V., ZULLO, A. J., SCHWEITZER, M. N., STATON, T. L., GALLO, E. M., CRABTREE, G. R. & GLIMCHER, L. H. 2009. The gene encoding early growth

response 2, a target of the transcription factor NFAT, is required for the development and maturation of natural killer T cells. *Nat Immunol,* 10**,** 306-13.

LEACH, D. R., KRUMMEL, M. F. & ALLISON, J. P. 1996. Enhancement of Antitumor Immunity by CTLA-4 Blockade. *Science,* 271**,** 1734.

LEE, C.-K., RAO, D. T., GERTNER, R., GIMENO, R., FREY, A. B. & LEVY, D. E. 2000. Distinct Requirements for IFNs and STAT1 in NK Cell Function. *The Journal of Immunology,* 165**,** 3571.

LEE, N., ZAKKA, L. R., MIHM, M. C., JR. & SCHATTON, T. 2016. Tumour-infiltrating lymphocytes in melanoma prognosis and cancer immunotherapy. *Pathology,* 48**,** 177-187.

LEVY, D. E. & DARNELL, J. E., JR. 2002. Stats: transcriptional control and biological impact. *Nat Rev Mol Cell Biol,* 3**,** 651-62.

LI, M. O. & FLAVELL, R. A. 2008a. Contextual Regulation of Inflammation: A Duet by Transforming Growth Factor-β and Interleukin-10. *Immunity,* 28**,** 468-476.

LI, M. O. & FLAVELL, R. A. 2008b. TGF-b: A Master of All T Cell Trades. *Cell,* 134**,** 392-404.

LI, M. O., WAN, Y. Y., SANJABI, S., ROBERTSON, A.-K. L. & FLAVELL, R. A. 2006. TRANSFORMING GROWTH FACTOR-β REGULATION OF IMMUNE RESPONSES. *Annual Review of Immunology,* 24**,** 99-146.

LI, S., MIAO, T., SEBASTIAN, M., BHULLAR, P., GHAFFARI, E., LIU, M., SYMONDS, A. L. & WANG, P. 2012. The transcription factors Egr2 and Egr3 are essential for the control of inflammation and antigen-induced proliferation of B and T cells. *Immunity,* 37**,** 685-96.

LI, S., SYMONDS, A. L., ZHU, B., LIU, M., RAYMOND, M. V., MIAO, T. & WANG, P. 2011. Early growth response gene-2 (Egr-2) regulates the development of B and T cells. *PLoS One,* 6**,** e18498.

LIAO, W., LIN, J.-X., WANG, L., LI, P. & LEONARD, W. J. 2011. Modulation of cytokine receptors by IL-2 broadly regulates differentiation into helper T cell lineages. *Nature Immunology,* 12**,** 551.

LIN, J.-X. & LEONARD, W. J. 2000. The role of Stat5a and Stat5b in signaling by IL-2 family cytokines. *Oncogene,* 19**,** 2566-2576.

LINSONG LI, YEE, C. & BEAVO, J. A. 1999. CD3- and CD28-Dependent Induction of PDE7 Required for T Cell Activation. *Science,* 283**,** 848-851.

LOVATO, P., BRENDER, C., AGNHOLT, J., KELSEN, J., KALTOFT, K., SVEJGAARD, A., ERIKSEN, K. W., WOETMANN, A. & ØDUM, N. 2003. Constitutive STAT3

Activation in Intestinal T Cells from Patients with Crohn's Disease. *Journal of Biological Chemistry,* 278**,** 16777-16781.

LU, Q. & LEMKE, G. 2001. Homeostatic Regulation of the Immune System by Receptor Tyrosine Kinases of the Tyro 3 Family. *Science,* 293**,** 306.

MALEK, T. R. 2008. The Biology of Interleukin-2. *Annual Review of Immunology,* 26**,** 453-479.

MARIE, J. C., LIGGITT, D. & RUDENSKY, A. Y. 2006. Cellular Mechanisms of Fatal Early-Onset Autoimmunity in Mice with the T Cell-Specific Targeting of Transforming Growth Factor-β Receptor. *Immunity,* 25**,** 441-454.

MARTINS, G. A., CIMMINO, L., LIAO, J., MAGNUSDOTTIR, E. & CALAME, K. 2008. Blimp-1 directly represses Il2 and the Il2 activator Fos, attenuating T cell proliferation and survival. *The Journal of Experimental Medicine,* 205**,** 1959-1965.

MEDZHITOV, R. 2001. Toll-Like Receptors and Innate Immunity. *Nature Reviews,* 1**,** 135-145.

MEDZHITOV, R. 2008. Origin and physiological roles of inflammation. *Nature,* 454**,** 428-435.

MENALLED, L. B. 2005. Knock-In Mouse Models of Huntington's Disease. *The American Society for Experimantal NeuroTheraputics,* 2**,** 465-470.

MIAO, T., SYMONDS, A. L. J., SINGH, R., SYMONDS, J. D., OGBE, A., OMODHO, B., ZHU, B., LI, S. & WANG, P. 2017. Egr2 and 3 control adaptive immune responses by temporally uncoupling expansion from T cell differentiation. *The Journal of Experimental Medicine,* 214**,** 1787.

MITCHELL, T. J. & JOHN, S. 2005. Signal transducer and activator of transcription (STAT) signalling and T-cell lymphomas. *Immunology,* 114**,** 301-312.

MITTELSTADT, P. R. & ASHWELL, J. D. 1998. Cyclosporin A-Sensitive Transcription Factor Egr-3 Regulates Fas Ligand Expression. *Molecular and Cellular Biology,* 18**,** 3744-3751.

MOLON, B., GRI, G., BETTELLA, M., GÓMEZ-MOUTÓN, C., LANZAVECCHIA, A., MARTÍNEZ-A, C., MAÑES, S. & VIOLA, A. 2005. T cell costimulation by chemokine receptors. *Nature Immunology,* 6**,** 465.

MOORE, K. W., DE WAAL MALEFYT, R., COFFMAN, R. L. & O'GARRA, A. 2001. Interleukin-10 and the Interleukin-10 Receptor. *Annual Review of Immunology,* 19**,** 683-765.

MORGAN, R. A., DUDLEY, M. E., WUNDERLICH, J. R., HUGHES, M. S., YANG, J. C., SHERRY, R. M., ROYAL, R. E., TOPALIAN, S. L., KAMMULA, U. S., RESTIFO, N. P., ZHENG, Z., NAHVI, A., DE VRIES, C. R., ROGERS-FREEZER, L. J., MAVROUKAKIS, S. A. & ROSENBERG, S. A. 2006. Cancer Regression in Patients After Transfer of Genetically Engineered Lymphocytes. *Science (New York, N.Y.),* 314**,** 126-129.

MORIGGL, R., TOPHAM, D. J., TEGLUND, S., SEXL, V., MCKAY, C., WANG, D., HOFFMEYER, A., VAN DEURSEN, J., SANGSTER, M. Y., BUNTING, K. D., GROSVELD, G. C. & IHLE, J. N. 1999. Stat5 Is Required for IL-2-Induced Cell Cycle Progression of Peripheral T Cells. *Immunity,* 10**,** 249-259.

MOTZ, G. T. & COUKOS, G. 2013. Deciphering and reversing tumor immune suppression. *Immunity,* 39**,** 61-73.

MURPHY, K. 2012. *Janeway's Immunobiology,* US, Garland Science.

NAGY, G., HUSZTHY, P. C., FOSSUM, E., KONTTINEN, Y., NAKKEN, B. & SZODORAY, P. 2015. Selected Aspects in the Pathogenesis of Autoimmune Diseases. *Mediators Inflamm,* 2015**,** 351732.

NATHAN, C. 2002. Points of control in inflammation. *Nature,* 420**,** 846-852.

NIE, Z., HU, G., WEI, G., CUI, K., YAMANE, A., RESCH, W., WANG, R., GREEN, DOUGLAS R., TESSAROLLO, L., CASELLAS, R., ZHAO, K. & LEVENS, D. 2012. c-Myc Is a Universal Amplifier of Expressed Genes in Lymphocytes and Embryonic Stem Cells. *Cell,* 151**,** 68-79.

O'DONOVAN, K. J., TOURTELLOTTE, W. G., MILBRANDT, J. & BARABAN, J. M. 1999. The Egr family of transcription-regulatory factors: Progress at the interface of molecular and systems neuroscience. *Trends Neurosci,* 22**,** 167-173.

O'SHEA, J. J., GADINA, M. & SCHREIBER, R. D. 2002. Cytokine Signaling in 2002: New surprises in the Jak/Stat Pathway. *Cell,* 109**,** S121-S131.

OBAR, J. J., MOLLOY, M. J., JELLISON, E. R., STOKLASEK, T. A., ZHANG, W., USHERWOOD, E. J. & LEFRANÇOIS, L. 2010. CD4+ T cell regulation of CD25 expression controls development of short-lived effector CD8+ T cells in primary and secondary responses. *Proceedings of the National Academy of Sciences,* 107**,** 193-198.

OGBE, A., MIAO, T., SYMONDS, A. L., OMODHO, B., SINGH, R., BHULLAR, P., LI, S. & WANG, P. 2015. Early Growth Response Genes 2 and 3 Regulate the Expression of Bcl6 and Differentiation of T Follicular Helper Cells. *J Biol Chem,* 290**,** 20455-65.

OHASHI, P. S. 2002. T-cell signalling and autoimmunity: molecular mechanisms of disease. *Nature reviews. Immunology,* 2**,** 427-438.

OLDSTONE, M. B. A. 1987. Molecular mimicry and autoimmune disease. *Cell,* 50**,** 819-820.

OLDSTONE, M. B. A., NERENBERG, M., SOUTHERN, P., PRICE, J. & LEWICKI, H. 1991. Virus infection triggers insulin-dependent diabetes mellitus in a transgenic model: Role of anti-self (virus) immune response. *Cell,* 65**,** 319-331.

OVCHARENKO, I., LOOTS, G. G., GIARDINE, B. M., HOU, M., MA, J., HARDISON, R. C., STUBBS, L. & MILLER, W. 2005. Mulan: Multiple-sequence local alignment and visualization for studying function and evolution. *Genome Research,* 15**,** 184-194.

OVERWIJK, W. W. & RESTIFO, N. P. 2001. B16 as a Mouse Model for Human Melanoma. *Current protocols in immunology / edited by John E. Coligan ... [et al.],* CHAPTER**,** Unit-20.1.

OWEN, J., PUNT, J., STRANFORD, S. & JONES, P. 2013. *Kuby Immunology,* US, Susan Winslow.

PANOPOULOS, A. D., ZHANG, L., SNOW, J. W., JONES, D. M., SMITH, A. M., EL KASMI, K. C., LIU, F., GOLDSMITH, M. A., LINK, D. C., MURRAY, P. J. & WATOWICH, S. S. 2006. STAT3 governs distinct pathways in emergency granulopoiesis and mature neutrophils. *Blood,* 108**,** 3682.

PARDOLL, D. M. 2012. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer,* 12**,** 252-264.

PARK, C., LI, S., CHA, E. & SCHINDLER, C. 2000. Immune Response in Stat2 Knockout Mice. *Immunity,* 13**,** 795-804.

PEREZ-CASTILLO, A., PIPAON, C., GARCIA, I. & ALEMANY, S. 1993. NGFI-A Gene Expression Is Necessary for T Lymphocyte Proliferation. *The Journal of Biological Chemistry,* 268**,** 19445-19450.

PERONA-WRIGHT, G., MOHRS, K. & MOHRS, M. 2010. Sustained signaling by canonical helper T cell cytokines throughout the reactive lymph node. *Nat Immunol,* 11**,** 520-6.

PETERSON, A. C., HARLIN, H. & GAJEWSKI, T. F. 2003. Immunization With Melan-A Peptide-Pulsed Peripheral Blood Mononuclear Cells Plus Recombinant Human Interleukin-12 Induces Clinical Activity and T-Cell Responses in Advanced Melanoma. *Journal of Clinical Oncology,* 21**,** 2342-2348.

PIPKIN, M. E., SACKS, J. A., CRUZ-GUILLOTY, F., LICHTENHELD, M. G., BEVAN, M. J. & RAO, A. 2010. Interleukin-2 and Inflammation Induce Distinct Transcriptional Programs that Promote the Differentiation of Effector Cytolytic T Cells. *Immunity,* 32**,** 79-90.

POIRIER, R., CHEVAL, H., MAILHES, C., GAREL, S., CHARNAY, P., DAVIS, S. & LAROCHE, S. 2008. Distinct functions of Egr gene family members in cognitive processes. *Frontiers in neuroscience,* 2**,** 47-53.

PRLIC, M., LEFRANCOIS, L. & JAMESON, S. C. 2002. Multiple Choices. *The Journal of Experimental Medicine,* 195**,** F49.

R.-BORLADO, L., REDONDO, C., ALVAREZ, B., JIMENEZ, C., CRIADO, L. M., FLORES, J., MARCOS, M. A. R., MARTINEZ-A, C., BALOMENOS, D. & CARRERA, A. C. 2000. Increased phosphoinositide 3-kinase activity induces a lymphoproliferative disorder and contributes to tumor generation in vivo. *The FASEB Journal,* 14**,** 895-903.

RABINOVICH, G. A., GABRILOVICH, D. & SOTOMAYOR, E. M. 2007. Immunosuppressive strategies that are mediated by tumor cells. *Annu Rev Immunol,* 25**,** 267-96.

RAMGOLAM, V. S. & MARKOVIC-PLESE, S. 2011. Regulation of suppressors of cytokine signaling as a therapeutic approach in autoimmune diseases, with an emphasis on multiple sclerosis. *J Signal Transduct,* 2011**,** 635721.

RANE, S. G. & REDDY, E. P. 2002. JAKs, STATs and Src kinases in hematopoiesis. *Oncogene,* 21**,** 3334-3358.

RAY, S., SONTHALIA, N., KUNDU, S. & SATYABRATA, G. 2013. Autoimmune Disorders: An Overview of Molecular and Cellular Basis in Today's Perspective. *Journal of Clinical & Cellular Immunology,* 01.

RENGARAJAN, J., MITTELSTADT, P. R., MAGES, H. W., GERTH, A. J., KROCZEK, R. A., ASHWELL, J. D. & GLIMCHER, L. H. 2000. Sequential Involvement of NFAT and Egr Transcription Factors in FasL regulation. *Cell Press,* 12**,** 293-300.

RIVERA, R. R., JOHNS, C. P., QUAN, J., JOHNSON, R. S. & MURRE, C. 2000. Thymocyte selection is regulated by the helix-loop-helix inhibitor protein, Id3 *Immunity,* 12**,** 17-26.

ROBERTA, M., , P. A., MAURICHI ANDREA , MOLLA ALESSANDRA , BERSANI ILARIA, BONO ALDO, BARTOLI CESARE, SANTINAMI MARIO , LOMBARDO CLAUDIA, RAVAGNANI FERNANDO, CASCINELLI NATALE, AND, P. G. & ANDREA, A. 2003. Lack of Terminally Differentiated Tumor-specific CD8+ T Cells at Tumor Site in Spite of Antitumor Immunity to Self-Antigens in Human Metastatic Melanoma. *Cancer Research,* 63**,** 2535-2545.

ROSENBERG, S. A. 2014. IL-2: The First Effective Immunotherapy for Human Cancer. *The Journal of Immunology,* 192**,** 5451.

ROSENBERG, S. A., RESTIFO, N. P., YANG, J. C., MORGAN, R. A. & DUDLEY, M. E. 2008. Adoptive cell transfer: a clinical path to effective cancer immunotherapy. *Nature reviews. Cancer,* 8**,** 299-308.

RUDD, C. E., TAYLOR, A. & SCHNEIDER, H. 2009. CD28 and CTLA-4 coreceptor expression and signal transduction. *Immunological reviews,* 229**,** 12-26.

RYAN, G. B. & MAJNO, G. 1977. Acute inflammation. A review. *The American Journal of Pathology,* 86**,** 183-276.

RYAN, G. B. & MAJNO, G. 1997. Acute inflammation: A review. *The American journal of pathology,* 86**,** 183-276.

SAFFORD, M., COLLINS, S., LUTZ, M. A., ALLEN, A., HUANG, C. T., KOWALSKI, J., BLACKFORD, A., HORTON, M. R., DRAKE, C., SCHWARTZ, R. H. & POWELL, J. D. 2005. Egr-2 and Egr-3 are negative regulators of T cell activation. *Nat Immunol,* 6**,** 472-80.

SAKUISHI, K., APETOH, L., SULLIVAN, J. M., BLAZAR, B. R., KUCHROO, V. K. & ANDERSON, A. C. 2010. Targeting Tim-3 and PD-1 pathways to reverse T cell exhaustion and restore anti-tumor immunity. *The Journal of Experimental Medicine,* 207**,** 2187.

SANJABI, S., ZENEWICZ, L. A., KAMANAKA, M. & FLAVELL, R. A. 2009. Anti- and Pro-inflammatory Roles of TGF-β, IL-10, and IL-22 In Immunity and Autoimmunity. *Current opinion in pharmacology,* 9**,** 447-453.

SARMA, J. V. & WARD, P. A. 2011. The Complement System. *Cell Tissue Res***,** 227-235.

SAUER, B. 1998. Inucible gene targeting in mice using the Cre/lox System. *Methods: A comparison to methods in Enzymology***,** 381-392.

SAVILL, J. 2001. Apoptosis in post-streptococcal glomerulonephritis. *Kidney International,* 60**,** 1203-1214.

SCHELLER, J., CHALARIS, A., SCHMIDT-ARRAS, D. & ROSE-JOHN, S. 2011. The pro- and anti-inflammatory properties of the cytokine interleukin-6. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research,* 1813**,** 878-888.

SCHUMANN, J., STANKO, K., SCHLIESSER, U., APPELT, C. & SAWITZKI, B. 2015. Differences in CD44 Surface Expression Levels and Function Discriminates IL-17 and IFN-gamma Producing Helper T Cells. *PLoS One,* 10**,** e0132479.

SCHUST, J., SPERL, B., HOLLIS, A., MAYER, T. U. & BERG, T. 2006. Stattic: a small-molecule inhibitor of STAT3 activation and dimerization. *Chem Biol,* 13**,** 1235-42.

SEIDEL, J. A., OTSUKA, A. & KABASHIMA, K. 2018. Anti-PD-1 and Anti-CTLA-4 Therapies in Cancer: Mechanisms of Action, Efficacy, and Limitations. *Frontiers in Oncology,* 8**,** 86.

SEILER, M. P., MATHEW, R., LISZEWSKI, M. K., SPOONER, C. J., BARR, K., MENG, F., SINGH, H. & BENDELAC, A. 2012. Elevated and sustained expression of the transcription factors Egr1 and Egr2 controls NKT lineage differentiation in response to TCR signaling. *Nat Immunol,* 13**,** 264-71.

SELA, U., DAYAN, M., HERSHKOVIZ, R., LIDER, O. & MOZES, E. 2008. A Peptide That Ameliorates Lupus Up-Regulates the Diminished Expression of Early Growth Response Factors 2 and 3. *The Journal of Immunology,* 180**,** 1584-1591.

SEO, N., HAYAKAWA, S., TAKIGAWA, M. & TOKURA, Y. 2001. Interleukin-10 expressed at early tumour sites induces subsequent generation of CD4(+) T-regulatory cells and systemic collapse of antitumour immunity. *Immunology,* 103**,** 449-457.

SERHAN, C. N. & SAVILL, J. 2005. Resolution of inflammation: the beginning programs the end. 6**,** 1191.

SETOGUCHI, R., HORI, S., TAKAHASHI, T. & SAKAGUCHI, S. 2005. Homeostatic maintenance of natural Foxp3(+) CD25(+) CD4(+) regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. *The Journal of Experimental Medicine,* 201**,** 723-735.

SHAO, H., KONO, D. H., CHEN, L.-Y., RUBIN, E. M. & KAYE, J. 1997. Induction of Early Growth Response (Egr) Family of Transcription Factors during Thymic Selection. *Journal of Experimental Medicine,* 185**,** 731-744.

SHARMA, M. D. 2007. Plasmacytoid dendritic cells from mouse tumor-draining lymph nodes directly activate mature Tregs via indoleamine 2,3-dioxygenase. *J. Clin. Invest.,* 117**,** 2570-2582.

SHUAI, K. & LIU, B. 2003. Regulation of JAK-STAT signalling in the immune system. *Nat Rev Immunol,* 3**,** 900-911.

SHULL, M. M., ORMSBY, I., KIER, A. B., PAWLOWSKI, S., DIEBOLD, R. J., YIN, M., ALLEN, R., SIDMAN, C., PROETZEL, G., CALVIN, D., ANNUNZIATA, N. & DOETSCHMAN, T. 1992. Targeted disruption of the mouse transforming growth factor-β1 gene results in multifocal inflammatory disease. *Nature,* 359**,** 693.

SIM, G. C. & RADVANYI, L. 2014. The IL-2 cytokine family in cancer immunotherapy. *Cytokine & Growth Factor Reviews,* 25**,** 377-390.

SINGH, P. K., PARSEK, M. R., GREENBERG, E. P. & WELSH, M. J. 2002. A component of innate immunity prevents bacterial biofilm development. *Nature,* 417**,** 552-555.

SINGH, R., MIAO, T., SYMONDS, A. L. J., OMODHO, B., LI, S. & WANG, P. 2017. Egr2 and 3 Inhibit T-bet-Mediated IFN-gamma Production in T Cells. *J Immunol,* 198**,** 4394-4402.

SNOW, J. W., ABRAHAM, N., MA, M. C., ABBEY, N. W., HERNDIER, B. & GOLDSMITH, M. A. 2002. STAT5 promotes multilineage hematolymphoid development in vivo through effects on early hematopoietic progenitor cells. *Blood,* 99**,** 95.

SPEISER, D. E., HO, P.-C. & VERDEIL, G. 2016. Regulatory circuits of T cell function in cancer. *Nature Reviews Immunology,* 16**,** 599.

STEPHEN, T. L., RUTKOWSKI, M. R., ALLEGREZZA, M. J., PERALES-PUCHALT, A., TESONE, A. J., SVORONOS, N., NGUYEN, J. M., SARMIN, F., BOROWSKY, M. E., TCHOU, J. & CONEJO-GARCIA, J. R. 2014. TRANSFORMING GROWTH FACTOR-BETA MEDIATED SUPPRESSION OF ANTI-TUMOR T CELLS REQUIRES FOXP1 TRANSCRIPTION FACTOR EXPRESSION. *Immunity,* 41**,** 427-439.

STUMHOFER, J. S., SILVER, J. S., LAURENCE, A., PORRETT, P. M., HARRIS, T. H., TURKA, L. A., ERNST, M., SARIS, C. J. M., O'SHEA, J. J. & HUNTER, C. A. 2007. Interleukins 27 and 6 induce STAT3-mediated T cell production of interleukin 10. *Nature Immunology,* 8**,** 1363.

SUMITOMO, S., FUJIO, K., OKAMURA, T. & YAMAMOTO, K. 2013. Egr2 and Egr3 are the unique regulators for systemic autoimmunity. *JAK-STAT,* 2.

TAKAKI, H., ICHIYAMA, K., KOGA, K., CHINEN, T., TAKAESU, G., SUGIYAMA, Y., KATO, S., YOSHIMURA, A. & KOBAYASHI, T. 2008. STAT6 Inhibits TGF-beta1-mediated Foxp3 induction through direct binding to the Foxp3 promoter, which is reverted by retinoic acid receptor. *J Biol Chem,* 283**,** 14955-62.

TIAN, T., OLSON, S., WHITACRE, J. M. & HARDING, A. 2011. The origins of cancer robustness and evolvability. *Integrative Biology,* 3**,** 17-30.

TIMOFEEVA, O. A., TARASOVA, N. I., ZHANG, X., CHASOVSKIKH, S., CHEEMA, A. K., WANG, H., BROWN, M. L. & DRITSCHILO, A. 2013. STAT3 suppresses transcription of proapoptotic genes in cancer cells with the involvement of its N-terminal domain. *Proceedings of the National Academy of Sciences of the United States of America,* 110**,** 1267-1272.

TIVOL, E. A., BORRIELLO, F., SCHWEITZER, A. N., LYNCH, W. P., BLUESTONE, J. A. & SHARPE, A. H. 1995. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity,* 3**,** 541-547.

TOPILKO, P., SCHNEIDER-MAUNOURY, S., LEVI, G., BARON-VAN EVERCOOREN, A., CHENNOUFI, A. B. Y., SEITANIDOU, T., BABINET, C. & CHARNAY, P. 1994. Krox-20 controls myelination in the peripheral nervous system. *Nature,* 371**,** 796-799.

TOSI, M. F. 2005. Innate immune responses to infection. *J Allergy Clin Immunol,* 116**,** 241-9; quiz 250.

TOUGH, A. L. B. A. D. F. 2002. Links between innate and adaptive immunity via type I interferon. *Current Opinion in Immunology,* 14**,** 432-436.

TOURTELLOTTE WG & J, M. 1998. Sensory ataxia and muscle spindle agenesis in mice lacking the transcription factor Egr3. *Nat Genet,* 20**,** 87-91.

TOURTELLOTTE, W. G., NAGARAJAN, R., AUYEUNG, A., MUELLER, C. & MILBRANDT, J. 1999. Infertility associated with incomplete spermatogenic arrest and oligozoospermia in Egr4-deficient mice. *Development,* 126**,** 5061-5071.

TURLEY, S. J. 2002. Dendritic cells: inciting and inhibiting autoimmunity. *Current Opinion in Immunology,* 14**,** 765-770.

TURVEY, S. E. & BROIDE, D. H. 2010. Innate immunity. *Journal of Allergy and Clinical Immunology,* 125**,** S24-S32.

ULLOA, L., DOODY, J. & MASSAGUE, J. 1999. Inhibition of transforming growth factor-[beta]/SMAD signalling by the interferon-[gamma]/STAT pathway. *Nature,* 397**,** 710-713.

VALENCIA, X., YARBORO, C., ILLEI, G. & LIPSKY, P. E. 2007. Deficient CD4⁺CD25^{high} T Regulatory Cell Function in Patients with Active Systemic Lupus Erythematosus. *The Journal of Immunology,* 178**,** 2579.

VAN ELSAS, A., HURWITZ, A. A. & ALLISON, J. P. 1999. Combination Immunotherapy of B16 Melanoma Using Anti–Cytotoxic T Lymphocyte–Associated Antigen 4 (Ctla-4) and Granulocyte/Macrophage Colony-Stimulating Factor (Gm-Csf)-Producing Vaccines Induces Rejection of Subcutaneous and Metastatic Tumors Accompanied by Autoimmune Depigmentation. *The Journal of Experimental Medicine,* 190**,** 355-366.

VELU, V., TITANJI, K., ZHU, B., HUSAIN, S., PLADEVEGA, A., LAI, L., VANDERFORD, T. H., CHENNAREDDI, L., SILVESTRI, G., FREEMAN, G. J., AHMED, R. & AMARA, R. R. 2009. Enhancing SIV-specific immunity in vivo by PD-1 blockade. *Nature,* 458**,** 206-210.

VESELY, M. D., KERSHAW, M. H., SCHREIBER, R. D. & SMYTH, M. J. 2011. Natural Innate and Adaptive Immunity to Cancer. *Annual Review of Immunology,* 29**,** 235-271.

VINAY, D. S. & KWON, B. S. 2012. Immunotherapy of Cancer with 4-1BB. *Molecular Cancer Therapeutics,* 11**,** 1062.

W, J. C., EDWARDS & GERALDINE 2006. B-cell targeting in rheumatoid arthritis and other autoimmune diseases. *Nature Reviews Immunology***,** 394-403.

WANG, L., TASSIULAS, I., PARK-MIN, K.-H., REID, A. C., GIL-HENN, H., SCHLESSINGER, J., BARON, R., ZHANG, J. J. & IVASHKIV, L. B. 2008. 'Tuning' of type I interferon-induced Jak-STAT1 signaling by calcium-dependent kinases in macrophages. *Nat Immunol,* 9**,** 186-193.

WANG, R., DILLON, CHRISTOPHER P., SHI, LEWIS Z., MILASTA, S., CARTER, R., FINKELSTEIN, D., MCCORMICK, LAURA L., FITZGERALD, P., CHI, H., MUNGER, J. & GREEN, DOUGLAS R. 2011. The Transcription Factor Myc Controls Metabolic Reprogramming upon T Lymphocyte Activation. *Immunity,* 35**,** 871-882.

WARNER, L. E., MANCIAS, P., BUTLER, I. J., MCDONALD, C. M., KEPPEN, L., KOOB, K. G. & LUPSKI, J. R. 1998. Mutations in the early growth response 2 (EGR2) gene are associated with hereditary myelinopathies. *Nature genetics,* 18.

WATERHOUSE, P., PENNINGER, J. M., TIMMS, E., WAKEHAM, A., SHAHINIAN, A., LEE, K. P., THOMPSON, C. B., GRIESSER, H. & MAK, T. W. 1995. Lymphoproliferative Disorders with Early Lethality in Mice Deficient in <em>Ctla-4</em&gt. *Science,* 270**,** 985.

WILLIAMS, J., ZHENG, Y. & GAJEWSKI, T. 2014. Egr2 targets identify a population of dysfunctional T cells in the tumor microenvironment with immune modulatory properties. *Journal for ImmunoTherapy of Cancer,* 2**,** P238.

WILLIAMS, J. B., HORTON, B. L., ZHENG, Y., DUAN, Y., POWELL, J. D. & GAJEWSKI, T. F. 2017. The EGR2 targets LAG-3 and 4-1BB describe and regulate dysfunctional antigen-specific CD8⁺ T cells in the tumor microenvironment. *The Journal of Experimental Medicine*.

WU, D., WANG, J., PAE, M. & MEYDANI, S. N. 2012. Green tea EGCG, T cells, and T cell-mediated autoimmune diseases. *Mol Aspects Med,* 33**,** 107-18.

WURSTER, A. L., TANAKA, T. & GRUSBY, M. J. 2000. The biology of Stat4 and Stat6. *Oncogene,* 19**,** 2577-2584.

YADAV, M., STEPHAN, S. & BLUESTONE, J. A. 2013. Peripherally induced tregs - role in immune homeostasis and autoimmunity. *Front Immunol,* 4**,** 232.

YANG, X. O., PANOPOULOS, A. D., NURIEVA, R., CHANG, S. H., WANG, D., WATOWICH, S. S. & DONG, C. 2007. STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. *J Biol Chem,* 282**,** 9358-63.

YU, A., ZHU, L., ALTMAN, N. H. & MALEK, T. R. 2009. A low IL-2R signaling threshold supports the development and homeostasis of T regulatory cells. *Immunity,* 30**,** 204-217.

ZENG, R., SPOLSKI, R., CASAS, E., ZHU, W., LEVY, D. E. & LEONARD, W. J. 2007. The molecular basis of IL-21 meidated proliferation. *Blood,* 109**,** 4135-4142.

ZHANG, X., ING, S., FRASER, A., CHEN, M., KHAN, O., ZAKEM, J., DAVIS, W. & QUINET, R. 2013. Follicular Helper T Cells: New Insights Into Mechanisms of Autoimmune Diseases. *The Ochsner Journal,* 13**,** 131-139.

ZHENG, Y., ZHA, Y., DRIESSENS, G., LOCKE, F. & GAJEWSKI, T. F. 2012. Transcriptional regulator early growth response gene 2 (Egr2) is required for T cell anergy in vitro and in vivo. *J Exp Med,* 209**,** 2157-63.

ZHOU, P., SHAFFER, D. R., ALVAREZ ARIAS, D. A., NAKAZAKI, Y., POS, W., TORRES, A. J., CREMASCO, V., DOUGAN, S. K., COWLEY, G. S., ELPEK, K., BROGDON, J., LAMB, J., TURLEY, S. J., PLOEGH, H. L., ROOT, D. E., LOVE, J. C., DRANOFF, G., HACOHEN, N.,

CANTOR, H. & WUCHERPFENNIG, K. W. 2014. In vivo discovery of immunotherapy targets in the tumour microenvironment. *Nature,* 506**,** 52.

ZHU, B., SYMONDS, A. L., MARTIN, J. E., KIOUSSIS, D., WRAITH, D. C., LI, S. & WANG, P. 2008. Early growth response gene 2 (Egr-2) controls the self-tolerance of T cells and prevents the development of lupuslike autoimmune disease. *J Exp Med,* 205**,** 2295-307.

ZHU, C., ANDERSON, A. C., SCHUBART, A., XIONG, H., IMITOLA, J., KHOURY, S. J., ZHENG, X. X., STROM, T. B. & KUCHROO, V. K. 2005. The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. *Nat Immunol,* 6**,** 1245-1252.

ZHU, J. & PAUL, W. E. 2008. CD4 T cells: fates, functions, and faults. *blood,* 112**,** 1557-1569.

ZHU, J., YAMANE, H. & PAUL, W. E. 2010. Differentiation of Effector CD4 T Cell Populations*. *Annual Review of Immunology,* 28**,** 445-489.

ZIPFEL, P. F. & SKERKA, C. 2009. Complement regulators and inhibitory proteins. *Nat Rev Immunol,* 9**,** 729-40.